REGULATION OF MACROPHAGE APOPTOSIS VIA BCL-2 FAMILY MEMBERS AND CERAMIDE

by

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Abstract

Apoptosis is an important mechanism involved in regulating the number of macrophages present at sites of inflammation. Several lines of evidence indicate that blocking macrophage apoptosis can increase atherosclerosis. We previously reported that oxidized LDL (oxLDL) can inhibit apoptosis in cultured bone marrow-derived macrophages in part by activating the phosphoinositide 3 kinase (PI3K)/protein kinase B (PKB) pathway and subsequent expression of pro-survival protein Bcl-X_L. Here we report that oxLDL also alters the levels of the pro-apoptotic protein, Bax. This effect of oxLDL on Bax regulation was at a post-transcriptional level, mediated by accelerated degradation via the ubiquitin-proteasome pathway. However, Bax knockout macrophages were not resistant to apoptosis following cytokine withdrawal, suggesting that the downregulation of Bax is only partially responsible for the pro-survival effects mediated by oxLDL in these cells. OxLDL is also able to increase the expression of the prosurvival relative, Mcl-1. The effect of oxLDL on Bax degradation and Mcl-1 expression was blocked by inhibitors of the PI3K/PKB pathway.

To investigate the upstream receptor(s) activated by oxLDL to mediate macrophage survival, we used pertussis toxin (PTX) to test whether G_i protein coupled receptors are involved. Unexpectedly, we found that PTX by itself selectively blocks macrophage apoptosis in a dose-dependent manner. PTX acts in part by inhibiting acid sphingomyelinase activity which in turn prevents generation of ceramide during apoptosis. A Gi activator peptide, mastoparan, increased ceramide levels in macrophage and induced apoptosis, but pre-treatment with PTX partially overrode mastoparan-induced apoptosis. PTX failed to prevent ASMase activation or apoptosis in

macrophages lacking toll-like receptor 4 (TLR4). Like oxLDL, the anti-apoptotic effect of PTX also activated the PI3K/PKB pathway which led to nuclear localization of the transcription factor NFκB and up-regulation of Bcl-X_L. These results indicate that G_i proteins, TLR4, ASMase and the PI3K/PKB pathway are crucial components for regulation of macrophage apoptosis.

We also looked at regulation of ceramide generation in response to apoptosis. Using ASMase-/- mice, we found that ceramide is still generated. Using inhibitors to enzymes involved in the *de novo* ceramide synthesis pathway, we concluded that both *de novo* synthesis and sphingomyelin hydrolysis can contribute to ceramide generation during macrophage apoptosis.

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List of Abbreviation

-/-	knockout
+/+	wildtype
Ab	antibody
AcLDL	acetylated low density lipoproteins
AIM	apoptosis inhibitor expressed by macrophages
ANT	adenine-nucleotide translocator
ASMase	acid sphingomyelinase
BMDM	bone marrow derived macrophages
BSA	bovine serum albumin
C1P	ceramide-1-phosphate
CAMP	cyclic AMP
CARD	caspase recruitment domain
Cdase	ceramidase
CK	cermamide kinase
CS	ceramide synthase
CTRL	control
CVD	cardiovascular disease
DAG	diacylglycerol
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
FBS	fetal bovine serum
GCS	glucosylceramide synthase
GM-CSF	granulocyte-macrophage colony stimulating factor
IB	immunoblot
IP	immunoprecipitation
KSR	kinase suppressor of Ras
LDL	low density lipoproteins
LPC	lysophosphatidylcoline
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage-colony stimulating factor
MEF	murine embryonic fibroblast
MMP	matrix metalloproteinases
NFκB	nuclear factor kappa B
NPD	Niemann-Pick disease
NSMase	neutral sphingomyelinase
OxLDL	oxidized low density lipoproteins

PA	phosphatidic acid
PARP	poly (ADP-ribose) polymerase
PI	propidium iodide
PI3K	phosphatidylinositol 3 kinase
PKB	protein kinase B
PLD	phospholipase D
PM	plasma membrane
PP1	serine/threonine protein phosphatases 1
PP2A	serine/threonine protein phosphatases 2A
PT	permeabilitity transition pores
PTX	pertussis toxin
PUFA	polyunsaturated fatty acid
S1P	sphingosine-1-phosphate
SK	sphingosine kinase
SM	sphingomyeline
SMS	sphingomyeline synthase
SPT	serine palmitoyltransferase
tBid	truncated Bid
TLR4	toll-like receptor 4
VCAM-1	vascular cell adhesion molecule-1
VDAC	voltage-depdendent anion channel

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1 Introduction

1.1 Atherosclerosis

1.1.1 The role of macrophages in the pathogenesis of atherosclerosis

Atherosclerosis remains the leading cause of death in Western societies. Early atherosclerotic lesions are characterized by intimal thickening and by expansion of the intima of arteries by lipid-laden foam cells [1]. Advanced lesions have a lipid core covered by a cap of fibrous tissue [2]. The rupture of advanced lesions can lead to thrombus formation that occludes the vessel lumen and results in acute myocardial infarction or stroke [3]. It is now recognized that increased cholesterol and inflammation work together to contribute to the pathogenesis of atherosclerosis [4-6].

Macrophages are believed to play a central role in all stages of atherosclerosis because of their role in cholesterol accumulation and because they are an essential constituent of innate immunity and inflammation [7]. The key *in vivo* evidence implicating macrophages in atherogenesis came from animal studies where plaque formation in the atherosclerosis prone apoE deficient mouse was almost abolished in the absence of macrophages [8]. In the initial stage, the up-regulation of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) on lipid activated endothelium[9], is thought to promote adhesion of blood monocytes, and facilitate their movement into the intima[10-12]. Secretion of chemokines like monocyte chemoattractant protein-1 (MCP-1), can also promote recruitment of monocytes into intima. Deficiency of MCP-1 [13] or its receptor, CCR2 [14] decreases macrophage numbers in the intima and also decreases lesion size. Conversely, over-expression of MCP-1 accelerates atherosclerosis development [15]. Bursill *et al.* have recently shown that inactivation of almost all CC-class chemokines

strongly inhibited atherosclerosis in ApoE-/- mice [16]. After migrating into the intima, monocytes differentiate into macrophages, which have high levels of expression of scavenger receptors. These receptors have many functions including the recognition and internalization of pathogens and apoptotic cells. However, they also recognize modified lipoproteins and contribute to the massive accumulation of cholesterol in macrophage foam cells [17, 18]. Activated macrophages may intensify and perpetuate the inflammatory process by the secretion of cytokines and chemokines.

In addition to its role in initial stages of atherosclerosis, the macrophage is a contributing factor to complications associated with later stages of the disease [19, 20, 21]. Macrophage foam cells are mechanically much weaker than fibrous regions of plaque, and they secrete matrix metalloproteinases (MMP) which digest the stabilizing connective-tissue elements of plaque [22]. Both of these effects make plaques more vulnerable to rupture. Not surprisingly, ruptured plaques commonly contain high numbers of macrophages in the shoulder region [23]. Macrophages also secrete pro-thrombotic tissue factor, which accelerates thrombus formation following rupture or erosion [24]. A study in cholesterol-fed rabbits showed that 6 months of feeding a cholesterol free diet resulted in disappearance of macrophages from plaque, and stabilization of lesions by connective tissue [25].

1.1.2 Effect of oxLDL on pathogenesis of atherosclerosis

In humans, most of the cholesterol in plasma is carried by low density lipoproteins (LDL). Increased LDL is found to correlate with increased risk of cardiovascular disease (CVD) [26]. However, it is thought that LDL must be somehow modified before it can induce foam-cell formation because the uptake of native LDL by

the LDL receptor is subject to feedback regulation by cell cholesterol levels [27]. Brown and Goldstein's group showed that chemical modifications of LDL that removed the positive charge of lysine amino groups generated ligands for scavenger receptors, and such modified LDLs could cause cultured macrophages to transform into foam cells [28]. Subsequent studies showed that oxidation of LDL also modified lysine amino groups, and caused rapid uptake of oxidized LDL (oxLDL) by scavenger receptors [29].While LDL is well protected from oxidation in the plasma compartment, it is thought to be susceptible to enzymatic and non-enzymatic modifications in the arterial intima where antioxidant defenses are less prevalent, and oxidant stress from macrophages, endothelial cells, and smooth muscle cells is quite intense. [30]. In addition to its interaction with scavenger receptors, oxLDL has been found to exhibit a number of other pro-atherogenic properties through its effects on macrophage recruitment, proliferation, apoptosis and survival.

1.1.3 Effect of oxLDL on macrophage recruitment

Recruitment of monocytes and their differentiation into macrophages are important determinants of macrophage numbers in plaque [31] and oxidized LDL (oxLDL) is believed to play a role in these processes [12]. It increases the expression of adhesion molecules and monocytes binding to endothelial cells [32, 33], induces the expression of MCP-1 on endothelial cells, and can act as a direct chemoattractant [34, 35].

1.1.4 Biological effect of oxLDL on macrophage proliferation

Another factor that controls the number of macrophages in plaques is the balance between macrophage proliferation, and macrophage death. Although cytotoxicity was

one of the first biological activities of oxLDL to be observed [36], more recent studies have shown that at least under some conditions, oxLDL can stimulate macrophage growth and inhibit apoptosis [37-40].

Traditionally viewed as terminally differentiated, macrophages have now been shown to be the predominant cell type in plaque that expresses proliferation markers [41, 42]. Evidence showing that macrophage proliferation is closely associated with progression of atherosclerosis comes from deletion of the retinoblastoma (Rb) tumor suppressor gene from macrophages in a murine model of atherosclerosis where mice without Rb showed increased macrophage proliferation and atherosclerotic lesion [43]. It has also been reported that in addition to their cholesterol-lowering effect, statins significantly inhibit macrophage proliferation [44].

Yui et al. first reported that oxLDL has the ability to promote macrophage proliferation [45]. They reported that internalization of lysophosphatidylcholine (lysoPC), a major phospholipid component in oxLDL, via macrophage scavenger receptors was essential for this growth response [46]. Internalization of lysoPC in macrophages caused activation of protein kinase C and autocrine release of GM-CSF, which this group believed was responsible for the macrophage proliferation[40, 47]. It was later suggested by Hamilton et al. that oxLDL "primes" macrophage proliferation in response to other factors, such as M-CSF or GM-CSF [37]. However, results from our laboratory showed that oxLDL stimulates macrophage growth without the involvement of lysoPC or GM-CSF [39, 48]. Instead, we demonstrated that oxLDL induces activation of phosphatidylinositol 3 kinase (PI3K) and that this activation plays a role in oxLDL-mediated macrophage proliferation [39].

1.1.5 Biological effect of oxLDL on macrophage apoptosis

Several reports in mouse models have shown an inverse correlation between macrophage apoptosis and rate of progression of early atherosclerotic lesions (reviewed by Tabas et al [49]). Apoptosis and necrosis of macrophages are observed in advanced stages of atherosclerosis, but high cellularity and proliferation are evident in early atherosclerotic lesions [50-52]. Whether macrophage death is advantageous or deleterious in atherosclerosis is not fully understood at present. Apoptosis of macrophages can be beneficial as removal of inflammatory cells from the plaque could attenuate the inflammatory response and decrease the synthesis of MMP. Loss of macrophages, however, also decreases the uptake of apoptotic bodies so that secondary necrosis occurs which contributes to the formation of an acellular lipid core, the hallmark of an advanced atherosclerotic lesion [53]. In addition to its pro-inflammatory effects, oxLDL has also been implicated in the induction of apoptosis in macrophages [54].

It has been shown that the mitochondrial pathway plays an important role in macrophage apoptosis induced by oxysterols, which are a major component of oxLDL [55, 56]. The balance between apoptotic and anti-apoptotic Bcl-2 family members is a major determinant of mitochondrial membrane integrity and a strong body of evidence implicates these proteins in apoptosis induced by oxLDL and oxysterols in macrophages. Harada et al. suggested a role for Bcl-2 in protection against oxysterol-induced apoptosis in macrophages [55]. More recently, Rusinol et al. have shown that increased degradation of protein kinase B (PKB) in response to oxysterols leads to increased activity of the pro-apoptotic Bcl-2 family members Bim and Bad, and down-regulation of Bcl-X_L followed by Bax mediated release of mitochondrial cytochrome C [57]. Berthier et al. have suggested

dephosphorylation of Bad by calcineurin and Bim displacement with subsequent association with Bcl-2 as additional mechanisms [58, 59].

There is also evidence pointing towards lysosomes as targets of oxLDL and oxysterols in induction of macrophage apoptosis. Yuan et al. reported compromise of macrophage lysosomal membrane integrity which accompanied apoptotic transformation [60]. The same group later showed that endocytosed oxLDL can destabilize the acidic vacuolar compartment but also cause the upregulation and translocation of lysosomal cathepsins [61, 62]. The role of lysosomes in oxysterol-mediated apoptosis was further emphasized by the finding that lysosomal dysfunction precedes apoptosis and that macrophage apoptosis was reduced by inhibitors of cathepsins B and L [63]. Although there is evidence that lysosome/cathepsin-triggered apoptosis merges with caspase activation and the mitochondrial apoptosis pathway [64, 65], the specific interactions of the two above-mentioned apoptotic arms (lysosomal and mitochondrial) have not been studied. For more detailed review on the mechanism employed by oxLDL to induce apoptosis in atherosclerosis, please refer to Martinet and Kockx [66].

1.1.6 Biological effect of oxLDL on macrophage survival

ApoE-/- mice lacking the pro-apoptotic protein p53 had less apoptosis of macrophages, and an increase in the size of early atherosclerotic lesions [67]. Paradoxically, knockout of the p53 target, p21, a cyclin-dependent protein kinase inhibitor that regulates entry into the cell cycle and inhibits apoptosis, increased macrophage apoptosis and protected mice against atherosclerosis [68]. This suggests that not all of the effects of p53 are due to p21. The importance of macrophage apoptosis in regulating the early development of atherosclerosis is further illustrated by a recent study demonstrating that

reduction of apoptosis due to Bax gene inactivation resulted in the larger atherosclerotic lesion area in LDLR-/- mice [69]. It was reported that oxLDL can induce expression of a macrophage survival protein, AIM (*a*poptosis *i*nhibitor expressed by *m*acrophages) which is abundant in lesions [70]. Furthermore, the absence of AIM dramatically reduced early atherosclerotic lesions in LDLR-/- mice [70]. These experiments indicate that the survival of macrophages, particularly during the early stages of atherosclerosis, may play a key role in determining whether lesions form and how quickly they progress.

Hamilton and his colleagues reported a pro-survival effect of oxLDL on bone marrow derived macrophages (BMDM) [37]. This effect of oxLDL was not reduced in mice with both M-CSF and GM-CSF genes inactivated, thus suggesting a direct role of oxLDL rather than an indirect process mediated through these cytokines. This group also showed that doses of oxLDL $\leq$ 50 μg/ml generally promoted survival in murine and human macrophages, whereas at higher concentrations, cell numbers declined [71]. We confirmed that oxLDL blocks apoptosis in BMDM and found that native LDL or acetylated LDL had no effect [38, 72]. In those papers, we also showed that high concentrations of oxLDL were toxic, and that soluble factors in the medium (such as GM-CSF or M-CSF) were not necessary for the anti-apoptotic effect of oxLDL.

1.1.7 Intracellular mechanisms employed in oxLDL-mediated macrophage survival

A range of signaling pathways have been implicated in the ability of oxLDL to activate vascular smooth muscle cells [73], endothelial cells [74, 75] and macrophages [7]. In this section we will focus on intracellular macrophage-specific events in particular on pro-survival events in relation to disease pathogenesis.

The serine/threonine protein kinase B (PKB) is activated downstream of phosphatidylinositol 3 kinase (PI3K) and this pathway presents a central point for transducing signals from oxLDL to components of the apoptotic machinery, such as IκB, and the Bcl-2 family members [76]. Extracellular signal-regulated protein kinase (ERK)1/2 is another candidate mediator for oxLDL-induced macrophage survival as it modulates cellular processes such as proliferation, differentiation, development, stress response, and apoptosis [77]. Alterations of both the PKB and ERK pathway have been detected in a number of diseases [77, 78]. It is known that oxLDL activates ERK in macrophages [79] and both ERK and PI3K have been implicated in the ability of oxLDL to promote macrophage survival [71]. We confirmed that oxLDL activates ERK1/2 in macrophages, but completely blocking this activation with ERK inhibitors had no effect on the pro-survival action of oxLDL. Only activation of PKB was essential for the inhibition of apoptosis in macrophages [72]. Minimally oxidized LDL is also reported to contribute to the survival of macrophage by activating the PI3K/PKB pathway [80]. As well, oxLDL immune complexes can also promote macrophage survival in a PKB-dependent manner [81]. Immunohistochemical analysis demonstrated PKB activation in murine atherosclerotic lesions, most of which was associated with macrophages [80].

Nuclear factor kappaB (NFκB) is another important signaling protein implicated in oxLDL mediated macrophage survival. Members of the NFκB/Rel family of transcription factors regulate many genes involved in atherogenesis such as those mediating inflammatory, anti-apoptotic and proliferative responses of cells [82]. Physiological evidence supporting the involvement of NFκB in atherosclerosis was suggested by the demonstration of active NFκB in macrophages, smooth muscle cells, and endothelial cells in human atherosclerotic lesions, but not in healthy vessels [83]. Our laboratory and others showed that concentrations of oxLDL below 75 ug/ml enhance NFκB activation in macrophages and promote cell survival [72, 84], but other groups have demonstrated inhibition of NFκB with high concentrations of oxLDL [85].

Sphingolipids also play a part in the development of atherosclerotic lesions [86]. Ceramide is a key sphingolipid that is implicated as a facilitator of apoptosis and its generation by sphingomyelinase is thought to cause aggregation of LDL trapped in arterial intima [87]. It has been found that secretory sphingomyelinase is more active at hydrolysing sphingomyelin in oxLDL compared to native LDL [88].

There are reports showing that oxLDL stimulates [89, 90] as well as inhibits [72] generation of ceramide in macrophages. Our group found that treatment of macrophages with oxLDL inhibited ceramide generation and that this was due to inhibition of acid sphingomyelinase (ASMase) by oxLDL [72]. Furthermore, it was recently demonstrated that inhibition of biosynthesis of ceramide significantly decreased atherosclerotic lesion area [91-94]. As well, the ceramide metabolite sphingosine-1-phosphate (S1P) was shown to have mitogenic and anti-apoptotic functions [95-97]. Hammad et al [98] recently reported that oxLDL immune complexes induced release of sphingosine kinase in U937 cells, which

increased the level of S1P in the medium and thereby enhanced macrophage survival. Another ceramide metabolite, ceramide-1-phosphate (C1P), was also found to be mitogenic and anti-apoptotic in macrophages [99]. Our laboratory showed that S1P as well as C1P, signal through similar pathways as oxLDL to promote macrophage survival [51, 100, 101].

1.1.8 Controversy regarding oxLDL's effect in causing survival and apoptosis

Cytotoxicity was one of the first properties of oxLDL to be reported, and there are numerous articles reporting the pro-apoptotic effects of oxLDL. However, several groups have also reported mitogenic as well as pro-survival properties for oxLDL. The reasons for the discrepant results are not fully known. One key difference is the concentration of oxLDL used for *in vitro* studies. High concentrations of oxLDL (>100ug/ml) are toxic for most cell types. However; several groups including our own have noticed that lower concentrations of oxLDL (5-75ug/ml) promote growth or survival in macrophages [37-40, 98, 102, 103].

The method used for preparation of oxLDL also affects its biologic properties. The techniques used to oxidize LDL include metal ion oxidation, enzymatic modification, UV irradiation, incubation of LDL with cells, or addition of aldehydes or products of polyunsaturated fatty acid (PUFA) autoxidation. Although a high level of apoptosis was observed in cells exposed to "minimally modified" LDL made with metal ions [104], incubation of LDL with cells over-expressing lipoxygenase produces a minimally modified LDL that actually promotes survival [80].

The degree of oxidation appears to affect the cytotoxicity of oxidized LDL, although this matter has not been fully characterized especially in relation to different

methods of oxidizing LDL [105]. Siow et al [106] have reported that moderately oxidized LDL was more toxic to vascular smooth muscle cells than minimally or extensively oxidized LDL. This was attributed to a higher content of lipid hydroperoxides found in moderately modified LDL. Similar results were observed with macrophages [104]. Using UV irradiation, Yuan et al have shown that increasing exposure time will result in a more cytotoxic/apoptotic oxLDL and this corresponded with increasing concentrations of hydroperoxides [60].

It is possible that both the pro-survival and the cytotoxic effects of oxLDL may be involved in the development of atherosclerotic plaques. In early lesions where oxLDL may exist in low concentration, it could promote macrophage survival and thereby increase the inflammatory response. On the other hand, in advanced lesions, there may be higher concentrations of more heavily oxidized LDL, which then results in macrophage apoptosis and subsequent plaque destabilization. Clearly, an understanding of the mechanisms by which oxLDL regulates macrophage survival and apoptosis is important for understanding the pathogenesis of atherosclerosis.

1.2 Apoptosis

1.2.1 Cell Death overview

Multi-cellular organisms require tight regulation of proliferation, differentiation and cell death to maintain proper development and tissue homeostasis [107]. In humans, dysregulation of these processes is involved in various diseases. For example, excessive cell death is thought to lead to neurodegenerative diseases while inhibition of apoptosis is implicated in many types of cancer or autoimmunity [108, 109].

There are two fundamentally distinct death pathways found in eukaryotes: necrosis and apoptosis. Necrosis occurs when the cells are killed by extreme trauma or injurious agents. Cells then swell and disrupt due to the inability of the plasma membrane to control ion flux. The uncontrolled release of cell contents to the surrounding causes local inflammation of the surrounding tissue [110]. As a pathological endpoint, this type of cell death would be expected to have some obvious adverse consequences for an organism. In contrast, apoptosis is the type of cell death that occurs when cells commit suicide in a controlled fashion, for example during development. During apoptosis, dying cells are quickly engulfed by neighboring phagocytes, which prevents the release of intracellular contents and minimizes the inflammatory response [111].

Apoptosis is characterized by chromatin condensation, DNA fragmentation, cell shrinkage, plasma membrane blebbing and exposure of phosphatidylserine in the outer leaflet of the plasma membrane [112], which is the signal for engulfment of apoptotic bodies by phagocytes [113]. The apoptotic pathway was initially defined in *C. elegans* and *D. melanogaster* [114, 115], but the molecular machinery of apoptosis is remarkably well conserved throughout evolution. The generation of transgenic and gene knockout mice

has facilitated our understanding of the apoptotic pathway in mammals, as reviewed by Ranger et al [116].

1.2.2 Caspases

Caspases are key effector components of apoptosis [113]. They belong to a family of cysteine proteases that use cysteine as the nucleophilic group and typically cleave peptide bonds C-terminal to aspartic acid residues in the substrate [117-119]. The caspases normally exist as inactive zymogens. Activation of caspases requires the proteolytic cleavage of the regulatory pro-domain and assembly into a hetero-tetramer [118]. While caspases 1 and 11 are involved in the processing of pro-inflammatory cytokines [120], the remaining caspases can be divided into the *initiator* caspases that include caspase 8 and 9 and the *effector* caspases, such as caspase 3, 6, and 7, that are activated as a result of the proteolytic activity of the initiator caspases. The effector caspases are the executioners of apoptosis as their processing of substrates such as poly (ADP-ribose) polymerase (PARP) or DNase inhibitor proteins [121] can subsequently lead to morphological changes associated with apoptosis [113].

Two major pathways can lead to caspase activation, the extrinsic and the intrinsic pathways [122]. The extrinsic apoptosis pathway is initiated at the cell surface through the binding of ligands such as FasL or TNFα to the corresponding death receptors to induce a conformational change [123]. This leads to interaction with adaptor proteins and is followed by the recruitment of pro-caspase 8. This undergoes auto-proteolytic activation and subsequent activation of effector caspases [124, 125]. Active caspase 8 can also amplify the apoptosis process by the cleavage of Bid, a Bcl-2 family member. Truncated Bid, tBid, is able to translocate to mitochondria and trigger the intrinsic apoptosis pathway [126].

Therefore, both pathways can lead to a central control and an execution stage where activation of caspases cascade occurs (

Figure **1.1**). The intrinsic pathway involves a permeability change in mitochondria, beginning with the release of apoptotic protease activating factors (Apafs) from mitochondria. Apaf-1 contains a caspase recruitment domain (CARD) that can interact with pro-caspase 9. By association with cytochrome c and ATP/dATP, Apaf-1 undergoes conformational changes that allow pro-caspase 9 to self-process and become active [127]. Together, Apaf-1, cytochrome c, ATP/dATP and caspase 9 form the apoptosome that then activates effector caspases [128]. Recent reports indicate there are mechanisms other than the two pathways mentioned above for the activation of caspase 2 [129], 4 [130], and 12 [131, 132].

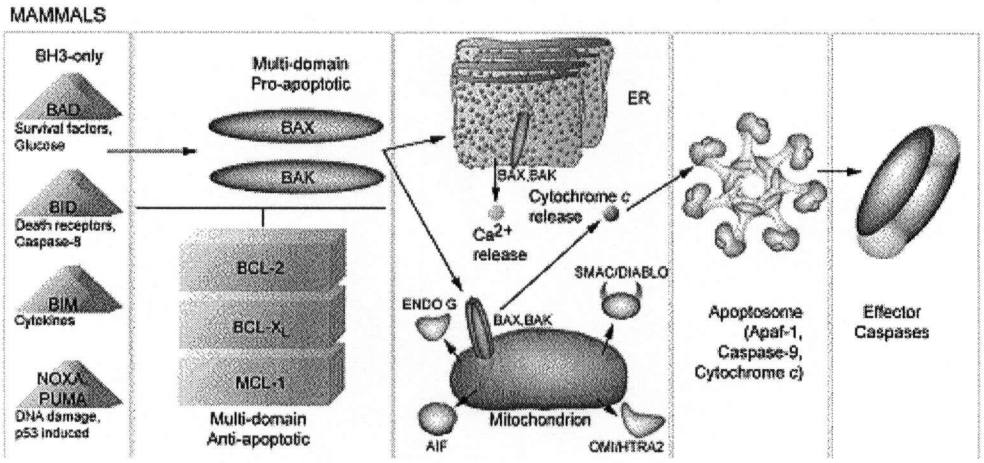


Figure 1.1 The intrinsic apoptotic pathway. Adapted from Danial et al [107].

1.2.3 Bcl-2 family members

Bcl-2 family members play a pivotal role in the intrinsic (mitochondrial) pathway of apoptosis. The "founding" protein, Bcl-2, was first discovered in human B cell follicular lymphoma cells carrying the t(14;18) chromosomal translocation [133]. Most lymphomas of this type have the breakpoint located in the Bcl-2 gene. Over-expression of this protein was later discovered to prolong cell survival by blocking apoptosis [134]. Bcl-2 members possess at least one of four conserved α-helical regions known as Bcl-2 homology domains (BH1-4) [135]. Based on their function, the members can be divided into two groups, the pro-survival and pro-apoptotic members. The pro-survival members can inhibit apoptosis triggered by a wide variety of stimuli and they mostly contain all four BH domains. The pro-apoptotic members can be divided into "multi-domain" and "BH3 only" members. The multi-domain proteins resemble Bcl-2, and contain BH1-3 domains. In contrast, the other pro-apoptotic members contain only the BH3 domain that is essential for killing[136-139]. Structural analysis of Bcl-X_L revealed that BH1-3 domains assemble with a hydrophobic groove that can accommodate the BH3 domain of pro-apoptotic members [140]. Commitment to life or death often is determined by opposing members of the Bcl-2 family. Pro- and anti-apoptotic proteins can heterodimerize and compromise one another's action [141, 142], and the balance of their corresponding concentration may be the key in determining whether cell death occurs.

The anti-apoptotic members include proteins such as Bcl-2, Bcl-X_L, and Mcl-1. Bcl-2 protects against diverse cytotoxic insults that can trigger apoptosis such as starvation of growth factors, loss of cell attachment to extracellular matrix, Fas-stimulation and cytotoxic T-cell killing [143,144]. Over-expression of Bcl-2 protein,

conferring a survival advantage, is frequently found in human cancers such as B-cell lymphomas [145] and breast cancer [146]. Although Bcl-2 -/- mice develop normally, accelerated lymphocyte death in thymus and spleen, distorted small intestine and neuronal disease have been observed [147].

BH3-only members can engage pro-survival proteins by the interaction of BH3 domains. The pro-apoptotic activity of BH3-only members is kept in check by either transcriptional control or post-translational modification [148]. They serve as sensors for initiating the intrinsic apoptotic pathway in response to selective stimuli. For example, Bid is engaged through the activation of death receptors [126], while Noxa and Puma respond to DNA damage [149, 150]. Bim and Bad can be activated by multiple stimuli including growth factor deprivation, detachment from the cell matrix [151], chemotherapeutic agents or UV treatments [55, 152]. Studies using knockout mice of BH3-only proteins in certain types of cells confer resistance to selective apoptotic stimuli. For example, loss of Bim renders lymphocytes resistant to paclitaxel, ionomycin and cytokine deprivation induced apoptosis [153] while loss of Bad in mammary epithelial cells confer some resistance to withdrawal of epidermal growth factor [154]. Moreover, Noxa-deficient cells are partially resistant to DNA-damaged induced apoptosis [155]. Overall, the redundancy of BH3-only proteins creates a robust control system that integrates responses to different stimuli.

After BH3-only proteins sense death stimuli, they need to activate Bax and Bak to initiate commitment to apoptosis [78, 156]. The first pro-apoptotic homolog, Bax, was first described as a protein that counteracted the pro-survival function of Bcl-2 [141].Over-expression of Bax or the addition of purified recombinant Bax accelerates apoptosis[141].

In healthy cells, Bax exists as a monomer in the cytosol or loosely associated with membranes [157]. Death stimuli cause a conformational change, allowing exposure of the hydrophobic grove that is otherwise hindered by the C-terminal helix of Bax [158]. This is immediately followed by the translocation and insertion of Bax proteins into the mitochondrial outer membrane as oligomers [159]. Inactive Bak exists as an integral membrane protein in mitochondria [160]. Bak is also induced to undergo conformational changes and oligomerization in response to apoptotic signals [161]. Bax-deficient mice display hyperplasia of thymocytes and B cells as well as abnormalities in the development of the male reproductive system[162]. Bak null mice show no developmental defects. However, when Bak- and Bax-deficient mice are intercrossed, a more marked phenotype is seen in the double-knockout [163]. Fewer than 10% of the animals survive into adulthood, and those that do display multiple developmental defects. Furthermore, the double knockout cells are resistant to multiple death stimuli [163-165]. It is evident from this that the combined pro-apoptotic functions of Bax and Bak are crucial for normal tissue development.

Bax and Bak in concert are an essential gateway for activation of caspases in the intrinsic apoptotic pathway [78], but there are debates as to how BH3-only proteins lead to the activation of Bax and Bak (Figure 1.2). The direct binding model suggests that the "activators" of BH3-only proteins such as Bid and Bim can directly activate Bax and Bak. Bad or Bik act as "sensitizers," that sequester the pro-survival proteins and allow unbound Bax and Bak to oligomerize [166, 167]. Inconsistent observations for binding of endogenous Bid and Bim to Bax or Bak lead to the suggestion that they operate at a "hit-and-run" fashion [159, 161]. The displacement model suggests that BH3-only proteins can

displace the binding of Bax or Bak to pro-survival proteins that sequester their active forms [168]. Chen et al [169] recently demonstrated the differential affinity of BH3-only proteins for pro-survival proteins. Using peptides mapped to BH3 sequence of BH3-only proteins, it was shown that certain molecules such as Puma and Bim can bind to all pro-survival proteins. Bad can counteract Bcl-2 and Bcl-X_L but not Mcl-1 while Noxa complements only by interacting with Mcl-1. By engineering Noxa to enhance promiscuous binding to all pro-survival proteins, effective killing occurred [169]. This was further supported by the observation of Willies et al [170] showing that both Noxa and Bad are required to neutralize Mcl-1 and Bcl- X_L respectively to drive efficient apoptosis mediated by Bak. A recent report showed that Bax and Bak can mediate apoptosis without discernable association with the putative BH3-only activators (Bim, Bid, and Puma), even in cells with no Bim or Bid and reduced Puma [171] while others showed mitochondrial permeabilization relies on BH3-only proteins engaging pro-survival Bcl-2 relatives and not Bak [172]. These results further support the notion that BH3-only proteins induce apoptosis at least primarily by engaging the multiple pro-survival relatives guarding Bax and Bak.

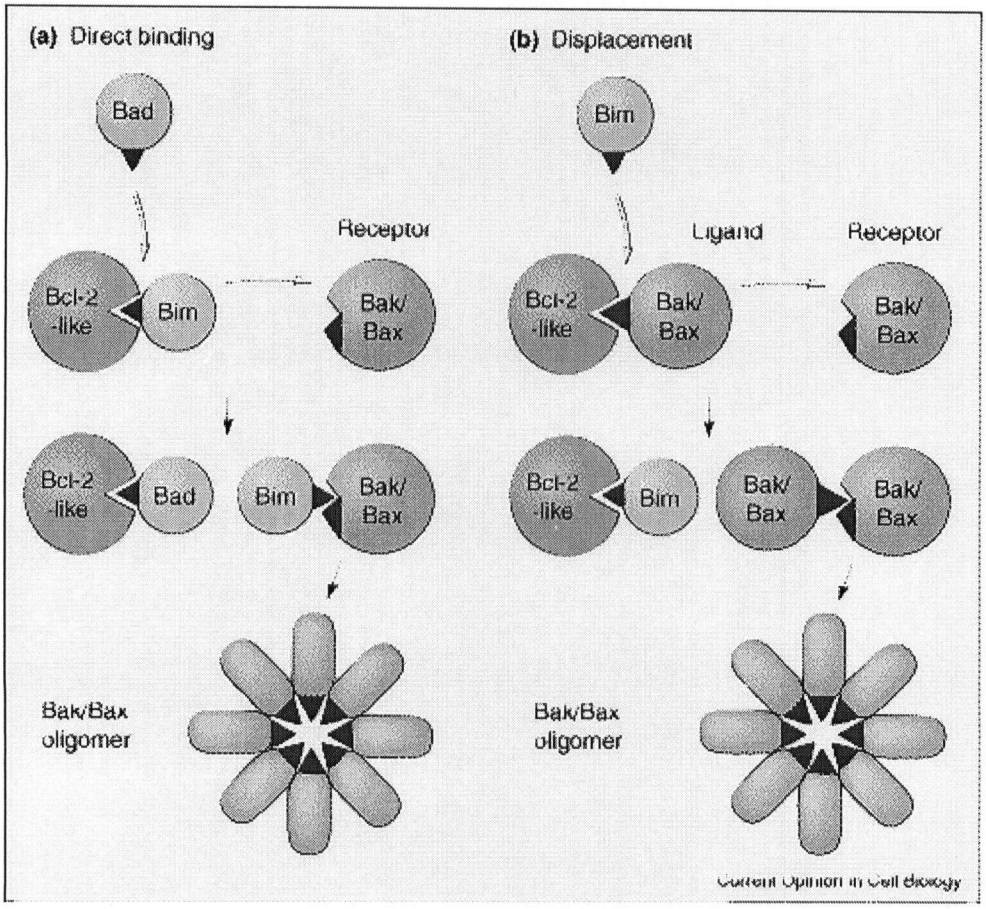


Figure 1.2 Models for how BH3-only proteins activate Bax and Bak. (a) Direct binding model. (b) Displacement model. Adapted from Willis et al [168].

1.2.4 Mitochondrial fission

The precise mechanism leading to mitochondrial permeabilization is still under debate. One hypothesis is that opening of permeability transition (PT) pores at contact sites between the inner and outer mitochondrial membranes results in the rupture of mitochondria. Voltage-dependent anion channel (VDAC), the adenine-nucleotide translocator (ANT), and the matrix chaperone cyclophilin D were initially proposed to participate in the formation of PT pores [173, 174] and Bcl-2 family members are shown to regulate this process [175]. However, this model has been challenged by the recent observation that the ANT-deficient mitochondria can still form PT and cells can die by

apoptosis without ANT [176]. Similar results were also reported recently where the basic properties of the PT formation in mitochondria were not affected by the absence of VDAC [177]. An alternative model suggests that Bax can directly modulate VDAC to control mitochondria permeability [178]. Based on the structural similarity between Bcl-X_L and pore-forming bacterial toxins [140], it is speculated that Bcl-2 family members themselves can form pores on the mitochondrial membrane that allows the leakage of cytochrome c. Bax, Bcl-2 and Bcl-X_L were shown to form a channel in liposomal membranes [65, 179-181]. Regardless of the exact mechanism, it is clear that Bcl-2 family proteins play an essential role in controlling mitochondrial membrane integrity, and that mitochondrial permeabilization results in the release of cytochrome c, which leads to caspase-mediated cell destruction [158].

1.2.5 Therapies targeting Bcl-1 family members

Defects in apoptosis resulting from mutations in Bcl-2 members are often observed in tumors [182-184], and this has prompted studies of anti-cancer drugs directly targeting pro-survival members. Given the functional importance of the BH3 domain in regulating apoptosis, two BH3 mimetics were designed. One, a 24-mer Bid BH3 peptide that is protease resistant and cell permeable, was shown to induce apoptosis in Jurkat cells. It also suppressed the growth of a transplanted leukemia *in vivo* [185]. The second is ABT-737, a molecule that tightly binds to the hydrophobic groove of a pro-survival protein. Behaving like Bad, it antagonizes the anti-apoptotic function of Bcl-2, Bcl-X_L, and Bcl-w but not Mcl-1. ABT-737 caused regression of certain tumors in mice [186]. Certo et al.[187] showed that priming of Bcl-2 with Bim or Bid enhanced killing of ABT-737, but recent reports argued that the level of Mcl-1 determines the sensitivity of certain types of

tumors toward ABT-737 [188, 189]. Nonetheless, ABT-737 has shown impressive single-agent toxicity in cell lines *ex vivo* and *in vivo*, and against primary human malignant cells [190].

These results encourage therapeutic designs for treating non-malignant diseases that also have defective Bcl-2 family members. Another strategy could involve targeting transcriptional and/or translational control of BH3-only proteins [148].

1.3 Ceramide and apoptosis

1.3.1 Overview of ceramide

Sphingolipids were originally considered to be just structural components of cell membranes, but some members of this family, especially ceramide, ceramide-1-phosphate, and sphingosine-1-phosphate (S1P), are now established as lipid second messengers that have important functions in cell proliferation, survival and apoptosis [99, 191, 192].

Sphingolipids are comprised of a long-chain sphingoid base, an amide-linked long-chain fatty acid and one of several head groups, which define the classes of sphingolipids. A hydroxyl head group gives rise to ceramide, phosphorylcholine to sphingomyelin (SM) and carbohydrates to glycosphingolipids [192]. Ceramide can be generated by hydrolysis of sphingomyelin (SM), by *de novo* synthesis, or by recycling sphingoid bases or breakdown of complex glycosphingolipids [193]. The acyl portion of ceramide is typically saturated, although mono-unsaturated forms exist, particularly in very-long-chain fatty acid species. A schematic representation of pathways for ceramide generation is presented in Figure 1.3.

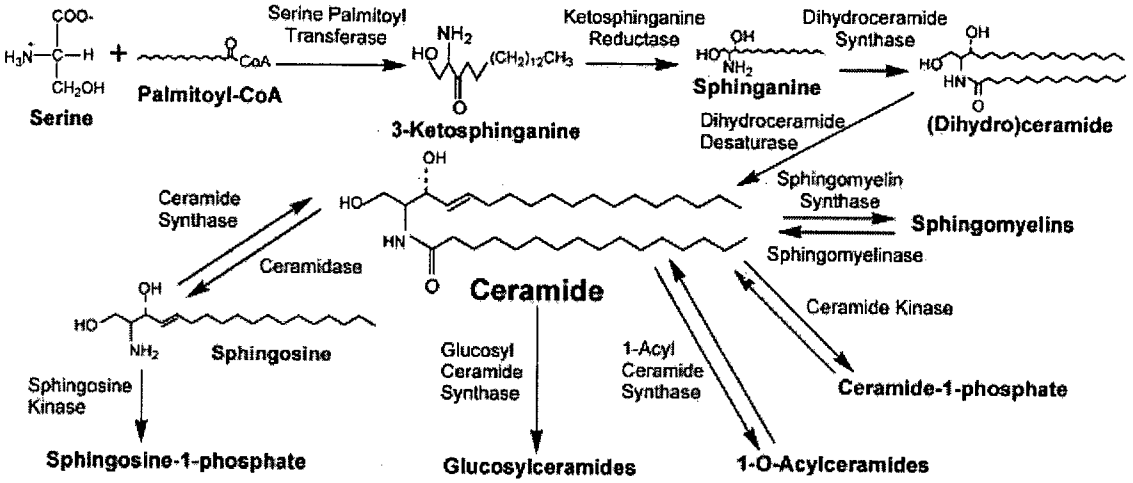


Figure 1.3 Ceramide metabolism. Adapted from Reynolds et al [192].

1.3.2 Regulation of ceramide metabolism in relation to apoptosis

Sphingomyelinase (SMase) hydrolyzes sphingomyelin to ceramide and phosphorylcholine [194]. There are three types of SMase and they are classified by the optimal pH for their activities. Alkaline SMase activity is found in intestinal mucosa and bile and may be involved in lipid digestion. As yet it has no defined role in apoptosis. Neutral and acid SMase have been found to respond to apoptotic stimuli [192, 195]. Acid SMase (ASMase) was originally considered an endosomal/lysosomal enzyme because of its pH optimum at 4.5-5.5 [196]. However, secretory sphingomyelinase is a product of the same gene and has been shown to act on extracellular substrates such as modified LDL [88, 197]. The outer leaflet of plasma membranes has a third form of ASMase that was initially detected in cells stimulated with CD95 or CD40 [198, 199]. Despite the neutral pH for the latter two locations, ASMase retains some activity, since an increase of the pH only changes the *Km* value of the enzyme [200]. All three types of ASMase require Zn^{2+} for activity [197]. Ceramide accumulation in the lysosomal compartment can exert positive

feedback on ASMase activity, and this can lead to enhanced apoptosis in human macrophages and fibroblasts [201].

Neutral SMase (NSMase) has a pH optimum at 7.4 and requires Mg^{2+}. To date, there are three mammalian isoforms identified and cloned [202] although NSMase 1 is thought to function more as a lyso-platelet activating factor-specific phospholipase C than as a *bona fide* NSMase [203]. NSMase can be activated in response to various apoptotic stimuli, including TNFα, Fas ligand, IL-1, IFNγ and chemotherapeutic agents [204, 205]. The activity of NSMase is postulated to be positively regulated by cytosolic phospholipase A_2 and negatively by glutathione levels in the cell [206]. Additionally, it has been observed that ceramide generation in macrophages results in the stimulation of NSMase activity [207].

The *de novo* pathway of ceramide synthesis has emerged as another key pathway of apoptosis regulation that is responsive to agonist stimulation. Two key enzymes in this pathway are serine palmitoyltransferase (SPT) and (dihydro)ceramide synthase (CS). SPT initiates the rate-limiting step in the pathway by condensing serine and palmitoyl CoA [208, 209]. Ceramide synthase is responsible for acylating sphinganine to generate dihydroceramide. Both enzymes in the pathway of *de novo* ceramide synthesis can be stimulated by chemotherapeutic agents and ionizing radiation [209-211]. This pathway can also be activated by the addition of free palmitoyl CoA and this has been proposed to play a role in diabetes and obesity, which result from increased levels of free fatty acids [212].

Under normal physiological conditions, ceramide is not a major end-product in this pathway; rather, it is a precursor for the synthesis of complex sphingolipids. Several

enzymes involved in synthesis of complex sphingolipids have been implicated in regulating apoptosis. SM synthase is responsible for transferring phosphorylcholine from phosphatidylcholine to ceramide and generating SM and diacylglycerol (DAG). A survival advantage has been attributed to increased SM synthase activity in several reports [213, 214] whereas apoptosis is induced when conversion of *de novo* ceramide to complex sphingolipids is inhibited [215]. Glucosylceramide synthase (GCS) glycosylates ceramide to glucosylceramide in the Golgi, and this can be further modified into more complex glycosphingolipids. GCS can increase cellular resistance to apoptosis induced by TNFα [216] as well as anticancer agents [217] by reducing ceramide levels. Interestingly, there is evidence of compartmentalization in that only *de novo* generated ceramide was found to be efficiently converted to glucosylceramide whereas ceramide accumulation induced by SM hydrolysis was not [218]. Ceramidase (CDase) catalyzes the formation of sphingosine by cleaving ceramide at the amide bond to remove the fatty acid. Over-expression of CDase has been shown to lower ceramide levels and reduce apoptosis [219]. Sphingosine generated by CDase can then be phosphorylated into sphingosine-1-phosphate (S1P) by sphingosine kinase (SK) [220]. Thus, in addition to ceramide clearance, CDase can also confer protection to apoptosis by shunting ceramide into S1P, which is known to promote cell survival and proliferation [100, 221-223]. Ceramide kinase is also implicated in shunting ceramide into a metabolite with very different biologic properties. It catalyzes the phosphorylation of ceramide to ceramide-1-phosphate (C1P), which has pro-survival effects [99 51, 101] and also plays a role in inflammation [224, 225], cell proliferation [226, 227] and phagocytosis [228].

Table 1 summarizes the subcellular localization of key enzymes of ceramide metabolism and lists some inhibitors. Many of the enzymes of ceramide metabolism are emerging as regulated switches controlling the levels of ceramide relative to those of other bioactive lipids such as DAG, S1P and C1P that oppose ceramide's actions in apoptosis [229-231]. The multiple pathways involved in ceramide metabolism have been shown to interact to regulate ceramide levels [215, 232-234].

Enzyme	Topology	Inhibitor	Status
Sphingomyelinase			
ASMase[196]		Desipramine[72]	
	Lysosomes/ Endosomes Secreted[88, 197] PM in Caveoli[238, 239]		Cloned [235-237]
NSMase[240]		Scyphostatin[241, 242] GW4869[243]	
NSMase1 [244]	ER/Golgi [245]		Cloned [245]
NSMase2 [202]	PM/ Golgi [246]		Cloned [247]
NSMase3			Cloned [248]
AlkSMase[249]	(Gastrointestinal tract)		
Enzymes involved in *de Novo* Synthesis			
Serine palmitoyltransferase [208]	ER	Myriocin/ Cycloserine	Cloned[250]
Ceramide Synthase	ER	Fumonisin B1	Cloned[251]
Dihydroceramide Desaturase	ER		Cloned[252]
Others			
SM Synthase	Golgi, PM, Nucleus, Mitochondria	D609[253]	Cloned[254, 255]
Ceramidase			
ACDase	Lysosomal	NOE	Cloned[256]
NCDase	Mitochondria/endosomes	C6UreaCER	Cloned[257]
AlkCDase	ER/Golgi	DMAPP	Cloned[258]
Glucosylceramide Synthase	Glogi	PDMP	Cloned[259, 260]
Ceramide Kinase	Microsomal fraction		Cloned[261]
Sphigosine Kinase	Cytoplasm/PM	N,N-dimethylSph B5334C, F12509A	Cloned [262-264]

Table 1: Enzymes of ceramide metabolism and key features.

1.3.3 Ceramide as second messenger to regulate apoptosis

Intracellular mediators for ceramide-induced apoptosis include protein kinases, phosphatases and cathepsin D [193, 265]. Cathepsin D is a lysosomal protease that was originally identified as a ceramide-binding protein that was activated by ceramide [266]. Activated cathepsin D is proposed to cleave Bid in response to TNFα-mediated ASMase activation and results in activation of caspases [267]. Kinase suppressor of Ras (KSR) [268, 269] and Raf-1 [270] have been suggested to be downstream targets of ceramide. Ceramide can mediate apoptosis through KSR to activate Raf-1 [268, 269, 271]. Another ceramide activated kinase is PKCζ. Several groups have shown that natural ceramide binds directly to and activates PKCζ [272] whose apoptotic effect might be exerted by negatively regulating PKB [273]. Ceramide accumulation can also activate serine/threonine protein phosphatases 1 (PP1) and PP2A [274, 275]. Ceramide-activated PP2A can counteract the anti-apoptotic effect of Bcl-2 by dephosphorylating Bcl-2, leading to its inactivation [276]. Ceramide activation of PP2A can also result in the inactivation of PKB [277, 278]. Ceramide can activate PP1 which in turn dephosphorylates Rb and causes cell cycle arrest [279]. Ceramide-activated PP1 can dephosphorylate serine/arginine-rich (SR) proteins [280], whose function is to regulate constitutive and alternative splicing, including that of the key apoptotic mediators caspase 9 and Bcl-X [281].

1.3.4 Ceramide as a modulator of membrane structure to regulate apoptosis

The functions of ceramide depend on its sub-cellular location and site of formation. Ceramide, SM and glycosphingolipids are highly enriched in caveolae and membrane lipid rafts [282]. ASMase translocates to the outer leaflet of the cell membrane where it is in close proximity to the bulk of cellular SM [238, 239]. Ceramide can be

generated there, and associate with membrane rafts where it acts to facilitate clustering and activation of TNF family receptors, such as Fas [238, 239]. In ASMase-deficient cells, it has been shown that this enzyme is required for death receptor clustering, and this in turn is essential for induction of apoptosis by the extrinsic pathway [283, 284]. ASMase activation and ceramide generation have also been reported to inactivate the PI3K/PKB survival signaling cascade [72, 285]. Inhibition of PI3K by ceramide is associated with recruitment of caveolin-1 to PI3K-associated receptor complexes in rafts. Antisense knockdown of caveolin dramatically reduces ceramide-induced PI3K deregulation in fibroblasts, suggesting that caveolin-1 is required for the inhibition of PI3K by ceramide [238]. Only 10-20% of cellular SM resides in the inner leaflet of the plasma membrane (PM). Relatively rapid SM hydrolysis at the cytosolic side of the PM following TNFα and CD40L is believed to be caused by NSMase via the adaptor protein FAN [286, 287]. The generation of ceramide from this pool is speculated to lead to alteration of cell surface morphology concomitant with the last phases of apoptosis [288].

ASMase overexpression in mitochondria causes ceramide generation there but not in other cellular compartments, and induces apoptosis [289]. Furthermore, the addition of exogenous ceramide to purified mitochondria inhibites oxidative phosphorylation and promotes cytochrome c release [290, 291]. Mitochondria contain enzymes regulating ceramide level, such as ceramide synthase [292, 293] and CDase [294]. Several apoptotic stimuli have been shown to induce apoptosis correlating with an increase in mitochondrial ceramide levels [295, 296]. Ceramide accumulation in mitochondria can induce changes in the electron transport chain leading to generation of reactive oxygen species that precede membrane permeability increases [297]. Siskind et al [298] have proposed that ceramide is

capable of directly forming a channel on mitochondria at the concentration observed during apoptosis. Phospholipid interactions after hydrolysis of SM to ceramide can have profound effects on membrane structure, including membrane blebbing, vesicle shedding, and apoptotic body formation [288].

1.3.5 Therapeutic implications

There is evidence implicating sphingolipid pathways in the pathogenesis of many diseases. For example, the neurodegenerative disorder Niemann-Pick Disease (NPD) types A and B, is the result of inherited deficiency of ASMase activity [299, 300] . Type A NPD is a severe infantile neuronopathic form that is usually fatal by age 3. Type B manifests as hepatosplenomegaly with minimal neurological involvement and patients often survive into adulthood [301]. Both disorders are due to mutations in the ASMase gene, but type B is associated with a small amount of residual ASMase activity. Drug resistance in some cancer cells was attributed to their failure to sustain high levels of ceramide in response to chemotherapy due to either the increased clearance or decreased degradation of complex sphingolipids [215, 302, 303]. Recently, apoptosis of β-islet cells induced by ceramide, whose synthesis is enhanced by free fatty acid overload, has been implicated in the pathogenesis of diabetes in obesity [304-306]. These examples illustrate how knowledge of sphingolipid metabolism can potentially provide better understanding of disease pathogenesis and offer a novel approach to pharmacological intervention.

1.4 Objectives

Macrophages are cells that are critical to the body's ability to repel pathogens and to remove damaged tissue and dying cells caused by normal growth and development. Apoptosis is an important mechanism involved in regulating the number of macrophages. The dysregulation of macrophage function is implicated in several human diseases such as rheumatoid arthritis, inflammatory bowel disease and atherosclerosis. Understanding how macrophage apoptosis is regulated can help develop novel therapeutic approaches to diseases.

We recently showed that oxLDL inhibits apoptosis in macrophages through the PI3K/PKB pathway and subsequent level of pro-survival protein Bcl-X_L [72]. We are interested in exploring if other Bcl-2 family members also play a role in macrophage apoptosis and if oxLDL is able to exert its anti-apoptotic function by regulating these proteins.

In previous studies, we showed that tyrosine phosphorylation as well as PI3K activation increase in response to oxLDL treatment in THP-1 cells [39]. To investigate the upstream receptor(s) activated by oxLDL to mediate macrophage survival, we propose using pertussis toxin (PTX) to test whether G protein coupled receptors are involved.

We also observed that during macrophage apoptosis the ASMase activity increases in parallel with ceramide generation [72]. Using mice deficient in ASMase, we seek to further elucidate the metabolic pathways that are responsible for ceramide generation in these cells.

2 Materials and methods

2.1 Materials

Antibodies to mouse Bak, Bax, vinculin and p85α isoform of PI3K were purchased from Upstate. Anti-cytochrome c, anti-PARP and annexin V-FITC conjugated antibody were from BD Pharmingen. Atni-Mcl-1 antibody was from Rockland. Antibody to the mitochondrial outer membrane receptor TOM20 was a kind gift from Dr. G.C. Shore, McGill University. Anti-ubiquitin was from Cell Signaling Technology. Anti-Bim was from Affinity BioReagent. Antibody recognizing active Bax (6A7) was purchased from Travegen. Antibodies against phospho-IκB, phospho-ERK and phospho-Ser473PKB were from Cell Signaling Technology. Lambda protein phosphatase was purchased from New England Biolab. Anti-Bcl-X_L and -actin were from Santa Cruz Biotechnology Inc. *Escherichia coli* diacylglycerol kinase, β-octyl glucoside, mastoparan, mastoparan 17, and all other inhibitors were supplied by Calbiochem. Pertussis toxin, β-oligomer, propidium iodide, glutathione, MG132, protease inhibitor cocktail, non-hydroxyl fatty acid ceramide, ceramide-1-phosphate, and RPMI 1640 medium were purchased from Sigma-Aldrich. Caspases FLICA kit was from Immunochemistry Technologies. Fetal bovine serum (FBS), random primer, SuperScript RNaseH-free reverse transcriptase, RNaseH and RNase-out were obtained from Invitrogen. Reagents required for 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) cell viability assays were purchased from Promega. Protein-G agarose beads were from Amersham Biosciences. QuantiTect SYBR green PCR kit and RNeasy mini kit were from Qiagen. Goat anti-mouse and anti-rabbit IgG, horseradish peroxidase secondary antibodies were from DAKO. Nitrocellulose

membranes and protein markers were purchased from Bio-Rad. BSA protein standards and BCA assay reagents were from Pierce. C2-ceramide, C2-dihydroceramide, cardiolipin were purchased from Avanti Polar. [γ-^{32}P]ATP, [^{3}H]serine, [14C]palmitoyl-CoA, [^{3}H]palmitate and [*N-methyl*-^{14}C] bovine sphingomyelin were purchased from Perkin-Elmer NEN.

2.2 Lipoprotein isolation, oxidation and acetylation

Low density lipoprotein (LDL, d=1.019-1.063) was isolated by sequential ultracentrifugation of EDTA-anticoagulated fasting plasma obtained from healthy normolipidemic volunteers as described in [307]. Oxidation was performed by incubating 200 μg/ml LDL with 5uM $CuSO_4$ in Dulbecco's PBS for 2, 5, or 24 hours at 37°C. The reaction was stopped by addition of 40 μM butylated hydroxytoluene and 300 μM EDTA. The modified LDL was then washed and concentrated using Centricon Plus-20 ultrafilters (Millipore, Bedford, MA) [307]. The protein concentrations of oxidized LDL were then determined using BCA protein assay. Unless otherwise stated, extensively oxidized LDL (incubated with copper for 24 hours) was used throughout the study. Acetylation of LDL was performed by the sequential addition of acetic anhydride [28].

2.3 Cell culture

CD1 and C57BL/6 mice were obtained from the UBC animal facility. Bax knockout mice were from the Jackson Laboratory. C57BL/10ScCr mice are a strain with a naturally occurring deletion of the *Tlr4* gene [308], and were purchased from the Jackson Laboratory (Bar Harbor, ME). ASMase knockout mice were obtained from Dr. R. Kolesnick.

Bone marrow was harvested from the femurs of 6-8 weeks old female CD1 mice as described [72]. Cells were plated overnight in RPMI 1640 supplemented with 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 100 units/ml penicillin/streptomycin and 10% L-cell conditioned medium as the source of M-CSF. Non-adherent cells were removed and cultured in the above medium until 80% confluence was reached (4-6 days). Peripheral blood mononuclear cells (PBMCs) from consenting normal donors were isolated on Ficoll-Paque^{TM}Plus (Pharmacia Biotech) gradients according to the manufacturer's protocol. PBMCs were resuspended in media (DMEM, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin) and dispensed onto tissue culture plates at a concentration of 1.5 x 10^6 cells per cm^2 and incubated for two hours in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by washing three times with warm Dulbecco's phosphate-buffered saline (DPBS). The adherent cell fractions were differentiated into macrophages by culturing for 5 to 6 days in media supplemented with 50 ng/ml recombinant human macrophage colony stimulating factor (R&D Systems). The macrophages were lifted using a rubber cell scraper and then seeded in 100 mm dishes at 1.0 x 10^5 cells per cm^2 and grown for 24 hours before treatment with oxLDL.

FDCP-1 cells, a murine factor dependent hematopoietic precursor cell line, were cultured in the same medium as macrophages, except that M-CSF was substituted with 2.5% conditioned medium from WEHI-3 cells as a source of IL-3.

2.4 Genotyping

ASMase-/- mice were genotyped by PCR [309]. Genomic DNA was mixed with an ASMase sense primer (PS; 5- AGCCGTGTCCTCTTCCTTAC-3') and two antisense

primers, one from within exon 2 of the ASM gene (PA l; 5'-CGAGACTGTTGCCAGACATC-3) and one from within *the neo* cassette (PA2; 5'-CGCTACCCGTGATATTGCTG-3'). Thirty cycles of PCR amplification, each consisting of 1 min at 93 °C , 1 min at 58 °C , and 1 min at 72 °C , were performed. In wild-type mice, a single band of 269 bp corresponding to the undisrupted ASM gene was amplified, while in ASM-/- mice a single band of 523 bp was amplified from the sense and *neo* primers.

2.5 Cell Viability assay

When 80% confluence was reached, BMDM were harvested using a rubber cell scraper. $5x10^4$ cells per well were seeded into 96-well plates and incubated overnight. Cells were then washed with PBS, and drugs in the absence or presence of PTX were added in 100 µl of the same medium except without M-CSF. At the end of the 24 hour incubation, 20 µl of MTS/PMS solution (prepared according to the manufacturer's instructions) was added to each microwell. The plate was incubated for 1-4 hours at 37°C and was read using an ELISA plate reader at 490 nm. We previously showed that the bioreduction rate of MTS is linearly correlated with the number of viable macrophages [39].

2.6 Immunofluorescence microscopy

Macrophages were plated on sterile glass coverslips and incubated for 18 h in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 10% L-cell conditioned medium. Cells were washed, and then cultured in the absence or presence of cytokine for 24 hours. The pan-caspase inhibitor Z-VAD was added at 100 µM to block later stages of apoptosis and

minimize detachment of cells. The coverslips were then washed twice with cold PBS and then fixed with 4% paraformaldehyde for 30 minutes at room temperature. Cell membranes were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes and blocked with blocking solution (10% FBS with 0.1% Triton X-100 in PBS) for 30 minutes at room temperature. Coverslips were then incubated with primary antibody in blocking solution for 30 minutes and then with AlexaFluor conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Molecular Probes) for 30 minutes in the dark. Cells were then examined on a Zeiss Axiophot fluorescence microscope equipped with a digital imaging system.

2.7 Flow cytometric analysis

One million cells were seeded in 6 well plates, incubated under the indicated conditions for 24 hours, harvested with a rubber scraper, and pelleted by centrifugation. To assess DNA fragmentation, cells were fixed with cold 70% ethanol for 10 minutes, and resuspended in propidium iodide staining solution (10 ug/ml RNaseA, 20 ug/ml PI, in PBS + 0.1% glucose). To quantify phosphatidylserine externalization, macrophages were incubated with annexin V-FITC according to the manufacturer's instructions. Measurement of caspase activation was carried out with a fluorescent-labeled indicator kit, FLICA (Immunochemistry), and assayed by flow cytometry according to manufacturer's instructions. Cells were analyzed by Beckman Coulter flow cytometer (EPICS XL-MCL) on the FL3 channel for DNA content, on the FL1 channel for FITC fluorescence with ten thousand events counted for each analysis.

2.8 Reverse transcription and Real time PCR

Total RNA was isolated from BMDM using RNeasy kit from Qiagen and reverse-transcribed using Superscript II® according to manufacturer's directions. Using the cDNA generated, Bax and actin were amplified by PCR. The amplification generates a 270-bp fragment for Bax (forward primer, 5'-AGATGAACTGGATAGCAATATGGA-3'; reverse primer, 5'-CCACCCTGGTCTTGGATCCAGACA-3') and a 138-bp fragment for actin (forward primer, 5'-AGAGGGAAATCGTGCGTGAC; reverse primer, 5'-CAATAGTGATGACCTGGCCGT). The amplification conditions were as follows: hold at 95°C for 10 minutes, then 40 cycles at 94°C for 30s, 55°C for 30s, 72°C for 30s. Final extension was performed at 72°C for 5 minutes. The PCR products were separated by electrophoresis in a 1.2% agarose gel, and stained with ethidium bromide. The cDNA was also used for real time PCR by using QuantiTect SYBR green PCR kit using the same amplification conditions as above.

2.9 Immunoblotting and immunoprecipitation

For immunoblotting whole cell lysates, 1.5 million cells were washed with PBS and lysed in 50 ul of ice-cold 20 mM Tris HCL pH 8.0, 1% NP40, 10% glycerol, 137 mM NaCl, 10 mM NaF (solubilization buffer A) supplemented with protease inhibitor cocktail and 200μM sodium vanadate. The cells were then sonicated for 5 seconds and centrifuged at 23,000 x g for 5 min. The supernatant was collected and assayed for protein concentration. The extracted proteins were adjusted to equal concentration and were boiled in SDS sample buffer for 5 min. 50 μg of cell lysate was loaded in each lane of a 12% SDS-polyacrylamide gel. For detection of active Bax, macrophages were lysed

with solubilization buffer B (10 mM HEPES, pH 7.4, 150 mM NaCl, and 1% Chaps). One microgram of 6A7 anti-Bax antibody was added to 500 μg of cell lysate for and incubated overnight at 4°C on an oscillating stage. Protein-G agarose beads (Amersham Bioscience) were added to the mixture for 1 hour at 4°C. The beads were spun down and washed 4 times with solubilization buffer B and immunoprecipitates were then dissolved in 2x sample buffer and loaded onto a 12% SDS-polyacrylamide gel. Transfers were done by semi-dry blotting onto nitrocellulose membranes. The membranes were blocked for one hour in 5% low fat dry milk in Tris-buffered saline with 0.05 % Tween 20 followed by overnight incubation at 4°C with appropriate antibody. Bound antibody was visualized with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies and enhanced chemiluminescence.

2.10 Lipid labelling for determination of ceramide levels

Radioactivity in ceramide was assayed after labeling of BMDM with 5 μCi/ml of [^{3}H]palmitate for 24 h in RPMI 1640 without or with10% FBS and 10% M-CSF. The cells were washed twice with PBS and scraped into 1 ml of methanol, which was then mixed with 1 ml of chloroform and 0.9 ml of 2M KCl, 0.2M H_3PO_4 [310]. The aqueous phase was discarded, and the chloroform phase was dried under nitrogen. Ceramides were isolated by TLC by using Silica Gel 60-coated glass plates developed with chloroform/methanol/acetic acid (9:1:1 by volume) for half their length and then with petroleum ether/diethyl ether/acetic acid (60:40:1 by volume). Lipids were visualized by iodine and identified by co-chromatography with authentic standards. Radioactivity was measured by scraping the corresponding bands from TLC plates and liquid scintillation counting.

2.11 Sphinomyelinase assay

For assay of sphingomyelinase (SMase) activity cell lysates were prepared by three cycles of freeze/thawing in TE buffer (20 mM Tris/HCl, pH 7.5, and 1 mM EDTA) containing protease inhibitor cocktail. DTT (1 mM) was also added for lysates to be used for NSMase assay, while 50 mM sodium acetate, pH 5.0 was included in the buffer for ASMase assay. The homogenate was centrifuged at 1,000 × *g* for 10 minutes and the supernatants were collected for assay of SMase activities. The activities of neutral and acid sphingomyelinases were determined exactly as described by Liu and Hannun [311] using [*N-methyl*-^{14}C] sphingomyelin as the substrate. The final reaction buffer for NSMase was 0.1% Triton X-100, 5 mM $MgCl_2$, 5 mM DTT and 0.1 M Tris/HCl, pH 7.4, and that for ASMase was 100 mM sodium acetate, pH 5.0 and 0.2% TritonX-100. After incubating for 1.5-2 h, the reaction was stopped by the addition of 0.5 ml of chloroform:methanol (2:1). The samples were vortexed and then centrifuged to separate the two phases. The upper phase, containing labeled phosphorylcholine released from sphingomyelin, was transferred to scintillation vials and counted by liquid scintillation counting. Negative controls containing no enzyme were run concomitantly.

2.12 Nuclear preparations and Electrophoretic Mobility Shift Assay

Cells were grown in 100 mm tissue culture plates until 80% confluent. Isolation of nuclei and radioactive labeling of an NF-κB EMSA probe was performed as previously described [312]. Nuclear extracts (10 μg) were preincubated for 15 min in binding buffer (20 mM HEPES, pH 7·9, 100 mM KCl, 10% glycerol, 1 mM DTT) containing 1 μg of poly dIdC (Amersham). The [^{32}P]-labeled probe (20,000 counts per minute) was then added and the reaction mixture incubated at room temperature for 30

min before electrophoresis in a 5% non-denaturing polyacrylamide gel in 0.25 X TBE (Tris 89 mM, boric acid 89 mM, EDTA 2 mM) at 200 V for 1.5 h. The gel was subsequently dried for 45 min and imaged using a Bio-Rad FX phospho-imager.

2.13 Diacylglycerol Kinase assay for ceramide mass

Ceramide levels were measured using the diacylglycerol kinase method as described previously [209, 313]. In brief, total cellular lipids were extracted with chloroform/methanol/2 M KCl, 0.2 M HCl, resuspended in a micellar solution of 1 mM cardiolipin, 1.5% N-octyl- β -D-glucopyranoside, 0.2 mM DETAPAC, 5 μg of diacylglycerol kinase and 10mM DTT. The reaction was initiated with 1 μCi of [γ-^{32}P]ATP diluted with unlabelled ATP to give a final concentration of 1 mM. After incubation for 45 min at 30°C, lipids were extracted and separated on Silica Gel 60 TLC plates with choloroform/methanol/2N NH_4OH (65:35:7.5). The plates were dried and redeveloped with chloroform/acetone/ methanol/acetic acid/water (50:20:10:10:5). Ceramide-1-phosphate spots were scraped from the plates and quantitated by scintillation counting. The assay was calibrated with a standard curve of authentic ceramide. Results were normalized to total lipid phosphate. To measure lipid phosphate, the chloroform phase of the cell extract was evaporated under N_2, and incubated at 180 °C for 30 min in 50ul of 70% $HClO_4$. Then 278 μl of H_2O, 55 μl of 2.5% ammonium molybdate, and 55 μl of 10% ascorbic acid were added and incubated for a further 15 min at 95 °C. Inorganic phosphate was detected by absorbance at 700 nm and quantified based upon a standard curve of glycerol phosphate.

2.14 Microsome preparation for in vitro serine palmitoyltransferase and ceramide synthase assays.

Microsomes were prepared by sonication of cell pellets in 50 mM HEPES, pH 7.4, 10 mM EDTA, 5 mM DTT, and 0.25 M sucrose supplemented with protease inhibitor cocktail from Sigma-Aldrich. The preparation was centrifuged at 1000 × *g*, and the resulting supernatant was then ultracentrifuged at 100,000 × *g*. The resultant pellet was suspended by homogenization in 50 mM HEPES, pH 7.4, 5 mM DTT buffer containing 20% glycerol to form microsomes for the assays. Protein concentration was determined using the Bio-Rad dye protein assay reagents with a standard curve of bovine serum albumin.

2.15 Serine palmitoyltransferase assay

Serine palmitoyltransferase was assayed as described previously [314]. Briefly, enzyme activity in 50-100 μg of microsomal membranes was determined in 100 mM HEPES (pH 8.3), 5 mM DTT, 2.5 mM EDTA (pH 7.0), and 50 μM pyridoxal 5'-phosphate. The reaction was initiated by the addition of 200 μM palmitoyl CoA and 3 μCi of L-[^{3}H]serine, with a final serine concentration of 1 mM. Reactions were incubated for 20 min at 37 °C prior to termination with 1.5 ml of chloroform/methanol (1:2) followed by 1 ml of chloroform and 1.8 ml of 0.5 N NH_4OH with sphinganine as carrier. Lipids were extracted as described previously and quantified by liquid scintillation counting [314].

2.16 Ceramide synthase assay

(Dihydro)ceramide synthase was assayed according to Bose *et al.* [210]. Briefly, 50-200 μg of microsomal membranes were incubated in 20 mM HEPES (pH 7.4), 2 mM

$MgCl_2$, 20 μM fatty acid-free bovine serum albumin, 2.5 uM DTT and 20 μM sphinganine. The reaction was then initiated by the addition of 2 μCi of [1-^{14}C]palmitoyl-CoA in the presence of 100 uM palmitoyl-CoA and allowed to incubate for 60 min at 37 °C. Lipids were extracted and ceramide was isolated and counted as described above.

2.17 Statistical analysis

Results were expressed as means ± SD. Statistical analysis was done by Student's *t*-test as appropriate. A *P* value of less than 0.05 was taken as significant.

3 OxLDL promotes macrophage survival by facilitating Bax degradation and increasing Mcl-1 expression[a]

3.1 Introduction

Accumulation of lipid laden macrophages (foam cells) in the intima of susceptible arteries is thought to play an important role in the development of atherosclerosis [41]. Foam cells are the hallmark of early and intermediate atherosclerotic lesions, pointing to a role in lesion formation and progression. In addition, macrophage-rich plaques are weaker than fibrous plaques because of the lipid deposits and because macrophages secrete matrix degrading metalloproteases (MMP) [315]. Several studies have demonstrated that macrophage-rich plaques have a significantly increased risk of rupture and thrombosis.

Oxidized LDL (oxLDL) has many biological properties which could promote atherogenesis, including the recruitment and retention of macrophages into the arterial intima. OxLDL can also induce proliferation of macrophages, and block macrophage apoptosis [20, 26]. OxLDL is present in atherosclerotic lesions [26], and could play a key role in expanding macrophage populations in the arterial intima.

The effect of oxLDL on macrophage apoptosis is mediated in part through Bcl-X_L, a member of the Bcl-2 family of regulators of apoptosis. This family is composed of proteins with an anti-apoptotic effect such as Bcl-2, Bcl-X_L, Mcl-1 and proteins with a pro-apoptotic effect, such as Bax, Bak, Bid, and Bad [107]. Bax was first shown to exert its pro-apoptotic effect by counteracting the pro-survival functions of Bcl-2 [142, 179, 316]. Bax can form oligomers on mitochondria which allow cytochrome c release [316]. Both ectopic

[a] A version of this chapter will be submitted for publication. Wang, S.W., Duronio, V. and Steinbrecher, U.P., Effect of oxLDL on Mcl-1 and Bax in macrophages.

expression of Bax and the addition of purified recombinant Bax can accelerate apoptosis [141]. The anti-apoptotic members on the other hand can protect cells from apoptosis by sequestering Bax. Bax is able to heterodimerize with Bcl-2 and mutagenesis studies have shown that this ability of Bcl-2 to bind Bax is required for its pro-survival effect [142]. Taken together, these observations suggest that Bax plays an important role in cell death.

The ubiquitin proteasome system is a conserved mechanism for controlling degradation of cellular proteins [317]. It is involved in many cellular processes, including apoptosis, since it targets proteins of the apoptotic machinery such as Bid [318], Bik [319], Bax [320], Bcl-2 [321], Bim [322], Mcl-1 [323]and XIAP[324]. Abnormalities of proteasome function have been implicated in the pathogenesis of several diseases, including atherosclerosis [325 326].

Mcl-1, a pro-survival Bcl-2 family protein, is essential for the homeostasis of early hematopoietic progenitors by ensuring cell survival [327]. Over-expression of Mcl-1 inhibits cell death induced by various apoptotic stimuli [328, 329] while elimination of Mcl-1 induces apoptosis [330]. Its association with Bim is implicated in apoptosis in multiple myeloma cells [331].

Although it is widely agreed that Bax and Bak are essential in executing apoptosis [163], the mechanism by which Bcl-2 family members interact and allow the activation of Bax and Bak still remain poorly understood [168]. One proposed model suggests that a subclass of BH3-only pro-apoptotic proteins, including Bim and truncated Bid (tBid), can bind directly not only to pro-survival proteins but also to Bax and Bak to activate them. This model further proposes that the other pro-apoptotic members bind to pro-survival proteins, and simply lower their capacity to sequester the activators [166, 187].

An alternative view supported by recent findings is that the BH3-only proteins exclusively displace the pro-survival proteins, overcoming their sequestration of Bax/Bak [169-172]. Thus, whether the BH3-only proteins activate Bax/Bak directly or indirectly (or both) remains to be established.

We have previously shown that oxLDL promotes survival of bone marrow derived macrophages by blocking apoptosis through the PI3K/PKB signaling pathway, which leads to activation of NFκB and subsequent upregulation of Bcl-X_L [38, 72]. The aim of the current study was to determine whether other Bcl-2 family members such as Bax, Mcl-1 and Bim levels are altered in macrophages upon treatment with oxLDL, and to define whether their interactions are required for the anti-apoptotic effects of oxLDL.

3.2 Results

3.2.1 Bax is involved in macrophage apoptosis following M-CSF withdrawal

Various apoptotic stimuli activate Bax by inducing a conformational change at its N-terminus, which then leads to its translocation to mitochondria to initiate apoptosis [157, 170, 332]. To determine whether Bax plays a role in macrophage apoptosis induced by M-CSF withdrawal, we used the antibody, 6A7, which is specific for the active conformation of Bax. BMDM were incubated without M-CSF for various times and were then lysed using CHAPS buffer to preserve Bax conformation [295], immunoprecipitated with 6A7, and then blotted with a conformation-independent anti-Bax antibody. Results in Figure 3.1A indicate that Bax is activated in BMDMs after 24 hours of cytokine withdrawal, coinciding with the significant number of cells undergoing apoptosis at that stage [333, 334].

Our next aim was to determine cellular localization of active Bax protein in the macrophages. In non-apoptotic cells, Bax is a soluble monomeric protein diffusely distributed in the cytoplasm [295]. There was no detectable staining for active Bax with 6A7 antibody in healthy control cells. However, when the macrophages were cultured without cytokines to induce apoptosis, active Bax was found in a pattern that colocalized with the mitochondrial outer membrane protein TOM 20 as seen in Figure 3.1B. These results confirm that there is a conformational change of Bax and redistribution to the mitochondria during apoptosis in bone marrow derived macrophages.

3.2.2 OxLDL reduces Bax protein levels in Macrophages

We have previously shown that oxLDL blocks apoptosis induced by growth factor withdrawal in BMDM, and that it does so partly by up-regulating expression of the pro-survival protein, Bcl-X_L [72]. In view of the key role of Bax in apoptosis, we hypothesized that oxLDL may also regulate Bax to promote survival. To test this, we incubated macrophages with oxLDL for 24 hours, and measured the level of Bax by immunoblotting. Figure 3.2A shows that there was a decrease in Bax after addition of oxLDL in BMDM while no significant change was observed in Bak. This was also observed in differentiated THP-1 cells (Figure 3.2B). The only significant down-regulation of Bax was observed in cells treated with heavily oxidized LDL (Figure 3.3). Native LDL, mildly oxidized LDL, and acetylated LDL had no significant effect. The increase in Bax relative to actin acetyl LDL may be an artifact due to the low-intensity actin band. Viability of cells incubated with acLDL was poor and although the amount of protein loaded was the same, the amount of actin per unit cell protein was evidently low. These results correlate with our previous findings that acetyl LDL and native LDL do not

confer resistance to macrophage apoptosis [38]. Bax was rapidly downregulated by oxLDL in mouse BMDM as well as in human monocyte-derived macrophages (Figure 3.4).

3.2.3 OxLDL regulates Bax by accelerating its degradation

To determine if oxLDL controls Bax expression at the level of transcription, mRNA was extracted from macrophages after 24 hours of treatment with or without M-CSF or oxLDL, and amplified by real-time PCR. Instead of decreasing, Bax mRNA in oxLDL treated cells increased 1.9 ± 0.2 fold compared to healthy control cells ($n=3$, $p<0.005$). This suggests that oxLDL promotes the decrease of Bax by a post-transcriptional mechanism. To determine if the rate of degradation of Bax is increased by oxLDL in macrophages, cells were pre-treated with 10 ug/ml cycloheximide for 1 hour to stop new protein synthesis and then oxLDL was added. As shown in Figure 3.5A, oxLDL induced a rapid drop in Bax levels and by 30 minutes it was roughly half of the starting level (Figure 3.5B). Cytokine withdrawal alone did not alter Bax levels during the 8-hour time course (Figure 3.5B).

3.2.4 Down-regulation of Bax by oxLDL is proteasome mediated

Bax has been reported to be degraded by the ubiquitin/proteasome-dependent pathway [170, 320], and oxLDL has been previously shown to affect this pathway [85, 335, 336]. To determine if the accelerated degradation of Bax involves proteasomes, 3 different proteasome inhibitors, ALLN, MG132 or lactacystin were added to BMDM in addition to oxLDL. Treatment with these inhibitors blocked the effect of oxLDL on Bax degradation (Figure 3.6A). Furthermore, Bax levels increased with increasing concentration of MG132 and ALLN in the presence of oxLDL (Figure 3.6B). As in 3.6A

also shows a second Bax band migrating at a slightly higher molecular weight, only in lysates from cells treated with oxLDL. It has been reported that Bax can be serine phosphorylated by PKB to promote its heterodimerization with pro-survival counterparts, Mcl-1 and Bcl-X_L [337]. Treatment of the samples with λ phosphatase did not eliminate the slower migrating band (data not shown). This negative result suggests that the band shift may not be due to phosphorylation.

To further elucidate the role of the proteasome pathway in the degradation of Bax, we treated the cells in the presence of oxLDL with ALLM, an inhibitor of calpains and cathepsins that is said to have no effect on proteasomal degradation. ALLM had no effect on cell viability in the presence of oxLDL (Figure 3.6C).

3.2.5 The PI3K/PKB pathway is involved in accelerated Bax degradation

Previous results from our laboratory showed that although oxLDL can cause activation of both ERK and PI3K/PKB pathway, only the latter pathway is vital for oxLDL-mediated BMDM survival [72]. PKB has been shown to regulate survival of pre-B hematopoietic cells by inhibiting the conformational change of Bax [338]. To examine whether the effect of oxLDL on Bax was mediated through the PI3K/PKB pathway, macrophages were treated with the selective PI3K inhibitor LY294002 in the presence of oxLDL. This drug partially restored the expression of Bax (Figure 3.7). In contrast, treatment with U0126 or SB203850, selective inhibitors of MEK kinase and p38 MAPK respectively, had no effect on reversing oxLDL mediated Bax down-regulation. This suggests that the PI3K/PKB pathway activated by oxLDL plays a role in down-regulating Bax expression.

3.2.6 Bax is only partially responsible for apoptosis induced by M-CSF withdrawal.

To assess the importance of Bax down-regulation in oxLDL-mediated survival events we compared macrophages obtained from Bax knockout and wild-type mice. We confirmed that Bax was undetectable by immunoblotting of BMDM lysates from knockout mice (Figure 3.8A). BMDM from these mice had an identical extent of apoptosis as BMDM from wild-type mice following cytokine withdrawal, and oxLDL was fully capable of blocking apoptosis (Figure 3.8B). This indicates that the pro-survival effect of oxLDL is not entirely due to its ability to down-regulate Bax.

3.2.7 Sequesteration of Bax by Mcl-1 is disrupted by cytokine withdrawal while oxLDL enhances this association

In many types of cells, Bax and Bak are thought to be kept in check by binding to pro-survival family members such as Bcl-2 and Mcl-1. However, as shown in Figure 3.9A, Bcl-2 does not sequester Bax or Bak in healthy BMDM. On the other hand, Mcl-1 associated with Bax in healthy BMDM, but when cells were deprived of M-CSF there was a substantial decrease in the association of Bax with Mcl-1 (Figure 3.9B). As shown in Figure 3.9C , the association of Mcl-1 with Bax rapidly decreases following cytokine withdrawal. However, despite the decreased level of Bax in the presence of oxLDL, Mcl-1 is still found to associate with Bax (Figure 3.9C).

3.2.8 OxLDL acts through the PI3K/PKB pathway to increase the level of Mcl-1

Mcl-1 is an important survival factor for multiple myeloma where its down-regulation has been shown to induce apoptosis [323]. Mcl-1 is a short-lived protein with a half-life between 30min and a few hours depending on the cell type [339, 340] and is reported to be regulated by proteasomal degradation during apoptosis [170]. Although Mcl-1 was not rapidly degraded in apoptotic macrophages (Figure 3.10A), its expression increased in response to oxLDL treatment (Figure 3.10B). Furthermore, this increase of Mcl-1 correlated with increased oxidation of LDL, while acetylated LDL (AcLDL) had no effect (Figure 3.11). Interestingly, like Bax, this effect of oxLDL is also under the control of PI3K/PKB pathway since inhibition of PI3K diminished oxLDL mediated Mcl-1 increase (Figure 3.10B last lane). This suggests that oxLDL regulates Bax and Mcl-1 in opposite directions through PI3K to promote cell survival.

3.2.9 Bim does not displace Mcl-1 from Bax

In order to induce apoptosis, BH3-only proteins are believed to activate Bax/Bak proteins and/or dissociate pro-survival Bcl-2 family members from Bax/Bak [168]. We chose the BH3-only protein Bim to investigate its role in macrophage apoptosis. Phosphorylation of Bim is reported to disrupt its interaction with Bax and contribute to cell survival [341]. We found that Bim was phosphorylated in healthy cells and this phosphorylation disappeared upon cytokine withdrawal. However, phosphorylation of Bim was not observed in oxLDL treated cells (Figure 3.12). Furthermore, we could not demonstrate Bax binding to Bim in macrophages incubated with or without M-CSF

(Figure 3.13). Mcl-1 co-immunoprecipitated with Bim but we found no change in association between Bim and Mcl-1 when cells were deprived of M-CSF (Figure 3.14).

3.3 Discussion

Dysregulation of apoptosis by Bcl-2 family members plays an important role in the pathogenesis of many diseases. Bax mutation in particular has been implicated in leukemias and colorectal cancer [342, 343]. It has been shown that advanced atherosclerotic lesions show higher Bax immunoreactivity and more TUNEL-positive cells [344] while IL-10 is able to enhance oxLDL-induced foam cell formation by up-regulating prosurvival members of the Bcl-2 family [345]. The importance of Bax regulation in atherosclerosis is further illustrated by a recent study demonstrating that reducing macrophage apoptosis by selective knockout of Bax resulted in accelerated lesion progression [69].

In this study, we show that oxLDL promotes Bax degradation via the proteasome pathway and increases levels of a pro-survival member of the Bcl-2 family, Mcl-1. In contrast, several previous studies have reported that oxLDL promotes apoptosis of cells [54, 346-348] and that Bax levels are elevated in the presence of oxLDL [344, 349]. We believe these discrepancies come from methods of oxidation of LDL, concentration of oxLDL and the cell types used in those studies. For example, we and others have shown that high concentrations of oxLDL [38, 350] or peroxide-rich oxLDL [72, 80] can be cytotoxic to cells.

Our observation that oxLDL can cause Bax degradation by proteasomes is in agreement with our previous findings that oxLDL treatment results in phosphorylation and proteasomal degradation of IκB, leading to activation of NFκB. Active NFκB has been shown to be present in atherosclerotic lesions [83]. Several reports also showed that

oxLDL was able to alter the activity of proteasome and HDL was able to counteract this effect [85, 335, 351].

The PI3K/PKB pathway has been shown to mediate macrophage survival by M-CSF [71, 352]. PKB can promote cell survival by directly phosphorylating regulatory components of the apoptotic machinery, such as forkhead transcription factors [353], IκB [354], or indirectly by changing the levels of expression of the genes that encode components of cell death, such as the Bcl-2 family members [76]. While both p38 MAPK [355] and PI3K [338] have been reported to regulate cell survival by preventing Bax translocation to mitochondria, oxLDL signals only through the latter pathway in primary macrophages (Figure 3.7). PI3K/PKB has been reported to be responsible for targeting various proteins for proteasomal degradation, such as p27Kip1[356], and Bim[357]. It is possible that Bax is selectively marked for proteasomal degradation by PKB in response to oxLDL signalling, similar to IκB. The observed reduction in the electrophoretic mobility of Bax in response to oxLDL may reflect a modification necessary for targeting to proteasomes.

Although there are reports that calpain and/or cathepsin can cleave Bax to generate a shorter fragment [358-360], this is unlikely to occur in the case of oxLDL-mediated cell survival. We tested the ability of the oxLDL to generate the 18KDa fragment of Bax by using an antibody directed against the C terminus of Bax. Our results (data not shown) did not reveal the presence of any such fragment suggesting the effect of oxLDL on Bax does not involve cathepsins or calpain. Furthermore, the short form of Bax was reported to enhance apoptosis [360] by acting like a BH3-only protein that binds strongly to Bcl-X_L thus leaving full-length Bax to translocate to mitochondria [361]. While inhibition of calpain and cathepsin with ALLM did not alter cell viability, the additional

inhibition of proteasome with ALLN prevented oxLDL's ability to promote cell survival (Figure 3.6C). Hence it is unlikely either calpain or cathepsin-mediated Bax degradation plays a role in the ability of oxLDL to promote cell survival.

Interestingly, survival of macrophages in the presence of M-CSF was not dependent upon degradation of Bax, indicating that there are likely multiple pathways that can promote survival. The fact that cells from Bax knockout mice were able to undergo apoptosis following cytokine withdrawal also supports the conclusion that other means of inducing apoptosis, e.g. via activation of Bak, can likely compensate for the complete lack of Bax. This is in accordance with the observation that Bax/Bak double knockout cells were resistant to many apoptotic stimuli (such as growth factor withdrawal, UV irradiation, drug treatment, TNFα), while single knockout of either of these two multidomain pro-apoptotic family members did not offer protection [163] because the deficiency in one is often compensated by an increase in the expression in the other protein [362].

Mcl-1, a pro-survival Bcl-2 family member, is critical to embryonic development since deletion of this gene results in peri-implantation embryonic lethality [363]. Over-expression of Mcl-1 is shown to inhibit cell death induced by various apoptotic stimuli [328, 329] while elimination of Mcl-1 induces apoptosis [330]. Furthermore, lymphocytes and hematopoietic stem cells lacking Mcl-1 expression undergo apoptosis and exhibit defective differentiation [327, 364]. We showed that extensively oxidized LDL upregulates Mcl-1 levels and that this is mediated through PI3K/PKB pathway. This is in accordance with reports that demonstrated that the PI3K/PKB pathway controlled the expression of Mcl-1 in primary human macrophages and that Mcl-1 was essential for macrophage

survival [365-367]. Our lab previously showed that PKB can increase Mcl-1 translation following cytokine stimulation to promote cell survival [277]. Hence it is possible this increase in Mcl-1 by oxLDL treatment may be due to increased mRNA translation and/or posttranslational stabilization of the protein.

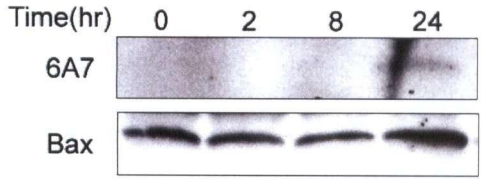
It is commonly accepted that the pro-survival proteins sequester Bak/Bax before apoptosis is induced [141, 172]. The anti-Mcl-1 immunoprecipitate shown in Figure 3.9B indicates that in macrophages undergoing apoptosis, less Bax is bound to Mcl-1. Immunoprecipitation with anti-Bax (Figure 3.9C) shows a similar pattern, with less Mcl-1 associated with Bax at 12 hr or 24 hr without oxLDL. OxLDL maintains a strong binding between Mcl-1 and Bax. It has been shown that along with Bcl-X_L, Mcl-1 keeps Bak in check in healthy cells [170]. We postulate that oxLDL promotes downregulation of Bax but not Bak, but that there is no change in cell viability because more Mcl-1 or Bcl-X_L is available to sequester Bak. This is the first report linking the expression of Mcl-1 to oxLDL's ability to promote macrophage survival.

Phosphorylation of Bim by cytokines such as M-CSF or IL3 is shown to regulate its apoptotic function by promoting its proteasomal degradation of the protein or its interaction with Bax [322, 341, 368]. Certain stresses, such as UV irradiation or cytokine withdrawal, induce the release of Bim from the dynein motor complex, allowing Bim to neutralize pro-survival Bcl-2 members [151] or directly activate other pro-apoptotic proteins [341]. Although we found Bim is phosphorylated in the presence of M-CSF (Figure 3.12), we did not observe Bim interacting with Bax (Figure 3.13). Others have proposed that BH3-only proteins displace the pro-survival relatives that constrain Bax/Bak rather than directly activate them to induce apoptosis [171]. However, with the finding that the

association between Bim and Mcl-1 not changing during apoptosis (Figure 3.14), we concluded that Bim is unlikely to mediate macrophage apoptosis through Bax, no matter which mechanism it employs to initiate apoptosis. There are other BH3-only proteins that can induce apoptosis by neutralizing the protective function of Mcl-1 or by activating Bax/Bak. For example, besides truncated Bid (tBid) and Puma, Noxa is reported to selectively displace Mcl-1 from binding to Bak [169, 170]. Further studies are required to determine the exact role of Mcl-1 in oxLDL-mediated macrophage survival.

In conclusion, these results support our previous findings showing that PI3K/PKB play an important role in oxLDL-mediated survival [38, 72]. Besides the previously shown role for PI3K/PKB in maintaining Bcl-X_L expression, oxLDL selectively promotes the proteasomal degradation of Bax and an increase in Mcl-1 expression, both mediated via the PI3K/PKB pathway (Figure 3.15).

A.



B.

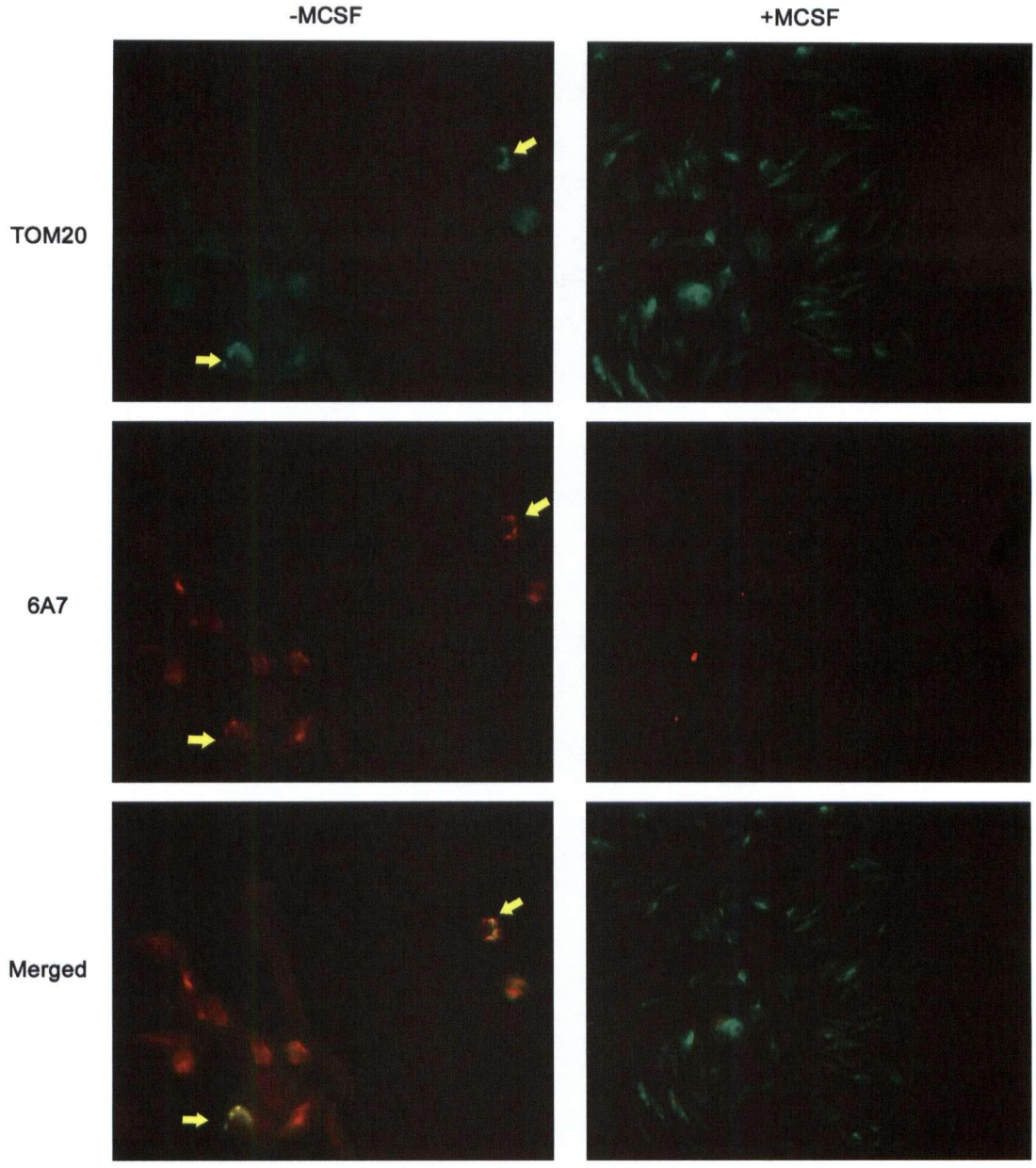


Figure 3.1 Bax undergoes conformational changes upon cytokine withdrawal. (A) Macrophages were cultured in the absence of cytokine for the indicated time. Cell lysates were generated with CHAPS buffer. Bax was immunoprecipitated with monoclonal antibody 6A7 which is specific for the active conformation of Bax, and immunoblotted with a polyclonal Bax antibody as described in Methods. Whole cell lysates were blotted for Bax to indicate the level of total Bax in each sample prior to immunoprecipitation. Data are representative of three independent experiments. (B) Macrophages were cultured in the absence or presence of cytokine for 24 hours. Z-VAD (100μM) was included to prevent caspase activation and the consequent shrinkage and detachment of cells. Cells were then immunostained for anti-active Bax 6A7 and TOM20. Data are representative of three independent experiments.

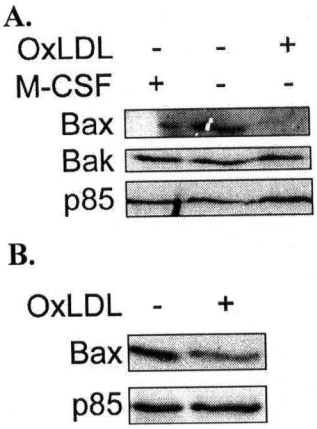
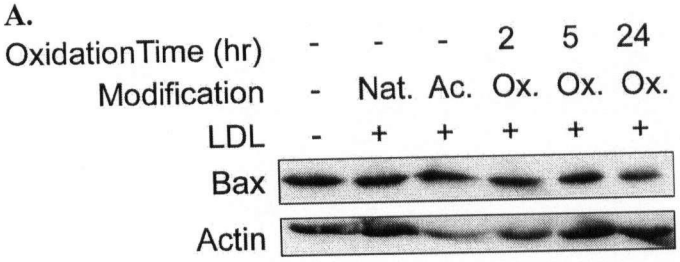


Figure 3.2 Expression of Bax is down-regulated by treatment with oxidized LDL in macrophages. (A) BMDM were cultured in the presence or absence of cytokine with or without oxLDL for 24 hours. Cell lysates were probed for total Bax, Bak and p85. Data are representative of five independent experiments. (B) THP-1 cells treated with 40nM PMA for 24 hours to induce differentiation. Then FBS was withdrawn and cells incubated with or without 25μg/ml oxLDL for 24 hour. Cells lysates were blotted for total Bax and for p85 as loading control. Data are representative of two similar experiments.

A.



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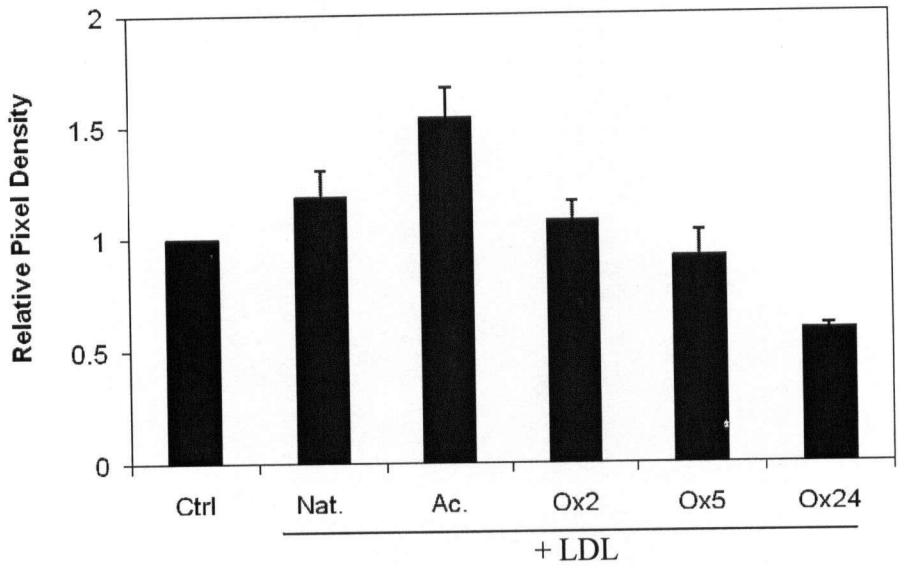


Figure 3.3 Only extensively oxidized LDL promotes a decrease in Bax protein. (A) BMDM were incubated for 24 h in the absence of M-CSF alone (control) or treated with native LDL (Nat.), acetyl LDL (Ac.), or LDL oxidized for the indicated times. Lysates were immunoblotted for total Bax and for actin as loading control. (B) Densitometric result of immunoblots that were normalized to the loading control and expressed as a ratio of the control. The data represent means ± S.D. of at least two independent experiments.

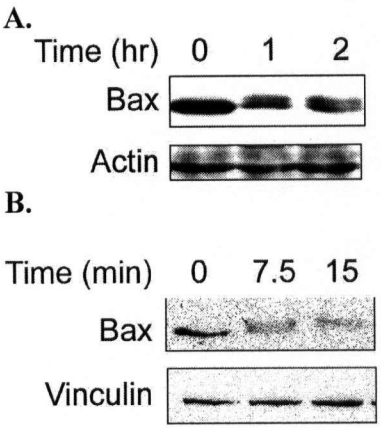
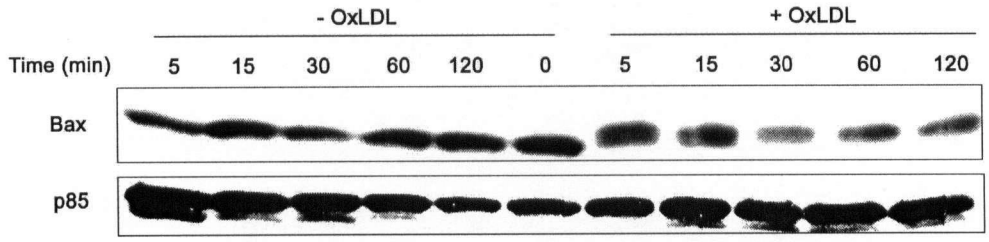


Figure 3.4 OxLDL also reduces Bax levels in human macrophages. (A) Murine BMDMs were incubated with 25ug/ml of oxLDL for the indicated time. Cell lysates were immunoblotted for total Bax and actin as loading control. Data are representative of five independent experiments. (B) Macrophages derived from human peripheral blood mononuclear cells were cultured in the absence of M-CSF for 4 hours before addition of oxLDL for the indicated time. Cell lysates were immunoblotted for total Bax and for vinculin as loading control.

A.



B.

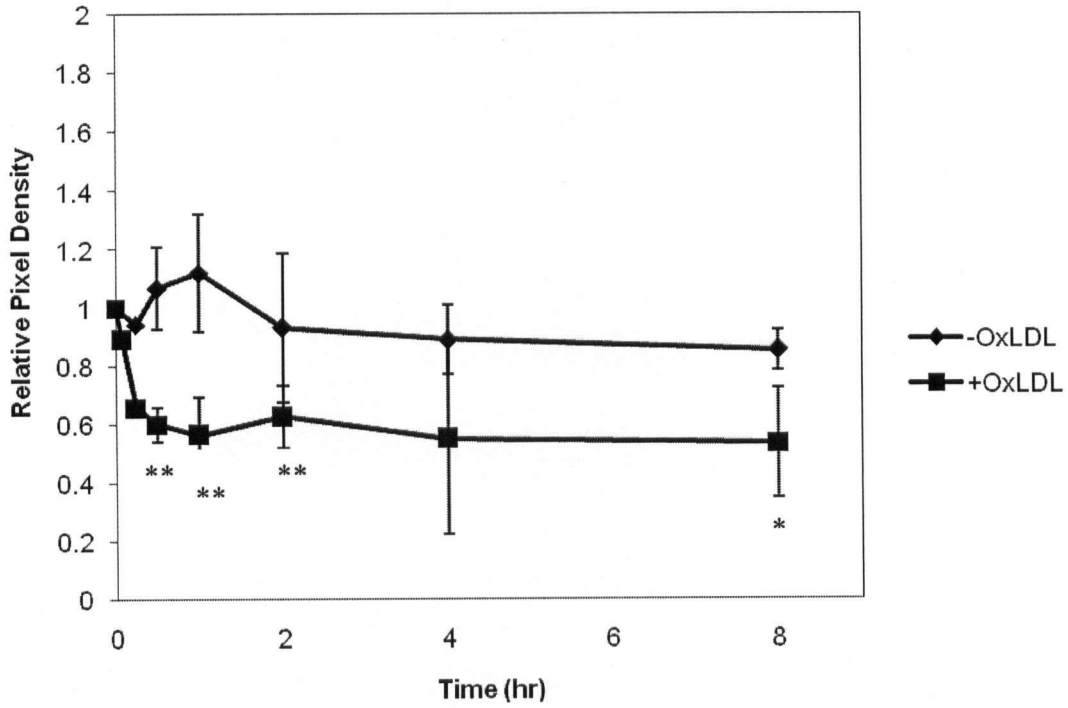
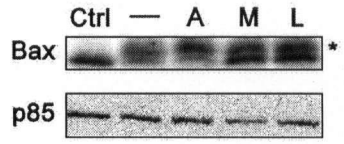


Figure 3.5 OxLDL facilitates Bax protein turn-over. (A) BMDM were pre-treated for 1h with 10 μg/ml of cyclohexamide before addition of oxLDL for the indicated time. Cell lysates were immunoblotted for total Bax and p85. Data are representative of three independent experiments. (B) Densitometric results of immunoblots expressed as a ratio of the time zero control, corrected for loading with p85. The data represent means ± S.D. of at least three experiments, except the 15 min time point is from one experiment only. *$p<0.05$,**$p<0.005$ vs. time zero.

A.



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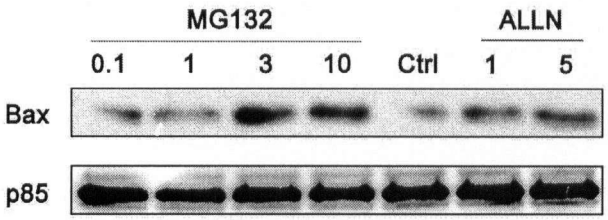

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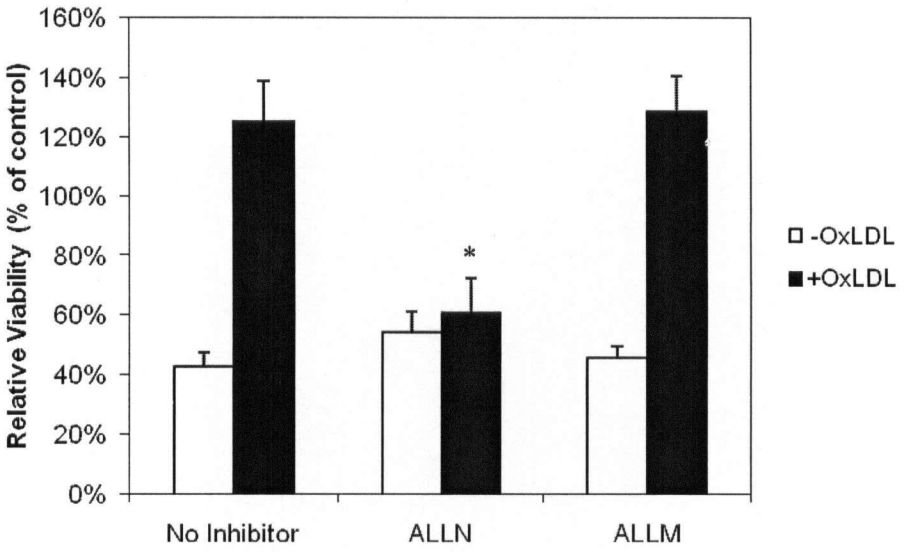
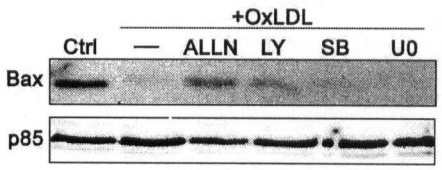


Figure 3.6 OxLDL induces Bax degradation via the proteasomal pathway. (A) BMDM were pre-treated with various inhibitors (A: 5 μM ALLN, M: 10 μM MG132, L: 10 μM Lactacystin) for 1 hour in the absence of M-CSF and then incubated with oxLDL for 24 hours. BMDM cultured in the absence of M-CSF served as control. Cell lysates were blotted with anti-Bax antibody and anti-p85. * shifted band indicating possible post-translational modification. Data are representative of three independent experiments. (B) Effects of MG132 and ALLN on Bax level in the presence of oxLDL. BMDM were pre-treated with varying concentrations of MG132 or ALLN for 30 minutes and then 25 μg/ml of oxLDL was added for 2 hours. Cell lysates were immunoblotted with anti-Bax and anti-p85 antibody. Data are representative of two independent experiments. (C) BMDM were treated without inhibitors or with ALLN or ALLM for 24 hours in the absence of M-CSF with or without the addition of oxLDL. Cell viability was expressed relative to cells cultured in the presence of M-CSF. The data represent means ±S.D. of at least three experiments done in triplicate. *$p<0.005$ vs. ox with no inhibitor.

A.



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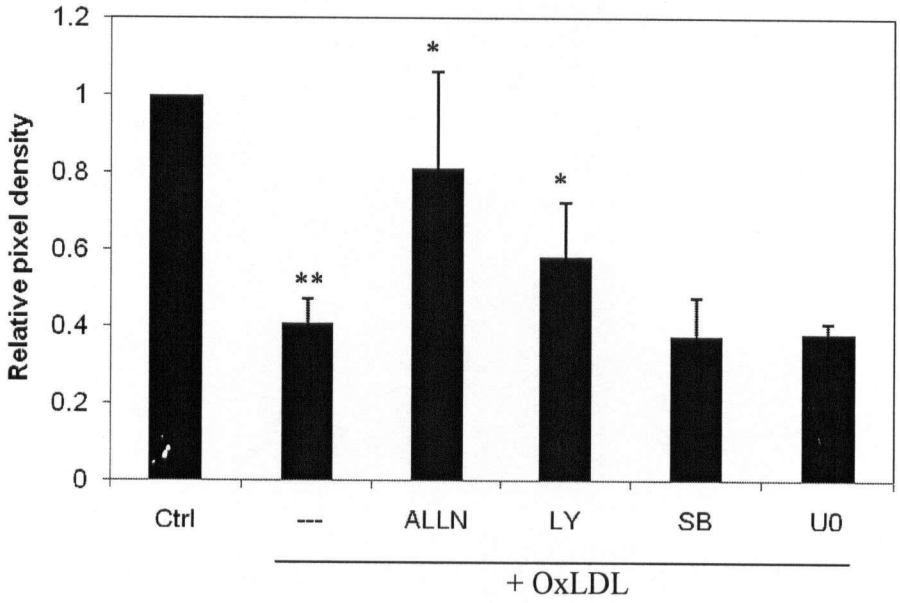
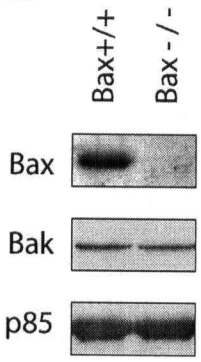


Figure 3.7 OxLDL signals at least partially through a PI3K dependent pathway leading to Bax degradation. (A) BMDM were pre-treated with various inhibitors for one hour in the absence of M-CSF (control) before addition of oxLDL for 24 hours. 5 μM ALLN; 20 μM LY294002; 30 μM SB203580; 10 μM U0126. Whole cell lysates were then blotted for Bax and for p85 as a loading control. Data are representative of three independent experiments. (B) Densitometric result of immunoblots expressed as a ratio of the control, corrected for loading as monitored by p85. The data represent means ± S.D. of at least three experiments. *$p<0.05$ vs. oxLDL alone, **$p<0.01$ vs. control.

A.



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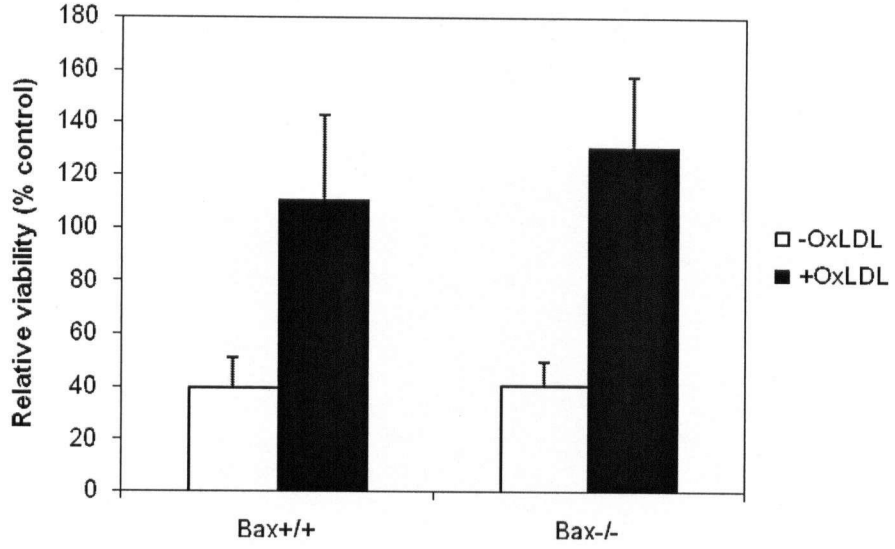


Figure 3.8 Bax knockout does not confer resistance to cytokine withdrawal induced cell death. (A) BMDM from Bax+/+ and Bax-/- mice were cultured in the presence of M-CSF. Cell lysates were harvested and immunoblotted for Bax, Bak and p85. Data is representative of three independent experiments. (B) BMDM were seeded at $5x10^4$ cells/well in 96-well plates. Cells were cultured in the absence of M-CSF with or without the addition of 25 μg/ml of oxLDL for 24 hours. Cell viability was then measured with the MTS assay, and is expressed relative to cells cultured in the presence of M-CSF. The data represent means ± S.D. of five experiments done in triplicate. *$p<0.005$ vs. control; **$p<0.001$ vs. no oxLDL.

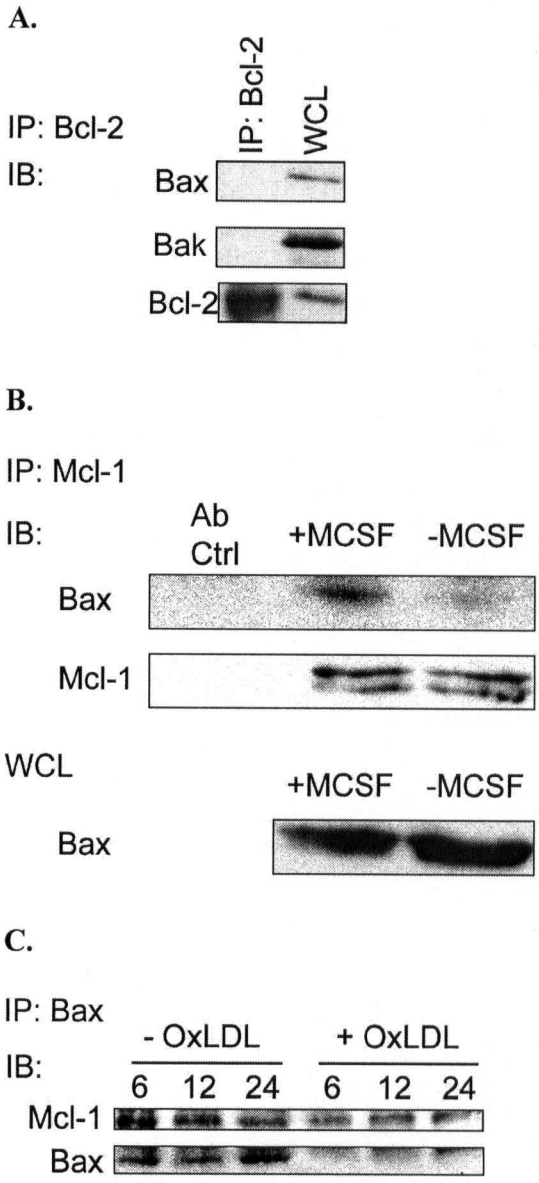


Figure 3.9 Mcl-1 but not Bcl-2 sequesters Bax and Bak. (A) BMDM were cultured in the presence of M-CSF and whole cell lysates were immunoprecipitated with anti-Bcl-2 antibodies and immunoblotted for Bax, Bak and Bcl-2. Data shown are representative of three independent experiments. (B) BMDM were cultured in the absence or presence of M-CSF for 24 hours. Whole cell lysates were blotted for Bax to check for imput and immunoprecipitated with anti-Mcl-1 antibodies and immunoblotted for Bax, and Mcl-1. Data shown are representative of three independent experiments. (C) BMDM were cultured in the absence of M-CSF with or without oxLDL for the indicated time. Whole cell lysates were immunoprecipitated with anti-Bax antibodies and immunoblotted for Mcl-1. Antibody control (ab ctrl) represents a sample of beads incubated with the respective antibodies, in the absence of cell lysates.

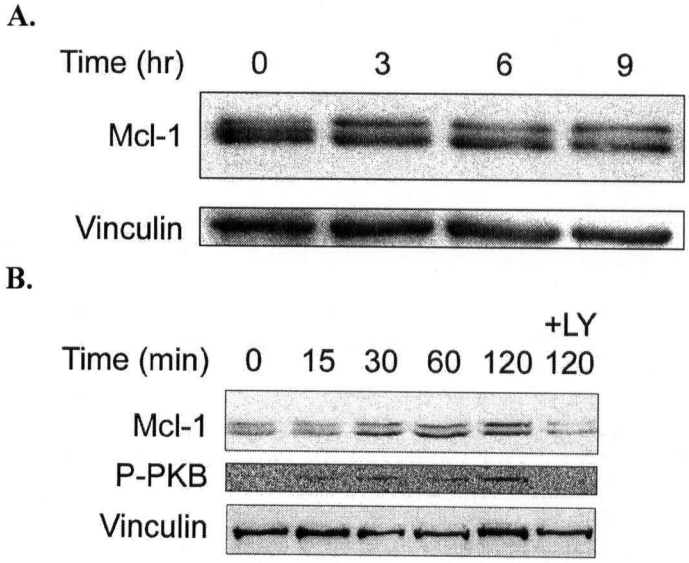
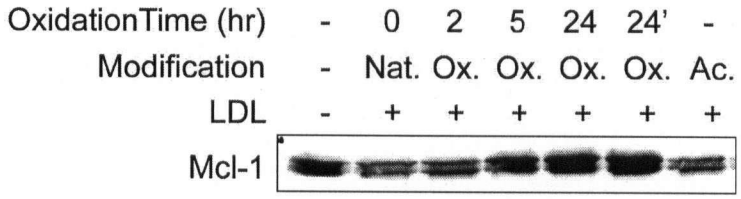


Figure 3.10 Mcl-1 level does not decrease in response to cytokine withdrawal but increases with oxLDL treatment. (A) BMDM were starved of M-CSF for the indicated time. The whole cell lysates were immunoblotted for Mcl-1 and vinculin, the loading control. Data shown are representative of three independent experiments. (B) BMDM were incubated for 2.5 hours without M-CSF. 20 μM of LY294002 was then added for 30 min, and finally oxLDL was added. After incubating with oxLDL for the indicated time, cells were lysed and blotted for Mcl-1, phospho-PKB (P-PKB) and vinculin. Data shown are representative of three independent experiments.

A.



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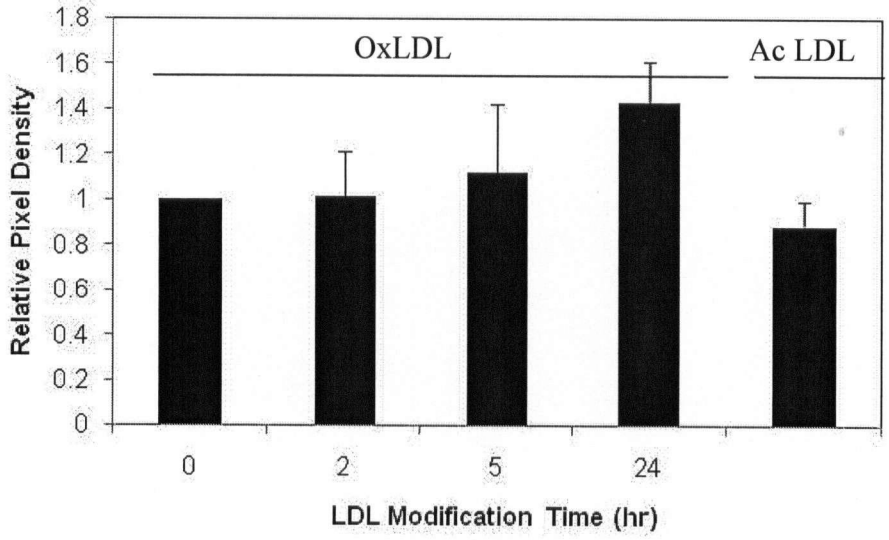

Figure 3.11 Mcl-1 expression is preserved by treatment of extensively oxidized LDL. (A) BMDM were incubated for 4h in the absence of M-CSF alone (control) or treated with LDL modified by oxidation or acetylation for the indicated times. Two different batches of 24hr-oxidized LDL were used. Lysates were immunoblotted for total Mcl-1. Data shown are representative of two independent experiments. (B) Densitometric result of immunoblot expressed as a ratio of the control. The data represents means ± S.D. of two experiments.

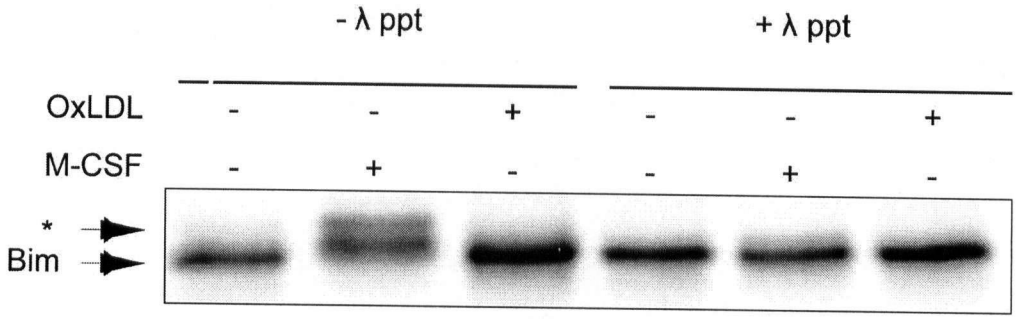

Figure 3.12 Bim is phosphorylated in the presence of M-CSF but not oxLDL. BMDM were cultured in the presence or absence of M-CSF +/- oxLDL for 24 hours. Cell lysates were divided into two sets. One is treated with λ phosphatase according to manufacture's instruction. Cell lysates were then run on the 10% low-bis SDS-PAGE gel before immunoblotted with anti-Bim antibodies. Data are representative of three experiments. * denotes phosphorylation of Bim.

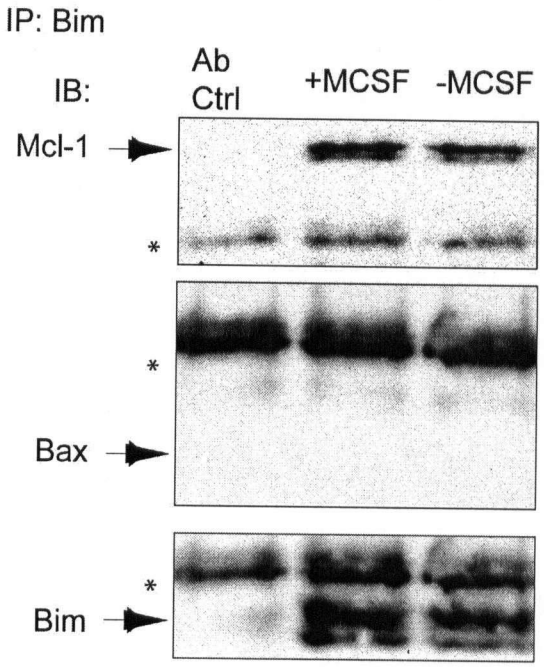

Figure 3.13 Interaction of Bim with Mcl-1 or Bax does not change during apoptosis. BMDM were cultured in the absence or presence of M-CSF for 24 hours before immunoprecipitation with anti-Bim antibodies. Then it was immunoblotted with anti-Mcl-1, Bax, Bak and Bim antibodies. Data are representative of three experiments. * denotes antibody light chains. Antibody control (ab ctrl) represents a sample of beads incubated with the respective antibodies, in the absence of cell lysates.

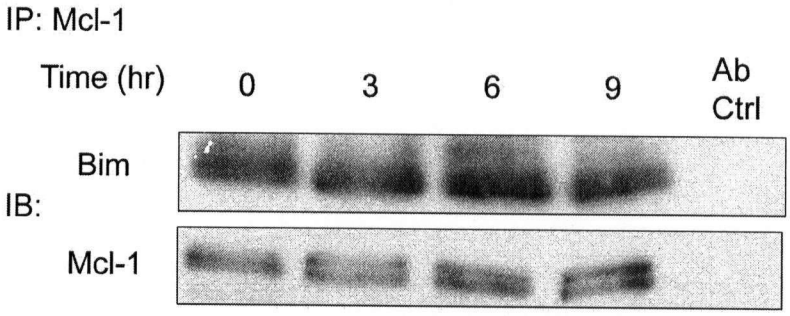

Figure 3.14 Mcl-1 association with Bim does not change during apoptosis. BMDM were cultured in the absence of M-CSF for the indicated time. Whole cell lysates were immunoprecipitated using anti-Mcl-1 antibodies and checked for association with Bim. Immunoprecipitation without cell lysates was used as the control (Ab ctrl).

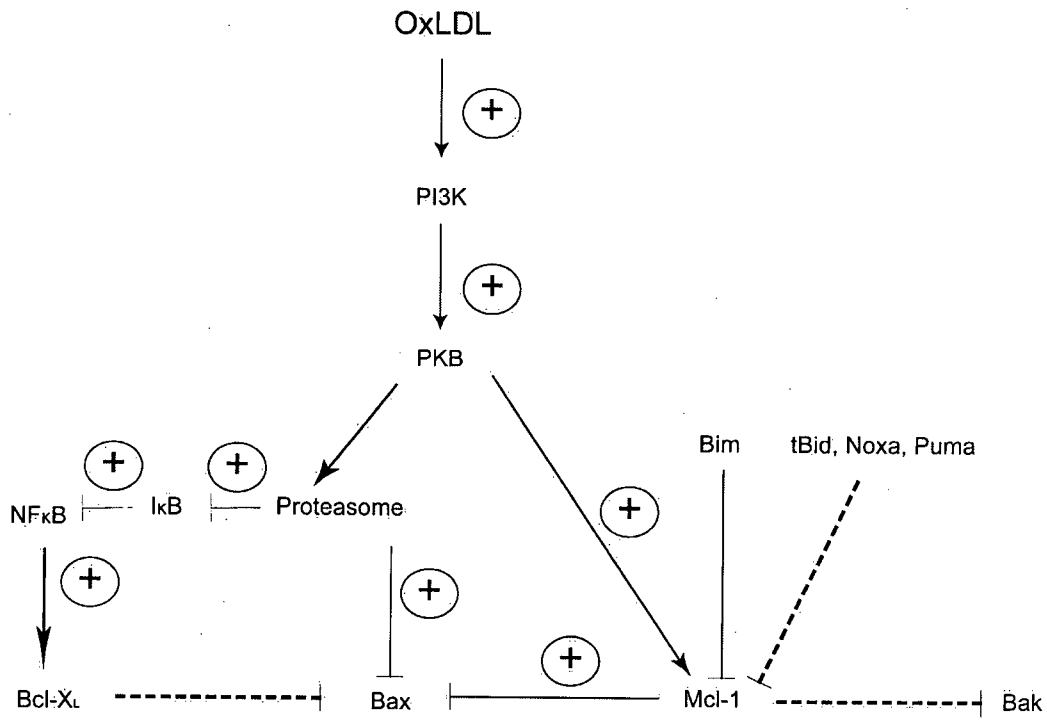

Figure 3.15 Proposed model of OxLDL regulation of Bcl-2 family members to mediate macrophage survival. OxLDL prevents macrophage apoptosis following M-CSF withdrawal by activating the PI3K/PKB pathway. PKB then mediates phosphorylation of IκB which marks the protein for its degradation by the proteasome and release NFκB. The freed NFκB is then available to maintain the expression of Bcl-X_L. PKB is also responsible for the proteasomal degradation of Bax and the upregulation of Mcl-1. Meanwhile, oxLDL also promotes Bax sequestration by Mcl-1. The dashed lines denote the postulated effect of oxLDL.

4 Pertussis toxin inhibits macrophage apoptosis via the PI3K/PKB pathway[b]

4.1 Introduction

In previous studies using mouse peritoneal macrophages we showed that oxidized low density lipoproteins (oxLDL), or lysophosphatidylcholine (LPC), stimulated the activity of phospholipase D (PLD). This enzyme activity generates phosphatidic acid (PA) from glycerophospholipids. PA is a potent mitogenic agent and second messenger that has been implicated in various pathophysiological processes, including atherosclerosis [369]. We found that PLD activation by oxLDL or LPC was inhibited by pertussis toxin, abbreviated PTX [48, 370]. PTX is a protein that is secreted by *Bordetella pertussis* and consists of five different subunits, designated S1 to S5. There are two S4 subunits, and so the complete toxin molecule is a hexamer [371]. PTX consists of an A subunit that carries the biologic activity and a B subunit that binds the complex to the cell membrane [371]. The S1 subunit constitutes the A protomer and the B oligomer is formed by the remaining five subunits [371]. PTX is a member of the family of ADP-ribosylating bacterial toxins. The S1 subunit of PTX ADP-ribosylates Cys352 of Gi. This modification of cysteine prevents G protein heterotrimers from interacting with receptors to block their coupling and activation. PTX has been widely used as a reagent to characterize the involvement of heterotrimeric G-proteins in cell signaling processes. The inhibitory effect of PTX on PLD activation by oxLDL or LPC suggested that Gi proteins were involved in this process.

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[b] A version of this chapter has been accepted for publication. Wang, S.W. et al (2007) Pertussis toxin promotes macrophage survival through inhibition of acid sphingomyelinase and activation of the phosphoinositide 3-kinase/protein kinase B pathway. Cellular Signaling 19(8):1772-83.

Some of the effects of PTX can be explained through actions other than G-protein inhibition. For example, PTX has been shown to use Toll-like receptor (TLR) 4 signaling to mediate some of its pathologic effects. It facilitates the break-down of the blood-brain barrier and is widely used as an adjuvant in experimental autoimmune encephalomyelitis [372]. TLRs are expressed on several cell types, including cells of the immune system [373]. One action of TLR4 signaling results in translocation of NF-kB, which induces transcription of a variety of genes, including those for pro-inflammatory cytokines. Therefore, some of the effects of PTX can be explained through actions other than G-protein inhibition.

In the present study, we show that PTX inhibits acid sphingomyelinase activation and the resulting accumulation of ceramides in bone marrow-derived macrophages. This is associated with inhibition of apoptosis that normally results from withdrawal of M-CSF from these cells. PTX caused phosphorylation of protein kinase B (PKB), activation of the transcription factor NFκB and up-regulation of Bcl-X_L, an anti-apoptotic Bcl-2 protein. These are the same mechanisms involved in the inhibition of apoptosis by oxLDL [72].

4.2 Results

4.2.1 Pertussis toxin can selectively protect macrophages from apoptosis induced by cytokine withdrawal

We have previously shown that BMDM undergo apoptosis if incubated in the absence of M-CSF for 24 hours or more, and that this is blocked by incubation with oxLDL [72]. To test if a G-protein-coupled membrane receptor(s) might be involved in oxLDL mediated cell survival, the G-protein inhibitor PTX, was used. Unexpectedly, we

found that control incubations done with PTX in the absence of cytokine or oxLDL also promoted cell viability in a dose-dependent manner in macrophages (Figure 4.1A). The concentration range of PTX used is in the same range as that reported previously for specifically inhibiting $G_{i/o}$ function in intact macrophages [374]. Furthermore, PTX treatment prevented DNA fragmentation (Figure 4.1B). This anti-apoptotic effect of PTX was cell type specific because FDCP-1, a mouse progenitor myeloid leukemia cell line whose survival is dependent on interleukin (IL)-3, did not respond to PTX (Figure 4.1).

4.2.2 Pertussis toxin inhibits ceramide generation in part by blocking acid sphingomyelinase activation after growth factor withdrawal

We previously showed that when bone marrow derived macrophages were cultured in the absence of M-CSF, acid sphingomyelinase (ASMase) activity and the level of ceramide increased and the cells became apoptotic [72]. We therefore tested the possibility that PTX promoted cell survival by inhibiting ASMase and ceramide generation. As shown in Figure 4.2A, ASMase activity was inhibited in a dose dependent manner by PTX. Ceramide levels increased by about 2.3 ± 0.4 fold (mean ± SD, n=4) in cytokine-deprived cells, and this was also blocked by PTX. The ability of ceramide to induce cell death was examined by treating the macrophages with exogenous C_2-ceramide. Figure 4.2B shows that C_2-ceramide caused a substantial decrease in macrophage viability. In addition, C_2-ceramide, but not its inactive analogue dihydro-C_2-ceramide obliterated the cytoprotective effect of PTX (Figure 4.2B). This supports the notion that ASMase-derived ceramides are likely to be responsible for the induction of apoptosis in BMDM incubated in the absence of M-CSF and that inhibition of ASMase is at least part of the mechanism whereby PTX exerts its anti-apoptotic action.

4.2.3 Mastoparan, a Gi agonist, induces cell death very rapidly.

Mastoparan, an activator of Gi_α subunits [375] that is found in wasp venom [376], acts directly on PTX-sensitive G protein [377] to stimulate guanine nucleotide exchange and GTP hydrolysis by a mechanism similar to that used by surface G protein-coupled receptors [378]. We employed mastoparan to determine if G protein activation induced macrophage apoptosis. Macrophages were treated with various concentrations of mastoparan in the presence of M-CSF. As seen in Figure 4.3A, mastoparan caused a sharp decrease in macrophage viability whereas the inactive analogue, mastoparan 17, had no effect. Flow cytometry results shown in Figure 4.3B demonstrate that DNA fragmentation and annexin V binding paralleled the changes in cell viability associated with the addition of mastoparan. Furthermore, as we have previously observed with oxLDL, caspases 3/7, 8, and 9 were all activated by treatment with mastoparan (Figure 4.3C). Finally, poly(ADP-ribose) polymerase (PARP), a substrate for activated caspase 3, was cleaved in response to mastoparan treatment but not in response to the inactive analogue, mastoparan 17 (Figure 4.3D). Although mastoparan has been reported to stimulate mitogenesis in the presence of growth factors including insulin-like growth factor-1, and platelet-derived growth factor [379], our results show that it induces apoptosis in macrophages.

4.2.4 Mastoparan activates ASMase in BMDM

Since apoptosis in macrophages incubated in the absence of M-CSF has been shown to be closely associated with increased ceramide levels and activation of ASMase [72], we tested to see whether apoptosis induced by mastoparan occurred through a similar mechanism. As shown in Figure 4.4A, ceramide accumulation was accompanied by ASMase activation, which was detected as early as 5 minutes after treatment with mastoparan. Ceramide levels increased over time after treatment with mastoparan but not with the inactive analogue mastoparan 17 (Figure 4.4B).

4.2.5 Pertussis toxin attenuates cell death induced by mastoparan

PTX-catalyzed ADP-ribosylation of Gi alpha can markedly inhibit mastoparan-stimulated GTPase activity [380] and some of its cellular and molecular effects [379, 381]. To examine whether PTX can overcome the pro-apoptotic effect of mastoparan, macrophages were pre-treated with PTX for 24 hours before the addition of mastoparan. ASMase activation was delayed by the addition of PTX (Figure 4.5A) and the increase in ceramide was attenuated (Figure 4.5B). Cell death after 4 or 8 hours of mastoparan treatment was prevented by PTX, as measured by DNA fragmentation. (Figure 4.5C). This further demonstrates that PTX-sensitive G-proteins play a role in macrophage apoptosis.

4.2.6 ADP-ribosylation is required for pertussis toxin to promote cell survival

The A subunit of the PTX holotoxin possesses the catalytic domain responsible for ADP-ribosylation of the α subunit of the heterotrimeric $G_{i/o}$ proteins. The B-oligomer

subunit is thought to mediate the binding of the toxin to cells [140]. To test if the enzymatic activity is required for PTX to provide cell survival, we compared the ability of the B-oligomer of PTX with that of the PTX holotoxin to promote survival of BMDM. As shown in Figure 4.6A, B-oligomer was unable to prevent the activation of ASMase or the increase in ceramide induced by growth factor withdrawal in macrophages. B-oligomer also did not prevent macrophage death (Figure 4.6B). These data support the conclusion that $G_{i/o}$ inhibition by ADP-ribosylation is required for the anti-apoptotic effects of PTX.

4.2.7 Toll-like receptor 4 may be involved in the anti-apoptotic effect of PTX in macrophages

It has been reported that TLR4 may be the receptor responsible for the effect of PTX on leukocyte recruitment [372]. We used macrophages from TLR4 deficient mice to test if TLR4 was responsible for transducing the pro-survival signal from PTX. After 24 hours of cytokine withdrawal, PTX failed to prevent apoptosis in BMDM lacking TLR4 (Figure 4.7A). Similar effects were observed after 48 hours (data not shown). PTX also did not prevent phosphatidylserine exposure, DNA fragmentation, ASMase activation or ceramide accumulation in TLR4 deficient cells (Figure 4.7B-D). This suggests that PTX acts at least partly through TLR4 to regulate ceramide production by ASMase and mediate cell survival.

4.2.8 Adenylyl cyclase is unlikely to contribute to macrophage apoptosis

PTX inhibits the activation of $G_{i\alpha}$,which normally prevents the generation of cAMP by adenylyl cyclase [382]. We and others have previously demonstrated that cAMP can inhibit apoptosis induced by various stimuli [383-385]. To test this possibility, 8-bromo-cAMP (a membrane-permeable analog of cAMP), or forskolin (a stimulator of adenylyl cyclase), were added to BMDM incubated in the absence of M-CSF. As shown in Figure 4.8A, modulating cAMP levels did not affect BMDM survival. This indicates that the effect of PTX on apoptosis is not through its effect on adenylyl cyclase.

4.2.9 The anti-apoptotic effect of PTX requires the activation of the PI3K/PKB pathway

Activation of ERK and PKB pathways has been reported to reduce apoptosis in several cell types [76, 77]. We previously reported that oxLDL stimulates both pathways in macrophages, but only the PI3K/PKB pathway was important for oxLDL-mediated survival [38, 39, 72]. Similarly, the PI3K pathway was important for sphingosine 1-phosphate-, or ceramide 1-phosphate-mediated survival in BMDM [100,51]. In the present study we tested to see if PTX could signal through these pathways to block apoptosis. As shown in Figure 4.8B, an increase in ERK1/2 phosphorylation was observed as early as 15 minutes after PTX treatment. However, the MEK inhibitors, UO126 and PD98095, did not significantly inhibit PTX-mediated macrophage survival (Figure 4.8C). PTX treatment caused phosphorylation of PKB at Ser473, which is a direct indication of its activation, after about 30 min of incubation (Figure 4.8D). The ability of PTX to prevent apoptosis was blocked by incubation of cells with the PI3K inhibitors, LY294002 and

wortmannin (Figure 4.8E). These results indicate that although PTX treatment results in the activation of both ERK1/2 and PKB, the pro-survival effect of PTX is mediated through activation of the PI3K/PKB pathway, similar to oxLDL [72].

4.2.10 Activation of NFκB is required for PTX to provide survival by regulating Bcl-X_L expression

The NFκB transcription factor is an important target of PKB [51, 386]. We previously demonstrated that M-CSF withdrawal results in NFκB inactivation and this is associated with decreased mRNA and protein levels of Bcl-X_L [51, 72]. We therefore determined whether PTX could regulate macrophage survival through activation of NFκB. First, we found that PTX stimulated phosphorylation of IκB (Figure 4.9A), which precedes the activation of NFκB [387]. We then examined the ability of PTX to induce the DNA binding activity of stimulated NFκB. This was performed using nuclear extracts and electrophoretic mobility shift assay (EMSA). Figure 4.9B shows that there is minimal basal binding of NFκB to DNA in apoptotic macrophages. In contrast, NFκB binding to DNA was significantly increased after exposure of the cells to PTX.

There are reports indicating that NFκB is the transcription factor for Bcl-X_L [388]. Over-expression of Bcl-X_L has been shown to provide protection toward apoptosis by preserving mitochondrial integrity [389]. We previously showed that oxLDL [72], sphingosine 1-phosphate [100] or ceramide 1-phosphate [51] can all enhance Bcl-X_L expression. As shown in Figure 4.9C, PTX restored Bcl-X_L expression to the same level as that in cells cultured in the presence of M-CSF. Taken together, these findings demonstrate that PTX is able to regulate the NFκB pathway, and the subsequent expression of anti-apoptotic Bcl-X_L. To evaluate whether NFκB was required for the inhibition of apoptosis by PTX, we tested

the effects of selective inhibitors on cell survival in the presence of PTX. As shown in Figure 4.9D, the NFκB inhibitors caffeic acid phenylethyl ester (CAPE) or SC-514 blocked the pro-survival effect of PTX in BMDM, suggesting that activation of this transcription factor is required for the antiapoptotic effect of PTX.

4.3 Discussion

There are several reports in the literature showing that some of the biological effects of oxLDL are sensitive to PTX. Whitman et al. demonstrated that uptake of acetylated LDL by macrophages is regulated by PTX-sensitive G proteins [390]. It was also reported that oxLDL-induced macrophage proliferation is mediated through a PTX-sensitive G-protein-coupled receptor [391] while another group documented that oxLDL induced cytotoxicity in vascular smooth muscle cells is mediated through PTX-sensitive G proteins [102]. Recent evidence showed that loss of a G-protein coupled receptor for lysophosphatidylcholine (LPC) resulted in macrophage accumulation in atherosclerotic lesions and that this may be mediated through the regulation of apoptosis [392]. These reports prompted this study to determine if PTX-sensitive G proteins are responsible for the inhibition of BMDM apoptosis by oxLDL. If that were the case, PTX would have induced apoptosis in cells incubated with oxLDL. The results presented here showed that PTX had the opposite effect, in that it protected BMDM from apoptosis induced by growth factor withdrawal, evidently by stimulating the same intracellular signaling pathways involved in the anti-apoptotic effect of oxLDL (Figure 4.10).

In previous studies we showed that apoptosis of BMDM induced by M-CSF withdrawal involves stimulation of ASMase and ceramide accumulation [72, 100]. Activation of ASMase seems to be essential for apoptosis in macrophages because

BMDM obtained from ASMase knockout mice were resistant to apoptosis upon M-CSF withdrawal (Please see next section, Figure 5.1). In addition, treatment of macrophages with C1P, which is a potent inhibitor of ASMase, prevented ceramide accumulation and blocked macrophage death [101], whereas incubation of the macrophages with the cell-permeable C_2-ceramide induced apoptosis. One major finding of the present studies is that PTX inhibits ASMase activation and the subsequent accumulation of pro-apoptotic ceramide. The second important observation is that PTX stimulates the PI3K/PKB pathway, which is a major mechanism by which growth factors promote cell survival. These effects of PTX are also consistent with previous observations by Testai et al [285] who have recently demonstrated that inhibition of PI3K leads to ASMase activation in oligodendrocytes.

Our results with mastoparan and B-oligomer suggest that Gi-proteins are involved in regulating ASMase activity and apoptosis in the macrophages. However, this is unlikely to be associated with an effect on the levels of intracellular cAMP since addition of 8-bromo-cAMP or stimulation of adenylyl cyclase with forskolin did not rescue macrophages from apoptosis.

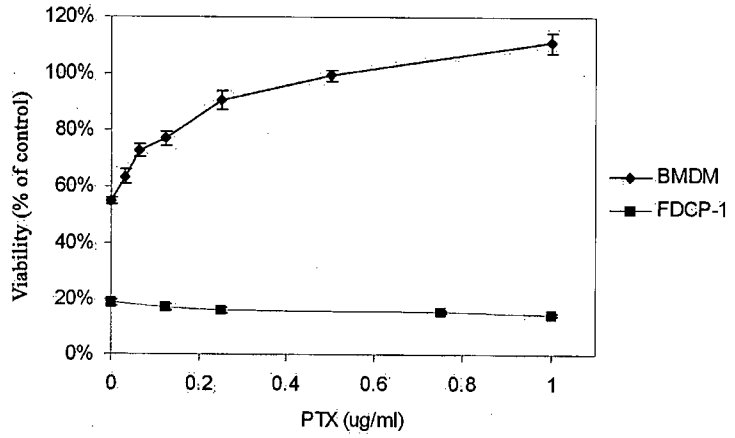
Some reports have suggested that ADP-ribosylation is not required for PTX to exert its function [56, 393, 394], but others showed that this action is essential for PTX to induce intracellular signaling [372, 390]. In this study, we show that in BMDM, ADP ribosylation of G_i proteins is required for PTX to provide macrophage survival, since the B oligomer of PTX was unable to mediate the same effects as intact PTX. We also show that PTX activates the PKB target NFκB, supporting our previous observation that PI3K is an important regulator of NFκB activation in BMDM [72, 100, 101]. Moreover, we found

that PTX upregulated Bcl-X_L, which is a downstream target of NFκB. This parallels the effects of oxidized LDL, sphingosine 1-phosphate, or C1P in BMDM, all of which cause upregulation of Bcl-X_L via activation of PI3K/PKB and inhibition of ASMase [51, 100, 101]. Inhibition of NFκB activation by the selective inhibitors CAPE or SC-514 abolished the anti-apoptotic effect of PTX suggesting that NFκB is required for PTX-mediated survival of macrophages. The above results provide the first evidence for a novel biological effect of PTX in the control of cell survival through inhibition of ASMase and stimulation of the PI3K/PKB/NFκB pathway in macrophages.

It has been reported that PTX can trigger a tyrosine kinase signaling cascade in myelomonocytic cells [395].There is evidence showing that tyrosine-kinase receptors and G proteins may converge on a common effector(s) necessary for the regulation of macrophage survival [396, 397]. This may be one of the mechanisms by which PTX signals to inhibit BMDM apoptosis. Another mechanism by which PTX might promote macrophage survival is through activation of TLR4 receptors. In fact, PTX has been found to co-immunoprecipitate with CD14 [398], which mediates the binding of lipopolysaccharide (LPS) to initiate signaling through TLR4. Also, it has been demonstrated that G_i proteins are coupled to the TLR4 signaling pathway in RAW264.7 cells [225], and that TLR4 is a receptor for PTX in nervous system autoimmune disease [372]. In the latter study, it was demonstrated that leukocyte recruitment induced by PTX is TLR4 dependent. PTX also induces dendritic cell maturation in a TLR4-dependent manner [56], and TLR4 stimulation activates NFκB through the PI3K/PKB pathway [399]. Our results are consistent with these observations in suggesting a cross-talk between TLR4 and Gi-protein signaling in the promotion of cell survival by PTX.

Recently a paper was published showing that ceramide activates TLR4 signaling, suggesting that this mechanism might allow pathogens to elicit TLR4 responses by perturbing sphingolipid receptors for virulence ligands [400]. LPS, a ligand for TLR4, is structurally similar to ceramide and stimulates some ceramide targets [401]. The fact that ceramide, and perhaps other related sphingolipids, can act as ligands for TLR4 adds a new dimension to the regulation of signal transduction processes by sphingolipids through toll-like receptors. However, results in Figure 4.7 suggest that at least under the conditions we used, TLR4 is not required for apoptosis or survival of BMDM in the absence or presence of MCSF.

A



B

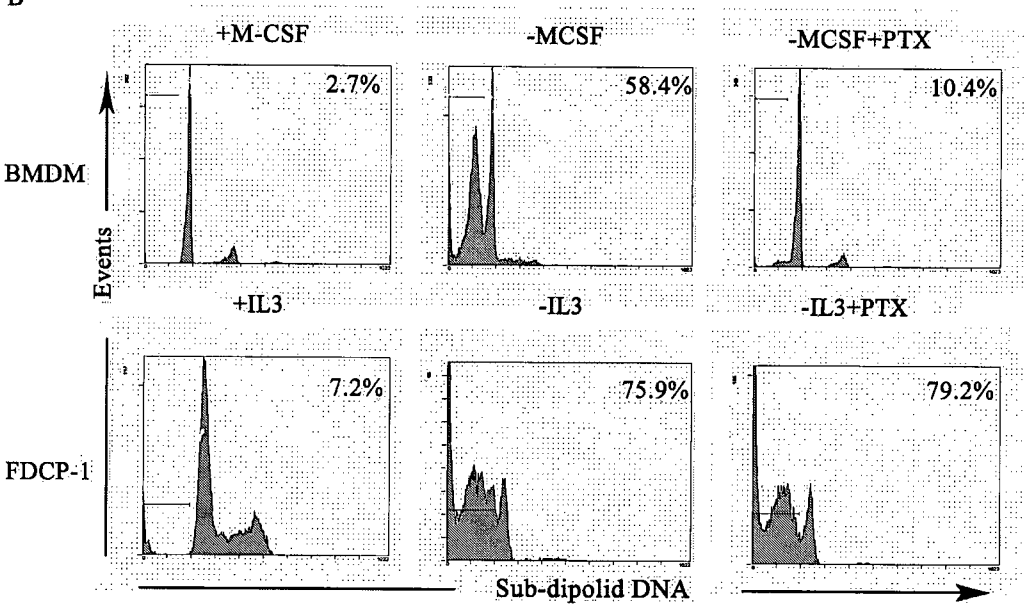


Figure 4.1 Pertussis toxin selectively protects macrophages apoptosis. (A) BMDM were seeded at $5x10^4$cells/well and FDCP-1 at $2x10^4$cells/well in 96-well plates. Cells were cultured in the absence of growth factors, but with PTX at indicated concentrations for 24 hours before adding MTS. Cell survival in the presence of M-CSF or IL-3 respectively was the reference for 100% survival. Data represent means ± SD of triplicate samples. Similar results were obtained in two replicate experiments. (B) BMDM or FDCP-1 cells were incubated in the presence or absence of respective growth factors with or without 0.5µg/ml of PTX for 24 hours. Cells were then stained for sub-diploid DNA with propidium iodide (PI) and analyzed by flow cytometry.

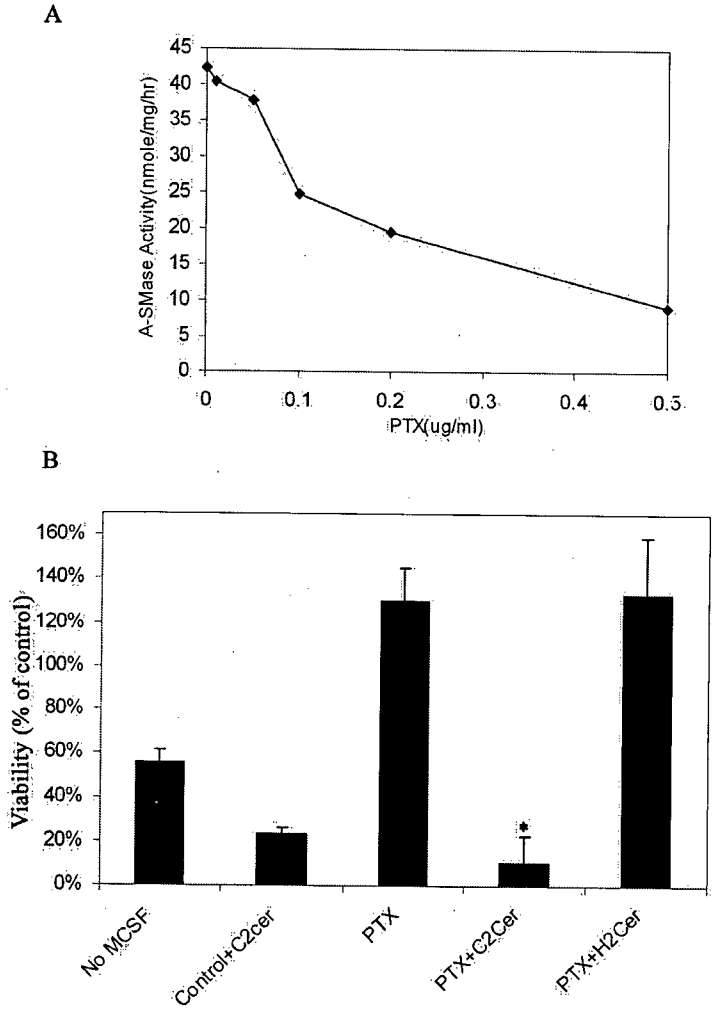
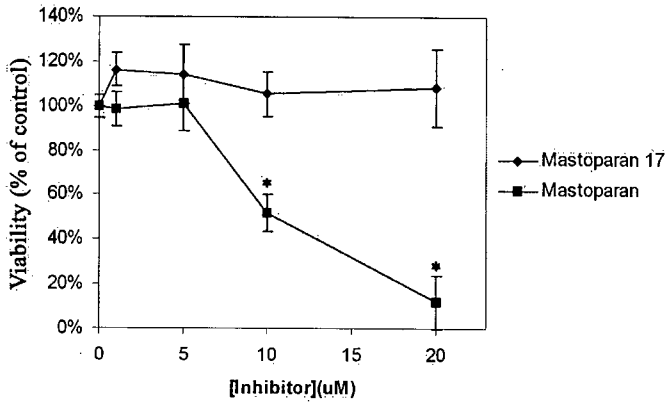
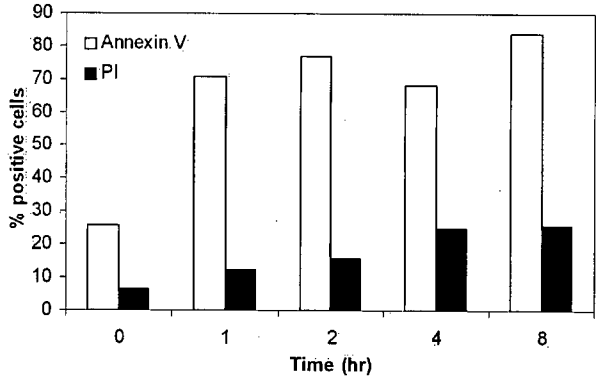


Figure 4.2 Pertussis toxin can inhibit ASMase activation and exogenous ceramide blocks the anti-apoptotic effect of pertussis toxin. (A) BMDM were cultured in the absence of M-CSF and with the indicated concentration of PTX for 24 hours. Lysates were assayed for ASMase activity. Results are representative of three experiments. (B) BMDM were cultured for 24 hours in the absence of M-CSF with or without 0.5μg/ml of PTX together with the indicated concentration of C_2-ceramide or dihydro-C_2-ceramide before the addition of MTS. Cell survival in the presence of M-CSF (control) was the reference for 100% survival. Data represent means ± SD of three experiments each in triplicate. *$p<0.005$ versus PTX alone.

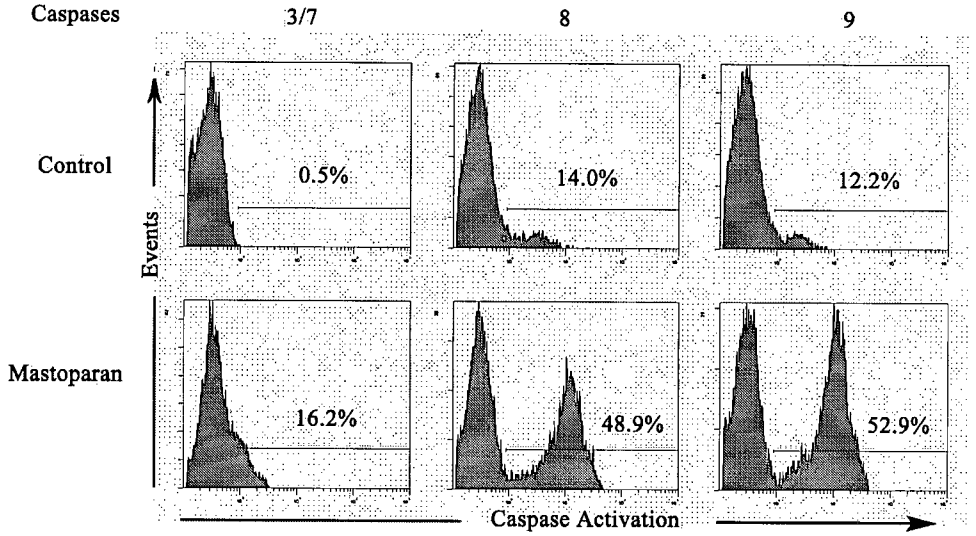
A


140%
120%
100%
80%
60%
40%
20%
0%
Viability (% of control)
0
5
10
15
20
[Inhibitor](uM)
Mastoparan 17
Mastoparan
*
*

B


90
80
70
60
50
40
30
20
10
0
% positive cells
Annexin V
PI
0
1
2
4
8
Time (hr)

C


Caspases
3/7
8
9
Control
Mastoparan
Events
0.5%
14.0%
12.2%
16.2%
48.9%
52.9%
Caspase Activation

D

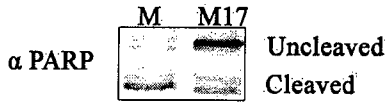
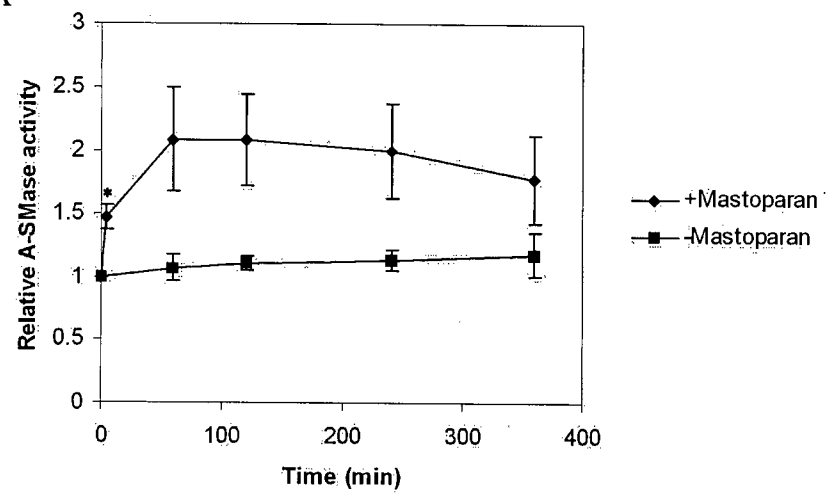

M
M17
α PARP
Uncleaved
Cleaved

Figure 4.3 Mastoparan induces apoptosis in macrophages. (A) BMDM were plated at $5x10^4$cells/well and incubated overnight. Mastoparan or mastoparan 17 was added in the presence of M-CSF and incubated for 24 hours before adding MTS. Cell survival with only M-CSF was the reference for 100% survival. Data represent means ± SD of three independent experiments performed in triplicate. *$p<0.005$ versus mastoparan 17. (B) BMDM were treated with 20μM mastoparan in the presence of M-CSF for the indicated time. Cells were then stained for sub-diploid DNA and for phosphatidylserine exposure. Results are representative of two experiments. (C) BMDM were incubated with or without 20μM of mastoparan for 4 hours, stained for activation of caspases 3/7, 8, or 9 and analyzed by flow cytometry. Results shown are representative of two experiments. (D) BMDM were cultured with 20μM of mastoparan or mastoparan 17 for 4 hours. Lysates were immunoblotted for PARP. Results are representative of two experiments.

A



B

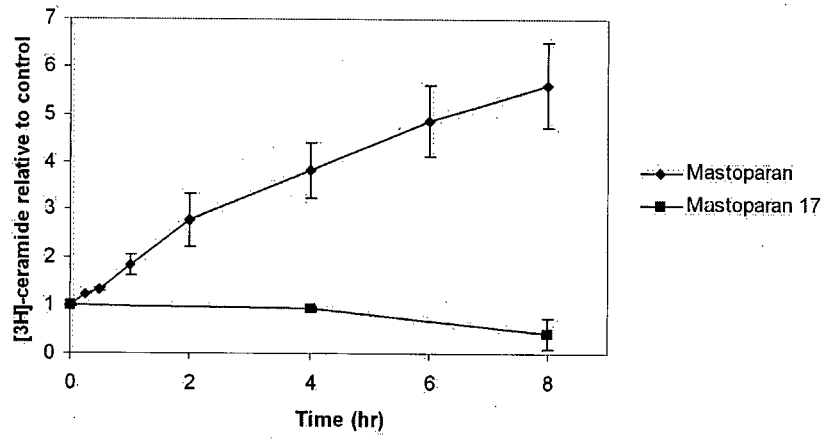
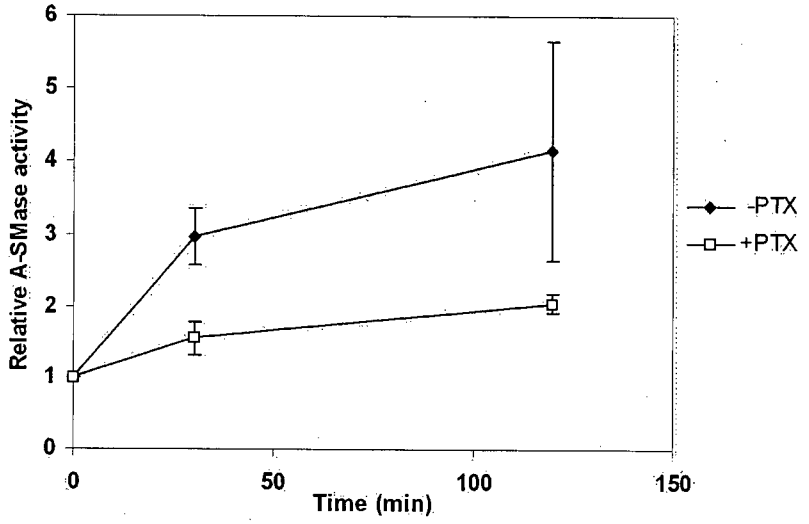
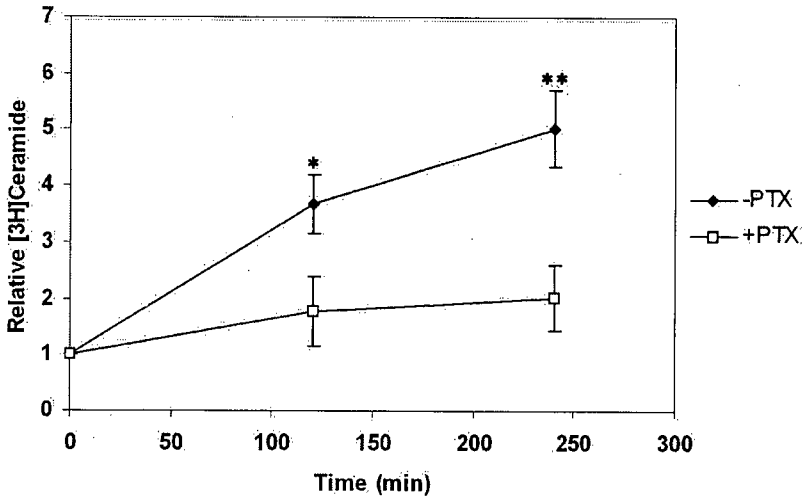


Figure 4.4 Mastoparan activates ASMase and increases ceramide levels in macrophages. (A) BMDM were incubated in medium containing M-CSF with or without 20μM of mastoparan, lysed by three cycles of freeze/thawing and assayed for ASMase. Results are means ± SD of two experiments, except the value at 5 minutes, which is the mean of three experiments. *$p<0.01$ versus zero minutes. (B) BMDM pre-labeled with [^{3}H]palmitate were treated with 20μM mastoparan or mastoparan 17 in medium containing M-CSF and ceramide levels were then determined. Data are means ± SD of two experiments done in duplicate. Results are the mean ± SD of two experiments.

A


Relative A-SMase activity
0
1
2
3
4
5
6
0
50
100
150
Time (min)
-PTX
+PTX

B


Relative [3H]Ceramide
0
1
2
3
4
5
6
7
*
**
0
50
100
150
200
250
300
Time (min)
-PTX
+PTX

C

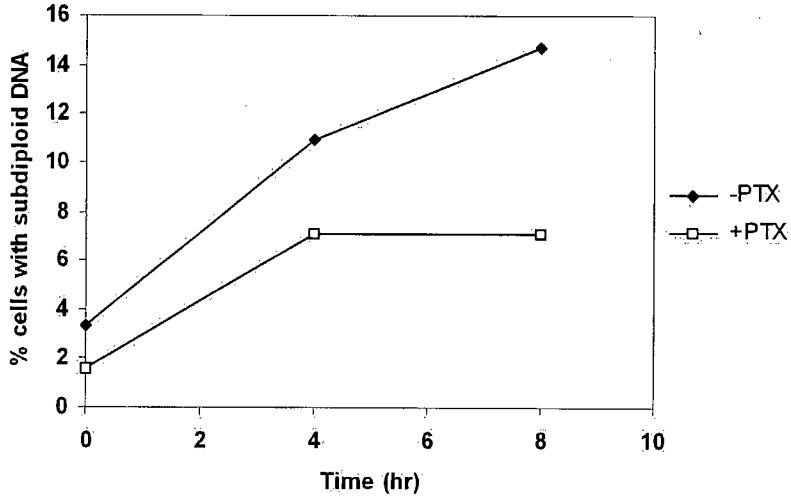
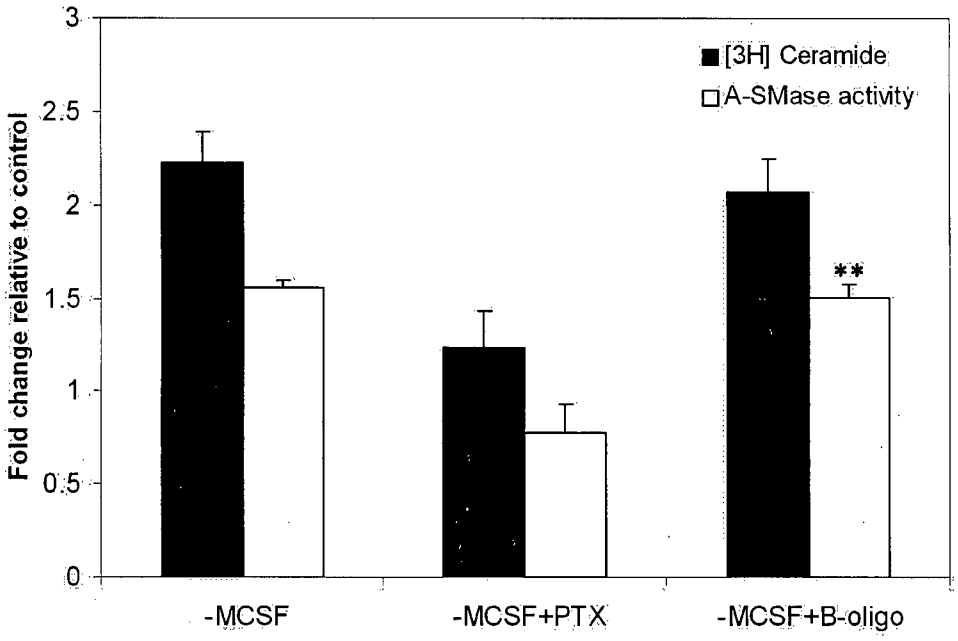

% cells with subdiploid DNA
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10
Time (hr)
-PTX
+PTX

Figure 4.5 Pertussis toxin confers partial resistance to mastoparan-induced cell death. (A) BMDM were pre-incubated with 1μg/ml PTX in the presence of M-CSF for 24 hours before the addition of 20μM of mastoparan. ASMase activity was then determined. Results are relative to time zero of mastoparan addition and are means ± SD of two experiments. (B) Cells were treated as in (A), and ceramide levels were determined. Results are relative to time zero of mastoparan addition and are means ± SD of three experiments done in duplicate. *$p<0.05$, **$p<0.01$ versus PTX treated at the respective times. (C) Cells were treated as in (A) and analyzed for sub-diploid DNA by PI staining and flow cytometry. Results are representative of two experiments.

A.


Fold change relative to control
3
2.5
2
1.5
1
0.5
0
[3H] Ceramide
A-SMase activity
**
-MCSF
-MCSF+PTX
-MCSF+B-oligo

B.

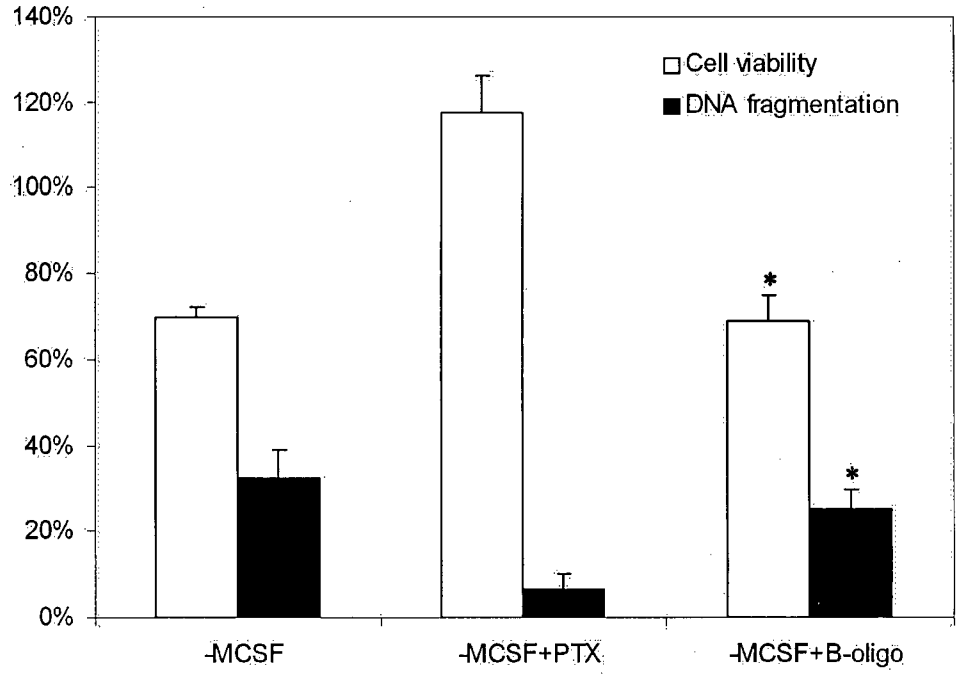
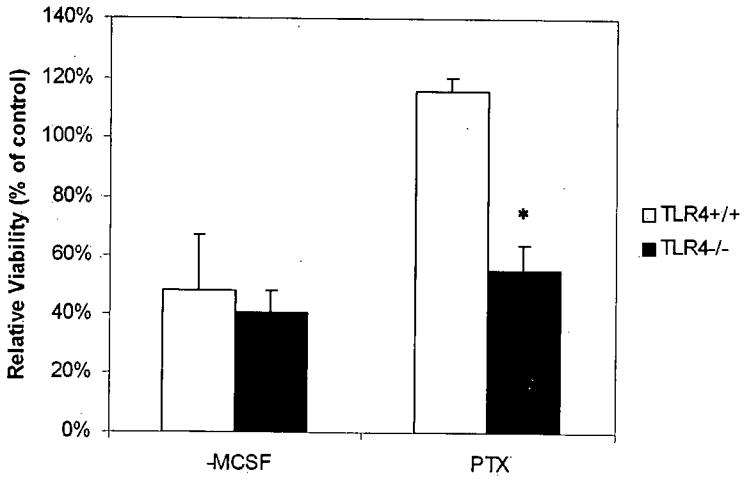
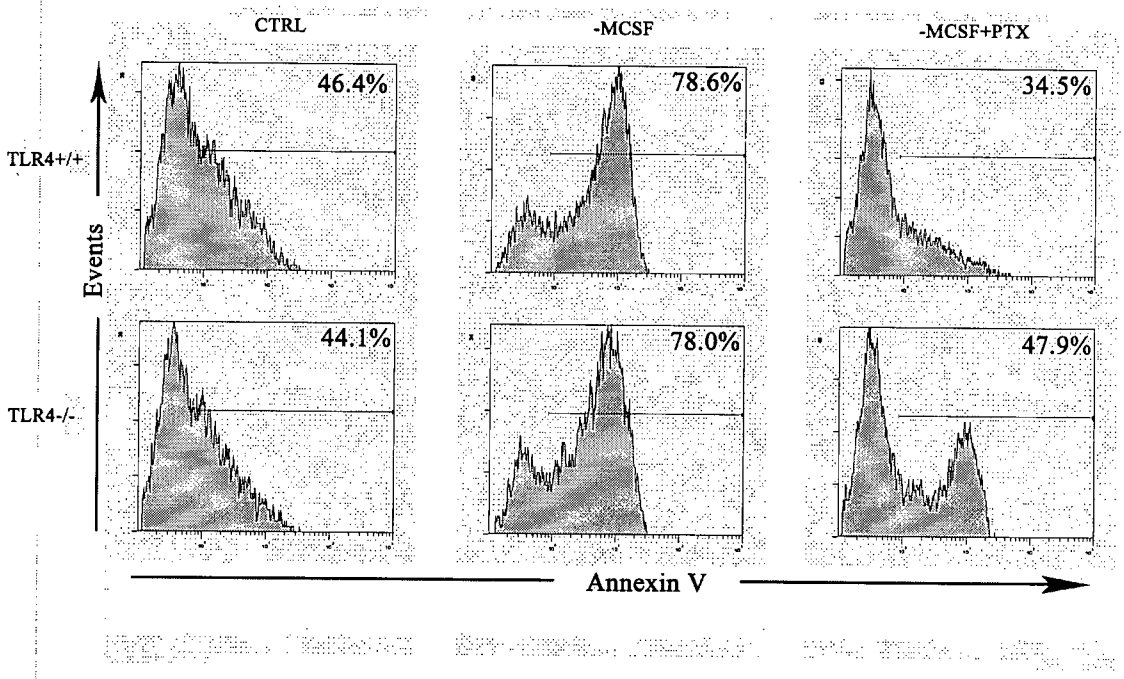

140%
120%
100%
80%
60%
40%
20%
0%
Cell viability
DNA fragmentation
*
*
-MCSF
-MCSF+PTX
-MCSF+B-oligo

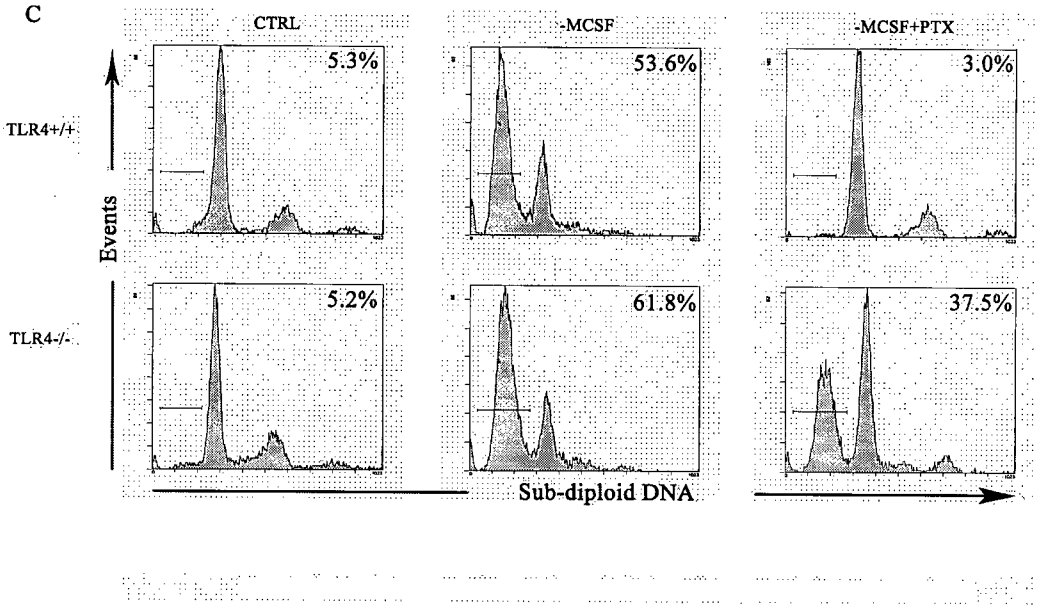
Figure 4.6 Enzymatic activity of PTX is required for inhibition of apoptosis. (A) BMDM were treated with 0.5μg/ml of PTX holotoxin or B-oligomer in the absence of M-CSF for 24 hours. Results for ASMase activity (open bars) and ceramide levels (solid bars) are expressed relative to cells incubated with M-CSF and are means ± SD of two (ceramide) or three (ASMase) experiments. **$p<0.01$ versus PTX treated. (B) For viability measurements, BMDM were seeded in 96-well plates and then incubated for 24 hours in the absence of M-CSF with 0.5μg/ml of PTX holotoxin or B-oligomer before adding MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means ± SD. Results are representative of two similar experiments performed in triplicate. For measuring cell death by DNA fragmentation, cells were treated as in (A), stained with PI and analyzed by flow cytometry. Data represent means ± SD of three experiments. *$p<0.05$ versus PTX.

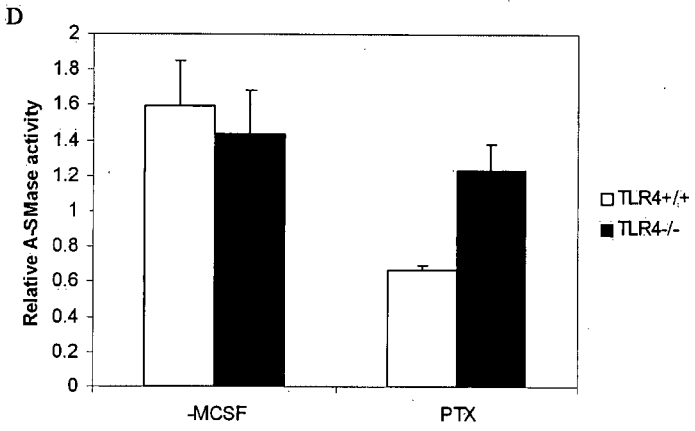
A


140%
120%
100%
80%
60%
40%
20%
0%
Relative Viability (% of control)
-MCSF
PTX
*
TLR4+/+
TLR4-/-

B


CTRL
-MCSF
-MCSF+PTX
46.4%
78.6%
34.5%
44.1%
78.0%
47.9%
TLR4+/+
TLR4-/-
Events
Annexin V


C
CTRL
-MCSF
-MCSF+PTX
TLR4+/+
TLR4-/-
Events
5.3%
53.6%
3.0%
5.2%
61.8%
37.5%
Sub-diploid DNA


D
Relative A-SMase activity
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-MCSF
PTX
TLR4+/+
TLR4-/-

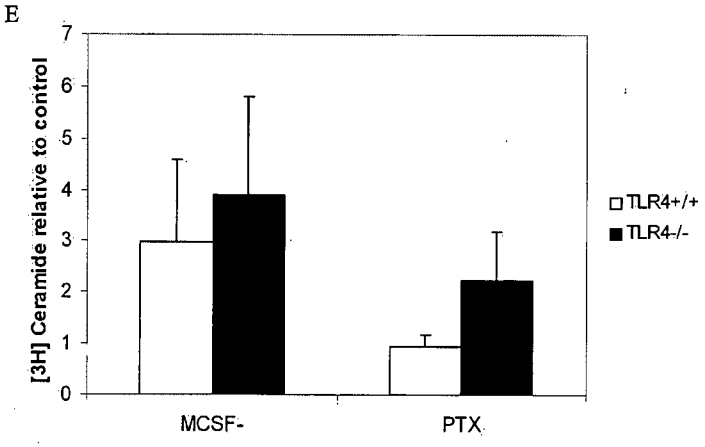
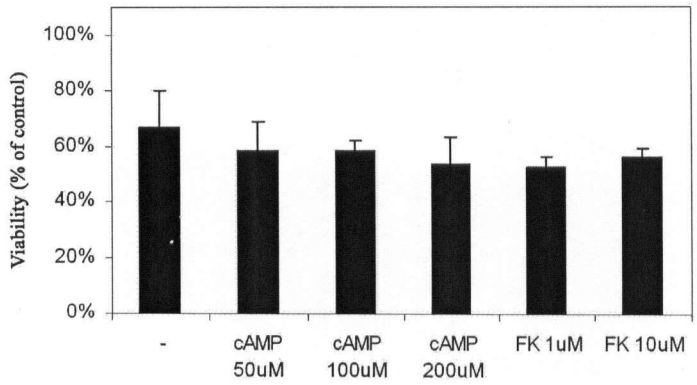
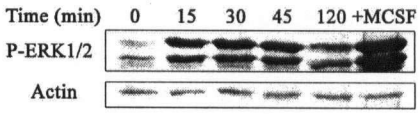

E
[3H] Ceramide relative to control
7
6
5
4
3
2
1
0
MCSF-
PTX
TLR4+/+
TLR4-/-

Figure 4.7 Pertussis toxin may signal through the TLR4 receptor to block apoptosis. (A) For viability measurements, BMDM were incubated with 0.5μg/ml of PTX in the absence of M-CSF for 24 hours before the addition of MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means ± SD of two experiments performed in triplicate. (B and C) BMDM from TLR4+/+ and TLR4-/- mice were incubated with 0.5μg/ml of PTX in the absence of M-CSF for 24 hours and then stained for phosphatidylserine exposure (B) or analyzed for sub-diploid DNA by flow cytometry (C). Results are representative of two similar experiments. (D) Macrophages were treated with 0.5μg/ml of PTX in the absence of M-CSF for 24 hours. Cells incubated with M-CSF were used as controls (CTRL). ASMase activity (D) or ceramide levels (E) were then determined. Results are expressed relative to control cells and are means ± SD of two (D) or three (E) experiments each in duplicate.

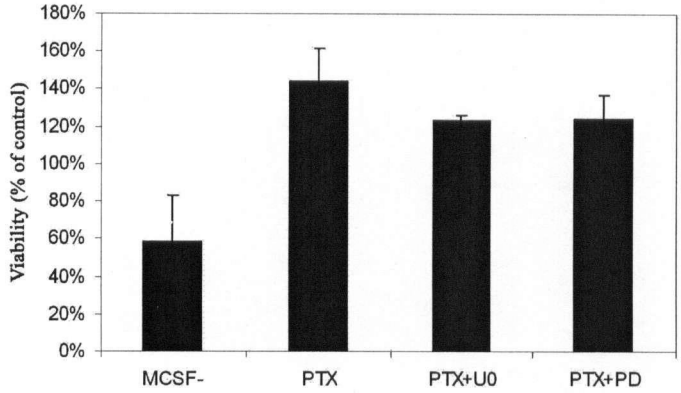
A


100%
80%
60%
40%
20%
0%
Viability (% of control)
-
cAMP 50uM
cAMP 100uM
cAMP 200uM
FK 1uM
FK 10uM

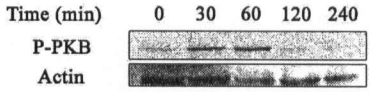
B


Time (min) 0 15 30 45 120 +MCSF
P-ERK1/2
Actin

C


180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
Viability (% of control)
MCSF-
PTX
PTX+U0
PTX+PD

D


Time (min) 0 30 60 120 240
P-PKB
Actin

E

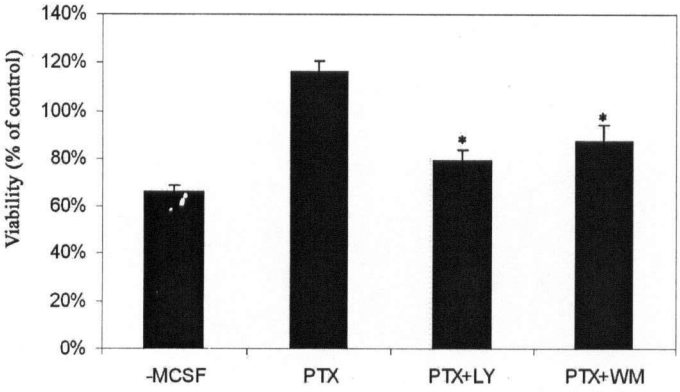
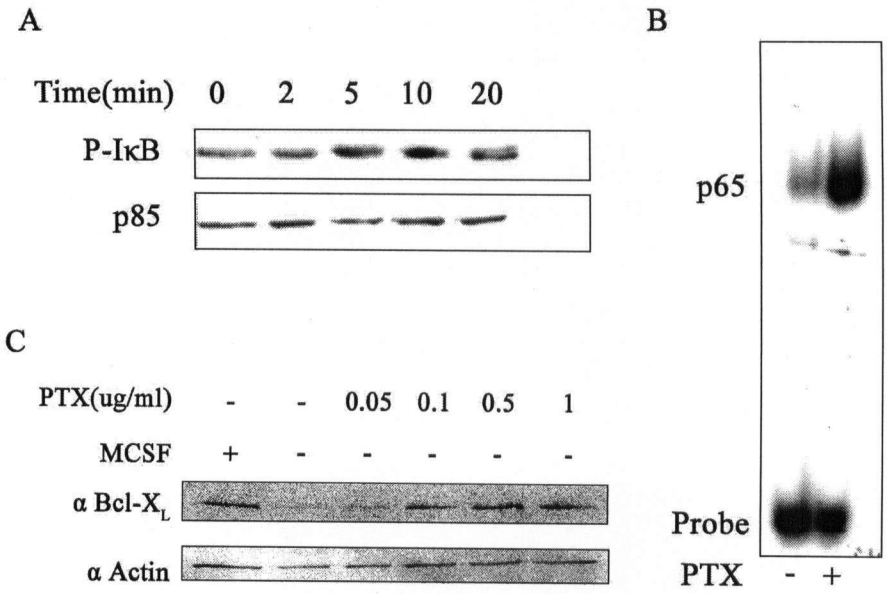

140%
120%
100%
80%
60%
40%
20%
0%
Viability (% of control)
*
*
-MCSF
PTX
PTX+LY
PTX+WM

Figure 4.8 PKB is the major pathway required for the anti-apoptotic effect of PTX. (A) BMDM were placed in M-CSF-deficient medium containing 0.5μg/ml of PTX with or without the indicated concentration of 8-bromo-cAMP (cAMP) or forskolin (FK) for 24 hours before addition of MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means ± SD of triplicates. Similar results were obtained in each of two replicate experiments. (B) BMDM were deprived of M-CSF for 4 hours before stimulation with 0.5μg/ml PTX for the indicated time. Cell lysates were blotted for phospho-ERK1/2 and actin as loading control. Results shown are representative of two experiments. (C) BMDM in M-CSF-deficient medium were treated with 0.5μg/ml of PTX with or without 2μM U0126 or 10uM PD98095 for 24 hours before addition of MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means ± SD of three experiments performed in triplicate. (D BMDM were deprived of M-CSF for 4 hours before addition of 0.5μg/ml of PTX for the indicated time. Cell lysates were blotted for phospho-PKB and actin as loading control. Results shown are representative of two experiments. (E) BMDM in M-CSF-deficient medium were treated with 0.5μg/ml of PTX with or without 5μM LY294002 or 100nM wortmannin for 24 hours before addition of MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means ± SD of three experiments performed in triplicate. *$p<0.001$ versus PTX alone.


A
Time(min) 0 2 5 10 20
P-IκB
p85
B
p65
Probe
PTX - +
C
PTX(ug/ml) - - 0.05 0.1 0.5 1
MCSF + - - - - -
α Bcl-X$_L$
α Actin

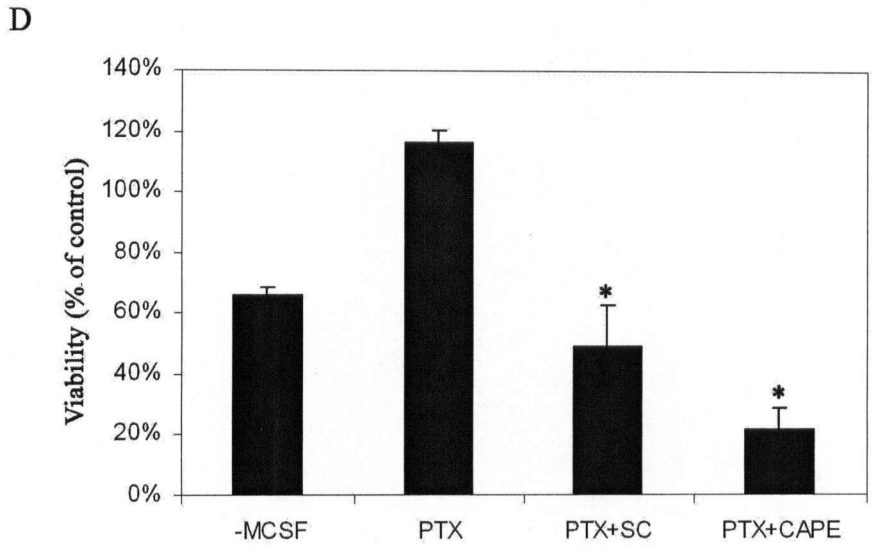

D
Viability (% of control)
140%
120%
100%
80%
60%
40%
20%
0%
-MCSF
PTX
PTX+SC
PTX+CAPE
*
*

Figure 4.9 Pertussis toxin signals through NFκB to mediate macrophage survival. (A) BMDM were deprived of M-CSF for 4 hours and then treated with 0.5μg/ml of PTX for the indicated time. Cell lysates were blotted for phospho-IκB and p85, as loading control. Similar results were obtained in each of three experiments. (B) Nuclear NFκB DNA binding activity was determined by EMSA after stimulation of BMDM for 6 hours with or without 0.5μg/ml PTX in the absence of M-CSF. Similar results were obtained in two experiments. (C) Macrophages were cultured in the presence or absence of M-CSF with the indicated concentration of PTX for 30 hours. Lysates were immunoblotted for Bcl-X_L, or for actin as loading control. Results shown are representative of two experiments. (D) BMDM were treated with 0.5μg/ml of PTX with or without SC514 or CAPE for 24 hours before addition of MTS. Cell survival in the presence of M-CSF was the reference for 100% survival. Data represent means ± SD of three experiments performed in triplicate. *$p<0.001$ versus PTX alone.

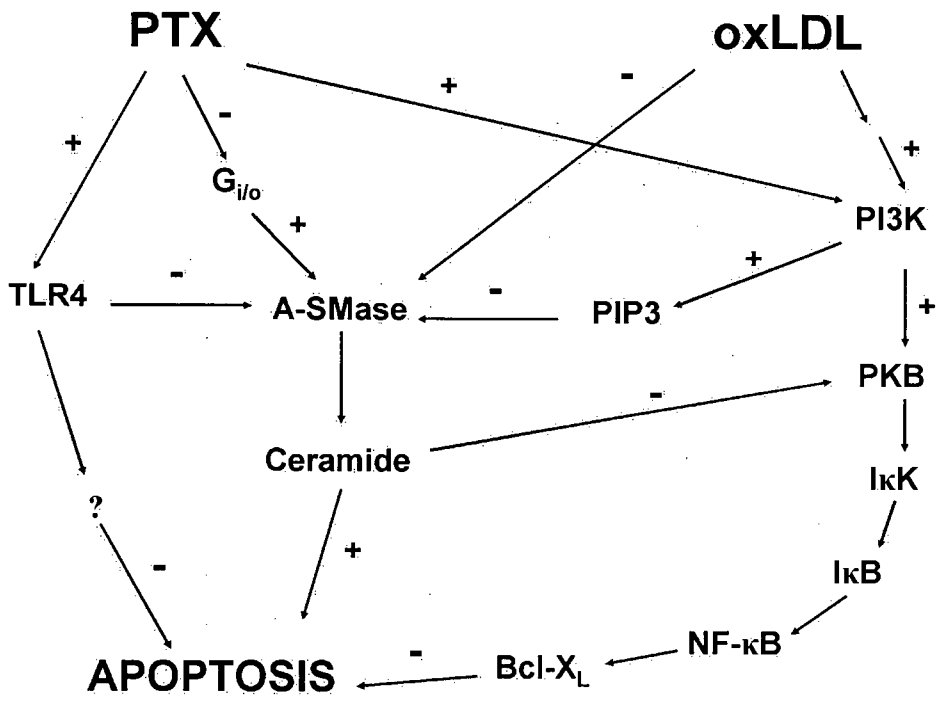


Figure 4.10 A working model of PTX induced macrophage survival. Like oxLDL, PTX prevents macrophage apoptosis following M-CSF withdrawal by at least two primary mechanisms. One is by activating the PI3K/PKB pathway. PKB-mediated phosphorylation of IκB leads to the release and activation of NFκB. This maintains Bcl-X_L expression, which leads to the inhibition of the caspase cascade and subsequent apoptosis. The second one is by inhibiting ASMase, thereby preventing ceramide generation, a process mediated through $G_{i/o}$ proteins. PTX also acts through TLR4 on unidentified targets to regulate apoptosis.

5 Regulation of ceramide generation during macrophage apoptosis

5.1 Introduction

Ceramide is a key mediator of apoptosis triggered by various stimuli such as ionizing radiation, TNFα, chemotherapeutic agents and Fas ligand [229, 402, 403]. Ceramide is thought to induce apoptosis either as a second messenger or by modulating membrane structure and dynamics. Some evidence suggests that ceramide may mediate raft clustering into macrodomains for transmembrane signaling, or alternatively, it may promote mitochondrial membrane permeability and channel formation for cytochrome c release [191].

Ceramide can be generated from the hydrolysis of sphingomyelin (SM) via the activity of sphingomyelinase (SMase). Acid sphingomyelinase (ASMase) is a lysosomal enzyme that belongs to a family that also includes neutral and alkaline SMases [196]. Deficient ASMase activity is the cause of human type A and B Niemann-Pick disease (NPD) in which SM degradation is impaired [301]. It has been shown that ASMase activity is essential for ceramide-mediated apoptosis. For example, cells from NPD patients or ASMase-/- mice were resistant to ionizing radiation with regard to ceramide generation and apoptosis [302, 404, 405]. Furthermore, ASMase-/- mice also showed defects in ceramide generation and apoptosis in lung endothelium [404] and throughout the central nervous system [406]. Interestingly, thymic cells from ASMase-/- mice remain sensitive to apoptosis induced by ionizing radiation [404].

Ceramide can also be produced from the *de novo* synthesis pathway regulated by enzymes such as serine palmitoyltransferase (SPT) and ceramide synthase (CS). SPT catalyzes the condensation of serine and palmitoyl-CoA while CS acylates sphinganine

to produce dihydroceramide, which is then desaturated to give ceramide. *De novo* synthesis of ceramide has been implicated in responses to TNFα [232], heat shock [407], exogenous ceramide [408], and several chemotherapeutic agents [209, 210].

Recently, we showed that ASMase activation and ceramide generation were involved in apoptosis of bone marrow-derived macrophages (BMDM) induced by growth factor withdrawal [72]. To further investigate ceramide production in cells undergoing apoptosis, we used BMDM generated from ASMase-/- mice. In the present study we demonstrate that loss of ASMase confers partial resistance to apoptosis, with less ceramide being generated in response to growth factor withdrawal. In addition, the *de novo* pathway of ceramide synthesis is implicated in the accumulation of ceramide in BMDM undergoing apoptosis.

5.2 Results

5.2.1 ASMase is only partly responsible for ceramide generated in response to M-CSF withdrawal in BMDM

We have previously shown that increased ceramide production following M-CSF withdrawal was due to the activity of ASMase [72]. Inhibition of ASMase activity by oxLDL or desipramine increased cell viability [72]. To further elucidate the role ASMase plays in the BMDM apoptosis, we used ASMase knockout mice generated in Dr. R. Kolesnick's laboratory [409]. As one would predict, in ASMase knockout cells, the increase in ceramide content as well as the cell death resulting from cytokine withdrawal were less than that in wild type cells (Figure 5.1). ASMase-/- BMDMs also showed less caspase 9 activation in response to M-CSF withdrawal (Figure 5.2B), and were partially protected from apoptosis as reflected by DNA fragmentation (Figure 5.2A).

5.2.2 Ceramide generation in ASMase-/- cells is unlikely to arise from degradation of sphingomyelin

It is noteworthy that in ASMase -/- cells, ceramide generation was only partially blocked even though these cells were expected to be totally deficient in ASMase. To verify that ASMase activity was undetectable in the knockout cells, we performed *in vitro* assays of SMase activity in cell lysates. Results in Figure 5.3A confirm that ASMase activity in wild type cells increased in response to M-CSF withdrawal while this activity was absent in ASMase-/- cells. Ceramide generation in response to stimuli such as TNF has been attributed to NSMase activity [410], and therefore we also assayed NSMase in the lysates. However, as seen in Figure 5.3B, NSMase activity in ASMase-/- cells is low and actually decreases in response to cytokine withdrawal so it is unlikely to account for the ceramide generation by means of SM hydrolysis in ASMase knockout cells.

5.2.3 Accumulation of ceramide mass from *de novo* synthesis upon M-CSF withdrawal

As we failed to find an increase in SMase activity in ASMase -/- macrophages after M-CSF deprivation, the obvious alternative explanation for increased ceramide radioactivity was that ceramide synthesis was increased. Ceramide production in response to death stimuli such as daunorubicin [210], etoposide [209], heat shock [407] or photodynamic therapy [215] has been reported to be due to accelerated *de novo* synthesis, not increased SM degradation. As demonstrated in Figure 5.4, ceramide mass increased rapidly after M-CSF withdrawal. Fumonisin B1 (FB1), an inhibitor of ceramide synthase, blocked the accumulation of ceramide after M-CSF withdrawal.

Congruent results were obtained when [^{3}H] palmitoyl-ceramide was monitored. Incubation of FB1 in the absence of M-CSF almost completely abolished the increase in [^{3}H] palmitoyl-ceramide in ASMase-/- BMDM (Figure 5.5). Incubation with SPT inhibitor, myriocin (Myr) also inhibited M-CSF withdrawal-induced ceramide increase in ASMase-/- BMDM. Both inhibitors also reduced the [^{3}H] palmitoyl-ceramide accumulation in wild type BMDM although not to the level seen in control cells incubated with M-CSF. The residual ceramide in these cells likely reflects the action of ASMase.

5.2.4 *De novo* production of ceramide is not dependent on serine palmitoyltransferase (SPT) but ceramide synthase (CS) activities

The findings that the production of ceramide upon cytokine withdrawal can be attenuated by inhibitors of SPT and CS led us to investigate the activities of SPT and CS. SPT is the rate limiting enzyme for *de novo* ceramide synthesis [208] and its activity has been shown to be required for ceramide increase during etoposide-induced apoptosis [209]. However, as shown in Figure 5.6, there was no change in SPT activity in BMDM at the end of 24- hour incubation in response to cytokine withdrawal and only a small increase in CS activity was observed. A possible explanation for this unexpected result is presented in the Discussion.

5.2.5 Ceramide-1-phosphate inhibits ceramide generation despite the absence of ASMase

We previously showed that ceramide-1-phosphate (C1P) can inhibit ceramide generation observed in BMDM when M-CSF is absent [101]. Exogenous C1P inhibited

ASMase in BMDMs at concentrations that also prevented apoptosis [101]. To determine whether C1P also inhibits *de novo* ceramide synthesis, we treated both ASMase+/+ and ASMase-/- BMDM with C1P. Ceramide accumulation was dramatically inhibited (Figure 5.7A). C1P also rescued cells from cytokine apoptosis after M-CSF withdrawal (Figure 5.7B).

5.3 Discussion

In this study, we have shown that in the absence of ASMase, BMDM generated less ceramide and were partially resistant to apoptosis after M-CSF withdrawal. However, there was still ceramide generation even though we confirmed there was no ASMase and a decrease in NSMase activity in ASMase-/- cells. We concluded that the ceramide accumulation was likely due to *de novo* synthesis in these cells.

Our observation that the resistance to apoptosis in ASMase-/- was not 100% (Figure 5.1) is probably because the role of ASMase in apoptosis is dependent upon the type of stress and may also be cell type specific. This is supported by the observation of Lozano et al that ASMase-/- murine embryonic fibroblasts (MEFs) were completely protected from radiation-induced apoptosis but only partially resistant to low serum induced cell death and offered no protection to staurosporine treatment [411]. Moreover, ASMase is essential for chemotherapy-induced apoptosis in oocytes [334] but not required for testicular ceramide production or for the ability of the germ cells to undergo apoptosis [412].

Without significant SMase activity in ASMase-/- cells, it is unlikely the ceramide accumulation is from SM hydrolysis. Using inhibitors for SPT and CS, FB1 and Myr, we confirmed that the *de novo* synthesis pathway also contributed to ceramide generation

upon cytokine withdrawal (Figures 5.4B & 5.5). However, results to measure SPT and CS activities at the end of the 24 h incubation were inconclusive, as the changes seemed insufficient to explain the observed increase in ceramide. There are reports demonstrating that the changes in ceramide level can be biphasic in certain conditions [234, 413]. Sumitomo et al [233] reported that etoposide induced early ceramide increase was due to the transient and rapid activation of *de novo* pathway while the ceramide level was sustained in the longer term by the activity of SMase. Thus it is possible that CS activity increased transiently in our model but we only measured a mild increase after 24 hours of cytokine withdrawal. This may also explain our observation that FB1 was able to significantly block ceramide mass accumulation within 6 hour as in Figure 5.5B but only attenuate ceramide generation in the ASMase+/+ at 24 hour as in Figure 5.4. Similarly, SPT activity was observed to be activated within 15 minutes by etoposide treatment in Molt-4 cells [209]. Although we did not observe a change in SPT activity (Figure 5.6), its inhibitor, myriocin reduced incorporation of [^{3}H] palmitate into ceramide (Figure 5.5). Hence, it is possible that SPT as well as CS were transiently activated in the first few hours.

Under normal pathways of sphingolipid synthesis, ceramide is considered an intermediate rather than an end product. It serves as a precursor for assembly of more complex sphingolipids such as sphingomyelin and glucosylceramide. Ceramide may accumulate if its conversion to complex sphingolipids is blocked, for example by inhibition of sphingomyelin synthase (SMS) and glucosylceramide synthase (GCS). Interestingly, ceramide itself is reported to inhibit SMS [414]. Whereas SPT and CS reside on the endoplasmic reticulum [415], SMS and GCS are located on the Golgi apparatus and/or plasma membrane [416, 417]. Therefore another regulatory point for accumulation of

newly synthesized ceramide during apoptosis in BMDM may be at the level of ceramide transport.

C1P was able to promote cell survival and block the ceramide generation despite the absence of ASMase (Figure 5.7). In addition to its effect on inhibiting ASMase activity in BMDM [101], C1P also stimulates PI3K to phosphorylate PKB to promote survival [51]. It was demonstrated that besides inhibition of ASMase, PI3K can also activate GCS and SMS to reduce ceramide production [418]. It is reasonable to expect that in the absence of ASMase, C1P still activates PI3K/PKB and possibly GCS and SMS to reduce ceramide production.

Ceramide synthesis and metabolism is a complex process. Besides the enzymes discussed here, there are still many regulatory enzymes that might be involved in modulating the concentration of this molecule. For example, cytokine withdrawal may activate ceramide kinase to produce C1P, which was shown by us and others to be pro-survival [51, 101]. A recent report demonstrated the involvement of dihydroceramide desaturase in cell cycle progression [419]. Another potential regulator is sphingosine kinase which generates the mitogenic metabolite sphingosine-1-phosphate that can inhibit ceramide production and block apoptosis in BMDM upon M-CSF withdrawal [100]. These additional effects could work in concert with ASMase to regulate ceramide levels and cell survival. For example, a recent observation showed that CS activation depended on ceramide generated by ASMase activity [234]. Therefore, although the individual contributions of either pathway of ceramide generation may vary with cell type, they appear to play complementary roles.

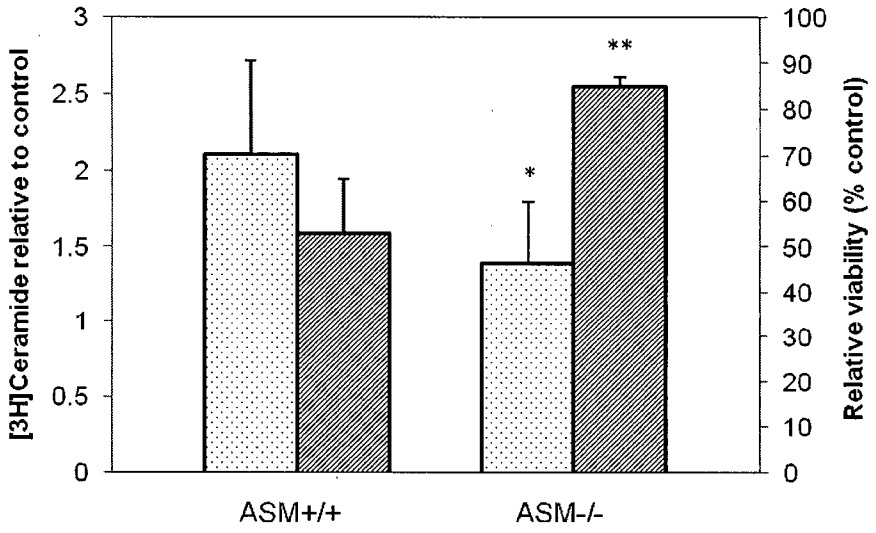


Figure 5.1 ASM deficiency confers partial resistance to cytokine withdrawal-induced apoptosis and ceramide increase. (A) ASMase +/+ and ASMase-/- BMDM were incubated with [^{3}H]palmitate and without M-CSF for 24 hours. Cells labeled in the presence of M-CSF served as the control. Ceramide was then isolated by TLC and counted. Radioactivity in ceramide relative to that in control cells was then calculated. Data are means ± SD of six independent experiments done in duplicate (*dotted bars*). BMDM from ASMase +/+ and ASMase-/- were seeded at 5x10^{4}cells/well and in 96-well plates overnight to allow cells to adhere. Cells were cultured in the absence of growth factors for 24 hours before adding MTS. Cell survival in the presence of the respective cytokine was the reference for 100% survival. Data represent means ± SD of three experiments performed in triplicate (*hatched line bars*). *$p<0.05$, **$p<0.01$ vs. ASM+/+ cells.

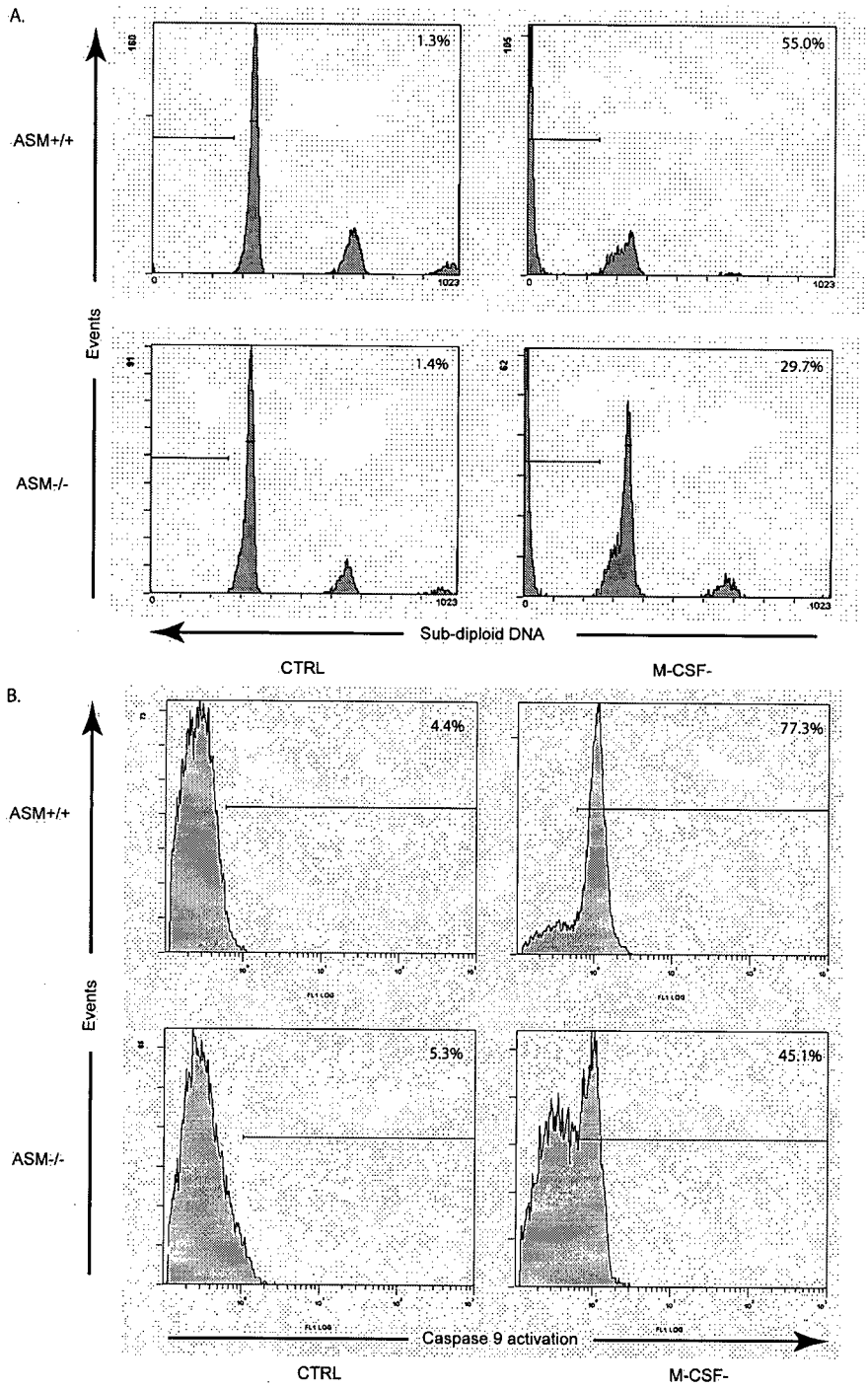
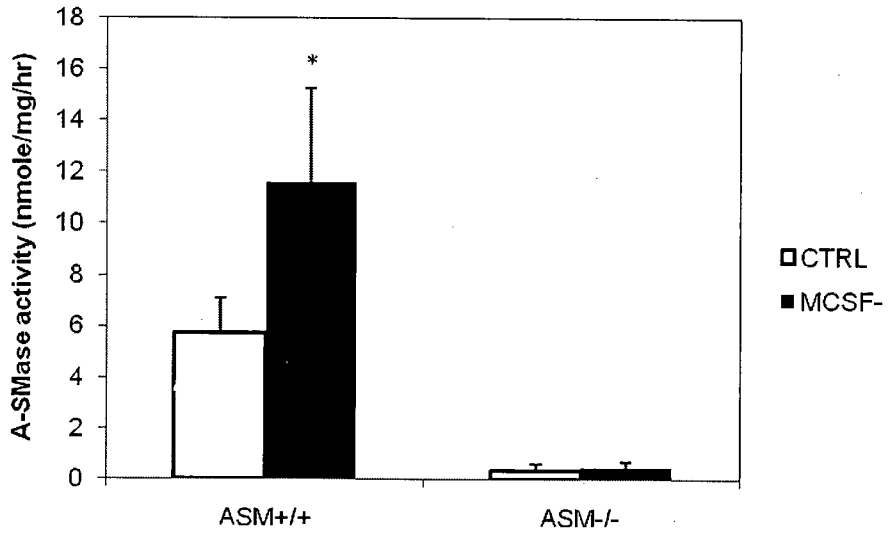


Figure 5.2 ASM deficiency confers partial resistance to cytokine withdrawal induced DNA fragmentation and caspase 9 activation. (A) BMDM from ASMase +/+ and ASMase-/- were incubated in the presence or absence of M-CSF for 24 hours. Cells were then stained for sub-diploid DNA with propidium iodide (PI) and analyzed by flow cytometry as described in Materials and Methods. (B) BMDM from ASMase +/+ and ASMase-/- were incubated with or without M-CSF for 24 hours, stained for activation of caspase 9 and analyzed by flow cytometry. Results shown are representative of two independent experiments.

A.



B.

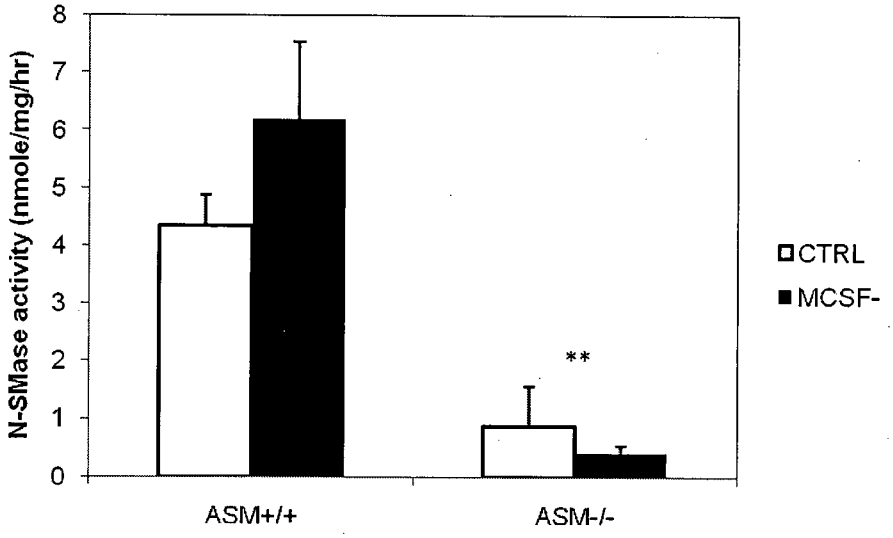


Figure 5.3 Ceramide generated in ASMase -/- BMDM is not due to SM hydrolysis. BMDM were cultured in the presence or absence of M-CSF for 24 hours. Lysates were assayed for ASMase (A) and NSMase (B) activity as described in Materials and Methods. Results were means ± SD of at least three independent experiments. *$p<0.05$ vs. control in ASM+/+ cells; **$p<0.01$ vs. ASM+/+ cells.

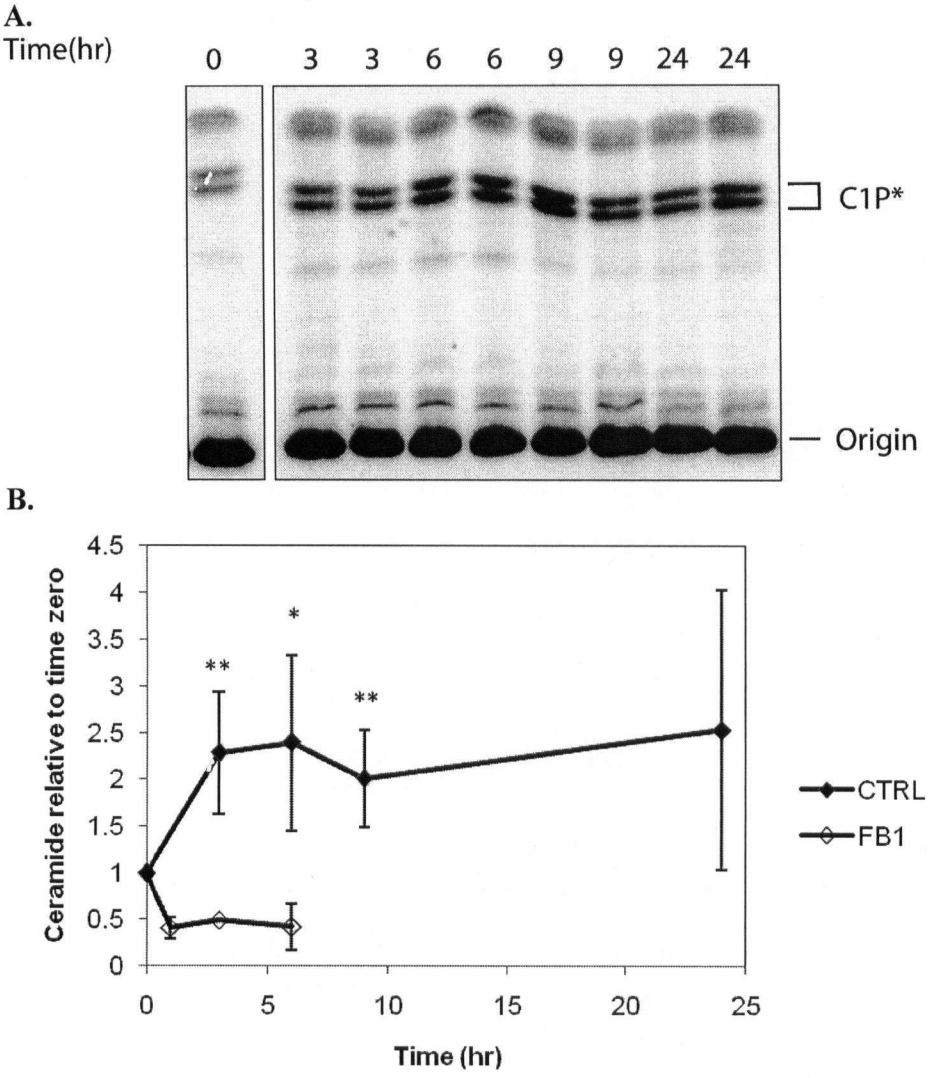


Figure 5.4 Time course for the change in ceramide mass after M-CSF withdrawal.
ASMase+/+ BMDM were cultured without M-CSF for 0 to 24 hours. At the indicated times, the cells were harvested, and ceramide mass was quantified using the diglyceride kinase assay and normalized to lipid phosphate. ^{32}P-labeled lipids were separated by TLC and quantified with a phosphorimager. (A) shows a scan from one of three independent experiments. (B) shows means ± SD of three experiments done in duplicate, except 24 hour time point is of two experiments and FB1 is of one experiment in duplicate. *$p<0.05$, **$p<0.01$ vs. time zero.

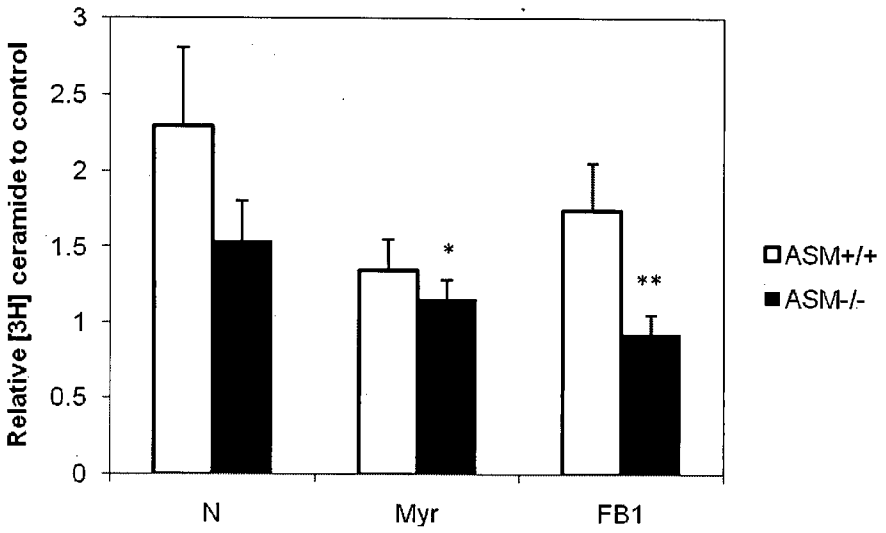


Figure 5.5 Inhibitors of the *de novo* ceramide synthesis pathway are able to block ceramide production in ASMase-/- BMDM. ASMase +/+ and ASMase-/- BMDM were incubated with [^{3}H]palmitate and without M-CSF for 24 hours in the absence or presence of 100 nM Myr or 50uM FB1. Ceramide was then isolated by TLC and counted. Cells incubated in the presence of M-CSF served as control. Radioactivity in ceramide relative to that in control cells was then calculated. Data are means ± SD of three independent experiments done in duplicate. *$p<0.05$, **$p<0.01$ vs. control.

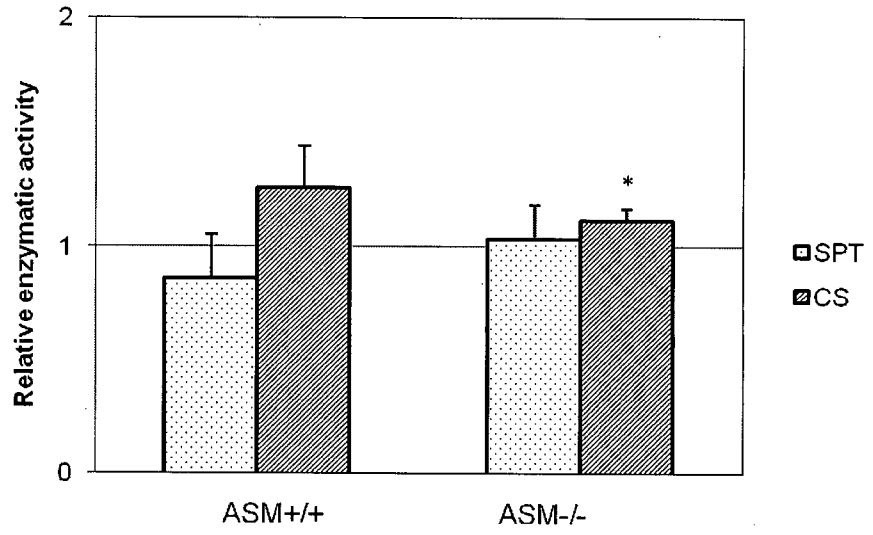
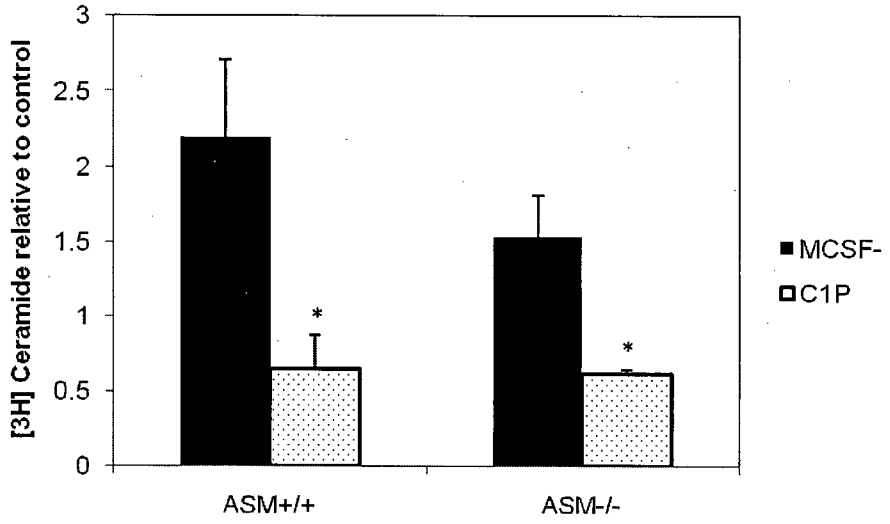


Figure 5.6 SPT is unlikely to be the enzyme responsible for the *de novo* synthesized ceramide during macrophage apoptosis. ASMase +/+ and ASMase-/- BMDM were cultured in the absence or presence of M-CSF for 24 hours. BMDM cultured in the presence of M-CSF are used as control. The microsomes were isolated and used to determine the *in vitro* SPT and CS activity as described in materials and methods. Data are expressed as fold change in the absence of MCSF relative to control and as means ± SD of five and three independent experiments for SPT and CS respectively.*$p<0.05$ vs. control.

A.



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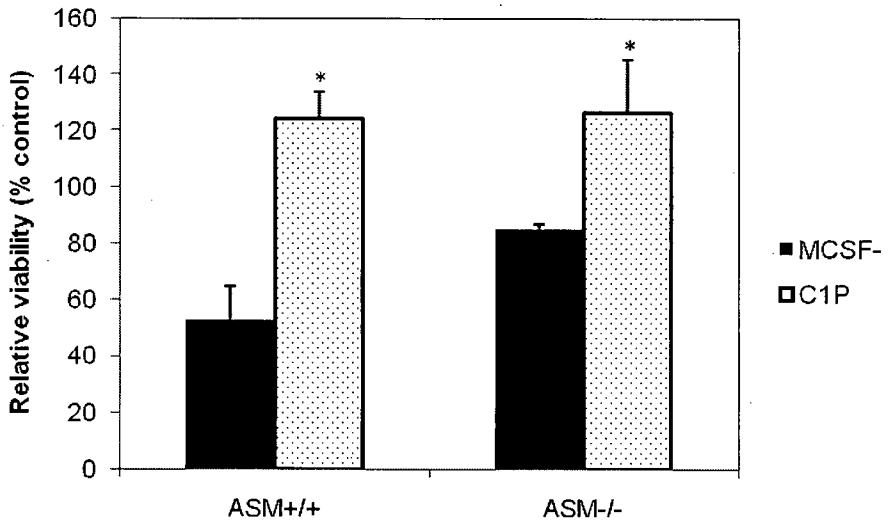

Figure 5.7 C1P can inhibit the ceramide generation and promote cell survival independent of ASMase. (A) For ceramide level, ASMase +/+ and ASMase-/- BMDM were labeled with [^{3}H]palmitate when M-CSF was withdrawn in the absence or presence of 30 uM C1P for 24 hours. Cells labeled in the presence with M-CSF served as control cells. Ceramide was then isolated by TLC and counted. Data are means ± SD of three independent experiments done in duplicate.*$p<0.05$ vs. MCSF-. (B) BMDM from ASMase +/+ and ASMase-/- were seeded at $5x10^4$cells/well and in 96-well plates overnight to allow cells to adhere. Cells were cultured in the absence of growth factors with or without 30 uM C1P for 24 hours before adding MTS. Cell survival in the presence of the respective cytokine was the reference for 100% survival. Data represent means ± SD of four experiments performed in triplicate (*closed bars*). *$p<0.01$, vs. MCSF-.

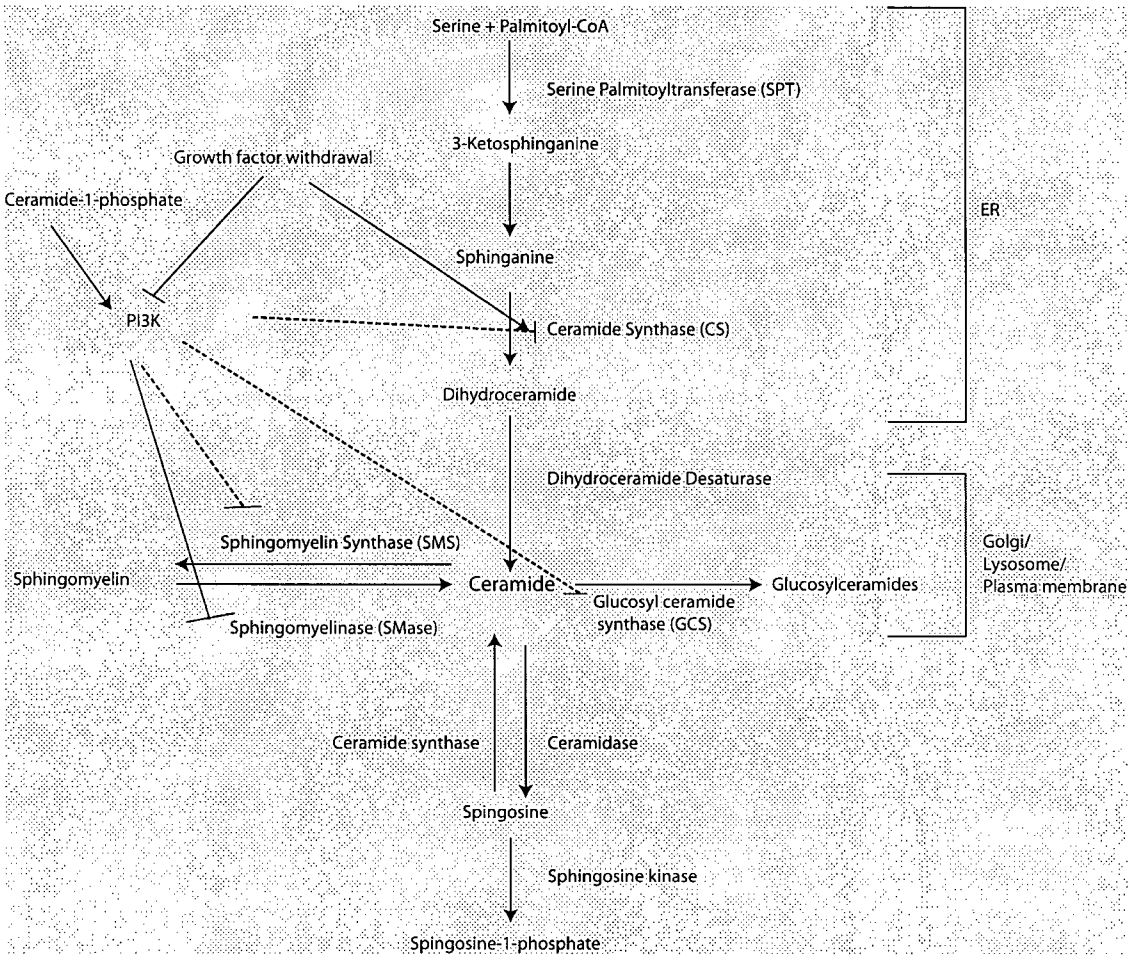

Figure 5.8 A working model of ceramide generation pathways in response to cytokine withdrawal. Ceramide generation induced by cytokine withdrawal can be regulated by the activation of sphingomyelinase. It can also be regulated by increasing *de novo* synthesis of ceramide. It is also possible ceramide accumulation is due to decrease synthesis of complex sphingolipids by inhibition of SMS and/or GCS activities. All these enzymatic activities may be controlled by PI3K.

6 Summary

At the outset of my studies, the aim was to investigate the events that regulated apoptosis of BMDM following cytokine starvation, as well as the survival of BMDM in the presence of oxidized LDL. In particular, the focus was on the family of proteins that are key to the regulation of the intrinsic pathway of apoptosis, the Bcl-2 family of proteins. An initial hypothesis pursued was that incubation with oxLDL may regulate the expression of multiple members of this family. In fact, we found that oxLDL induced Bax degradation as well as increased Mcl-1 expression in BMDM to promote cell survival. Both of these effects were mediated through the PI3K/PKB pathway.

A recent model proposed that BH3-only proteins promote apoptosis by displacing pro-survival Bcl-2 family members resulting in the release of Bax and Bak. Since we did not observe any change in the interaction between Bim and Mcl-1, it is possible that Bim may act to displace other pro-survival family members to cause the release of Bax and Bak. Because Bcl-2 was not found to interact with Bax and Bak, Bcl-X_L may be a candidate to explore, especially since we showed that Bcl-X_L is involved in oxLDL mediated macrophage survival against cytokine withdrawal induced apoptosis [72]. Other BH3-only proteins, such as Bad or Noxa may also play a role in macrophage apoptosis. In UV induced apoptosis, Bad was shown to displace Bcl-X_L while Noxa was shown to bind to Mcl-1, allowing Bak to be free to induce apoptosis [170]. OxLDL may promote macrophage survival by regulating these proteins and their interactions.

Regulation of Mcl-1 level by oxLDL is also worth exploring for therapeutic purpose in atherosclerosis. In cancer therapy, several reports have pointed out that the Mcl-1 level in cancer cell lines is the determinant of the efficacy of the BH3 mimetic

chemotherapeutic agent, ABT-737 [188, 420]. OxLDL may regulate Mcl-1 levels at the translational level by facilitating its translation. It may also increase the stability of Mcl-1 proteins. It was recently demonstrated that a BH3 like protein, MULE, is able to bind to Mcl-1 and promote its degradation [421]. It would be interesting to see which mechanism oxLDL employs to regulate Mcl-1 level.

Next, a collaborative study with Dr. Anton Gómez-Muñoz followed up on an unexpected observation with PTX in BMDM. In the initial experiment, we used PTX to test if part of the survival effect of oxLDL involved G-protein coupled signalling. This hypothesis appeared to be incorrected, as incubation of PTX with BMDM in the absence of serum or oxLDL promoted cell survival. In this thesis, I demonstrated that this effect of PTX was mediated through the PI3K/PKB signalling cascade, G_i proteins and TLR4.

Finally, our laboratory has been interested in a number of aspects of sphingomyelin and ceramide metabolism. We were able to obtain ASMase-deficient mice, from which we isolated BMDM. These macrophages had enhanced survival compared to wild-type BMDM, probably because they can not generate ceramide from sphingomyelin when starved of cytokine. However, the ASMase-deficient cells still generated some ceramide under these conditions, due to an increase in ceramide synthesis. Experiments in these cells allowed us to demonstrate that ASMase and *de novo* ceramide synthesis work in concert to regulate ceramide generation during macrophage apoptosis. This was important because apoptosis induced by some other stimuli was abrogated in cells from the ASMase-/- mice [411].

While we showed the enzymatic inhibitors for SPT and CS can reduce ceramide generation in response to cytokine withdrawal, *in vitro* enzymatic activities after 24 hour

withdrawal were not impressive. We propose this may be due to the transient activation of these enzymes. A shorter time course to probe for transient activation of these enzymes should be investigated. Enzymes downstream of the *de novo* pathway for synthesis of complex sphingolipids, such as GCS and/or SMS may also be involved in macrophage apoptosis (Figure 5.8). Their activities may be reduced during apoptosis and therefore contribute to the increased ceramide levels. Furthermore, like ASMase, oxLDL may also be able to regulate enzymes in the *de novo* pathway to inhibit ceramide generation to promote survival and this regulation may also be under the control of the PI3K/PKB pathway.

It is interesting how various agnoists can contribute to macrophage survival through different mechanisms. OxLDL is able to do so by down-regulating the pro-apoptotic Bax and increasing the anti-apoptotic Mcl-1 expression. PTX is able to promote survival not only through GPCR but also partially through TLR4. C1P is able to promote survival through the inhibition of ceramide generation even in the absence of ASMase. Each of these individual events cannot completely explain the physiological response of the macrophage, but PI3K/PKB seems to play a central role in regulating all these events. In the future, being able to quantify the importance of each event and how PI3K/PKB can selectively control the outcome of macrophage apoptosis versus survival will provide a valuable tool for diseases intervention such as for atherosclerosis.

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