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THE ROLE OF CASPASES IN LYTIC MECHANISMS OF TUMOR  
NECROSIS FACTOR-MEDIATED CYTOLYSIS

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## ABSTRACT

TNF activates several signaling pathways, some lead to cell death by apoptosis or necrosis. For our cell lines (B/C-N, 10ME, and L88.3), which represent a tumor progression model, it has not been determined whether TNF induces apoptosis or necrosis. B/C-N is not tumorigenic and represents normal cells; it is TNF-resistant. 10ME forms tumors only in immunodeficient animals and thus is intermediate on the pathway to cancer; it is TNF-sensitive. L88.3 forms tumors in normal mice and thus is cancerous; it is TNF resistant. TNF resistant cells can be rendered TNF-sensitive by addition of transcription or translation inhibitors. Recent findings indicate that TNF-induced cytolysis involves several initiator and effector caspases. Caspase comprise a family of proteases known to be involved apoptotic cell death. Thus, we investigated the correlation between TNF-mediated cytolysis and caspases activation in our cell lines.

Our findings indicated that TNF induces activation of two initiator caspases (caspase-8 and caspase-9) and an effector caspase (caspase-3). Caspase-8 was activated first and may be involved in activating caspase-9. This suggests there is a link between the death receptor pathway (associated with caspase-8) and the mitochondrial pathway (associated with caspase-9). Activity levels of caspases correlated with TNF sensitivity of each cell line. TNF-induced resistance mechanisms also affect the level of caspase activities. In TNF-resistant cell lines B/C-N and L88.3, addition of a translation inhibitor caused an increase in TNF-mediated cytolysis and caspase activity. However, in order to see an increase in TNF-mediated cytolysis, activity of caspase-3, -8 and -9 had to reach a threshold-level. When caspase activities were inhibited, TNF treatment

activated a caspase-independent lytic pathway, perhaps involving generation of oxygen radicals or activation of phospholipase A<sub>2</sub>. In TNF-sensitive 10ME, inhibition of caspases caused a switch from apoptotic death to necrotic death in some cells. These experiments also revealed the presence of caspase-dependent and caspase-independent resistance mechanisms.

Thus, the data indicate that TNF induces apoptosis through a caspase-dependent lytic mechanism. When caspase activities are inhibited, TNF induces a caspase-independent lytic mechanism. TNF also induces caspase-dependent and caspase-independent resistance mechanisms.

## TABLE OF CONTENTS

Acknowledgements .....	iii
Abstract.....	v
Table of contents.....	vii
List of Tables.....	xi
List of Illustrations.....	xii
List of Abbreviations .....	xvi
CHAPTER 1: INTRODUCTION.....	1
1.1 TUMOR NECROSIS FACTOR.....	1
1.1.1 <i>Background</i> .....	1
1.1.2 <i>TNF receptors</i> .....	2
1.1.3 <i>TNF signaling pathway through TNF-R1</i> .....	4
A. TNF-R1 signaling through adaptor molecules .....	8
B. Protective mechanism through NF- $\kappa$ B .....	11
1.2 CASPASES.....	14
1.2.1 <i>Background</i> .....	14
1.2.2 <i>Structure of caspases</i> .....	14
1.2.3 <i>Activation of caspases</i> .....	15
1.2.4 <i>Caspases and apoptosis</i> .....	17
1.2.5 <i>Caspases mediated cleavage of cellular proteins</i> .....	20
1.2.6 <i>Caspase inhibitors</i> .....	24

A. Natural or endogenous inhibitors .....	24
B. Synthetic peptide inhibitors .....	25
1.3 HEAT SHOCK PROTEINS .....	27
1.3.1 <i>Background</i> .....	27
1.3.2 <i>HSPs and cell death</i> .....	30
1.4 TUMOR PROGRESSION MODEL .....	32
1.5 OBJECTIVES .....	33
CHAPTER 2: MATERIALS AND METHODS.....	35
2.1 <i>Medium and cell lines</i> .....	35
2.2 <i>Reagents</i> .....	35
2.3 <i>Heat shock treatment and caspase inhibitors pretreatment</i> .....	36
2.4 <i>In vitro chromium release assay</i> .....	37
2.5 <i>Caspase enzyme activity assay</i> .....	39
2.6 <i>Microscopic observation of target cell morphology</i> .....	42
CHAPTER 3: CASPASE ACTIVATION IS ONE OF THE LYTIC PATHWAYS UTILIZED IN TNF-MEDIATED CYTOTOXICITY.....	43
3.1 <i>Caspase activities peaked around 10 to 12 hours of TNF         treatment</i> .....	48
3.2 <i>Increase in TNF-mediated cytolysis due to addition of CHX         correlates with increase in caspase activation</i> .....	53
3.2.1 B/C-N .....	54
3.2.2 10ME .....	57

3.2.3 L88.3 .....	60
3.2.4 L929 .....	62
3.2.5 Discussion: increase in TNF-mediated cytotoxicity due to addition of CHX correlates with increase in caspase activities .....	65
3.3 <i>Preincubation with pan-caspase inhibitor z-VAD-fmk suppressed caspase activities in B/C-N, 10ME, L88.3 and L929; however, z-VAD-fmk increased TNF-mediated cytotoxicity in B/C-N, 10ME, and L929, but not in L88.3</i> .....	67
3.4 <i>For TNF sensitive cell lines, preincubation with z-VAD-fmk caused TNF-mediated cytotoxicity to switch from apoptotic death to necrotic death</i> .....	85
3.5 <i>Summary and discussion</i> .....	92
CHAPTER 4 A SPECIFIC PHOSPHOLIPASE A <sub>2</sub> INHIBITOR, QUINACRINE, AND THE OXYGEN-RADICAL SCAVENGER BUTYLATED HYDROXYANISOLE (BHA) REDUCES TNF-MEDIATED CYTOTOXICITY .....	
100	
4.1 <i>TNF-induced cytotoxicity involves activation of phospholipase A<sub>2</sub> in B/C-N, 10ME, and L88.3 cell lines</i> .....	100
4.2 <i>BHA reduces TNF-mediated cytotoxicity of TNF sensitive cells</i> .....	103
4.3 <i>BHA reduces TNF-mediated cytotoxicity in cells sensitized to TNF by preincubation with the pan-caspase inhibitor z-VAD-fmk</i> .....	106



4.4 <i>BHA has no effect in reducing TNF-mediated cytotoxicity in L88.3</i> .....	111
4.5 <i>Summary and discussion</i> .....	111
CHAPTER 5 THE EFFECT OF HEAT-SHOCK TREATMENT ON	
TNF-MEDIATED CYTOLYSIS AND CASPASES ACTIVITIES.....	118
5.1 <i>Heat shock treatment at 42 °C for two hours prior to addition</i> <i>of TNF reduces TNF-mediated cytotoxicity of B/C-N, 10ME, and</i> <i>L88.3 cell lines, but not L929 cell line.....</i>	119
5.2 <i>Heat shock-induced reduction in TNF-mediated cytotoxicity was not due</i> <i>to suppression of caspases activities in B/C-N, 10ME, and L88.3</i> .....	121
5.3 <i>Summary and Discussion</i> .....	125
CHAPTER 6 CONCLUSION.....	130
REFERENCES .....	134

## LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Caspase activities observed with TNF treatment at 5, 7, 10, and 12 hours .....	49
2. Summary of lytic pathway and resistance pathway induced by TNF in B/C-N, 10ME, L88.3, and L929 cell lines .....	133

## LIST OF ILLUSTRATIONS

<u>Figure</u>	<u>Page</u>
1. Binding of TNF to TNF-R1 causes several different signaling pathways to be activated .....	9
2. Generation of target cell lines at different developmental stages of progression model .....	32
3. 10ME and L929 cell lines are sensitive to TNF-mediated cytotoxicity while B/C-N and L88.3 are not .....	45
4. For B/C-N, addition of CHX increased TNF-mediated cytotoxicity and caspase activities .....	55
5. For 10ME, addition of CHX increased TNF-mediated cytotoxicity and caspase activities .....	58
6. For L88.3, addition of CHX increased TNF-mediated cytotoxicity and caspase activities .....	61
7. L929 shows a time-dependent increase in TNF-mediated cytotoxicity .....	63
8. Pan-caspase inhibitor z-VAD-fmk, but not caspase-3, -7, and -8 inhibitor z-DEVD-fmk increased TNF-mediated cytotoxicity in B/C-N .....	69
9. Pan-caspase inhibitor z-VAD-fmk increased TNF-mediated cytotoxicity to the level of TNF plus CHX in 10E, but caspase-3, -7, and -8 inhibitor z-DEVD-fmk showed no effect on TNF-mediated cytotoxicity .....	72
10. Preincubation with z-VAD-fmk or z-DEVD-fmk reduced TNF mediated cytotoxicity in L88.3 .....	73

11. Pan-caspase inhibitor z-VAD-fmk increased TNF-mediated cytolysis to the level of TNF plus CHX in L929, but caspase-3, -7, and -8 inhibitor z-DEVD-fmk showed some inhibitory effect on TNF-mediated cytolysis .....	75
12. Caspase inhibitors z-VAD-fmk and z-DEVD-fmk suppressed caspase activities compared to TNF treatment in B/C-N, 10ME, L88.3 and L929 .....	77
13. Pan-caspase inhibitor z-VAD-fmk increased TNF-mediated cytolysis, but not caspase-3, -7, and -8 inhibitor z-DEVD-fmk in B/C-N .....	79
14. Preincubation with pan-caspase inhibitor z-VAD-fmk and caspase-3, -7, and -8 inhibitor z-DEVD-fmk caused decrease in TNF-mediated cytolysis in L88.3 .....	82
15. Preincubation with pan-caspase inhibitor z-VAD-fmk, but not with caspase-3, -7, and -8 inhibitor z-DEVD-fmk, caused increase in TNF-mediated cytolysis in 10ME .....	82
16. Preincubation with pan-caspase inhibitor z-VAD-fmk, but not with caspase-3, -7, and -8 inhibitor z-DEVD-fmk, caused increase in TNF-mediated cytolysis in L929 .....	83
17. Preincubation with z-VAD-fmk plus TNF caused necrotic death in L929 cell line.....	87
18. Preincubation with z-VAD-fmk alone caused no morphological changes in L929 cell line.....	88

19. Preincubation with z-VAD-fmk plus TNF caused switch from apoptotic to necrotic death in the 10ME cell line .....	90
20. Preincubation with z-VAD-fmk alone caused no morphological changes in the 10ME cell line.....	91
21. Addition of specific PLA <sub>2</sub> inhibitor quinacrine reduced TNF mediated cytolysis in B/C-N, 10ME, and L88.3 cell lines.....	102
22. Addition of 100 $\mu$ M BHA reduces TNF-mediated cytolysis in TNF-sensitive cell lines 10ME and L929.....	105
23. For B/C-N, the increase in TNF-mediated killing due to addition of the caspase inhibitor z-VAD-fmk can be blocked by BHA .....	107
24. For 10ME, the increase in TNF-mediated killing due to addition of the caspase inhibitor z-VAD-fmk was not due to increase in generation of oxygen radicals.....	108
25. For L929, the increase in TNF-mediated killing due to addition the of caspase inhibitor z-VAD-fmk was not due to increase in generation of oxygen radicals.....	110
26. For L88.3, BHA did not decrease TNF-mediated killing in the presence or absence of the caspase inhibitor z-VAD-fmk.....	112
27. Prior heat-shock treatment at 42°C for 2 hours before addition of TNF caused reduction in TNF-mediated cytolysis in B/C-N, 10ME, and L88.3 cell lines.....	120

28. Heat-shock treatment of L929 at 42oC for 2 hours prior to TNF treatment did not reduce TNF-mediated cytolysis, and could increase TNF-mediated cytolysis during 5 hours and 7 hours chromium release assay.....	122
29. Prior heat-shock treatment caused increase in caspases activities at 5 hours and 7 hours of TNF treatment, but decrease in caspases activities at 10 hours of TNF treatment .....	124
30. Prior heat shock-treatment reduced TNF-mediated cytolysis in B/C-N, 10ME, and L88.3 at 5 hours and 7 hours time point .....	126

## LIST OF ABBREVIATIONS

Ac-YVAD-CHO: acetyl Tyr-Val-Ala-Asp aldehyde

Ac-DEVD-CHO: acetyl Asp-Glu-Val-Asp aldehyde

Act D: actinomycin D

Apaf-1: apoptotic protease-activating factor-1

BHA: butylated hydroxyanisole

CARD: caspase recruitment domain

Caspase: cysteinyl aspartate-specific proteinase

*ced-3*: *Caenorhabditis elegans* cell-death gene

CHX: cycloheximide

DED: death effector domain

DD: death domain

DR: death receptor

FADD: Fas-associated protein with death domain

FAK: focal adhesion kinase

HSP: heat shock protein

IAP: inhibitor of apoptosis protein

ICE: interleukin-1 $\beta$ -converting enzyme (caspase-1)

ICH-1: *Ice* and *ced-3* homologue-1 (caspase-2)

I $\kappa$ B: immunoglobulin kappa light chain B

IKK: I $\kappa$ B kinase

kDa: kilodalton

LPS: lipopolysaccharide

MAPKKK or MAP3K: mitogen-activated protein kinase kinase kinase

NF- $\kappa$ B: nuclear factor- $\kappa$ B

NIK: NF- $\kappa$ B inducing kinase

PKC: protein kinase C

PLA<sub>2</sub>: phospholipase A<sub>2</sub>

RAIDD: RIP-associated ICH-1/CED-3 homologous protein with a death domain

RIP: receptor interacting protein

ROI: reactive oxygen intermediates

ROS: reactive oxygen species

sHSP: small heat shock protein

SODD: silencer of death domain

TNF: tumor necrosis factor

TNF-R1: tumor necrosis factor-receptor 1

TRAF: TNF receptor-associated factor

TRADD: TNF receptor-associated death domain

z-VAD-fmk: benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone

z-DEVD-fmk: benzyloxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone



## CHAPTER 1

### INTRODUCTION

#### 1.1 TUMOR NECROSIS FACTOR

##### 1.1.1 *Background*

There are two homologous forms of tumor necrosis factor: tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and tumor necrosis factor- $\beta$  (TNF- $\beta$ ), also known as lymphotoxin- $\alpha$  (LT- $\alpha$ ). These cytokines exhibit similar biological activities. TNF- $\alpha$  (TNF from now on) was originally found to induce necrosis of some tumors *in vivo* and *in vitro* (Carswell et al., 1975). TNF is associated with direct cytotoxic effects on tumor cells, the stimulation of immunocompetent cells, the induction of cellular proliferation and differentiation, and antiviral activity. It is also associated with development of septic shock syndrome, tissue injury, cachexia, inflammation, insulin resistance, allograft rejection, graft vs. host disease, hemorrhagic necrosis of tumors, adult respiratory distress syndrome, pathogenesis of multiple sclerosis, meningitis, brain tumor, and cerebral edema (Hotamisligil et al., 1993; Sugarman et al., 1985; Tracey and Cerami, 1993).

TNF is a 157 amino acid protein, and TNF- $\beta$  is 171 amino acids in length. Human TNF is synthesized as a 233 amino acid precursor containing a hydrophobic sequence that is proteolytically cleaved to produce the mature 157 amino acid form (17 kDa). A cell-membrane-associated form of TNF (26 kDa) that also possesses bioactivity, is proteolytically cleaved by a membrane metalloproteinase to release the active secreted (soluble) form of TNF (17 kDa) (Gearing et al., 1994). The secreted biologically active form of TNF is a noncovalently bound trimer (the monomer is inactive).

TNF, which was originally found to cause hemorrhagic necrosis of certain tumors, is produced primarily by activated macrophages. However, it is now known to be produced by other cell types as well. TNF is produced by certain B lymphoblastoid cells, T lymphocytes, natural killer (NK) cells, mast cells, astrocytes, Kupffer cells, granuloma cells, glial cells, adipocytes, and smooth muscle cells. Its production is stimulated by LPS from Gram-negative bacteria, viruses, protozoa, mitogens, various cytokines (e.g. interleukins, and interferons), tumor cells, and phorbol esters (Aggarwal, 1992; Tracey and Cerami, 1993)

#### 1.1.2 *TNF receptors*

Biological function of TNF is exerted through binding to its high-affinity cell receptors. There are two TNF receptors: TNF-R1 (also known as TNF-R60), and TNF-R2 (also known as TNF-R80) (Aggarwal et al., 1985). These receptors are also known to exist in soluble form generated *in vivo* by proteolytic cleavage of the surface receptor. A wide variety of cell types express both receptors on the cell surface. Although TNF is best known for its cytotoxic activity, it has been shown that these cell surface receptors are on both TNF sensitive and TNF resistant cells, and sensitivity to TNF-mediated cytotoxicity is not due to a lack of receptors or their inability to internalize and degrade TNF (Tsujimoto et al., 1985). Internalized TNF receptors are not recycled and thus, protein-synthesis is required to replace receptors that are internalized.

The membrane-bound form of the TNF receptors consists of three components: 1) an extracellular domain that corresponds to the soluble form of TNF receptors, 2) a

transmembrane segment, and 3) an intracellular domain. The intracellular domain of the TNF receptors lacks enzymatic activities. Signaling through TNF receptors is done by binding of adapter proteins/molecules to the intracellular domain of TNF receptors by protein-protein interaction. Most cells express both TNF receptors and the surface expression of these receptors can be up regulated by interferon- $\gamma$ , interleukin-2 (IL-2), activators of protein kinase A, and concanavalin A.

TNF-R1 signals the majority of TNF's activities. In the murine system, TNF- R1 is associated with cytotoxicity and production of a steady-state level of manganous superoxide dismutase (Mn-SOD) (Tartaglia et al., 1991). In human tumor cell lines, TNF-R1 is also associated with cytotoxicity (Higuchi and Aggarwal, 1994). It was also shown to mediate early DNA fragmentation as well as inducing monocytoid differentiation in certain human cell lines (Greenblatt and Elias, 1992). Human TNF-R1 has been shown to initiate the majority of known components of the TNF-signaling cascade such as the stimulation of protein kinase C, sphingomyelinase, and phospholipase A<sub>2</sub> as well as the production of the second messengers, diacylglycerol and ceramide (Wiegmann et al., 1992). TNF-R2 is associated with the proliferation of thymocytes and cytotoxic T cells in the murine system (Tartaglia et al., 1991). In some human tumor cell lines, DNA fragmentation and differentiation needs the presence of both TNF-R2 and TNF-R1 (Higuchi and Aggarwal, 1994). TNF-R2 binds soluble TNF poorly, hence, it may be activated by membrane-bound TNF only (Grell et al., 1995).

The soluble form of TNF receptors (sTNFR) is known to bind TNF in the circulation and to compete with cell surface TNF receptors for binding of TNF (Tartaglia

et al., 1991; Tartaglia and Goeddel, 1992; Tartaglia et al., 1993; Tracey and Cerami, 1993). Van Zee et al. (1992) and Mohler et al. (1993) have shown that soluble forms of both TNF receptors were induced under inflammatory or during endotoxemia conditions and were responsible for neutralizing TNF-mediated cytotoxicity by binding to TNF.

Recently it was shown by Jiang et al. (1999) that, under normal conditions, the intracellular domain of TNF-R1 is associated with a widely expressed 60 kDa protein termed silencer of death domain (SODD). When the authors looked for SODD mRNA expression by Northern blots, they found that mRNA was expressed in all human tissues. When TNF binds to TNF-R1, SODD is released from the receptor and this allows adapter molecules to bind to TNF-R1 and to start a signaling cascade. SODD was shown to be rapidly released from TNF-R1 after TNF treatment, but began to reassociate with TNF-R1 after about 10 minutes. Thus, SODD acts as a silencer of TNF signaling. SODD is also found to associate with another TNF receptor family member, DR3 (receptor which binds to Apo3L, a ligand that is closely related to TNF), but not with other death receptors (Fas, DR4, DR5 or TNF-R2). (Fas, also known as CD95 or Apo-1, binds Fas ligand. Both DR4 and DR5 receptors bind Apo2L/TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.)

### 1.1.3 *TNF signaling pathway through TNF-R1*

TNF-mediated cytotoxicity only requires binding of TNF to a few receptors. When TNF binds to TNF-R1, it induces two signals: 1) activation of a protein-synthesis independent cytotoxic mechanism, and 2) activation of a protein-synthesis dependent

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### 1.1.3 *TNF signaling pathway through TNF-R1*

TNF-mediated cytotoxicity only requires binding of TNF to a few receptors. When TNF binds to TNF-R1, it induces two signals: 1) activation of a protein-synthesis independent cytotoxic mechanism, and 2) activation of a protein-synthesis dependent

resistance mechanism. Most normal cells are TNF resistant. This protein-synthesis dependent resistance mechanism can be blocked by transcription inhibitors, such as Actinomycin D (Act D), or the translation inhibitors, such as cycloheximide (CHX), because these agents block the resistance mechanism but have little effect on the protein-synthesis independent lytic mechanism.

Over the years, activation of several signaling pathways have been associated with TNF-R1. Binding of TNF to TNF-R1 was shown to activate pertussis toxin-sensitive G protein which regulate the activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and the *de novo* synthesis of cyclooxygenase in murine osteoblast-like cells (Yanaga et al., 1992).

In some cells TNF treatment causes protein phosphorylation through kinases and phosphatases. TNF caused rapid stimulation of several cytosolic serine/threonine protein kinases (casein kinase-2, myelin basic protein kinases-1 and -2) in Swiss 3T3 and L929 cells (Van Lint et al., 1992). TNF induced the association of several phosphoproteins with TNF-R1, but not with TNF-R2 (VanArsdale and Ware, 1994). This TNF-R1 receptor complex showed a TNF-dependent serine protein kinase activity, and caused rapid phosphorylation in a TNF concentration-dependent manner. A protein phosphorylation step is associated with inhibition of TNF-mediated cytotoxicity, because the tyrosine phosphatase inhibitor vanadate can inhibit TNF cytotoxicity (Totpal et al., 1992). TNF treatment is also associated with activation of protein kinase C (PKC) (Wiegmann et al., 1992). Effects of PKC are cell line dependent. PKC could cause activation of transcription factor AP-1, adherence of lymphocyte, production of

cytokines, enhance TNF-mediated cytotoxicity, and modulation of TNF resistance (Schutze et al., 1994).

TNF has also been shown to activate certain signaling pathways that contribute to lytic mechanisms such as activation of phospholipases, generation of ceramide, and mitochondria-dependent generation of reactive oxygen intermediates (ROI). Activation of a phospholipase pathway is unique to TNF-mediated apoptosis and is not known to be involved in other pathways leading to apoptosis, such as Fas-mediated apoptosis (Enari et al., 1996). In TNF-sensitive cell lines, TNF causes activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and release of arachidonic acid (Mutch et al., 1992). Another group showed that TNF causes activation of phospholipase D (De Valck et al., 1993). In TNF resistant cell lines, it was found that TNF is either unable to induce PLA<sub>2</sub> activation, or TNF activates PLA<sub>2</sub>, but due to a defect in arachidonic acid biosynthesis, arachidonic acid release is blocked (Hayakawa et al., 1993; Hollenback et al., 1992; Reid et al., 1991). When these TNF-resistant cell lines were treated with TNF in the presence of a transcription inhibitor (e.g. Actinomycin D) or a translation inhibitor (e.g. cycloheximide or emetine), these cells became TNF sensitive and, at the same time, showed PLA<sub>2</sub> activity with arachidonic acid release (Mutch et al., 1992; Hollenback et al., 1992).

Ceramide is a secondary messenger in the sphingomyelin pathway. Sphingomyelin is catabolized by the sphingomyelin-specific forms of phospholipase C, called sphingomyelinases, to generate ceramide and phosphorylcholine. TNF-induced ceramide generation function in diverse pathways. It seems to play a role in signaling both the proinflammatory and apoptotic effects of TNF. It was shown that TNF-induced

generation of ceramide enhanced activation of the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) and caused growth inhibition of Jurkat T cells (Dbaibo et al., 1993). Later it was found that TNF induces two distinct types of sphingomyelinases (a membrane-associated neutral N-sphingomyelinase, and an endosomal acidic A-sphingomyelinase) leading to different signals via ceramide generation (Wiegmann et al., 1994). Ceramide generated by N-sphingomyelinase directs the activation of proline-directed serine/threonine protein kinases(s) and phospholipase A<sub>2</sub>. A-sphingomyelinase generated ceramide triggers the activation of NF- $\kappa$ B. Recently, synthetic cell-permeable ceramide was shown to cleave and activate caspase-3 and to induce apoptosis (Mizushima et al., 1996). Generation of ceramide by TNF treatment also showed activation of caspase-3 and induction of apoptosis (Monney et al., 1998). (The role of caspase-3 is discussed in detail below.)

Generation of reactive oxygen radicals in the mitochondria has been linked to TNF-mediated cytotoxicity. It was shown that structural mitochondrial damage accompanied TNF-mediated cytotoxicity. TNF treatment caused early degeneration of the mitochondrial ultrastructure but showed no pronounced damage on other cellular organelles (Schulze-Osthoff et al., 1992). Addition of antioxidants and iron chelators provided protection from this TNF-mediated cytotoxicity. It was shown by confocal microscopy and flow cytometry that intracellular ROIs were formed after addition of TNF to cultured cells (Goossens et al., 1995). This ROI generation was observed exclusively under condition when cells were sensitive to TNF-mediated cytotoxicity. Addition of ROI scavenger (e.g. butylated hydroxyanisole, BHA) blocked the formation



of free radicals and cytotoxicity. It was also shown that ROI formation induced by TNF in the presence of Actinomycin D caused DNA damage (Shoji et al., 1995). Similar to other studies, addition of a ferric iron chelator and a sulfhydryl reagent both prevented DNA damage and cell killing suggested generation of oxygen radicals contribution to DNA damage.

#### A. TNF-R1 signaling through adapter molecules

Binding of TNF to TNF-R1 also activates caspases and NF- $\kappa$ B through the use of adapter molecules. Binding of TNF trimers to TNF-R1 causes trimerization of receptors, and activates two conflicting signals. One signal activates apoptosis and another signal activates protective mechanisms that inhibit apoptosis. An apoptotic signal is activated through involvement of a caspase cascade; a protective signal involve activation of transcription factor NF- $\kappa$ B (Figure 1). Another transcription factor AP-1 is also known to be activated when TNF binds to TNF-R1. AP-1 is a heterogeneous collection of dimeric transcription factors comprising Jun, Fos and ATF subunits, whose activity is regulated by multiple mechanisms. Both NF- $\kappa$ B and AP-1 induce genes involved in chronic and acute inflammatory responses.

The cytoplasmic domains of the TNF family of receptors do not have enzymatic activity. Instead, signal transduction is induced by aggregation of intracellular adapter molecules through protein-protein interactions. When soluble TNF binds to TNF-R1, it causes SODD to detach from TNF-R1. This allows trimerization of TNF-R1, and that allows adapter molecules to bind to the intracellular domain of TNF-R1. The

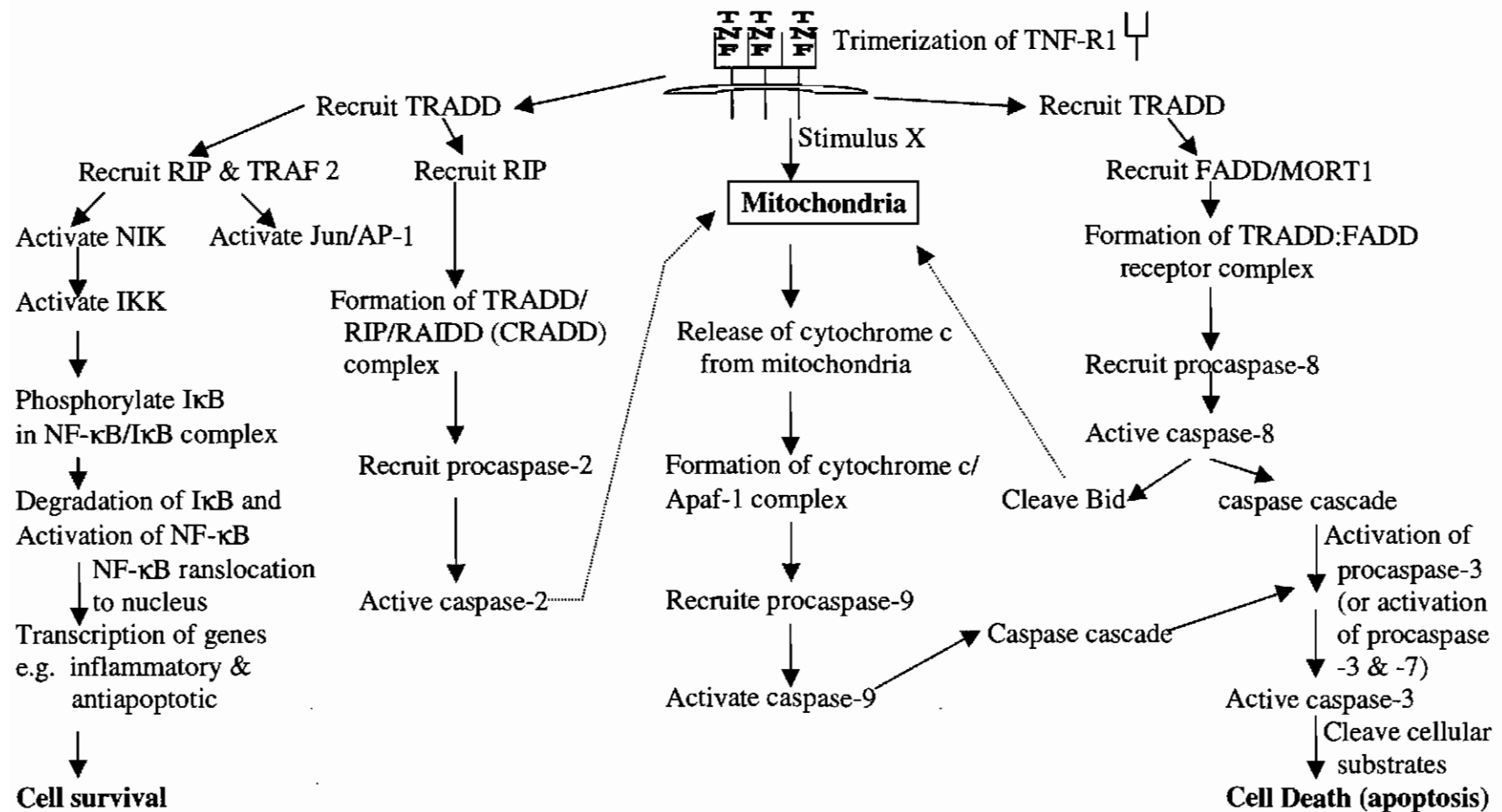


Figure 1 Binding of TNF to TNF-R1 causes several different signaling pathways to be activated.

cytoplasmic domains of TNF-R1 contains a motif called a “death domain” (DD), while the cytoplasmic domains of TNF-R2 lacks DD. Binding of trimeric TNF to TNF-R1 causes an adapter molecule, TNFR-associated death domain (TRADD), which also contains DDs, to bind to the DD of TNF-R1 (Hsu et al., 1995). TRADD then recruits two proteins, RIP (receptor interacting protein) and FADD (Fas-associating protein with death domain). FADD contains DDs at its C-terminal (which associates with DD of TRADD), and a death effector domain (DED) at its N- terminal (Boldin et al., 1996; Chinnaiyan et al., 1996). The DED of FADD allows it to bind to a DED in the procaspase-8 and to initiates apoptosis (Bolding et al., 1996). Activation of procaspase-8 activates a caspase cascade which, in the absence of a resistance mechanism, resulting in apoptosis (Figure 1).

RIP contains a DD at its N-terminal, and a serine/threonine kinase domain at C-terminal. It binds to TRADD through the DD, and is required for activation of NF- $\kappa$ B by TNF (Ting et al., 1996; Hsu et al., 1996a) (Figure 1). TRADD also interacts with TNFR-associated factor-2 (TRAF2) which then activates NF- $\kappa$ B and JNK/AP-1 (Liu et al., 1997; Natoli et al., 1997). TRAF2 associates with the N-terminal half of TRADD (Hsu et al., 1996a & 1996b). TRAF2 and RIP also interact with each other possibly to stabilize the complex. This complex will associate with a mitogen-activated protein kinase kinase kinase (MAPKKK or MAP3K), also known as NF- $\kappa$ B-inducing kinase (NIK) (Figure 1). NIK complexes with NIK-interacting protein kinase called I $\kappa$ B kinase  $\alpha$  (IKK- $\alpha$ ) or I $\kappa$ B kinase  $\beta$  (IKK- $\beta$ ). This NIK and IKK interaction causes signal-induced phosphorylation of NF- $\kappa$ B inhibitor I $\kappa$ B at the critical serine residues (Regnier et al., 1997; Woronica et

al., 1997). This causes I $\kappa$ B to dissociate from NF- $\kappa$ B and that allows translocation of NF- $\kappa$ B from the cytoplasm into the nucleus. In the nucleus, NF- $\kappa$ B binds to specific DNA sequence and facilitates transcription of inflammatory and antiapoptotic genes.

RIP can associate with another adapter molecule called RIP-associated ICH-1/CED-3-homologous protein with a death domain (RAIDD), also known as caspase and RIP adapter with death domain (CRADD) (Duan and Dixit, 1997; Ahmad et al., 1997). RAIDD contains DD at C-terminal and a caspase recruitment domain (CARD). This allows RAIDD to bind to RIP through DD domain and binds to procaspase-2 through the CARD domain to initiates apoptosis (Duan and Dixit, 1997; Ahmad et al., 1997; Hofmann et al., 1997; Chou et al., 1998).

#### B. Protective mechanism through NF- $\kappa$ B

Transcription factor NF- $\kappa$ B has been known to be involved in gene expression induced in cells by pathogens or inflammatory cytokines, and is also known to play an important role in development of the immune system (Baeurele and Hendel, 1994). It was first identified as a tissue-restricted factor that activated the immunoglobulin kappa ( $\kappa$ ) light chain intron enhancer in certain murine B cells (Sen and Baltimore, 1986a; Sen and Baltimore, 1986b). NF- $\kappa$ B is made up of two protein subunits: the DNA binding subunit p50, and the DNA and I $\kappa$ B binding subunit p65. Normally, NF- $\kappa$ B is in the cytoplasm of unstimulated cells, bound to an inhibitory protein called I $\kappa$ B. I $\kappa$ B prevents DNA binding as well as nuclear translocation of NF- $\kappa$ B by binding to the subunit p65. Upon removal of I $\kappa$ B, NF- $\kappa$ B rapidly translocates from the cytoplasm to the nucleus.

Binding of TNF to TNF-R1, interleukin-1 (IL-1) to IL-1 receptor, or bacterial lipopolysaccharide (LPS) binding to a Toll-like receptor (TLR) causes signal that activate NF- $\kappa$ B. TNF binding to TNF-R1 leads to phosphorylation of I $\kappa$ B, and causes it to dissociate from NF- $\kappa$ B, allowing translocation of NF- $\kappa$ B into nucleus (Figure 1). Binding of NF- $\kappa$ B to specific sequence on DNA allows transcription of genes necessary for inflammation, antiapoptotic proteins, and regulation of cell growth.

Activation of NF- $\kappa$ B has been shown to protect various cell types from apoptosis. It has been shown that the activation of NF- $\kappa$ B protected cells from apoptosis induced by tumor necrosis factor (TNF), ionizing radiation, daunorubicin (a cancer chemotherapeutic compound), or Fas-mediated T cell apoptosis. Inhibition of NF- $\kappa$ B translocation enhances apoptotic killing by these agents (Beg and Baltimore 1996; Wang et al., 1996; van Antwerp et al., 1996; Dudley et al., 1999). NF- $\kappa$ B activation also protects hippocampal neurons from apoptosis induced by the oxidative insults, FeSO<sub>4</sub> and amyloid  $\beta$ -peptide (Mattson et al., 1997). In addition, activation of NF- $\kappa$ B has been shown to suppress p53 (tumor-suppressor gene)-independent apoptosis induced by the proto-oncogene Ras (Mayo et al., 1997). Researchers have shown that a high NF- $\kappa$ B activity and increased expression of inhibitor of apoptosis protein-1 (IAP-1) protected vascular smooth muscle cells from apoptosis (Erl et al., 1999). Their result suggests that IAP-1 is transcriptionally regulated by NF- $\kappa$ B. NF- $\kappa$ B activation is also associated with protection from cadmium- and reactive oxygen species-mediated apoptosis in kidney proximal tubule cells by up-regulating the drug efflux pump, multi-drug resistance P-glycoprotein (mdr1) (Thevenod et al., 2000).

Despite these findings, the target genes that determine cell survival in response to NF- $\kappa$ B activation were largely unknown until recently. Genes for zinc-finger protein A20 (which is induced after TNF treatment *in vitro*) and the manganous superoxide dismutase (Mn-SOD) have been proposed as candidates for target genes of NF- $\kappa$ B activation (Baichwal and Baeuerle, 1997; van Antwerp et al., 1998). Recently, pretreatment of hippocampal neuron cell cultures with agents that activate NF- $\kappa$ B (TNF and C2-ceramide) has shown to protect these cells from apoptosis induced by oxidative stress caused by FeSO<sub>4</sub> and amyloid  $\beta$ -peptide (Mattson et al., 1997). Activation of NF- $\kappa$ B by pretreatment of these neuron cells with TNF is associated with production of Mn-SOD and suppressing peroxynitrite formation and membrane lipid peroxidation. He and Ting (2002) showed that zinc finger protein A20 is a NF- $\kappa$ B-inducible gene, and is involved in protection from TNF-mediated apoptosis by disrupting the recruitment of the TRADD and RIP to TNF-R1.

IAP has also been implicated in signal transduction pathways that prevent TNF-induced apoptosis. Two research groups have shown that NF- $\kappa$ B activation induces c-IAP1 and c-IAP2 to suppress apoptosis (Chu et al., 1997; Wang et al., 1998). NF- $\kappa$ B controls transcriptional activation of genes for c-IAP1, c-IAP2, TNF receptor-associated factor 1 (TRAF1), and TRAF2. This in turn blocks the activation of caspase-8, leading to suppression of TNF-induced apoptosis.

## 1.2 CASPASES

### 1.2.1 *Background*

Caspases (cysteinyl aspartate-specific proteinases) are cysteine proteases, which play a central role in apoptotic death. They are constitutively expressed in mammalian tissues as inactive procaspases. They were implicated in apoptosis by genetic analysis in the nematode *Caenorhabditis elegans* (*C. elegans*). Caspases have an absolute requirement for aspartic acid adjacent to the site of cleavage in a cellular substrate. (Caspases cleave their substrates C-terminal of an aspartic acid.) Each caspase has a specific tetrapeptide recognition sequence N-terminal to the caspase cleavage site. Currently there are fourteen mammalian caspases (named caspase-1 to caspase-14). Out of these 14 caspases, caspase-11 and -12 are known only in mice. They have distinct roles in apoptosis and inflammation (Salvesen and Dixit, 1997; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). Caspases-1, -4, -5, -11, -12, -13, and -14 are involved in inflammation/cytokine activation. Caspases-2, -3, -6, -7, -8, -9, and -10 are involved in apoptosis. In apoptosis, caspases function as initiators and effectors of apoptotic cell death in response to proapoptotic signals. Caspases-2, -8, -9 and -10 are initiator caspases involved in upstream or early steps of apoptosis. And Caspases-3, -6, and -7 are effector caspases involved in the later stages of apoptosis.

### 1.2.2 *Structure of caspases*

Caspases share similarities in amino acid sequence, structure, and substrate specificity. They are constitutively and ubiquitously expressed as catalytically inactive

proenzymes/procaspases. Structurally, all procaspases are composed of a variable-length prodomain at the N-terminus and a highly homologous protease domain at the C-terminus. This protease domain consists of two subunits: a large subunit of approximately 20 kDa (p20) and a small subunit of approximately 10 kDa (p10). In some procaspases, there is a short linker (about 10 amino acids) between the large and small subunits. Prodomain for initiator caspases and inflammatory caspases are over 100 amino acids long, while prodomain for effector caspases are usually less than 30 amino acids long. These long prodomains of initiator caspases and inflammatory caspases contain a DED and a CARD. Procaspases associate with the adapter molecules through the DEDs and play important roles in procaspase activation. CARDS are present in caspase-1, -2, -4, -5, -9 and the adapter molecules RAIDD and Apaf-1 (Hofmann et al., 1997; Zou et al., 1997). Procaspase-9 binds to Apaf-1 through CARD-CARD interaction (Chou et al., 1998; Zhou et al., 1999). Also procaspase-2 binds to RAIDD through CARD-CARD interaction (Hofmann et al., 1997; Chou et al., 1998). The short prodomains of effector caspases are unlikely to mediate protein-protein interactions. Instead, these short prodomains seem to inhibit caspase activation.

### 1.2.3 *Activation of caspases*

Procaspases are activated by proteolytic processing at specific aspartate residues. There is an aspartate cleavage site that separates the prodomain from the large subunit, and a linker region containing one or two aspartate cleavage sites to separate the large and small subunits. Two cleavage events are required to produce fully functional



caspases. The first proteolytic cleavage separates a caspase into a large subunit with a prodomain and a small subunit. The second proteolytic cleavage removes the N-terminal prodomain from a large subunit. An active caspase is a tetramer composed of two large and two small subunits. Once a caspase is activated, it can activate its own precursor or activate downstream procaspases.

Activation of initiator procaspases such as procaspase-8 and procaspase-9 seems to require binding to their specific adapter molecules. This allows procaspases to be in close proximity to each other and to be activated by their oligomerization (Muzio et al., 1998; Yang et al., 1998a). In procaspase-8, it was found that autocleavage could occur through oligomerization (Yang et al., 1998b). Procaspase-9 was shown to be activated without proteolytic processing. Instead, its activation is dependent on cytosolic factors (Stennicke et al., 1999). When expressed in a yeast system, procaspase-2 can be activated by autoprocessing through dimerization that requires both the prodomain and the C-terminal residues (Butt et al., 1998). Procaspase-2 could also be activated by caspase-3 during TNF-mediated apoptosis in the presence of CHX (Li H et al., 1997). Later, it was shown that under UV-induced apoptosis, procaspase-2 was activated by caspase-9 and caspase-3, but under TNF-induced apoptosis, procaspase-2 was activated by caspase-3 alone (Paroni et al., 2001).

Activation of effector procaspases are usually mediated by initiator caspases, but can also be activated by other proteases. Procaspase-3 and -7 were shown to be activated by initiator caspases-8, -9, and -10 (Duan et al., 1996; Li P et al., 1997; Orth et al., 1996; Stennicke et al., 1998). Activation of procaspase-3 could also be mediated by a serine

protease, granzyme B, used by cytotoxic T lymphocytes to kill their targets (Darmon et al., 1995). Procaspase-3 could not be autoactivated by oligomerization like initiator caspases. Recently it was reported that procaspase-3 activation is under an intrinsic “safety catch” regulatory tripeptide (Asp-Asp-Asp) contained within a linker region between the large and the small subunits (Roy et al., 2001). Removal of this regulatory tripeptide resulted in substantially enhanced autocatalytic maturation of procaspase-3 to caspase-3. At the same time removal of this regulatory tripeptide increased vulnerability to proteolytic activation by initiator caspases such as caspase-9 and granzyme B.

#### 1.2.4 *Caspases and apoptosis*

A cell dying from apoptosis shows very different characteristics compared to cells dying from necrosis. Some of the morphological features seen in apoptotic cells are membrane blebbing followed by cytoplasmic condensation (cell shrinkage), which is accompanied by an increase in cell density, compaction of cytoplasmic organelles, and condensation of the nuclear chromatin. A dying cell also separates from its neighbors, however, membrane integrity is intact. Eventually the nuclear membrane disintegrates and DNA fragmentation occurs. Without releasing their intracellular contents, the dying cells will split into many membrane-bound bodies called “apoptotic bodies”, containing a variety of organelles. These apoptotic bodies are quickly absorbed by adjacent cells, not by phagocytic cells. No inflammatory response is associated with apoptosis.

Biochemical characteristic of apoptotic cells are externalization of phosphatidylserine residues from the inner leaflet to the outer leaflet of the cell membrane, reduction in the

mitochondrial transmembrane potential, intracellular acidification, production of reactive oxygen species, selective proteolysis of cellular proteins, and degradation of DNA into internucleosomal fragments.

In contrast, cells dying by necrosis show swelling, alteration in cytosolic and nuclear structures, but no chromatin condensation. Membrane integrity is usually lost during necrosis. Eventually cell membrane ruptures due to cell swelling and internal materials are spilled into the surrounding area to induce an inflammatory response, causing chemotaxis of phagocytic cells to the area. Usually necrosis is caused by a general failure of cellular homeostasis due to physically or chemically induced damage. Unlike apoptosis where a single cell can be induced to die, necrosis causes group of cells usually adjacent to each other to die.

Caspases can be activated by many proapoptotic stimuli. Generally there are two pathways that cause activation of initiator caspases leading to activation of effector caspases. One is a death receptor pathway and another is a mitochondrial pathway (Figure 1). Recently, the third pathway involving the endoplasmic reticulum (ER) was found. In this pathway, caspase-12 is involved in the apoptosis that results from stress in the ER (Nakagawa et al., 2000). Activation of procaspase-12 in the endoplasmic reticulum was achieved by translocation of cytosolic caspase-7 to the ER surface, and cleavage of procaspase-12 to generate active caspase-12 (Rao et al., 2001). This resulted in an increase in cell death. It was further determined that active caspase-12 can activate procaspase-9 in a cytochrome c independent manner leading to activation of procaspase-3 (Morishima et al., 2002).

In a death receptor pathway, when a ligand is bound to a death receptor such as TNF-R1 or Fas, there is recruitment of procaspases to the receptor/adaptor molecule complex (membrane-associated death complex) to initiate caspase-induced apoptosis. This is generally done through recruitment of procaspase-8 or procaspase-2 (Figure 1). Activation of procaspase-8 results in activation of the effector caspases-3, -6, or -7, leading to cellular substrate cleavage and cell death. Recently, it was shown that death receptor-mediated apoptosis initiated by caspase-8 could amplify apoptosis through mitochondria (Figure 1). Li et al. (1998) showed cleavage of BID, a “BH3 domain only” member of a Bcl-2 protein family, by activated caspase-8 in Fas induced apoptosis. Cleaved BID then translocates to mitochondria and induces mitochondria to release cytochrome c and activate caspase-9 leading to apoptosis. BID activation by caspase-8 was also shown in TNF-induced apoptosis (Gross et al., 1999). Recruitment of procaspase-2 through RAIDD/CRAIDD to TNF-R1 causes activation of procaspase-2 by caspase-3 (Li H et al., 1997). Paroni et al. (2001) showed that activation of caspase-8 eventually led to activation of caspase-2 through activation of caspase-3. Activated caspase-2 was shown to feed into the mitochondrial pathway by causing mitochondria to release cytochrome c and induce apoptosis via activation of caspase-9 (Paroni et al., 2001; Lassus et al., 2002). Hence, caspase-2 also leads to mitochondria activation. In the caspase-8 activation of BID and caspase-2 induced apoptosis, mitochondria are amplifiers of caspase activity rather than initiators of caspase activation.

In the mitochondrial pathway, apoptotic stimuli, such as DNA damaging agents or cellular stress, can trigger the mitochondrial release of cytochrome c to the cytoplasm.

Released cytochrome c binds to Apaf-1, forming a complex. Procaspase-9 is recruited to this cytochrome c/Apaf-1 complex to initiate apoptosis (Figure 1).

#### 1.2.5 *Caspases mediated cleavage of cellular proteins*

Effector caspases such as caspase-3, -6, and -7 have been shown to cleave different cellular proteins to carry out apoptosis. All of the cellular substrates/proteins that are cleaved by these effector caspases contain the tetrapeptide-recognition sequence amino-terminus of the specific caspase cleavage site. Some cellular substrates cleaved by effector caspases contribute to apoptosis (of the three effector caspases, caspase-3 appears to be most important for apoptosis). There are other cellular substrates cleaved by caspase-3 that are found in cells undergoing apoptosis, but are not known to be associated with apoptotic characteristics.

Some of the cellular substrates cleaved by caspase-3 that lead to apoptotic features such as DNA fragmentation are the poly (ADP-ribose) polymerase (PARP), the cleavage of inhibitor of caspase-activated deoxyribonuclease (ICAD), and the DNA fragmentation factor (DFF) (Lazebnik et al., 1994; Nicholson et al., 1995; Tewari et al., 1995; Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998). PARP is a DNA repair enzyme. Cleavage of this enzyme inactivates a DNA repair system, and has been suggested to lead to DNA fragmentation. DFF is a novel cytosolic protein that is activated by caspase-3 cleavage and induces DNA fragmentation (Liu et al., 1997). Cleavage of ICAD by caspase-3 allows it to dissociate from a caspase-activated deoxyribonuclease (CAD). CAD exists as an inactive complex with ICAD in the

cytoplasm of living cells. Released CAD translocates to the nucleus and cause DNA fragmentation in apoptotic cells (Enari et al., 1998; Sakahira et al., 1998).

Cleavage of  $\alpha$ -fodrin, lamin, gelsolin, keratin, and p21-activated kinase-2 (PAK2) were shown to cause membrane blebbing, chromatin condensation, and nuclear fragmentation. Cleavage of  $\alpha$ -fodrin by caspase-3 caused membrane blebbing (Cryns et al., 1996). Lamin was shown to be cleaved by caspase-6 (which was activated by caspase-3) (Srinivasula et al., 1996; Takahashi et al., 1996). Nuclear lamina, generated by polymerization of lamins, forms a proteinaceous meshwork located between chromatin and the inner nuclear membrane. Thus cleavage of lamin can be responsible for chromatin condensation and nuclear fragmentation. Gelsolin was shown to be cleaved by caspase-3 *in vitro* and *in vivo* (Kothakota et al., 1997; Yamashita et al., 1999). Gelsolin is a severing protein that regulates the length of actin filaments by breaking them into short pieces or blocking their ends. Expression of the gelsolin cleavage product in multiple cell types caused the cells to round up, detach from the plate, and undergo nuclear fragmentation. Keratins are major components of intermediate filaments of simple epithelial cells. During apoptosis, keratin 18 (K18) intermediate filaments reorganized into granular structures. K18 was proteolytically cleaved by caspase-6 into 2 fragments (26 kDa and 22 kDa). Caspase-3 and -7 further cleaved the 22-kD fragment into a 19 -D fragment (Caulin et al., 1997). Thus cleavage of keratins may contribute to cell shrinkage. Cleavage of PAK2 was shown in apoptotic cells induced through Fas receptor pathway in Jurkat cell line. Cleavage generated a constitutively active PAK2 fragment by caspase-3 in anti-Fas mediated apoptotic Jurkat T cells (Rudel and Bokoch,

1997). PAKs are serine-threonine kinases whose activity is regulated by the small guanosine triphosphatases (GTPases) Rac and Cdc42. PAKs regulate morphological and cytoskeletal changes in a variety of cell types. Hence cleavage of PAKs probably contributes to cell blebbing and cell shrinkage.

Some of anti-apoptotic proteins that are cleaved by the effector caspases are retinoblastoma (Rb) protein, Bcl-2 protein, and tyrosine kinase focal adhesion kinase (FAK). It was shown that Rb protein, an important cell cycle regulator with a known anti-apoptotic function, was specifically cleaved by a caspase-1-like protease in TNF and staurosporine-induced apoptosis (Janicke et al., 1996). The extent of Rb cleavage correlated directly with TNF-induced apoptosis in tumor cell lines HeLa D98 and MCF-7. Cleaved Rb bound cyclin D3 and inhibited the transcriptional activity of E2F-1 (transcription factor), but failed to bind to the regulatory protein MDM2 (Janicke et al., 1996). This inability of cleaved Rb to bind to the regulatory protein MDM2 may lead to apoptosis. Bcl-2, an integral intracellular membrane protein can inhibit programmed cell death induced by multiple insults in a wide variety of cell types. It was shown that Bcl-2 was cleaved at Asp34 by caspase-3 *in vitro* in cells overexpressing caspase-3, after induction of apoptosis by Fas ligation or after interleukin-3 (IL-3) withdrawal from IL-3-dependent cell lines. It appears that the cleaved Bcl-2 fragment promoted apoptosis (Cheng et al., 1997).

FAK, a tyrosine kinase, localizes to focal adhesions and associates temporally and spatially with integrins. FAK plays an important role in regulating focal adhesion formation in adherent cells. It was shown that inhibition of FAK in fibroblasts resulted in

apoptosis. Wen et al. (1997) showed that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) cleaved FAK sequentially into two different fragments (85 kDa and 77 kDa). Using purified caspases and Baculovirus expressed FAK *in vitro*, they demonstrated that first FAK cleavage might be caused by caspase-3 or caspase-7 (with caspase-7 approximately 100-fold more active in generating the first FAK 85-kDa fragment than caspase-3), and the second cleavage by caspase-6 or by a related caspase into a 77 kDa fragment.

Activation of NF- $\kappa$ B is associated with transcription of antiapoptotic genes along with transcription of inflammatory genes. Caspase-3 was shown to promote apoptosis by cleaving NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  at its N-terminal. This truncated form of I $\kappa$ B $\alpha$  acted as a dominant inhibitor that could still bind to NF- $\kappa$ B, but inhibited NF- $\kappa$ B activation and caused sensitization to cell death (Reuther et al., 1999).

Cleavage of some proteins, such as cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) and protein kinase C  $\delta$  (PKC  $\delta$ ) can activate them to induce apoptosis. cPLA<sub>2</sub> was shown to be cleaved by TNF-induced activation of caspases. Caspase-3 or a closely related protease, was most likely involved in cleavage of cPLA<sub>2</sub> since it contains a caspase-3 recognition sequence and the caspase-3 inhibitor Ac-DEVD-CHO inhibited cPLA<sub>2</sub> cleavage (Wissing et al., 1997). This activated cPLA<sub>2</sub> contributed to apoptosis. Proteolytic activation of PKC  $\delta$  by caspase-1 was deduced in human U937 cells treated with ionizing radiation using tetrapeptide caspase-1 inhibitor, YVAD (Emoto et al., 1995), and by *in vitro* caspase-3 cleavage experiment (Ghayur et al., 1996). Inhibition of apoptosis by overexpression of Bcl-2 or Bcl-x<sub>L</sub> was associated with a blocking of PKC  $\delta$  cleavage. Also, Ghayur et al.



(1996) demonstrated that overexpression of the cleaved PKC  $\delta$  catalytic kinase fragment in cells was associated with chromatin condensation, nuclear fragmentation, induction of sub-G1 phase DNA and lethality.

In TNF-induced apoptosis, one of the adapter molecules associated with the TNF:TNFR signaling complex, RIP, the death domain kinase, was shown to be cleaved by caspase-8. This resulted in the blockage of TNF-induced NF- $\kappa$ B activation. RIPc, one of the cleavage products, enhanced interaction between TRADD and FADD and increased cells' sensitivity to TNF (Lin et al., 1999).

#### 1.2.6 Caspase inhibitors

##### A. Natural or endogenous caspase inhibitors

Several viral proteins are endogenous inhibitors of mammalian caspases and can prevent apoptosis. Baculovirus p35 protein was shown to inhibit apoptosis by acting as a competitive inhibitor for certain caspases. In *C. elegans* it is a competitive inhibitor of CED-3 (a caspase-1 homolog) (Xue and Horvitz, 1995). It can also inhibit Fas and TNF-induced apoptosis in human cell lines by blocking PARP cleavage (Beidler et al., 1995). Other have shown that Baculovirus p35 irreversibly inhibits caspases-1, -2, -3, and -4 (Bump et al., 1995). Another natural inhibitor of caspases is cytokine response modifier A (CrmA), a 38 kDa serpin produced by the cowpox virus. CrmA shows highest affinity to caspase-1, and second highest for caspase-8 (Zhou et al., 1997). CrmA can also inhibit caspases-4, -5, -9, and -10 (Garcia-Calvo et al., 1998).

There are some cellular inhibitors of apoptosis. FLICE-inhibitory protein (FLIP), which has a viral form and mammalian forms, inhibits Fas-induced apoptosis (Irmeler et al., 1997). FLIP is predominantly expressed in muscle and lymphoid tissues. Two forms of FLIP (FLIP<sub>S</sub> and FLIP<sub>L</sub>) interact with the adapter protein FADD and casapse-8 to block apoptosis. Thus FLIP inhibits caspase-adapter interactions. Mammalian IAPs were shown to inhibit activation of caspases. cIAP-1 and cIAP-2 were shown to inhibit apoptosis by binding to TRAF1 and TRAF2 to block receptor signaling (Uren et al., 1996). X-linked IAP (XIAP) was shown to inhibit activation of caspase-3 and -7 in mitochondria-induced apoptotic pathway *in vitro* (Deveraux et al., 1997). A year later, Deveraux et al. (1998) showed that XIAP, cIAP1 and cIAP2 blocked cytochrome c-induced activation of procaspase-9, thus preventing the proteolytic activation of procaspases-3, -6, and -7. However, XIAP, cIAP1 and cIAP2 did not block caspase-8-induced activation of procaspase-3.

#### B. Synthetic peptide inhibitors of caspases

The most potent peptide inhibitors contain a peptide-recognition element corresponding to that found in endogenous cellular substrates. The knowledge of these specific peptide-recognition sequences enabled the development of cleavage-site-directed inhibitors of caspase-1 and caspase-3. Short peptide inhibitors, such as benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (z-VAD-fmk), acetyl Tyr-Val-Ala-Asp aldehyde (Ac-YVAD-CHO), and acetyl Asp-Glu-Val-Asp aldehyde (Ac-DEVD-CHO), are designed based on the known specific recognition sequences adjacent to the

cleavage site of different caspases. Z-VAD-fmk was initially developed as a cell-permeable irreversible inhibitor of caspase-1-like protease. Ac-YVAD-CHO was initially developed as a reversible competitive inhibitor of caspase-1. And Ac-DEVD-CHO was initially developed as a reversible competitive inhibitor of caspase-3. To date, Ac-YVAD-CHO the most specific caspase-1 inhibitor ( $K_i = 0.76$  nM) (Garcia-Calvo et al., 1998).

Ac-DEVD-CHO, is a relative potent inhibitor of both caspase-1 ( $K_i = 17$  nM) and caspase-3 ( $K_i = 0.35$  nM), with a 49-fold preference for caspase-3 (Thornberry, 1997). Its peptide component is identical to the P1-P4 sites in at least two endogenous caspase-3 substrates (Nicholson and Thornberry, 1997). Originally, it was developed as a tetrapeptide aldehyde containing the P1-P4 amino-acid sequence of the PARP cleavage site. It inhibited the PARP cleavage activity in apoptotic osteosarcoma cell extracts with a 50% inhibitory concentration ( $IC_{50}$ ) of 0.2 nM (Nicholson et al., 1995). Later, Garcia-Calvo et al. (1998) found that it is also a potent inhibitor for caspase-7 ( $K_i = 1.6$  nM) and caspase-8 ( $K_i = 0.92$  nM), not just for caspase-3 ( $K_i = 0.23$  nM).

Z-VAD-fmk was originally developed as an irreversible inhibitor of ICE-like proteases. (Peptidyl-fluoromethylketones (fmk) are irreversible inhibitors of cysteine proteases.) However, recently it was shown to work as a pan-caspase inhibitor. The sequence of the peptide is based on substrate cleavage sites. VAD is the caspase-1 recognition and cleavage site in progenitor interleukin- $1\beta$ . The peptides based on this sequences partially inhibited purified caspase-1 in Fas-mediated apoptosis (Pronk et al., 1996). Z-VAD-fmk contains an Asp in the P1 position, which has been shown to be

critical for caspase-1-like proteases inhibition (Thornberry et al., 1992; Dolle et al., 1994). The tripeptide analog z-VAD-CH<sub>2</sub>OC(O)-2,6-Cl<sub>2</sub>Ph, 3, showed a substantially faster rate of inactivation for caspase-1 compared to tetrapeptide Ac-YVAD-CHN<sub>2</sub> (Dolle et al., 1994). Another group found that z-VAD-fmk inhibited both murine caspase-1 and human caspase-3, with caspase-1 showing the faster rate of inactivation (Armstrong et al., 1996). The selectivity of z-VAD-fmk for caspase-1-related proteases is further indicated by its lack of detectable inhibition of granzyme B, a mammalian serine protease with specificity for Asp also in the P1 position. Currently z-VAD-fmk is known as an irreversible inhibitor of caspase-1 to caspase-9 (Garcia-Calvo et al., 1998). Z-VAD-fmk inhibits caspase-1 the best, followed by caspase-8>caspase-9>caspase-5>caspase-7>caspase-3>caspase-6>caspase-4>caspase-2.

### 1.3 HEAT SHOCK RESPONSE

#### 1.3.1 *Background*

The first heat shock response was discovered in *Drosophila melanogaster* as the characteristic puffs in the salivary gland chromosomes when heat shocked (Ritossa, 1962). Since then, heat shock proteins (HSPs) have been found to be present in all organisms from bacteria to mammals. HSPs are classified into several groups based on their molecular size. Currently there are five major classes of HSPs: HSP100, HSP 90, HSP 70, HSP60 (GroEL), and small HSPs (sHSPs such as HSP 27/28). Mammalian HSPs have been classified into four major families: sHSPs, HSP 60, HSP 70, and HSP 90.

Some of these HSPs are expressed constitutively (e.g. HSP 60, HSC 70, and HSP 90). Some are induced (e.g. HSP 70 and HSP 27) by some kind of environmental stimulus that causes stress to an organism or a cell. The majority of these proteins play a role as molecular chaperones or as components of proteolytic systems. As molecular chaperones in a cell, they participate in proper folding of newly synthesized proteins, and/or guide proteins to move to their proper destination throughout the cell. Under a stressful condition, these HSPs can associate with partially unfolded proteins and help to refold these proteins into a proper conformation. HSPs also indirectly participate in the degradation of badly damaged proteins resulting from stressful conditions.

A brief review of the properties of different HSP families follows (reviewed in Morimoto et al., 1994; Garrido et al., 2001). The HSP 100 protein family has been universally conserved from prokaryotes to eukaryotes. In yeast and *E. coli*, these proteins are required for thermotolerance of extreme temperatures. Some proteins in this family are actively involved in the proteolytic pathway.

The HSP 90 protein family is found in the cytosol and ER. These proteins are abundant, constitutively expressed and essential for cell growth. Under stress condition, their expression level can be increased. They are mostly seen in association with certain protein kinases (e.g. pp60<sup>src</sup>) and transcription factors (e.g. glucocorticoid receptors). HSP 90 binds to these molecules and suppresses their function until it escorts them to their proper final destination or until the proper ligand arrives. HSP 90 also has chaperone function by preventing the aggregation of some denatured polypeptides.

HSP 70 protein family is the most conserved class of HSPs. There are several forms in the HSP 70 protein family. The most common HSP 70s in human cells are a constitutively expressed form of HSP 70 protein family, HSC 70, and a stress inducible form of HSP 70 protein family, HSP 70. Other HSP 70 protein family, such as HSP75 is found in mitochondria, and a glucose-regulated protein of about 78 kDa (GRP 78) is found in the endoplasmic reticulum. HSC 70 normally function as an ATP-dependent molecular chaperones by assisting the folding of newly synthesized polypeptide. During stress, inducible HSP70 is synthesized to enhance the ability of stressed cells to deal with the increased concentration of unfolded or denatured proteins. HSP 70s are also known to negatively autoregulate the heat-shock response by interfering with the function of the heat shock factor responsible for heat shock gene transcription.

HSP 60, also known as chaperonin, is usually in association with another HSP 60 family member HSP10. HSP 10 binds to HSP 60 to regulate the substrate binding and the ATPase activity of HSP 60. They are restricted to eubacteria, mitochondria, and chloroplasts. HSP 60 is shown to bind to unfolded polypeptide to prevent protein disaggregation and also dissolve some protein aggregates. It can also facilitate the proteolytic degradation of misfolded or denatured proteins in an ATP-dependent manner.

Small heat shock proteins, such as HSP 27, are expressed in many cell types and tissues at specific stages of development and differentiation. Unlike other HSPs, HSP 27 is an ATP-independent chaperone. HSP27/28 and crystallin family members can prevent polypeptide aggregation. They can also undergo phosphorylation in response to stress and mitogenic signals and are involved in cellular thermotolerance.

### 1.3.2 *HSPs and cell death*

Many researches have shown that induction of HSPs under stress protect cells from dying. Early research has shown that cells that survive the initial heat-shock treatment at or above 42 °C acquire transient thermotolerance, and the sensitivity of these cells to the subsequent hyperthermia is substantially reduced (Hahn and Li, 1990; Li and Hahn, 1987). It has also been shown that nonlethal heat shock treatment of cells at 39 - 43 °C, or induction of stress, can protect different tumorigenic cell lines from TNF-mediated cytotoxicity, and other cell-mediated cytotoxic effectors such as CTL and activated monocytes (Jaattela et al., 1989; Gromkowski et al., 1989; Sugawara et al., 1990; Jaattala, 1990). More recently, heat shock proteins, especially HSP70 and HSP 27, have been implicated in increased resistance to apoptosis (Samali and Cotter, 1996; Li et al., 1996; Buzzard et al., 1998). In addition, inhibition of HSP synthesis can lead to apoptosis (Gorman et al., 1999).

Expression of sHSPs, such as HSP 27, and  $\alpha$ B-crystallin, have been associated with protection from TNF and oxidative stress-induced cytotoxicity (Mehlen et al., 1995). These proteins have also been shown to have antiapoptotic activity. Garrido et al. (1999) showed that HSP 27 could prevent activation of procaspase-9 and -3 in etoposide-induced apoptosis in U937, but did not prevent the release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria into the cytosol. Kamradt et al. (2001) showed that under normal condition another sHSP,  $\alpha$ B-crystallin, inhibits autoproteolytic maturation of partially processed caspase-3 intermediate, thus preventing generation of a fully active caspase-3.

The induction of HSP 70 is known to protect cells from TNF-induced cytotoxicity (Kushner et al., 1990; Jaattela et al., 1992; Kim et al., 1997; Jaattela et al., 1998). Overexpression of HSP 70 has been shown to inhibit TNF-induced activation of phospholipase A<sub>2</sub> (Jaattela, 1993). Jaattela et al. (1998) showed that HSP70 blocked TNF, staurosporine and doxorubicin-induced apoptosis by interfering with post caspase-3 activity at the arachidonic acid release step (Jaattela et al., 1998). Li et al. (2000) showed that the antiapoptotic effect of HSP 70 occurred downstream of cytochrome c release and upstream of caspase-3 activation in a cell free system. HSP 70 was also shown to inhibit caspase-dependent and caspase-independent apoptosis in Jurkat T cells (Creagh et al., 2000). HSP70 inhibited activation of caspase-3 in caspase-dependent apoptosis. HSP 70 inhibited loss of mitochondrial membrane potential, release of cytochrome c from mitochondria to cytosol, and increase in intracellular calcium level for both caspase-dependent and caspase-independent apoptosis. And lastly, HSP 70 was shown to interact with AIF, a caspase-independent apoptotic effector, and prevented AIF-induced chromatin condensation of purified nuclei in a cell-free system (Ravagnan et al., 2001). The data indicated that HSP 70 could bind AIF and neutralize its apoptotic effect.

Unlike sHSP or HSP 70, HSP 60 and HSP10 have a proapoptotic role. In Jurkat and Hela cells, camptothecin treatment caused HSP 60 and HSP 10 to be released from mitochondria and associate with procaspase-3 to promote its activation (Samali et al., 1999; Xanthoudakis et al., 1999).

HSP 90 was shown to bind to Apaf-1 and inhibit cytochrome-c mediated activation of caspase-9 under normal conditions (Pandey et al., 2000). When cells were



treated with a DNA-damaging agent such as staurosporin to induce apoptosis, HSP 90 dissociated from Apaf-1 and allowed the normal cytochrome c/Apaf-1/procaspase-9 complex to form, leading to activation of procaspase-9.

#### 1.4 TUMOR PROGRESSION MODEL

The B/C-N, 10ME, and L88.3 murine cell lines were developed by Patek et al. (1978) to represent the progression of a cell from normal to a cancerous phenotype. The murine tumor progression model is depicted in Figure 2.

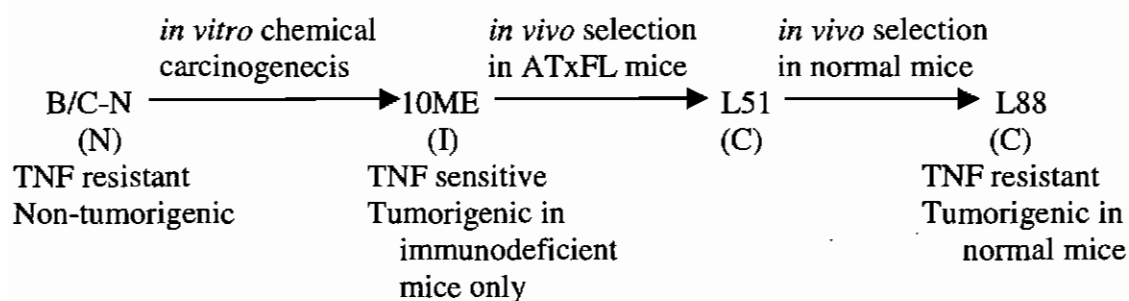


Figure 2 Generation of target cell lines at different developmental stages of tumor progression model

The normal (N) cell line, represented by B/C-N, is a cloned fibroblastic cell line derived from Balb/C fetal tissue (primary fibroblast). It shows continuous growth in culture and expresses no known transformed phenotype either *in vivo* or *in vitro*. It is anchorage-dependent and contact-inhibited.

The intermediate (I) cell line, represented by 10ME HD A.5 R.1 (10ME), is a cloned, methylcholanthrene-transformed (tumorigenic fibroblast) cell line derived from

B/C-N. Isolation of 10ME from 3-methylcholanthrene-11, 12-dihydroepoxide treated B/C-N was done by selection for anchorage-independence growth in 0.3% agarose. 10ME is transformed but susceptible to rejection as demonstrated by its ability to form tumors in immune deficient hosts [athymic nude mice or adult mice thymectomized, lethally X-irradiated then reconstituted with fetal liver cells (ATXFL)]. It is susceptible to immune and nonimmune effector mechanisms in normal host, thus, it cannot form tumor in normal syngeneic mice.

Cancer (C) cell lines, represented by L51 and L88, are uncloned cell population. Selection of these cells were done *in vivo*. L51 was established from the tumors that arose in ATxFL mice after subcutaneous injection of  $10^7$  10ME cells. L88 was established from tumors in normal mice after the subcutaneous injection of L51 cells. L88 exhibits anchorage-independent growth in 0.3% agar, and growth as foci of contact-noninhibited cells on plastic surfaces. L88 cells are resistant to rejection, hence, form tumors in normal syngeneic mice. L88.3 cells used in this study are a subclone of the L88. Like L88, L88.3 is TNF-resistant and forms tumors in normal syngeneic mice.

## 1.5 OBJECTIVES

TNF can induce several different signaling pathways leading to apoptosis or necrosis, depending on the cell lines used. Researches have shown that caspases play a central role in the execution of apoptosis. Several initiator and effector caspases were shown to be activated by TNF-mediated cytotoxicity. The most common initiator caspase activated by TNF were either caspase-8 or caspase-9, and the most common effector

caspase activated by TNF was caspase-3. Additionally, it has been shown that nonlethal heat-shock treatment of cells, or induction of stress, can protect different tumorigenic cell lines from TNF-mediated cytotoxicity, and certain cell-mediated cytotoxic effectors (Jaattela et al., 1989; Gromkowski et al., 1989; Sugawara et al., 1990; Jaatola, 1990). Hence, heat-shock treatment may confer protection from TNF-mediated cytotoxicity by preventing activation of caspases in B/C-N, 10ME, and L88.3 cell lines.

This study was directed at better understanding some of the biochemical pathways involved in TNF-induced cell death. Moreover, using our unique lineage of cells representing steps on the pathway from normal to cancerous, understanding why some cells are sensitive to TNF-mediated lysis whereas others are not, may provide insight into the development of cancer and may provide a rational basis for the development of anti-cancer therapeutics.

Thus, we designed experiments to answer the following questions.

- 1) Are initiator caspases, caspase-8, -9, and an effector caspase, caspase-3 involved in TNF-mediated cytotoxicity in B/C-N, 10ME, and L88.3 cell lines?
- 2) Presuming caspases are involved, are caspases the only lytic pathway activated in TNF-mediated cytotoxicity? Additionally, since TNF could induce apoptotic or necrotic death we investigated the role of oxygen radical generation and activation of PLA<sub>2</sub>.
- 3) Does heat-shock pretreatment at 42 °C for two hours differentially protect B/C-N, 10ME, and L88.3 cell lines from TNF-mediated cytotoxicity? And, if so, is the protection conferred by heat-shock pretreatment due to inhibition of caspase activation (inhibition of a lytic mechanism)?

## CHAPTER 2

### MATERIALS AND METHODS

#### *2.1 Medium and cell lines*

All murine cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, and 100 units of penicillin and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO). Cells were grown at 37 °C with 5% CO<sub>2</sub> in an incubator. Murine cell lines B/C-N, 10ME, and L88.3 were derived from Balb/c mice by Patek et al. (1978). These cell lines are fibroblastic cell lines, and represent different stages of tumor progression. For characteristics and development of these cell lines, please see chapter 1 section 1.4. L929 is a murine fibrosarcoma cell line (Sanford et al., 1948).

#### *2.2 Reagents*

Recombinant murine TNF was provided by Genentech (San Francisco, CA). One lytic unit is the amount of TNF which can lyse 50% of the L929 cells in a standardized assay. Cycloheximide (CHX), butylated hydroxyanisole (BHA), and quinacrine were purchased from Sigma Chemical Co. (St. Louis, MO). CHX, a translation inhibitor, was dissolved in supplemented DMEM and used at a final concentration of 10 µg/ml. BHA, an oxygen radical scavenger, was used at a final concentration of 100 µM. BHA was dissolved in DMSO (Sigma, St. Louis, MO) at stock concentration of 300 mM and aliquots were frozen until use. Quinacrine, a specific PLA<sub>2</sub> inhibitor, was dissolved in

supplemented DMEM and used at a final concentration of 1  $\mu$ M. Caspase inhibitors z-VAD-fmk and z-DEVD-fmk were purchased from ICN Biomedicals, Inc. (formerly Enzyme Systems Products, Livermore, CA) and used at a final concentration of 20  $\mu$ M. Z-VAD-fmk and z-DEVD-fmk were dissolved in DMSO (Sigma, St. Louis, MO) at stock concentration of 20 mM and aliquots were frozen until use.

### 2.3 *Heat shock treatment and caspase inhibitors pretreatment*

For heat shock treatment, target cells were counted and plated in a 60 mm tissue culture dish (petri dishes that are vacuum gas plasma-treated to facilitate adhesion of cells) with 4 ml of supplemented DMEM the night before the experiment. On the day of the experiment, cells were heat shocked at 42 °C with 5% CO<sub>2</sub> for 2 hours. If used in an *in vitro* chromium-release assay, these heat-treated targets were incubated with 100  $\mu$ Ci/ml <sup>51</sup>chromium (<sup>51</sup>Cr) for 80 – 90 minutes at 37 °C following 2 hours of heat shock treatment. If used in a caspase enzyme activity assay, cells were plated in a 100 mm tissue culture dish, after 2 hours of heat shock treatment, cells were incubated at 37 °C with 100 units TNF/plate for various lengths of time.

For preincubation with caspase inhibitors z-VAD-fmk or z-DEVD-fmk, target cells were counted and plated in a tissue culture dish with supplemented DMEM the night before. For an *in vitro* chromium-release assay, targets were incubated with <sup>51</sup>Cr (100  $\mu$ Ci/ml) for 80 – 90 minutes at 37 °C. The target cells were then washed, counted, and diluted to 2 x 10<sup>5</sup> cells/ml in supplemented DMEM with z-VAD-fmk or z-DEVD-fmk at a final concentration of 20  $\mu$ M. Targets were plated in 96-well tissue culture microtiter

plates containing 50  $\mu$ l/well of cells (i.e.,  $10^4$  cells) and inhibitors, and incubated at 37 °C for one hour. Appropriate concentrations of TNF (50  $\mu$ l/well) were added at the end of one hour preincubation with z-VAD-fmk or z-DEVD-fmk.

When z-VAD-fmk or z-DEVD-fmk was used in caspase activity assays, they were added to the 100 mm tissue-culture-treated dishes at a final concentration of 20  $\mu$ M and incubated at 37 °C for one hour. At the end of one-hour preincubation with z-VAD-fmk or z-DEVD-fmk, TNF was added at 100 units/dish (1,000 U TNF/ml) to these 100 mm tissue-culture-treated dishes and incubated at 37 °C for various lengths of time.

#### *2.4 In vitro Chromium release assay*

On the day of the experiment, target cells were incubated in radioactive sodium chromate ( $^{51}\text{Cr}$  100  $\mu\text{Ci/ml}$ ) for 80 to 90 minutes in a tissue-culture-treated dish in Hank's balanced salt solution (HBSS). During this incubation period, the sodium chromate diffuses into the target cells. The chromate binds to macromolecules in the cells and cannot diffuse out. After the incubation period,  $^{51}\text{Cr}$  not taken up by live target cells is removed by washing with supplemented DMEM media. Serum proteins in the medium will bind free  $^{51}\text{Cr}$ -isotope left in a plate. Chromium-labeled targets were diluted to  $2 \times 10^5$  cells/ml with supplemented DMEM. Mixing of  $^{51}\text{Cr}$  labeled targets (50  $\mu$ l containing  $10^4$  cells) with 50  $\mu$ l of effectors (e.g. TNF or cytotoxic cells) at appropriate concentrations causes lysis of target cells. In these experiments, various concentrations of TNF (0.01 unit – 100 units/well in DMEM) are added to 96-well tissue culture

microtiter plates containing a final volume of 0.1 ml/well. The amount of  $^{51}\text{Cr}$  released into the supernatant is proportional to the number of cells killed.

The amount of  $^{51}\text{Cr}$  released was determined at 5 hours, 7 hours or 18 hours after targets and TNF were mixed in a well. The percent (%) specific  $^{51}\text{Cr}$  release, equivalent to target lysis, was calculated by the following formula:

$$\% \text{ specific lysis} = \frac{(\text{Sample cpm} - \text{spontaneous cpm})}{(\text{total cpm} - \text{spontaneous cpm})} \times 100$$

Total cpm is the total amount of radioactivity in the well. Spontaneous cpm refers to the amount of radioactivity released from the targets in the absence of TNF. Percentage spontaneous release is calculated by the following formula:

$$\% \text{ spontaneous release} = \frac{\text{Sample cpm (no TNF)}}{\text{total cpm}} \times 100$$

Each data point is the average of triplicate samples. Triplicate samples rarely vary more than plus or minus 10% from the average. Spontaneous release did not go above 35% in all the experiments. Generally difference in 10% or more lysis between different treatments is considered significant.

Although there is little difference between identically treated samples in a given assay ( $\pm 10\%$ ), there can be significant differences in the absolute amount of killing on a given day. However, the rank order of the results are very consistent and this consistency provides the confidence in the overall experimental results. Thus, for example, although the absolute amount of cell lysis for B/C-N, 10ME and L88.3 may vary from day to day, we consistently find that B/C-N is always poorly lysed by TNF, L88.3 is lysed more than B/C-N, however the level of lysis is still low, and 10ME is always lysed much more than

B/C-N and L88.3. These kind of result are not particularly amenable to statistical analysis but the relative consistency of the repeated experiments make them quite amenable to experimentation and serious analysis. Thus, this is the standard for mammalian cell cytotoxicity assays.

In an experiment where cycloheximide (CHX) was added to the assay, it was added at 50  $\mu$ l/well (final concentration = 10  $\mu$ g/ml) 2 hours after the targets and TNF were mixed. It was found that addition of CHX 2 hours after the targets and TNF were mixed caused maximum effect on TNF-mediated cytolysis by blocking a protein-synthesis-dependent resistance mechanism (Patek et al., 1987).

In an experiment where BHA was added to the assay, the stock solution was diluted with supplemented DMEM, and added at 50  $\mu$ l/well (final concentration = 100  $\mu$ M) right after TNF was mixed with the target cells.

In an experiment where quinacrine was added to the assay, it was added at 50  $\mu$ l/well (final concentration = 1  $\mu$ M) immediately after TNF was mixed with the target cells.

## 2.5 *Caspase enzyme activity assay*

*In vitro* caspase enzymatic activity is based on the principle that caspases are synthesized as inactive procaspases and their activation is induced by apoptotic stimuli received by the target cells. Thus, in the untreated cells, activation of procaspases to become active caspases is at basal level. However, in the cells that are treated to induce apoptosis, procaspases are proteolytically cleaved to become active caspases. Only the



activated caspases can proteolytically cleave substrates. Three steps are involved in measurement of a caspase enzymatic activity. First, target cells are treated to induce apoptosis. Then these treated cells are lysed to collect their intracellular contents. The next step is to test cell lysates for caspase (protease) activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroanilide (p-NA). A caspase-specific peptide (a substrate for an active caspase) is based on each caspase's known specific tetrapeptide cleavage recognition sequence of its cellular substrate(s). Lastly, spectrophotometric quantitation of the released chromophore, p-NA, due to cleavage of the caspase-specific peptide is measured at 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction (development of a yellow color).

Detection of caspase-8, -9, and -3 activities were measured using caspase colorimetric assay kits from R & D Systems (Minneapolis, MN). The kit provided cell lysis buffer, dilution buffer, reaction buffer (2x), DTT, and an appropriate caspase colorimetric substrate. For detection of caspase-8 activity, IETD-pNA (p-nitroaniline) substrate was provided in the kit. For detection of caspase-9, LEHD-pNA substrate was provided in the kit. For detection of caspase-3, DEVD-pNA substrate was provided in the kit.

For the preparation of cell lysates, cells were counted and plated in 100 mm tissue culture dishes with supplemented DMEM the night before the assay. After appropriate pretreatment (heat shock or caspase inhibitors), TNF was added to these plates at 100 units/plate and incubated at 37 °C for various lengths of time. If CHX was used, it was

added to the targets two hours after addition of TNF. CHX was used at a final concentration of 10 µg/ml. At the end of the TNF treatment, the cells were washed and counted in HBSS. After the total cell count was determined for each sample, cells were centrifuged at 250 x g for 10 minutes. The supernatant was removed, and appropriate amounts (25 µl per 1 x 10<sup>6</sup> cells) of cold cell-lysis buffer was added to each sample. The sample was then incubated on ice for 10 minutes. Cell lysates were centrifuged at 10,000 x g for one minute. The supernatant was transferred to a new microfuge tube and kept on ice to be used in a caspase colorimetric assay. Fifty µl of cell lysate, 50 µl of reaction buffer (mixed with DTT), and 5 µl of appropriate caspase-colorimetric substrate were mixed in a well of a flat-bottomed 96-well ELISA plate. The plate was incubated at 37 °C for two hours. The results were read on a microplate reader at 405 nm, and the increase in each caspase activity was expressed as a ratio of fold increase of (an OD of a treated sample)/(an OD of an untreated control).

Similar to the <sup>51</sup>Cr-release assays, there can be significant difference in results from “identical” caspase activity assays from day to day. However, like the <sup>51</sup>Cr-release assays, repeated assays give the same relative results such that there is confidence that a particular treatment always has the same relative effects on various cell lines. Although this is not the ideal situation, it is a limitation of studies on mammalian cell lines where minor differences in cell density, serum, temperature, CO<sub>2</sub> concentration and any number of unknown influences have effects on the behavior of the cells.

## *2.6 Microscopic observation of target cell morphology*

Target cells were counted and plated in 35 mm tissue culture treated dishes with supplemented DMEM the night before the various treatments. Targets were either not treated (untreated control), treated with 100 units TNF/dish, treated with z-VAD-fmk only at 20  $\mu$ M final concentration, or treated with z-VAD-fmk for one hour followed by 100 units TNF/dish for various lengths of time up to 6 hours at 37 °C. The untreated control and treated target cells were observed hourly under an inverted microscope at a total magnification of 200x. A digital camera was used to take pictures of these untreated and treated targets.

## CHAPTER 3

### CASPASE ACTIVATION IS ONE OF THE LYTIC PATHWAYS UTILIZED IN TNF-MEDIATED CYTOTOXICITY

When TNF binds to TNF-R1, it induces two signals: 1) activation of a cytotoxic mechanism which is protein-synthesis-independent, and 2) activation of a resistance mechanism which is protein-synthesis dependent. In TNF-sensitive cells, when TNF binds to TNF-R1, the protein-synthesis-independent lytic mechanism is activated while the protein-synthesis-dependent resistance mechanism is poorly activated. In TNF-resistant cells, when TNF binds to TNF-R1, the protein-synthesis-independent lytic mechanism is activated along with the protein-synthesis-dependent-resistance mechanism. Most TNF-resistant cells become TNF-sensitive when transcription inhibitors, such as Actinomycin D (Act D), or translation inhibitors, such as cycloheximide (CHX), are added, because these agents block resistance but have little effect on the protein-synthesis-independent lytic mechanism.

TNF can mediate apoptotic or necrotic death, depending on the cell lines used (Laster et al., 1988). Recently, signal transduction mediated through TNF receptors (TNF-R) has been elucidated as discussed in Chapter 1 (Figure 1). However, understanding the downstream signaling events remain unresolved due to the complexity of the signaling pathways and variations from one cell type to another. Several caspases have been implicated in TNF-mediated apoptosis including both the initiator caspases (caspase-2, -8, and -9) and the effector caspases (caspase-3, and -7) (Duan et al., 1996;

Higuchi et al., 1997, Li H et al., 1997; Li P et al., 1997 ; Zou et al., 1997; Kuida et al., 1998; Jaeschke et al., 1998; Yamashita et al., 1999; Sun et al., 1999). Activation of caspase-8 indicates activation of a death receptor-mediated signaling pathway leading to a caspase cascade culminating in activation of caspase-3 and apoptotic death in the target cells. Activation of caspase-9 indicates activation of the mitochondrial signaling pathway. In the mitochondrial signaling pathway, cytochrome c is released from mitochondrial membranes into the cytoplasm, followed by the binding of cytochrome c to Apaf-1. Then a complex of cytochrome c/Apaf-1/caspase-9 is formed, initiating the caspase cascade leading to activation of caspase-3 and apoptotic death of the target cells (see Figure 1). Hence, we investigated which caspase pathways are activated during TNF-mediated cytotoxicity of the cell lines B/C-N, 10ME, and L88.3. Since these cell lines represent different stages of tumor progression (Figure 2), the data might give insight into the effects of malignant transformation on a cells' sensitivities to host protective mechanisms, such as TNF.

TNF cytotoxicity is typically measured in an 18-hour chromium-release assay. B/C-N, 10ME, and L88.3, along with the prototypic TNF target cell line L929, were used in these studies. When treated with TNF alone, the TNF-sensitive cell line L929 showed 52% killing at 1 unit of TNF and reached 61% killing at 100 units of TNF (Figure 3). This is a typical trend seen in L929 cell line, and is expected because 1 unit of TNF is, by definition, supposed to cause 50% killing in L929 cells.

The B/C-N cell line, which represents a “normal” (N) cell line in our tumor progression model (Figure 2), is TNF resistant. When treated with TNF alone, only

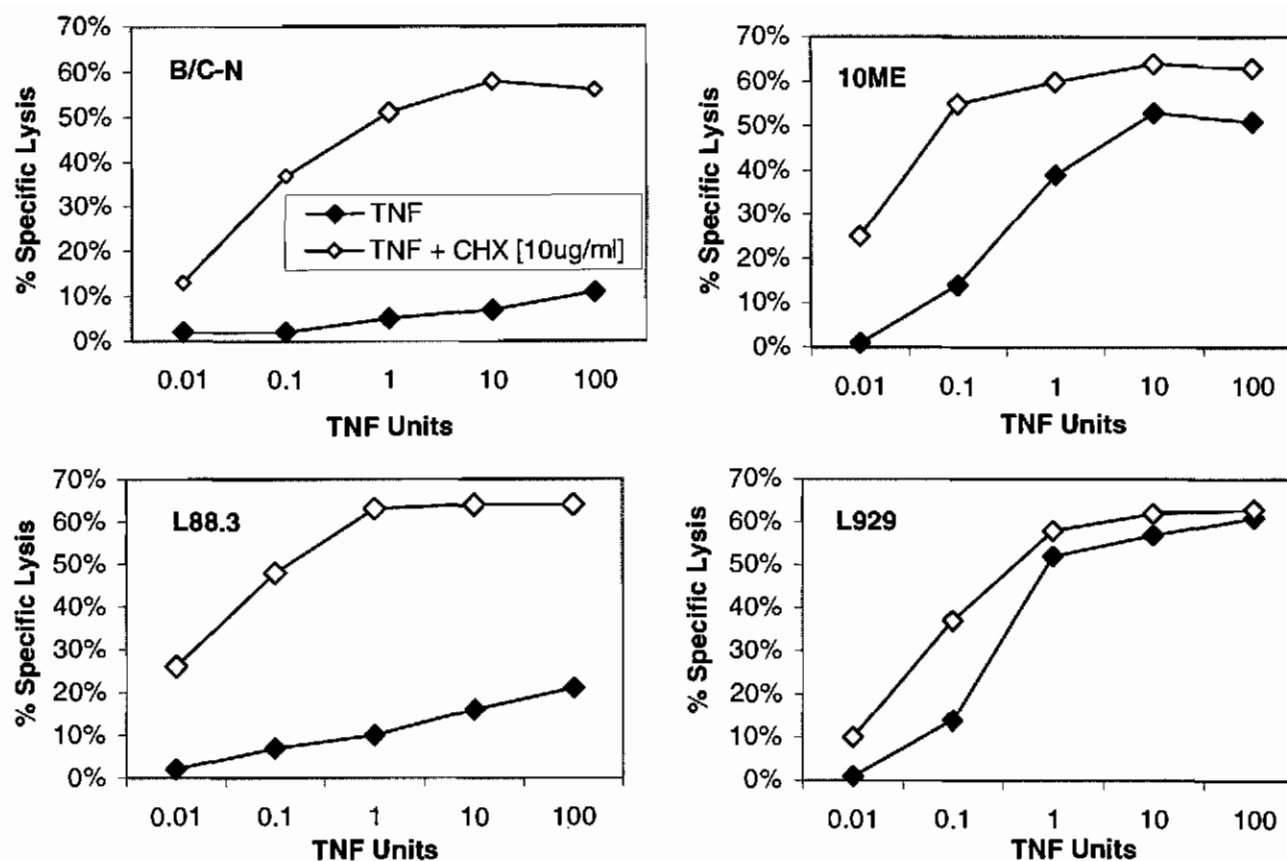


Figure 3 10ME and L929 cell lines are sensitive to TNF-mediated cytotoxicity while B/C-N and L88.3 are not. Addition of CHX can render B/C-N and L88.3 to become TNF sensitive. B/C-N represents a “normal” (N) cell line, 10ME represents an “intermediate” (I) cell line, and L88.3 represents a “cancer” (C) cell line in our tumor progression model. L929 is a prototype TNF-sensitive cell line used as a positive control. TNF-mediated cytotoxicity was measured by 18-hour chromium release assay. CHX was added 2 hours after mixing TNF and targets (method described in Chapter 2 Material and Methods).

11% of the cells were killed even at 100 units of TNF (Figure 3). However, addition of 10 µg/ml of CHX two hours into the 18 hour TNF assay (TNF + CHX), only required 0.01 unit of TNF to reach 13% of killing. Also the TNF + CHX dose-response curve for B/C-N was very similar to the curve for the TNF-sensitive cell line L929 treated with TNF alone (Figure 3). One unit of TNF + CHX showed 51% killing of B/C-N. TNF-mediated cytolysis went up to 58% at 10 units of TNF and decreased to 56% at 100 units of TNF. Hence, B/C-N is normally TNF resistant, but TNF + CHX treatment rendered B/C-N TNF sensitive. Thus, these results indicated that TNF treatment activated protein-synthesis-dependent TNF resistance pathway in B/C-N. Blocking that pathway with CHX rendered the cells sensitive to TNF-mediated lysis.

The 10ME cell line, which represents an “intermediate” (I) cell on the pathway to cancer, is TNF sensitive. Its sensitivity to TNF is very similar to that of L929 (Figure 3). For 10ME, TNF-mediated cytotoxicity reached 51% at 1 unit of TNF, and peaked at 10 units of TNF with 58% of cell death. Then killing decreased to 56% at 100 units of TNF. Addition of 10 µg/ml CHX increased killing of 10ME, but not as much as for B/C-N. 10ME required 10 units of TNF to reach 58% killing by TNF. However, in TNF + CHX, 10ME required only 0.1 unit of TNF to reach 55% killing. TNF-mediated cytolysis reached 64% at 10 units of TNF in the presence of CHX and decreased to 63% in 100 units of TNF. Therefore, 10ME is another TNF-sensitive cell line, like L929, and addition of CHX to the TNF assay showed intermediate increase in TNF-mediated killing. These results indicated that TNF treatment activated protein-synthesis-

independent lytic pathway, as well as some level of a protein-synthesis-dependent TNF-resistant pathway in 10ME.

The L88.3 cell line, which represents the cancer (C) phenotype in our model (Figure 2), is TNF resistant, but not as resistant as B/C-N. Twenty one percent of L88.3 cells were killed at 100 units of TNF (Figure 3). However, for TNF + CHX, it only required 0.01 unit of TNF to reach 26% killing. L88.3 treated with TNF + CHX also showed similar killing as L929 treated with TNF alone. In the presence of CHX, TNF-mediated cytotoxicity reached 48% at 0.1 unit of TNF, then killing went up to 64% for 10 units and 100 units of TNF. Thus, similar to B/C-N, L88.3 is TNF resistant, but became TNF sensitive with TNF + CHX treatment. These results indicated that TNF treatment of L88.3 activated a protein-synthesis-dependent resistant pathway, and a protein-synthesis-independent lytic pathway; although there is some lysis of L88.3 by TNF, the resistance pathway protects most of the cells.

As mentioned earlier, the TNF-sensitive cell line L929 showed 52% killing at 1 unit of TNF and reached 61% killing at 100 units of TNF (Figure 3). Addition of 10  $\mu$ g/ml of CHX showed least increase in killing compared to B/C-N, 10ME, and L88.3 cell lines. L929 required 10 units of TNF to reach 57% killing in TNF, but, in TNF + CHX, L929 required 1 unit of TNF to reach 58% killing. Thus, L929 is a TNF-sensitive cell line, and of the four cell lines examined here, it showed the least increase in killing by the addition of the protein synthesis inhibitor CHX. Hence, TNF treatment activated a protein-synthesis-independent lytic pathway in L929, but failed to activate a high level of a protein-synthesis-dependent resistance mechanism.



### 3.1 *Caspase activities peaked around 10 to 12 hours of TNF treatment.*

In order to determine whether caspases were activated during TNF-mediated cytotoxicity of our cell lines, a chromogenic caspase enzyme activity assay was utilized. Activation of two initiator caspases, caspase-8 and caspase-9, and one effector caspase, caspase-3, were detected by mixing cell lysates with caspase specific chromogenic substrates. The ratio of the caspase activity of treated over the caspase activity of untreated cells, expressed as a fold increase, was used to determine treatment effect. A fold increase of 1.0 means the caspase activity of TNF-treated cells and the caspase activity of untreated cells was the same.

For the TNF-resistant cell line B/C-N, a 5-hour treatment with 100 units of TNF/dish did not cause activation of caspase-8, -9, or -3 (Table 1). There were no increase for any of the three caspases, since the level of activities for caspase-8, caspase-9 and caspase-3, were about the baseline level (1.0-fold increase). These results suggested that the protein-synthesis-dependent resistance mechanism was already activated after 5 hours of TNF treatment. This is consistent with the CHX data showing that addition of CHX to the TNF assays after 2 hours of TNF treatment causes diminished CHX effects, indicating that that a 2-hour treatment is sufficient to begin activation of the protein-synthesis-dependent resistance mechanism.

At 7 hours of TNF treatment of B/C-N, caspases-8 and -9 activity levels were still at baseline, but caspase-3 activity showed a slight increase. Caspase-3 showed 1.6-fold increase (Table 1). By 12 hours of TNF treatment, all three caspases showed some increase in activities compared to 5 hours and 7 hours of TNF treatment. Both initiator

Table 1

Caspase activities observed with TNF treatment at 5, 7, 10 and 12 hours

Target cells	Length of TNF treatment	Fold increase		
		Caspase-8	Caspase-9	Caspase-3
B/C-N (N line)	5 hr	1.0	0.9	1.1
	7 hr	1.1	1.0	1.6
	12 hr	1.5	1.5	3.3
10ME (I line)	5 hr	1.3	1.2	2.5
	7 hr	1.7	1.6	3.2
	12 hr	1.5	1.9	4.3
L88.3 (C line)	5 hr	1.2	1.3	1.7
	7 hr	1.5	1.4	2.1
	10 hr	1.7	1.7	3.0
L929	5 hr	1.3	1.3	1.6
	7 hr	1.5	1.0	2.2
	12 hr	2.2	2.8	7.1

caspace-8 showed similar levels of activation at 12 hours of TNF treatment: caspace-8 showed a 1.5-fold increase and caspace-9 showed 1.5-fold increase over the caspace activity of the untreated cells (Table 1). For the effector caspace, caspace-3, activity showed 3.3-fold increase (Table 1). These increases in three caspace activities were still lower than the caspace activities detected in TNF-sensitive cell lines 10ME and L929 after similar treatments (Table 1). Since the cells B/CN is not lysed, these levels of caspace activities are not sufficient to kill the cells. Caspace activities for all three caspaces decreased after 12 hours of TNF treatment (data not shown). These data suggested that TNF-mediated cytolysis activated both TNF receptor signaling pathway (caspace-8-mediated) and the mitochondrial signaling pathway (caspace-9-mediated) leading to apoptotic death in B/C-N. Caspace activities did not increase significantly until 12 hours of TNF treatment in B/C-N, and decreased after 12 hours of TNF treatment. These data also showed that TNF-resistant characteristic of B/C-N resulted in low levels of TNF-induced caspace activity.

In the TNF-sensitive cell line 10ME, increase in caspace-3 activity could be detected within 5 hours of TNF treatment. Fold increases for both caspace-8 and caspace-9 were still close to the baseline. Caspace-3 was up 2.5-fold (Table 1). After 7 hours of TNF treatment, activity of all three caspaces had increased compared to the untreated and the 5-hour TNF treatment. By 12 hours of TNF treatment, caspace-8 increased 1.5-fold, and caspace-9 increased 1.9-fold and caspace-3 increased 4.3-fold (Table 1). The 10ME cell line showed decrease in all three caspaces activities after 12 hours of TNF treatment (data not shown).

Another TNF-sensitive cell line, L929, showed a similar pattern of time-dependent increase in TNF-induced caspase activities. For L929, 5 hours of TNF treatment showed baseline level of caspase activities in caspase-8 and caspase-9 (1.3-fold increase for both caspase-8 and caspase-9). Caspase-3 showed slight increase compared to the untreated control at 1.6-fold increase. For L929, TNF treatment for 7 hours caused some increase in caspase-3 activity, but caspase-8 and -9 levels remained similar to the untreated control or 5 hours of TNF treatment. At 12 hours of TNF treatment, all three caspases showed significant increase. Caspase-8 showed a 2.2-fold increase. Caspase-9 showed a 2.8-fold increase. And caspase-3 showed a 7.1-fold increase. All three caspase activities decreased after 12 hours of TNF treatment (data not shown).

Between the two TNF-sensitive cell lines, L929 showed higher caspases activation compared to 10ME at 12 hours of TNF treatment. This trend may reflect the higher TNF-mediated killing of L929 compared to 10ME seen in the 18-hour chromium-release assay (Figure 3). For both of the TNF-sensitive cell lines, 10ME and L929, caspase activities for all three enzymes peaked at 12 hours of TNF treatment. 10ME showed a rise in caspases activities steadily from 5 hours to 12 hours of TNF treatment. However, in L929, rise in caspases activities was delayed compared to 10ME. It showed a large increase between 7 hours to 12 hours of TNF treatment (Table 1). These increases in both caspase-8 and caspase-9 activities suggested that TNF-mediated cytolysis in 10ME and L929 also involves both the TNF receptor signaling pathway as well as the mitochondrial signaling pathway leading to apoptotic death.

In another TNF-resistant cell line, L88.3, treatment with TNF for 5 hours showed low caspase activities (Table 1) compared to TNF-sensitive cell lines 10ME and L929. Both caspase-8 and caspase-9 activities were close to the baseline level. Caspase-3 showed 1.7-fold increase. At 7 hours of TNF treatment, caspase-8 and caspase-9 activities were similar to the activity levels at 5 hours of TNF treatment. Caspase-3 showed 2.1-fold increase at 7 hours (Table 1). At 10 hours of TNF treatment, all three caspases showed increase compared to the untreated control. Caspase-8 showed 1.7-fold increase. Caspase-9 showed 1.7-fold increase. And caspase-3 showed 3-fold increase. All three caspases activities decreased after 10 hours of TNF treatment (data not shown). L88.3 showed a similar caspases activity pattern as another TNF-resistant cell line B/C-N. These data also indicated that in L88.3, TNF-mediated cytolysis activated both TNF receptor signaling pathway as well as the mitochondrial signaling pathway.

These data clearly indicated that caspases-8, -9, and -3 activities are involved in TNF-mediated cytolysis in B/C-N, 10ME, L88.3, and L929. These caspase activities peaked at 12 hours for cell line B/C-N, 10ME, and L929, but peaked at 10 hours for cell line L88.3 (Table 1). The level of caspase activation reflected these cell lines' susceptibility to TNF-mediated cytolysis (Figure 3). B/C-N, the cell line most resistant to TNF-mediated cytolysis, showed the least activities of the three caspases (caspase-8, -9, and -3) when compared to other cell lines. All three caspases activities in B/C-N were lower due to activation of its protein-synthesis-dependent TNF resistance mechanism by 5 hours of TNF treatment.

In L88.3, another TNF-resistant cell line, all three caspases activities were detected. Their activity levels were between that of B/C-N and TNF-sensitive cell lines of 10ME and L929. This is consistent with the idea that the level of cell lysis is directly related to the level of caspase activity since L88.3 showed intermediate levels of caspase activities (more than B/CN and less than 10ME and L929) and is of intermediate sensitivity to TNF-mediated lysis (Figure 3).

Activation of the two initiator caspases (caspase-8 and -9) seemed to increase simultaneously in all four cell lines used. Both caspases are known to activate caspase-3 and our data are consistent with that. These data also indicate that both the TNF-receptor signaling pathway and the mitochondrial-signaling pathway are activated in all four cell lines used.

### *3.2 Increase in TNF-mediated cytolysis due to addition of CHX correlates with increase in caspase activities.*

As shown in Figure 3, TNF-resistant cell lines B/C-N and L88.3 become TNF-sensitive with the addition of 10 µg/ml of CHX two hours into the 18-hour TNF cytotoxicity assay (i.e., a chromium-release assay). If caspase activity was central to TNF-mediated cytotoxicity, we thought this reversal in TNF resistance due to the addition of CHX might correlate to increase in caspases activities. Therefore, chromium-release assays and caspase activity assays were conducted at 5 and 7 hours after TNF treatment to see whether there is a correlation between the increased killing by TNF in the presence of a protein synthesis inhibitor and an increase in caspase activities.

### 3.2.1 *B/C-N*

For B/C-N, we found there was a correlation between the increase in TNF-mediated cytolysis and an increase in caspase activities caused by the addition of CHX (Figure 4). This phenomenon has been shown in other TNF-resistant cell lines which become TNF-sensitive with addition of a transcription inhibitor or a translation inhibitor (Binder et al., 1999; Khwaja and Tatton, 1999). In 5-hour chromium-release assays, addition of 10 µg/ml of CHX caused a slight increase in TNF-mediated cytolysis compared to TNF alone (Figure 4). Addition of CHX two hours into the 5-hour assay revealed a small increase in the activity of caspase-8 and caspase-9. However, the addition of CHX to the assay resulted in a 2-fold increase in caspase-3 compared to TNF alone; at 5 hours of TNF treatment, caspase-3 activity increased 1.5-fold, whereas 5 hours of TNF treatment plus CHX caused a 3.4-fold increase. This 3.4-fold increase in caspase-3 activity at 5 hours of TNF plus CHX is equivalent to the peak of caspase-3 activity seen with 12 hours of TNF treatment (3.3-fold increase). Thus, addition of CHX to TNF treatment seems to speed up the time course for TNF-mediated cytolysis as well as caspase activities in a TNF-resistant cell line B/C-N. This may explain the large increase in caspase-3 activity for 5 hours of TNF treatment plus CHX without corresponding large increase in caspase-8 and caspase-9 activities. Khwaja and Tatton (1999) showed that in TNF-resistant cell lines U937 and NIH 3T3, addition of CHX to TNF treatment caused a large increase in caspase-8 activity two hours into the treatment, while treatment with TNF alone barely showed increase in caspase-8 activity up to 6 hours of TNF treatment. Thus, a large

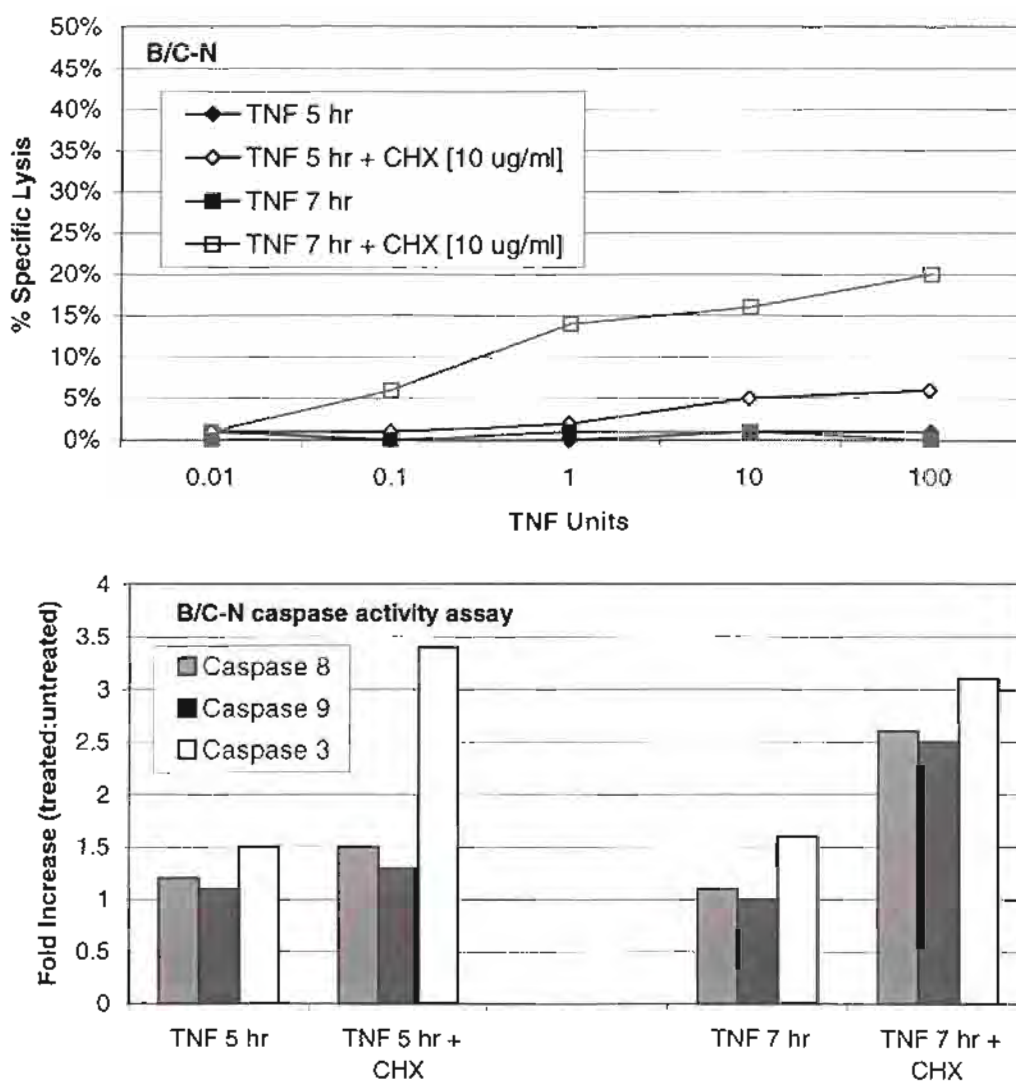


Figure 4 For B/C-N, addition of CHX increased TNF-mediated cytotoxicity and caspases activities. TNF-mediated cytotoxicity was measured by 5- and 7-hour chromium-release assays with or without addition of 10  $\mu$ g/ml CHX two hours after targets and TNF were mixed. For caspase activities, targets were incubated with 100 units TNF for 5 or 7 hours with or without addition of 10  $\mu$ g/ml of CHX two hours after TNF was added. Caspase activity was measured using kits from R &D System (see Chapter 2 for details).



increase in caspase-8 and caspase-9 activities probably occurred earlier than 5 hours of TNF treatment plus CHX.

As at 5 hours, in the 7-hour chromium-release assay TNF did not mediate cytolysis of B/C-N. However, TNF plus CHX for 7 hours showed the most TNF-mediated cytolysis, up to 20% at 100 units TNF (Figure 4). As for the 7-hour caspase activity assays, B/C-N treated for 7 hours with TNF showed a similar level of caspase-8 and caspase-9 activities that differed little from the baseline levels. In the absence of CHX, caspase-3 levels, after 7 hours of TNF treatment, were similar to the levels at 5 hours (Figure 4). These low levels of caspase activity correlated well with the low cytotoxicity measured in the chromium-release assays. Cells treated for 7 hours with TNF plus CHX showed a large increase in caspase-8 and caspase-9 activities compared to similarly treated cells without CHX or cells treated for 5 hours with both TNF and CHX. Caspase-8 increased to 2.6-fold in the presence of TNF and CHX. Caspase-9 increased to 2.45-fold when CHX was added. These large increase in caspase-8 and caspase-9 activity levels in B/C-N treated with TNF plus CHX were closer to the caspase-8 and caspase-9 activity levels seen in the TNF-sensitive cell line L929 treated for 12 hours with TNF (Table 1). For B/C-N in the 7-hour assay, caspase-3 increased 1.6-fold for TNF alone, and 3.1-fold for TNF 7 hours plus CHX. Although at 7-hour treatment with TNF plus CHX manifests an increase in caspase-8 and caspase-9 activities as compared to the 5-hour treatment, caspase-3 activity decreased slightly from 5 to 7 hours. This decrease could be due to the increased caspase-8 and caspase-9 activities running out of substrate (due to suppression of *de novo* protein synthesis by CHX), along

with a rapid turnover of active caspase-3. It is also possible that there is an increase in caspase-3 activity at a later time point due to increased caspase-8 and caspase-9 activities at 7 hours of treatment with TNF plus CHX.

### 3.2.2 10ME

In the TNF-sensitive cell line 10ME, addition of CHX to the 18-hour cytotoxicity assay showed a moderate increase in cytolysis above the already high toxicity of TNF alone (Figure 3). Addition of CHX at T = 2 hours into the 5-hour chromium-release assay showed some increase in TNF-mediated cytolysis (13% killing at 100 units TNF) compared to TNF treatment without CHX (3% killing at 100 units TNF) (Figure 5). For the 5 hour-caspase assay, addition of 10 µg/ml of CHX two hours into the 5-hour TNF assay showed a increase in caspase-3 activity, however, caspases-8 and -9 activities remained at the baseline levels (Figure 5). Caspase-3 increased significantly with addition of CHX (1.4-fold above baseline in TNF alone to 2.6-fold for TNF plus CHX). However, the increase in caspase-3 activity seen in 10ME was much lower than that seen in B/C-N (Figure 4). These differences seen between B/C-N and 10ME might help explain the protein-synthesis-dependent TNF resistance mechanism observed in B/C-N; it is conceivable that caspase activities are not fully activated in B/C-N because of the protein-synthesis-dependent resistance mechanism. However, in the presence of protein synthesis inhibitors, there is full activation of all the caspases and this results in maximum cell death. In other words, B/C-N fully expresses its protein-synthesis-independent lytic mechanism only in the presence of protein synthesis inhibitors.

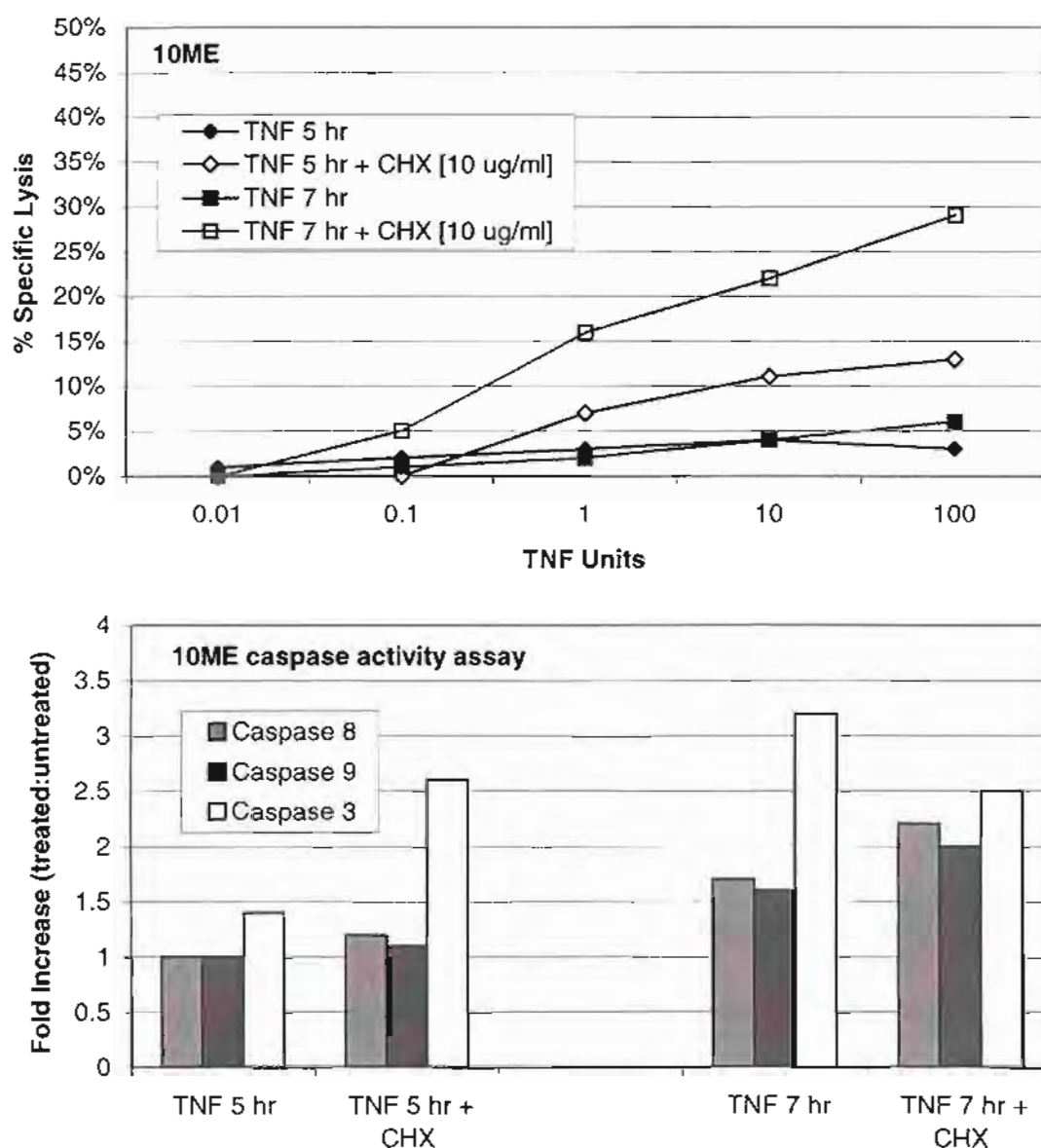


Figure 5 For 10ME, addition of CHX increased TNF-mediated cytotoxicity and caspases activities. TNF-mediated cytotoxicity was measured by 5- and 7-hour chromium-release assays with or without addition of 10  $\mu$ g/ml CHX two hours after targets and TNF were mixed. For caspase activities, targets were incubated with 100 units TNF for 5 or 7 hours with or without addition of 10  $\mu$ g/ml of CHX two hours after TNF was added. Caspase activity was measured using kits from R & D System (see Chapter 2 for details).

As with B/C-N, CHX can cause a dramatic change in the level of TNF-mediated killing of 10ME. This is best observed after 7 hours of exposure to TNF where TNF alone cause almost no killing but the addition of CHX to the TNF resulted in 29% cytotoxicity (Figure 5). As for caspase activities, 7 hours after the TNF treatment, the data reveal an increase in all three caspases compared to TNF treatment for 5 hours (Figure 5). Although caspase-8 remained at baseline levels after 5 hours in TNF, it increased 1.7-fold by 7 hours. Similarly, at 7 hours caspase-9 activity increased 1.6-fold above baseline and 5 hour levels. At 7 hours of TNF treatment, caspase-3 level increased to 3.2-fold above that of untreated cells whereas it had only increased 1.4-fold at 5 hours. These increases in caspase activities are early indicators that 10ME will be lysed in the course of the 18 hours assay period.

Seven hours of TNF plus CHX showed an even more dramatic increase in caspase-8 and -9 activities. In the presence of CHX, TNF-induced caspase-8 activity rose to 2.2-fold above baseline. Caspase-9 activity increased 2.0-fold. These increases in caspase-8 and caspase-9 activities after 7 hour of TNF plus CHX treatment were higher than the activity levels observed for 10ME after 12 hours of TNF treatment (Table 1). In contrast to the increases in caspase-8 and caspase-9 that resulted from TNF and CHX treatment, caspase-3 activity remained about the same at 5 and 7 hours of treatment. Although the caspase-3 activity was high 7 hours after TNF treatment began, addition of CHX caused a decrease in caspase-3 activity from 3.2-fold above background in the presence of TNF to 2.5-fold above background in the presence of TNF plus CHX. The

level of caspase-3 activity after 7 hours in TNF and CHX remained essentially unchanged from cells with the same treatment over only 5 hours.

Perhaps the lower caspase-3 activity seen in the 7-hour treatment with TNF plus CHX versus TNF alone is the result of the early (i.e., 5 hour) activation of caspase-3 and the exhausting of the procaspase-3 pool due to suppression of *de novo* protein synthesis by CHX. The opposite is also possible; the rise in caspase-8 and caspase-9 at 7 hours TNF plus CHX may indicate that a rise in caspase-3 activity would be seen at later time points.

### 3.2.2 L88.3

Although normally TNF-resistant, L88.3, like B/C-N, became TNF-sensitive when the protein synthesis inhibitor CHX was added 2 hours into an 18-hours chromium-release assay for cytotoxicity (Figure 3). Likewise, L88.3 also increased caspase activation in the presence of CHX (Figure 6). Thus, although no lysis of L88.3 was detectable after 5 hours of TNF treatment, similar treatment in the presence of CHX resulted in some degree of killing (14%). Caspase-8, and caspase-9 activities were slightly increased by the addition of CHX to the 5-hour TNF assays (Figure 6). Caspase-3 activity increased to 3.0-fold with addition of CHX to the 5-hour TNF assays. After 7 hours of TNF treatment, L88.3 was still not measurably lysed. However the addition of CHX to the assay caused a marked increase in killing such that at 100 units TNF, the level of killing was 40% (Figure 6). The low level of L88.3 killing by TNF (in the absence of CHX) was paralleled by the low levels of caspase-8 and caspase-9 activities

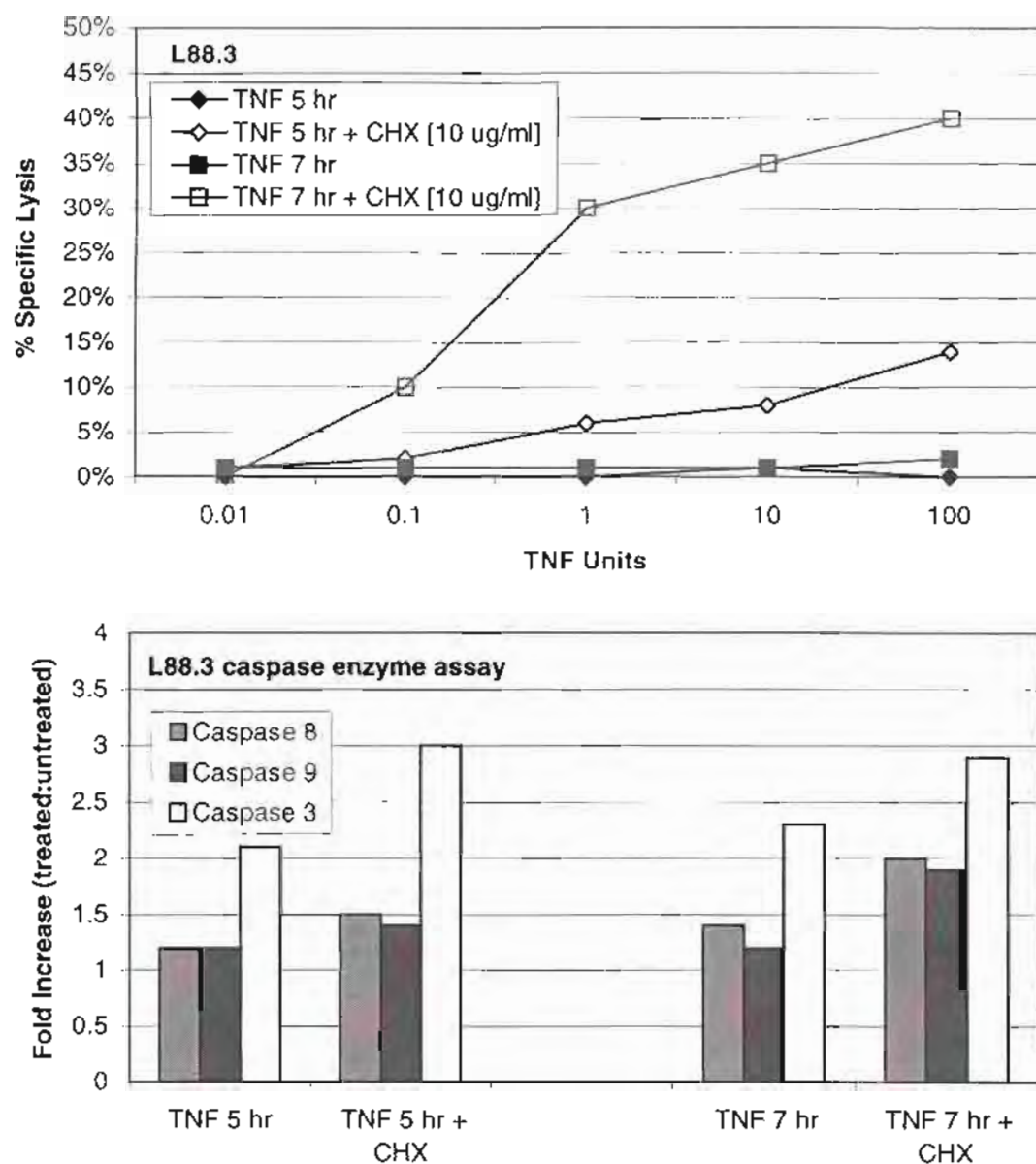


Figure 6 For L88.3, addition of CHX increased TNF-mediated cytolysis and caspases activities. TNF-mediated cytolysis was measured by 5- and 7-hour chromium-release assays with or without addition of 10  $\mu$ g/ml CHX two hours after targets and TNF were mixed. For caspase activities, targets were incubated with 100 units TNF for 5 or 7 hours with or without addition of 10  $\mu$ g/ml of CHX two hours after TNF was added. Caspase activity was measured using kits from R & D System (see Chapter 2 for details).

(Figure 6). Caspase-3 activity was significantly higher (more than 2-fold above untreated cells) but there was little difference between the amount of caspase-3 activity at 5 and 7 hours. However, in the presence of CHX there was a significant increase in caspase-8 and caspase-9 activities between 5 and 7 hours of TNF treatment. Also, in the presence of TNF and CHX, there was a significant increase in caspase-3 activity at both 5 and 7 hours. Again, it is not clear why the rapid rise in caspase-8 and caspase-9 was not paralleled by a comparable rise in caspase-3. As suggested before, it could be that in the presence of CHX, the procaspase-3 pool is exhausted and cannot be renewed or it is possible that the level of caspase-3 will rise at later times.

#### 3.2.4 L929

Similar to the cell lines B/C-N, 10ME and L88.3, TNF-mediated lysis of L929 was very low after 5 hours of TNF treatment. However, it rose sharply after that and was relatively high at 7 hours of TNF treatment (Figure 7) and at 18 hours of TNF treatment (Figure 3). Moreover, the effects of CHX on the TNF-mediated lysis were not as dramatic as with the other cells. However, L929 showed similar trends to the other cell lines with regard to the effects of CHX on TNF-induced caspase activation. At 5 hours of TNF treatment, caspase-8 and caspase-9 activity remained at background levels. Caspase-3 activity was up slightly to 1.4-fold over the baseline (Figure 7). At 7 hours of TNF treatment, all three caspase activities remained essentially the same as the activity levels of 5 hours of TNF treatment. Yet, TNF-mediated cytolysis showed increase at 7 hours of TNF treatment (Figure 7).

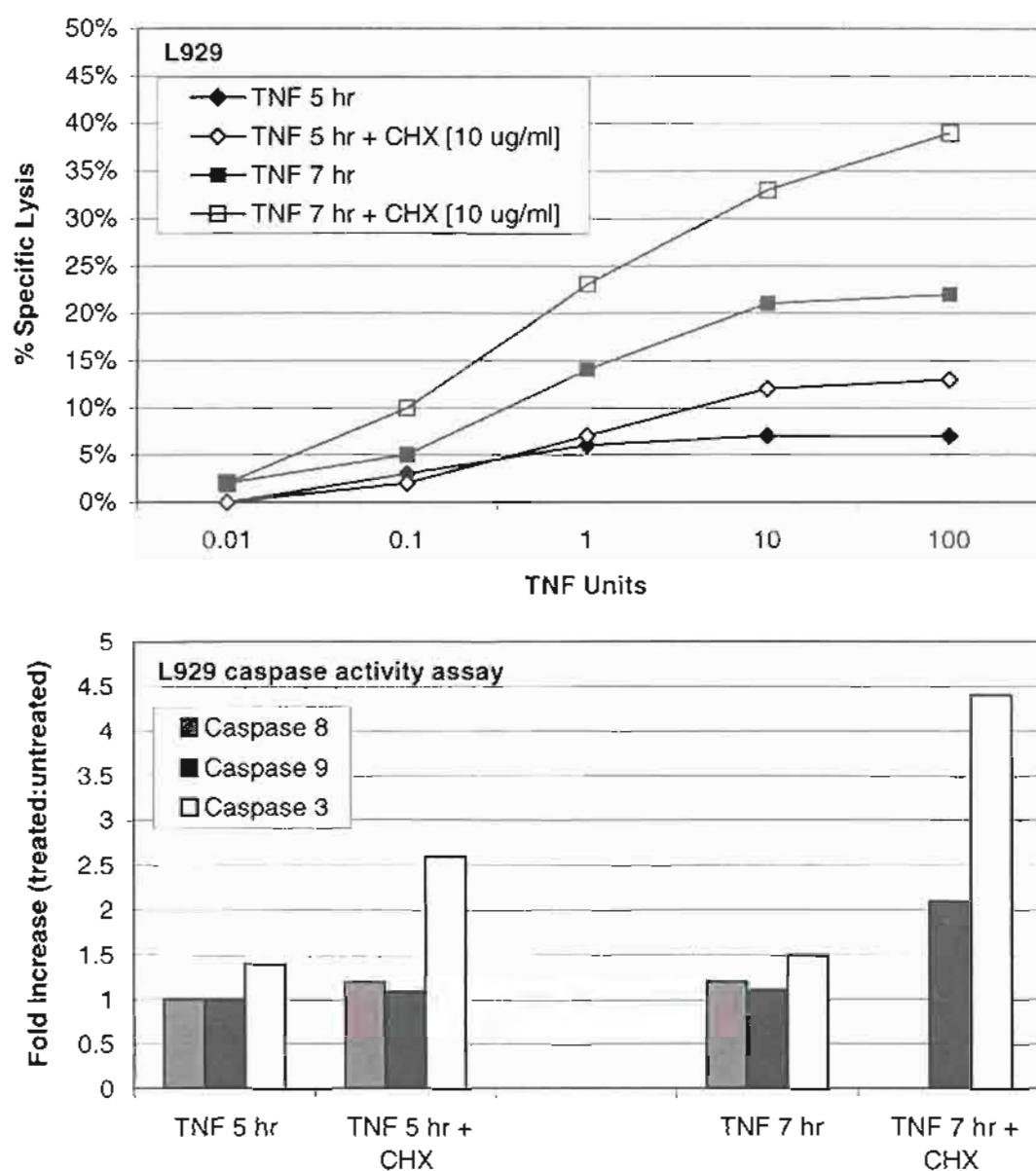


Figure 7 L929 shows a time-dependent increase in TNF-mediated cytotoxicity. Addition of CHX increased TNF-mediated cytotoxicity and caspase activities. TNF-mediated cytotoxicity was measured by 5- and 7-hour chromium-release assays with or without addition of 10  $\mu$ g/ml CHX two hours after targets and TNF were mixed. For caspase activities, targets were incubated with 100 units TNF for 5 or 7 hours with or without addition of 10  $\mu$ g/ml of CHX two hours after TNF was added. Caspase activity was measured using kits from R & D System (see Chapter 2 for details).



These results suggest that increase in TNF-mediated cytolysis of L929 might not depend on caspase-8 and caspase-9 and the small increase in caspase-3 is inconsistent with the results from other cell lines. Thus, cytolysis at 5 and 7 hours of TNF treatment may not be solely due to increased caspase activities. As shown in Table 1, caspase-8, caspase-9, and caspase-3 activities did not show dramatic increase until 12 hours of TNF treatment in L929. These data suggest that for L929 caspase-mediated TNF cytolysis occurs later in the time course of TNF-mediated killing compared to B/C-N, 10ME, and L88.3. Some researchers have shown that TNF-mediated cytotoxicity in L929 correlates with activation of phospholipases and release of arachidonic acid (Brekke et al., 1992 and Brekke et al., 1994). Goossens et al. (1995) showed that TNF could generate reactive oxygen intermediates (ROI) leading to necrotic death in L929. Thus, killing of L929 might be more dependent on these cytotoxic pathways in the early time point of TNF-mediated cytolysis.

The addition of CHX to the 5-hour TNF assay had little effect on caspase-8 and caspase-9 activity but manifested a rise in caspase-3 activity. Seven hours of TNF treatment plus CHX increased killing to 39% at 100 units TNF. This increase was paralleled by a large increase in caspase-9 and caspase-3 activities. This high level of killing resulted in too few cells to run assays for all three caspases so the caspase-8 assay was not done at this time point. Caspase-9 activity was chosen to represent the initiator caspases since in earlier assays increases in caspase-9 seemed to correlate best with high caspase-3 levels. At 7 hours of TNF treatment, caspase-9 activity increased from 1.1-fold above background in the absence of CHX to 2.1 -fold above background in the presence

of CHX. Similarly, caspase-3 activity increased from 1.5-fold above background in the absence of TNF to 4.4-fold in the presence of CHX. Although these are quite high levels of the caspases, they remain less than the levels observed in L929 at 12 hours of TNF treatment (Table 1).

*3.2.5 Discussion: increase in TNF-mediated cytotoxicity due to addition of CHX correlates with increase in caspase activities.*

Despite the different sensitivities of B/C-N, 10ME and L88.3 to TNF lysis after 18 hours of treatment, these data showed that TNF-mediated lysis of the cell lines was very low during the first 7 hours of TNF treatment (Figures 4 – 6). All three cell lines are resistant to killing by TNF in this short assay period. Addition of CHX caused a substantial increase in TNF-mediated killing only after 7 hours of TNF treatment. However, in L929 cell line, there was a large rise in cell lysis between 5 and 7 hours of TNF treatment (Figure 7).

As for the caspase activities, TNF-resistant cell lines B/C-N and L88.3 showed modest increase in caspase-3 activities between the 5 and 7 hour assays, but caspase-8 and -9 activities remained close to the baseline (Figures 4 and 6). However, for B/C-N and L88.3, TNF treatment plus CHX caused a large increase in all three caspase activities at 7 hours of TNF treatment. The level of increase in three caspase activities seen at 7 hours of TNF treatment in the presence of CHX were either equivalent or above the peak caspase activities seen at 10 or 12 hours of TNF treatment (Table 1, Figures 4 and 6). These data suggest that TNF-induced protein-synthesis-dependent resistance mechanism

could interfere with caspase activities in B/C-N and L88.3 cell lines. These data also suggest that increase in caspase-3 activity alone was not sufficient to cause a large increase in TNF-mediated cytolysis. All three caspase activities have to increase to a threshold (at least 1.5-fold to 2-fold above background) in order for a substantial increase in TNF-mediated cytolysis to occur.

In the TNF-sensitive cell line 10ME, caspase-3 showed a larger increase in activity from 5 to 7 hours of TNF treatment, however caspase-8 and -9 activities showed only modest increase (Figure 5). These increase in caspase activities at 7 hours of TNF treatment compared to B/C-N and L88.3 cell lines probably reflects the TNF sensitivity of 10ME cell line, and is likely a predictor of the increased killing seen at later time points. In L929 cell line, which is also a TNF-sensitive cell line, activity levels of three caspases did not show an increase from 5 to 7 hours of TNF treatment (Figure 7). In fact, their activity levels remained close to the baseline. Caspase activity data at 5 and 7 hours of TNF treatment for L929 suggests that increase in TNF-mediated cytolysis observed during these time points were not mediated by caspases. As suggested earlier, it may involve activation of PLA<sub>2</sub> or generation of oxygen radicals.

Addition of CHX also increased caspases activities in 10ME and L929, just like in B/C-N and L88.3 cell lines. Both cell lines showed large increase in caspase-3 activities only at 5 hours of TNF treatment in the presence of CHX, but showed large increase in all three caspases at 7 hours of TNF treatment in the presence of CHX (Figures 5 and 7). However, unlike B/C-N and L88.3 cell lines, the level of increase in caspase-3 activity never reached the level of peak caspase activities seen at 12 hours of TNF treatment in

10ME and L929. These results also suggest that all three caspase activities have to reach a threshold level before a substantial increase in TNF-mediated cytolysis could occur. Based on our results, all three caspases have to reach at least 1.5-fold above, preferably 2-fold above the baseline in order for a substantial increase in TNF-mediated cytolysis to occur.

*3.3 Preincubation with pan-caspase inhibitor z-VAD-fmk suppressed caspases activities in B/C-N, 10ME, L88.3 and L929; however, z-VAD-fmk increased TNF-mediated cytolysis in B/C-N, 10ME, and L929, but not in L88.3.*

Pan-caspase inhibitor z-VAD-fmk and caspase-3, -7, and -8 inhibitor Ac-DEVD-CHO have been shown to inhibit apoptosis in many cells. Z-VAD-fmk is a broad-specificity irreversible caspase inhibitor which can inhibit caspase-1 through caspase-9, but inhibits caspase-1 the best followed by caspase-8, caspase-9, caspase-5, caspase-7, caspase-3, caspase-6, caspase-4, and caspase-2 (Garcia-Calvo et al., 1998). Z-VAD-fmk has been shown to inhibit apoptosis by blocking the activation of procaspase-3 into its active form (Slee et al., 1996), and to inhibit chromatin condensation and DNA fragmentation in primary cultures of rat hepatocytes (Cain et al., 1996). Ac-DEVD-CHO has also been shown to inhibit the cleavage of poly(ADP ribose) polymerase (PARP) leading to DNA fragmentation (Nicholson et al., 1995), and inhibit TNF-induced activation of cPLA<sub>2</sub> and apoptosis (Wissing, et al., 1997). However, Ac-DEVD-CHO has poor cell permeability. Z-DEVD-fmk has the same inhibitory characteristic as ac-DEVD-CHO, except it contains fluoromethyl ketone (fmk) instead of aldehyde amino

acid sequences. These fmK sequences enhance cell permeability as well as making the peptide inhibitor an irreversible inhibitor (Nicholson and Thornberry., 1997).

Since caspase-8, -9, and -3 activities were detected in B/C-N, 10ME, L88.3, and L929 cell lines, we wanted to see if one hour preincubation of target cells with pan-caspase inhibitor z-VAD-fmk or caspase-3, -7, and -8 inhibitor z-DEVD-fmk could inhibit TNF-mediated cytolysis in B/C-N, 10ME, and L88.3 cell lines.

Preincubation with 20  $\mu$ M z-VAD-fmk, the pan caspase inhibitor, for 1 hour before addition of TNF caused increase in TNF-mediated cytolysis in B/C-N, and 10ME, but not in L88.3 as measured by 18-hour chromium-release assay (Figures 8 – 10). An increase in TNF-mediated cytolysis by preincubation with z-VAD-fmk in L929 had been shown before (Vercammen et al., 1998). Vercammen et al. (1998) showed that preincubation with z-VAD-fmk and z-DEVD-fmk rendered L929 more sensitive to TNF-mediated cytolysis. Hence, it was not surprising to see the same trend in this experiment for L929 cell line (Figure 10).

In B/C-N, addition of CHX 2 hours into an 18 hour cytotoxicity assay resulted in maximum killing (73% at 100 units TNF), again demonstrating that a protein-synthesis-dependent mechanism was key in protecting B/C-N from TNF-mediated lysis (Figure 8). The pan-caspase inhibitor z-VAD-fmk also caused a significant increase in lysis (49% at 100 units TNF). This indicates that there are components of the lytic mechanism that are not caspase-dependent (e.g., mediated by phospholipases or ROI). This is also consistent with the idea that one component of the resistance mechanism includes *de novo* synthesis of a caspase that can be blocked by either a protein synthesis inhibitor or a caspase

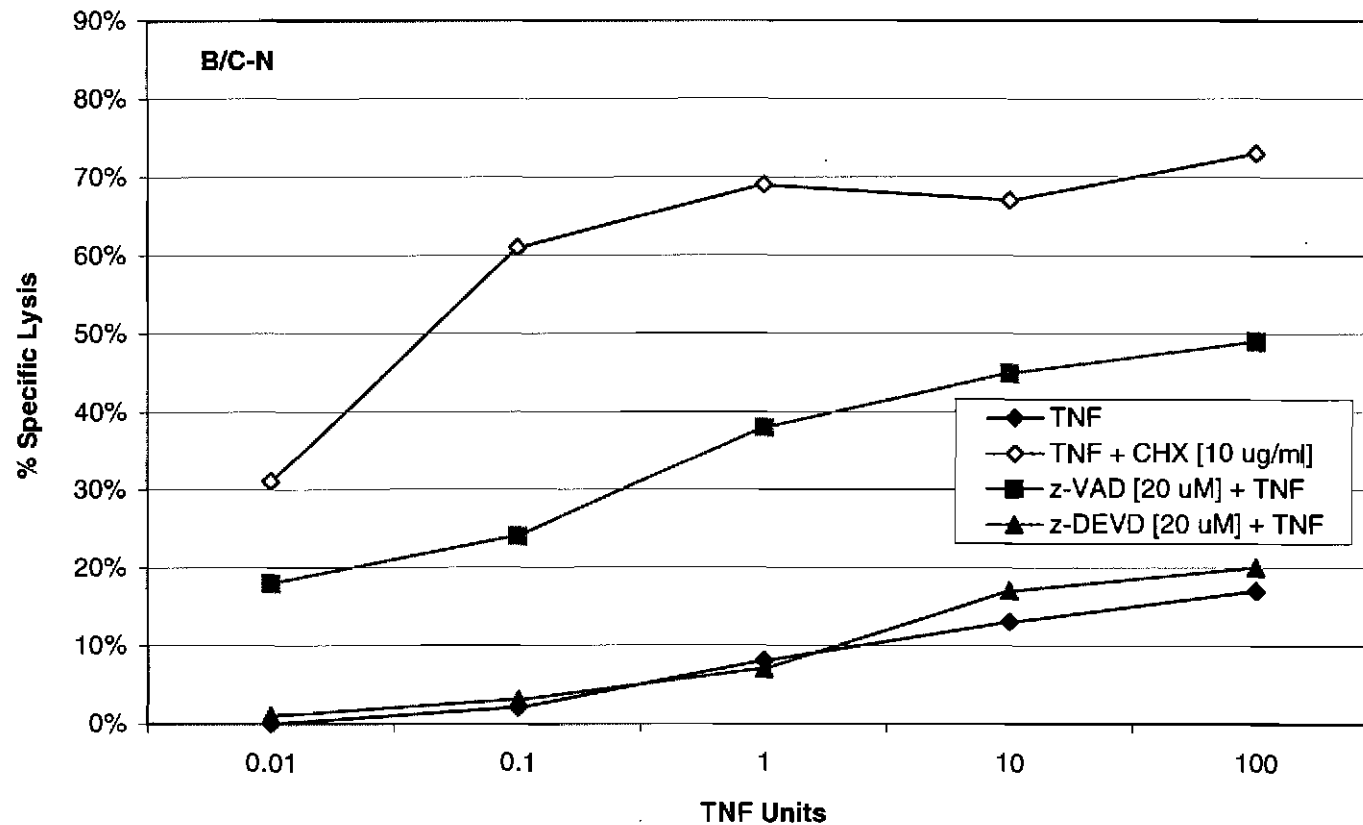


Figure 8 Pan-caspase inhibitor z-VAD-fmk, but not caspase-3, -7, and -8 inhibitor z-DEVD-fmk, increased TNF-mediated cytotoxicity in B/C-N. Eighteen-hour chromium-release assay was used to measure TNF-mediated cytotoxicity. Targets were either preincubated with or without 20  $\mu$ M of z-VAD-fmk or 20  $\mu$ M z-DEVD-fmk before addition of TNF. The translation inhibitor CHX was added 2 hours after targets and TNF were mixed (see Chapter 2 for details).

inhibitor. Since CHX was more effective at increasing the TNF mediated lysis of B/C-N than z-VAD-fmk, it appears that there are also components of the resistance mechanism that are not caspases-dependent.

The caspase-3, caspase-7, and caspase-8 inhibitor, z-DEVD-fmk, had little effect on the killing of B/C-N at any dose of TNF (Figure 8). Even at 100 units TNF, z-DEVD-fmk plus TNF showed only 20% killing when TNF alone showed 17% killing. This suggests that caspase-3, caspase-7, and caspase-8 are not involved in the lytic process or that B/C-N's protein-synthesis-dependent resistance mechanism blocks their activities. The data from the previous section (Figure 4 – 7) showing a correlation between TNF-induced expression of caspases-8, -9, and -3 and the level of cell killing indicate their involvement in the lytic process. The rather low expression of all three caspases in B/C-N treated with TNF (Figure 4) is consistent with the idea that B/C-N is resistant to killing because of the low expression of these enzymes. Thus, it is not too surprising that a caspase-3, -7 and -8 inhibitor (i.e., z-DEVD-fmk) has little effect on killing. This further suggests that the pan-caspase inhibitor, z-VAD-fmk, that increases killing (Figure 8), functions by blocking enzymes other than caspase-3, caspase-7, and caspase-8. Since inhibition of protein synthesis has a greater effect on the susceptibility of B/C-N to TNF than does the pan-caspase inhibitor, we suggest that B/C-N express two TNF resistance mechanisms; one mechanism that is caspase-dependent and one that is protein-synthesis-dependent but caspase-independent.

The effects of the pan-caspase inhibitor, z-VAD-fmk, were more dramatic on 10ME than on B/C-N. At 100 unit of TNF, preincubation with z-VAD-fmk plus TNF

showed 72% killing while TNF plus CHX showed 65% killing; TNF alone showed 47% killing at 100 units of TNF and pretreatment with z-DEVD-fmk plus TNF showed 40% killing (Figure 9). For B/C-N, z-VAD-fmk increased TNF-mediated cytolysis, but not to the level of TNF plus CHX. This suggests that there are caspase-dependent and caspase-independent resistance mechanisms for B/C-N. However, for 10ME, the pan-caspase inhibitor increased lysis to the level seen with CHX. This suggests that there is no caspase-independent resistance mechanism and that protein synthesis is required for expression of the caspase-dependent resistance. Since 10ME was derived from B/C-N, one can speculate that the mutation in the TNF-resistant B/C-N that created TNF-sensitive 10ME caused a loss in the caspase independent resistance mechanism, thus rendering 10ME TNF-sensitive.

The caspase-3, caspase-7 and caspase-8 inhibitor, z-DEVD-fmk, also had no effect on the TNF-mediated killing in 10ME, as it was seen in B/C-N (Figure 9). This suggests that these enzymes are involved in neither the lytic process nor the resistance mechanism. However, assays for caspase-3 activity (Figure 5) showed a corresponding increase in caspase activities and cell death, indicating caspases are involved in TNF-mediated cell death. Thus, the role of caspase-3 in the killing of 10ME remains unclear.

The effects of caspase inhibitors on the TNF-mediated lysis of L88.3 are unique. Both the pan-caspase inhibitor and the caspase-3, -7 and -8 inhibitor lessened the toxic effects of TNF whereas the protein synthesis inhibitor, CHX increased TNF-mediated lysis. When L88.3 was preincubated for one hour with z-VAD-fmk plus TNF, it showed 16% killing at 100 unit of TNF while TNF alone showed 40% killing (Figure 10). But



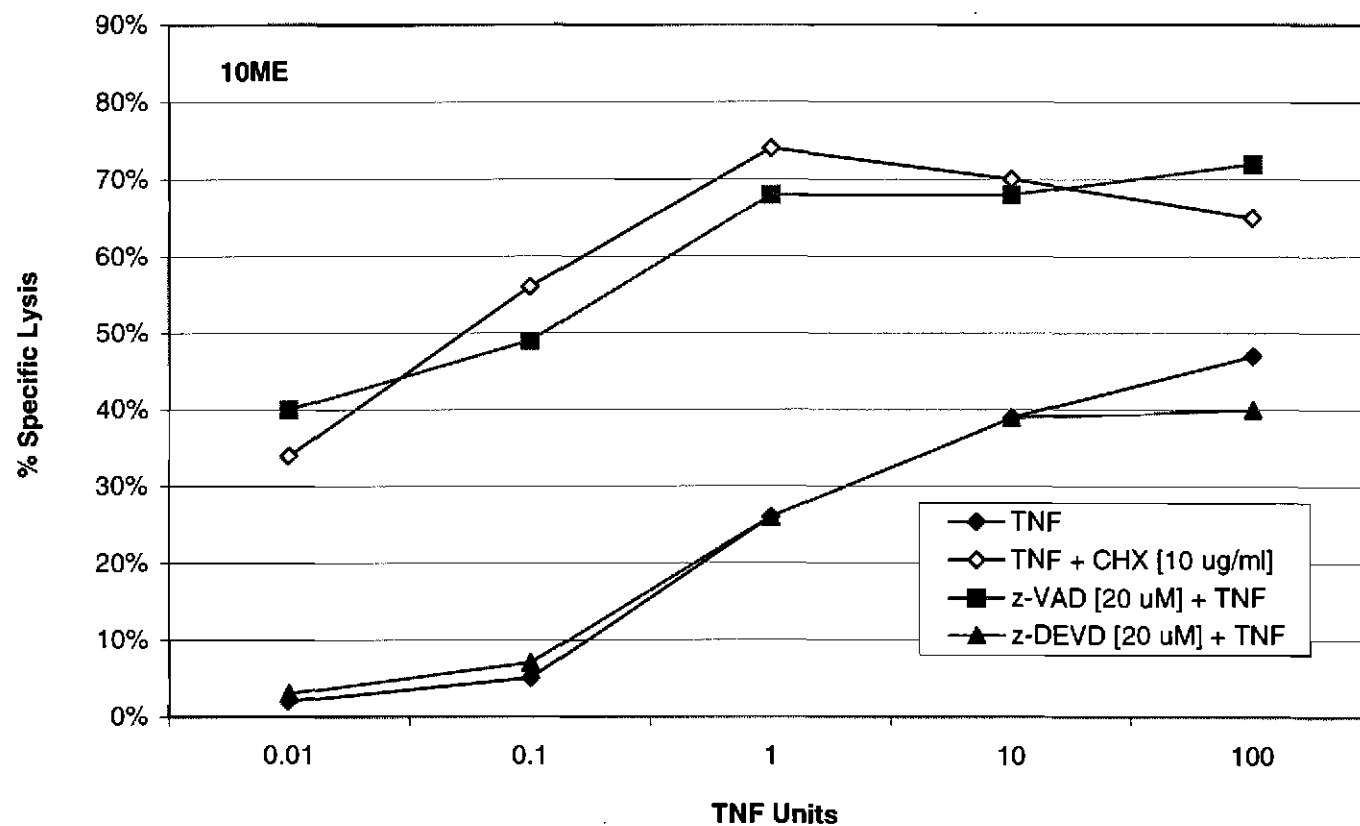


Figure 9 Pan-caspase inhibitor z-VAD-fmk increased TNF-mediated cytolysis to the level of TNF plus CHX in 10ME , but caspase-3, -7, and -8 inhibitor z-DEVD-fmk showed no effect on TNF-mediated cytolysis. Eighteen-hour chromium-release assay was used to measure TNF-mediated cytolysis. Targets were either preincubated with or without 20  $\mu$ M of z-VAD-fmk or 20  $\mu$ M z-DEVD-fmk before addition of TNF. A translation inhibitor CHX was added 2 hours after targets and TNF were mixed (see Chapter 2 for details).

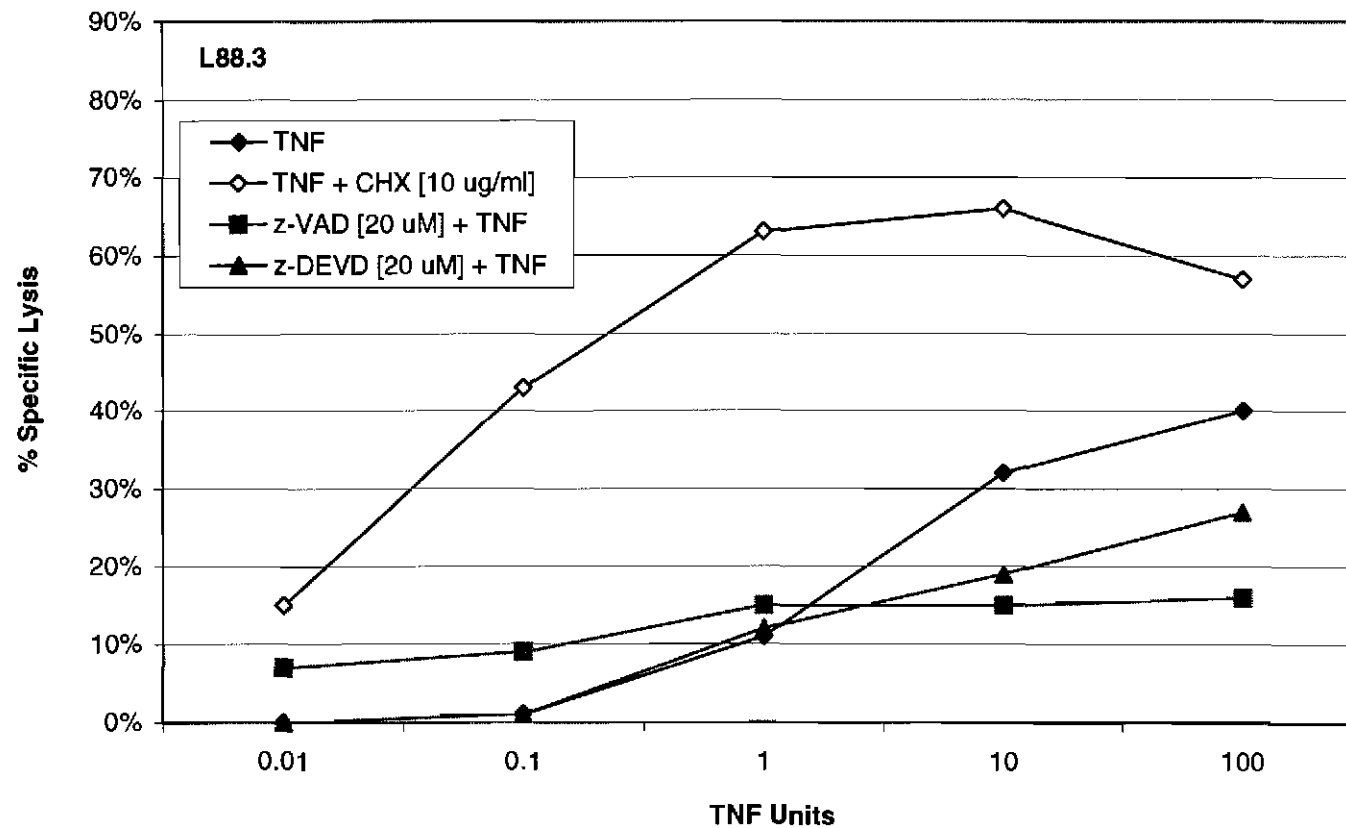


Figure 10 Preincubation with z-VAD-fmk or z-DEVD-fmk reduced TNF-mediated cytotoxicity in L88.3. Eighteen-hour chromium-release assay was used to measure TNF-mediated cytotoxicity. Targets were either preincubated with or without 20  $\mu$ M of z-VAD-fmk or 20  $\mu$ M z-DEVD-fmk before addition of TNF. A translation inhibitor CHX was added 2 hours after targets and TNF were mixed (see Chapter 2 for details).

TNF plus CHX increased killing to 57 %. When L88.3 was preincubated with z-DEVD-fmk plus TNF, it showed 27% killing at 100 units TNF (Figure 10). These data suggests that the TNF resistance expressed by L88.3 is protein-synthesis-dependent but does not involve caspase activity. Thus, when L88.3 was derived from 10ME, it became resistant to TNF by turning off the protein-synthesis-dependent caspase-mediated resistance mechanism and turning on a protein-synthesis-dependent resistance mechanism that does not require caspase activity.

The cell line L929 is quite sensitive to the effects of TNF. Thus, at concentrations of 10 units or more per well, maximum lysis was observed (Figure 11). However, at subsaturating doses of TNF the effects of the various drugs could be observed. For TNF plus CHX, highest killing was observed at 1 unit of TNF (79% killing). Similarly, for z-VAD-fmk pretreatment plus TNF, highest killing was observed also at 1 unit of TNF (80% killing) (Figure 11). For z-DEVD-fmk pretreatment plus TNF, highest killing was observed at 10 units TNF (66% killing) (Figure 11). At the lower doses of TNF, L929 behaves much like the other TNF-sensitive cell line, 10ME. That is, the protein synthesis inhibitor, CHX, and the pan-caspase inhibitor, z-VAD-fmk, significantly and equally enhanced the killing of L929. As mentioned earlier, in the L929 cell line, increase in TNF-mediated cytotoxicity by preincubation with z-VAD-fmk and z-DEVD-fmk were shown before (Vercammen et al., 1998). Hence, it was not surprising to see the similar trend in this experiment (Figure 11).

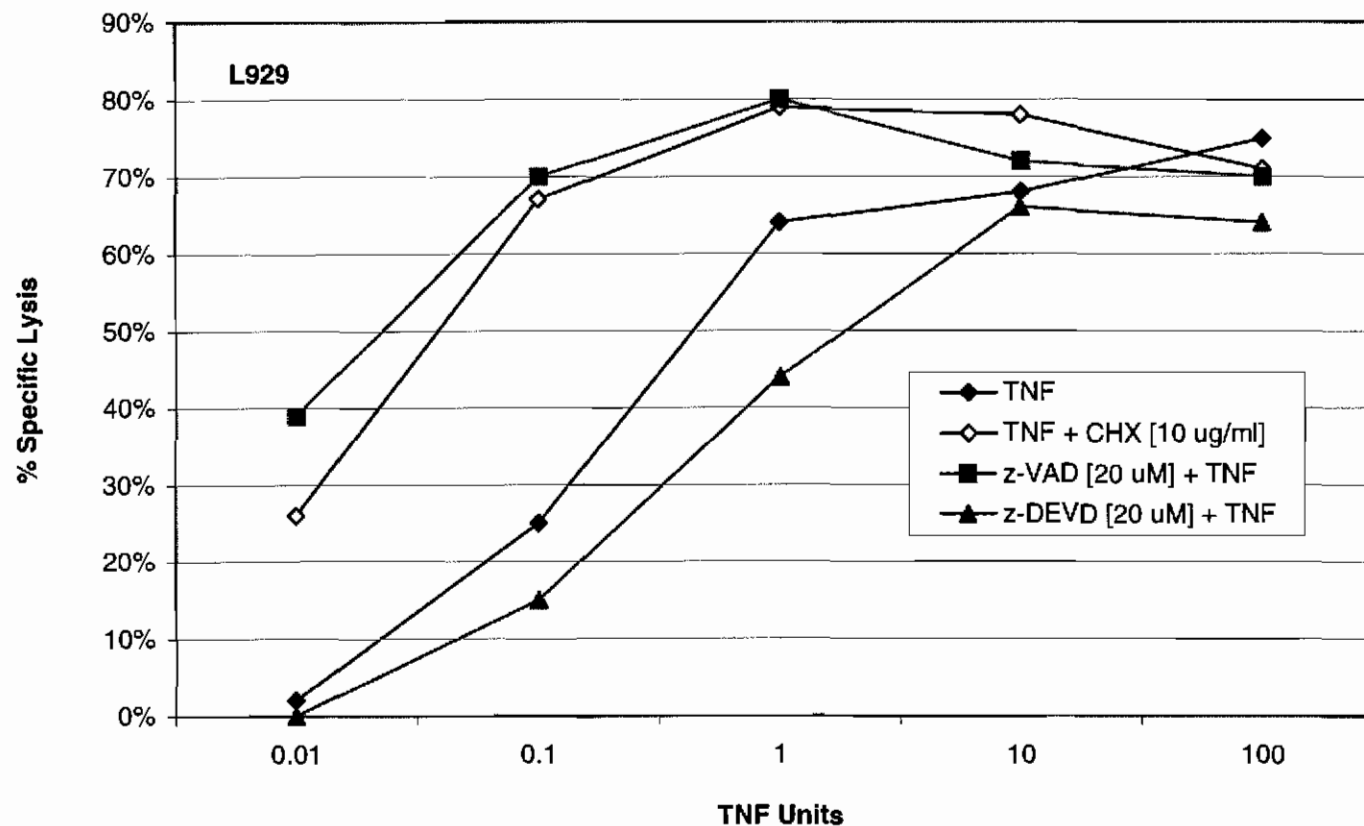


Figure 11 Pan-caspase inhibitor z-VAD-fmk increased TNF-mediated cytolysis to the level of TNF plus CHX in L929, but caspase-3, -7, and -8 inhibitor z-DEVD-fmk showed some inhibitory effect on TNF-mediated cytolysis. Eighteen-hour chromium-release assay was used to measure TNF-mediated cytolysis. Targets were either preincubated with or without 20 µM of z-VAD-fmk or 20 µM z-DEVD-fmk before addition of TNF. A translation inhibitor CHX was added 2 hours after targets and TNF were mixed (see Chapter 2 for detail).

The caspase-3, -7 and -8 inhibitor, z-DEVD-fmk, had some inhibitory activity on the cytotoxicity assay. Thus, it appears that L929 expresses a low level of a TNF resistance that can be overcome with high dose of TNF. This resistance mechanism appears to be both caspase-dependent and protein-synthesis-dependent. The simplest model is to view these two observations as a manifestation of one phenomenon. That is, the resistance is dependent on caspase activity and that activity is protein-synthesis-dependent.

In order to determine whether the increase in TNF-mediated cytolysis seen with pan-caspase inhibitor z-VAD-fmk preincubation was due to caspase inactivation or due to some other mechanism, caspase activity assays and chromium-release assays at 5 and 7 hours of TNF treatment were investigated. For caspase activity assays, targets were preincubated with z-VAD-fmk or z-DEVD-fmk for one hour before TNF were added to the target cells.

As seen in Figure 12, preincubation with pan-caspase inhibitor z-VAD-fmk plus TNF or caspase-3, -7 and -8 inhibitor z-DEVD-fmk plus TNF did show lower caspase activities, especially for caspase-3 activity, when compared to TNF alone. This was true in both the 5 and 7 hours caspase activity assays in B/C-N, 10ME, and L88.3. Among B/C-N, 10ME, and L88.3, levels of caspase-8, caspase-9, and caspase-3 activities remained very similar between z-VAD-fmk pretreated plus TNF or z-DEVD-fmk pretreated plus TNF at both 5 and 7 hours of TNF treatment, while targets treated with TNF alone for 5 to 7 hours showed time-dependent increase in these caspase activities. Reduction in caspase activities due to caspase inhibitors was not absolute since

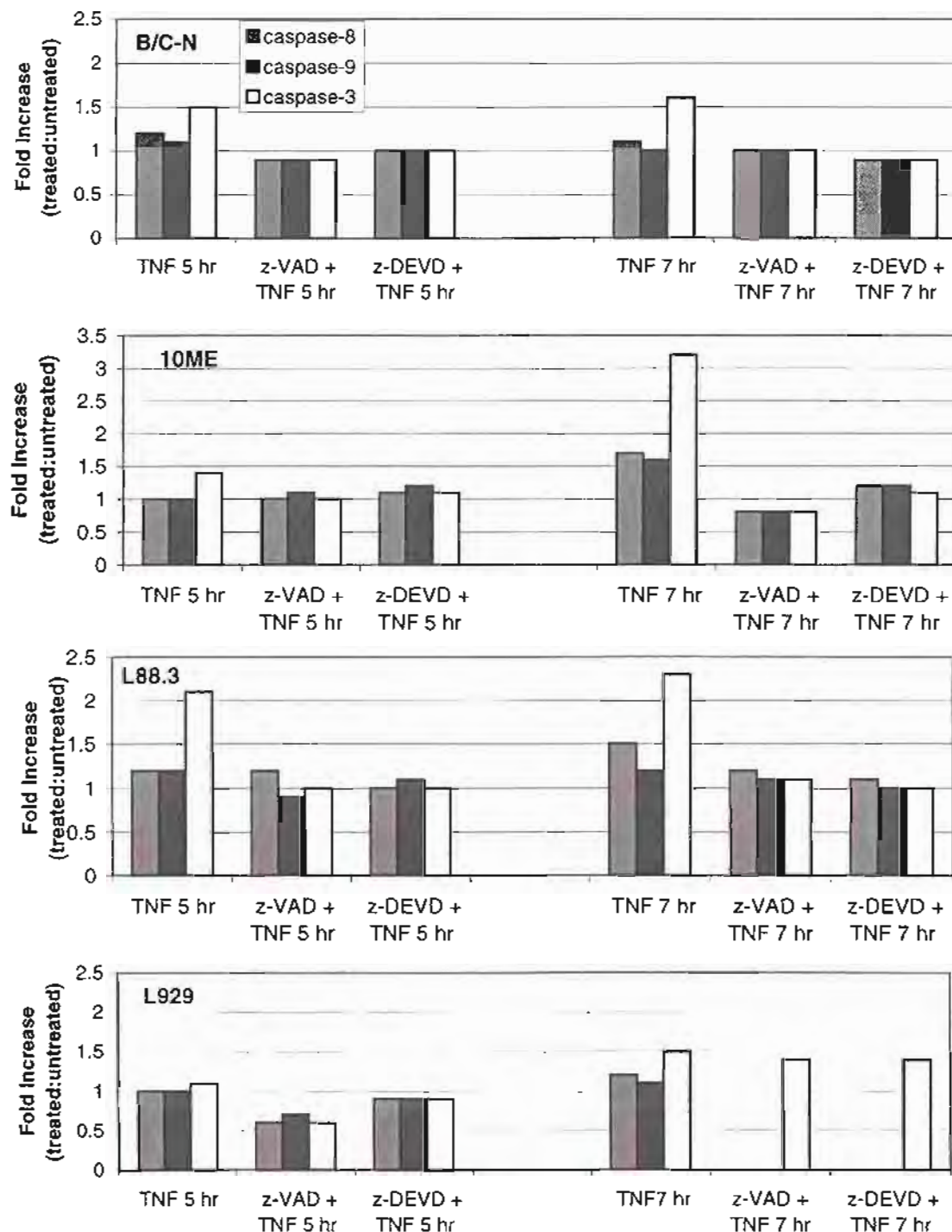


Figure 12 Caspase inhibitors z-VAD-fmk and z-DEVD-fmk suppressed caspase activities compared to TNF treatment in B/C-N, 10ME, L88.3, and L929. Target cells were pretreated with 20  $\mu$ M of z-VAD-fmk or z-DEVD-fmk for one hour before addition of 100 units of TNF. Activities were measured by the caspase enzyme activity assay kit (see Chapter 2 for details.)

caspase-8, -9, and -3 activities were either slightly below or above 1.0-fold increase when compared to the untreated control. For L929, 5 hours TNF treatment with z-VAD-fmk and z-DEVD-fmk showed similar results to those of B/C-N, 10ME, and L88.3. For 7 hours of TNF treatment, there was some increase in caspase-3 activity for z-VAD-fmk plus TNF and z-DEVD-fmk plus TNF, when compared to 5 hours time points (Figure 12). However, for L929, these values were still lower compared to 7 hours of TNF treatment alone. Hence, these data validated that preincubation with z-VAD-fmk and z-DEVD-fmk were suppressing caspases activities compared to the TNF treatment alone.

Preincubation with z-VAD-fmk or z-DEVD-fmk did lower caspases activity levels when compared to TNF. Yet, these reductions in caspases activities did not correlate with the 5 and 7 hours cytotoxicity assays (Figure 13 - 16). There were increase in TNF-mediated cytolysis for z-VAD-fmk pretreatment plus TNF in 5 and 7-hours chromium-release assays when compared to TNF alone. Increase in killing for z-VAD pretreatment plus TNF compared to TNF was more prominent in 7-hour chromium-release assays. As seen before, z-DEVD-fmk pretreatment plus TNF appeared to have no effect on TNF-mediated cytolysis.

In the TNF-resistant cell line B/C-N, a z-VAD-fmk-induced increase in TNF-mediated cytolysis was not noticeable until 7 hours of TNF treatment (Figure 13). At 7 hours of TNF treatment, B/C-N showed 17% killing for z-VAD-fmk plus 100 units TNF, but showed only 7% killing for TNF alone, and 2% killing for z-DEVD-fmk plus TNF (Figure 13). In another TNF-resistant cell line, L88.3, z-VAD-fmk effect is minimal on TNF-mediated cytolysis. Even at 7 hours of TNF treatment, preincubation

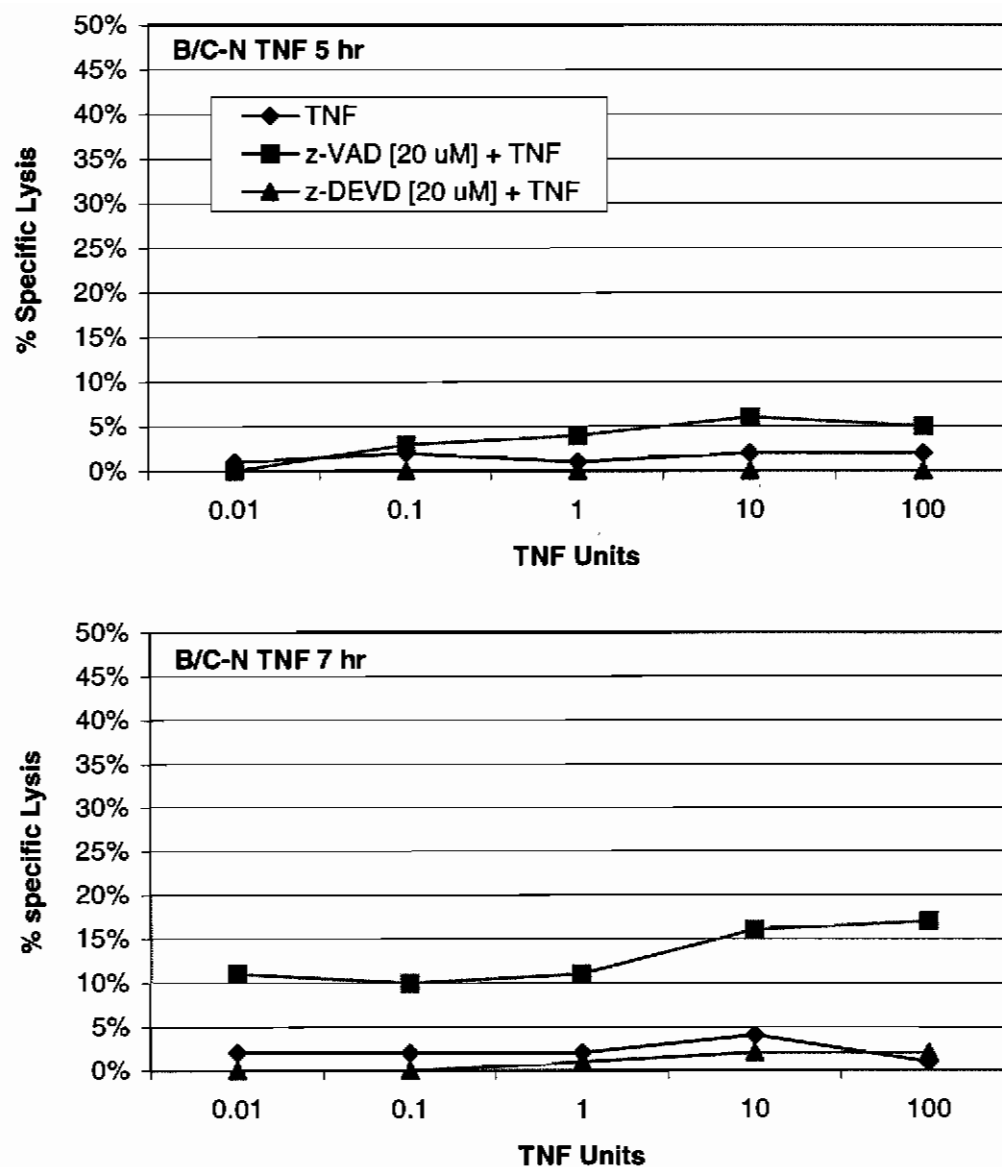


Figure 13 Pan-caspase inhibitor z-VAD-fmk increased TNF-mediated cytolysis, but not caspase-3, -7 and -8 inhibitor z-DEVD-fmk in B/C-N. TNF-mediated cytolysis was measured by the chromium-release assay (see Chapter 2 materials and methods). Targets were preincubated with z-VAD-fmk 20  $\mu$ M or z-DEVD-fmk 20  $\mu$ M for one hour before TNF were added.



with z-VAD-fmk plus TNF only caused 11% killing at 100 units of TNF for L88.3 (Figure 14). TNF alone and z-DEVD-fmk pretreatment plus TNF showed 2% killing at 100 units of TNF. These results for L88.3 are in concordance with the 18-hour chromium-release assay (Figure 10) showing pretreatment with z-VAD-fmk or z-DEVD-fmk causes reduction in TNF-mediated cytotoxicity in L88.3.

In the TNF-sensitive cell lines 10ME and L929, z-DEVD-fmk had no effect on TNF-mediated cytotoxicity. However, the effects of z-VAD-fmk sensitization on TNF-mediated cytotoxicity were noticeable by 5 hours of TNF treatment (Figure 15 – 16). At 5 hours of TNF treatment, 10ME showed 15 % killing with z-VAD-fmk pretreatment at 100 units TNF compared to 5% for TNF alone (Figure 15). By 7 hours of TNF treatment, preincubation with z-VAD-fmk plus TNF showed 34% killing in 10ME at 100 units TNF while TNF alone showed 11% killing (Figure 15). Sensitization to TNF-mediated cytotoxicity due to preincubation with z-VAD-fmk was more pronounced in L929. In L929, 5 hours of TNF treatment showed 35% killing for z-VAD-fmk pretreatment at 100 units TNF, while TNF alone caused only 7% killing (Figure 16). By 7 hours of TNF treatment, z-VAD-fmk pretreatment plus TNF caused 47% killing compared to 19% killing by TNF alone (Figure 16).

These data showed that in B/C-N, 10ME, L88.3, and L929 preincubation with both the pan-caspase inhibitor z-VAD-fmk or caspase-3, -7 and -8 inhibitor z-DEVD-fmk, lowered caspase-8, -9, and -3 activities when compared to TNF alone (Figure 12).

Despite the reduction in caspase activities seen for preincubation with both caspase

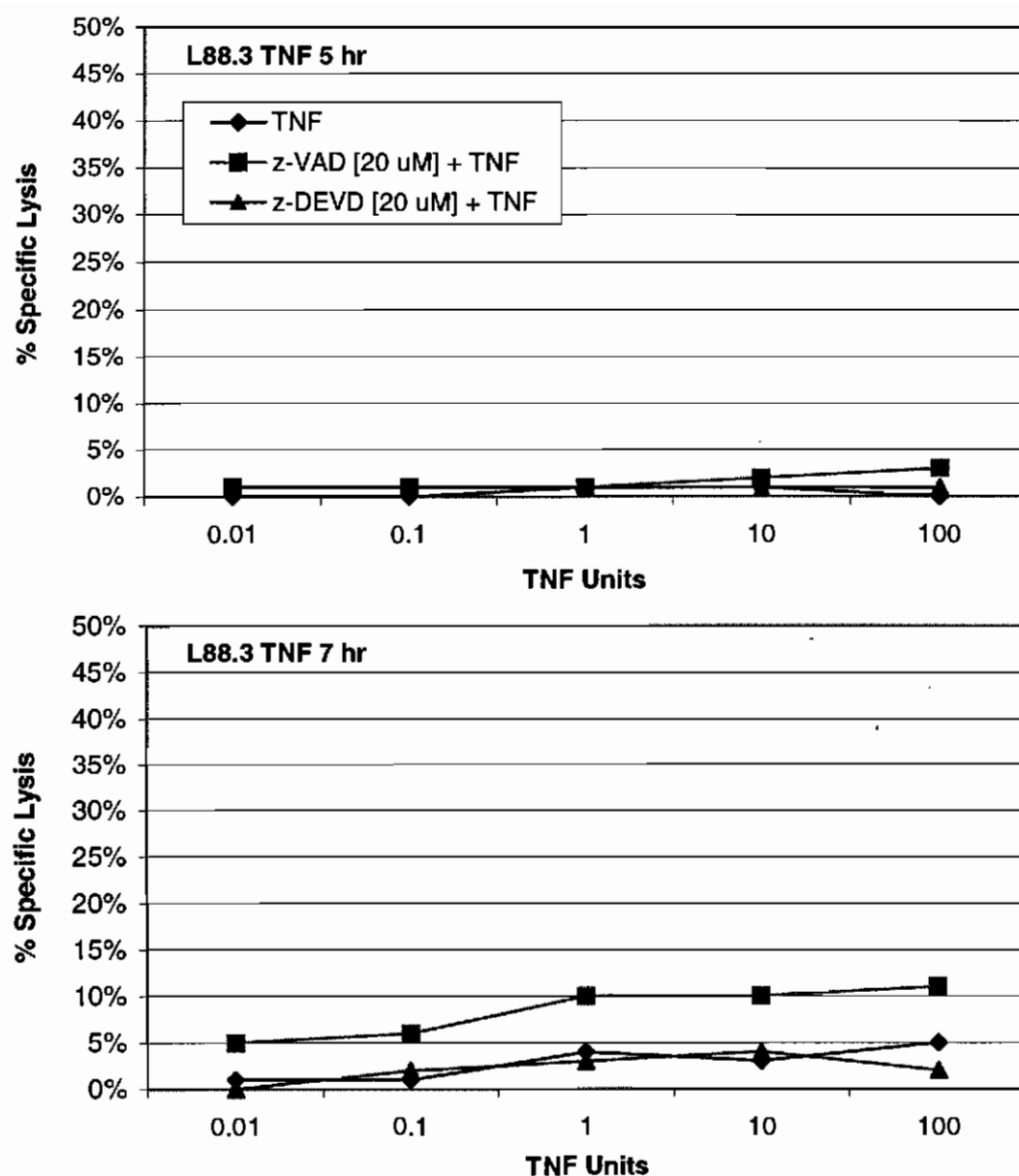


Figure 14 Preincubation with pan-caspase inhibitor z-VAD-fmk and caspase-3, -7 and -8 inhibitor z-DEVD-fmk caused decrease in TNF-mediated cytotoxicity in L88.3. TNF-mediated cytotoxicity was measured by the chromium-release assay (see Chapter 2 materials and methods). Targets were preincubated with z-VAD-fmk 20  $\mu$ M or z-DEVD-fmk 20  $\mu$ M for one hour before TNF were added.

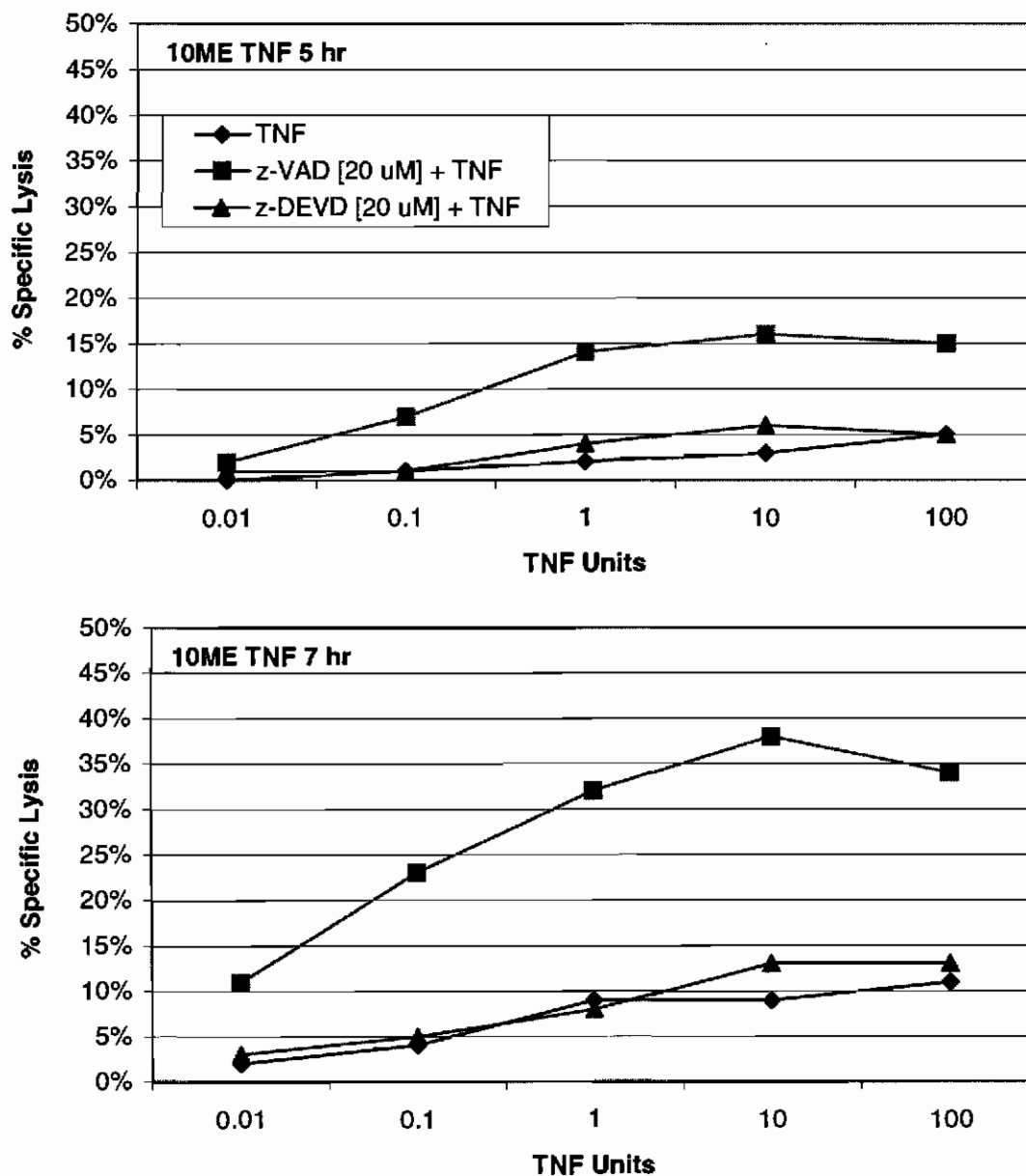


Figure 15 Preincubation with pan-caspase inhibitor z-VAD-fmk, but not with caspase-3, -7 and -8 inhibitor z-DEVD-fmk, caused an increase in TNF-mediated cytolysis in 10ME. TNF-mediated cytolysis was measured by the chromium-release assay (see Chapter 2 materials and methods). Targets were preincubated with z-VAD-fmk 20  $\mu$ M or z-DEVD-fmk 20  $\mu$ M for one hour before TNF were added.

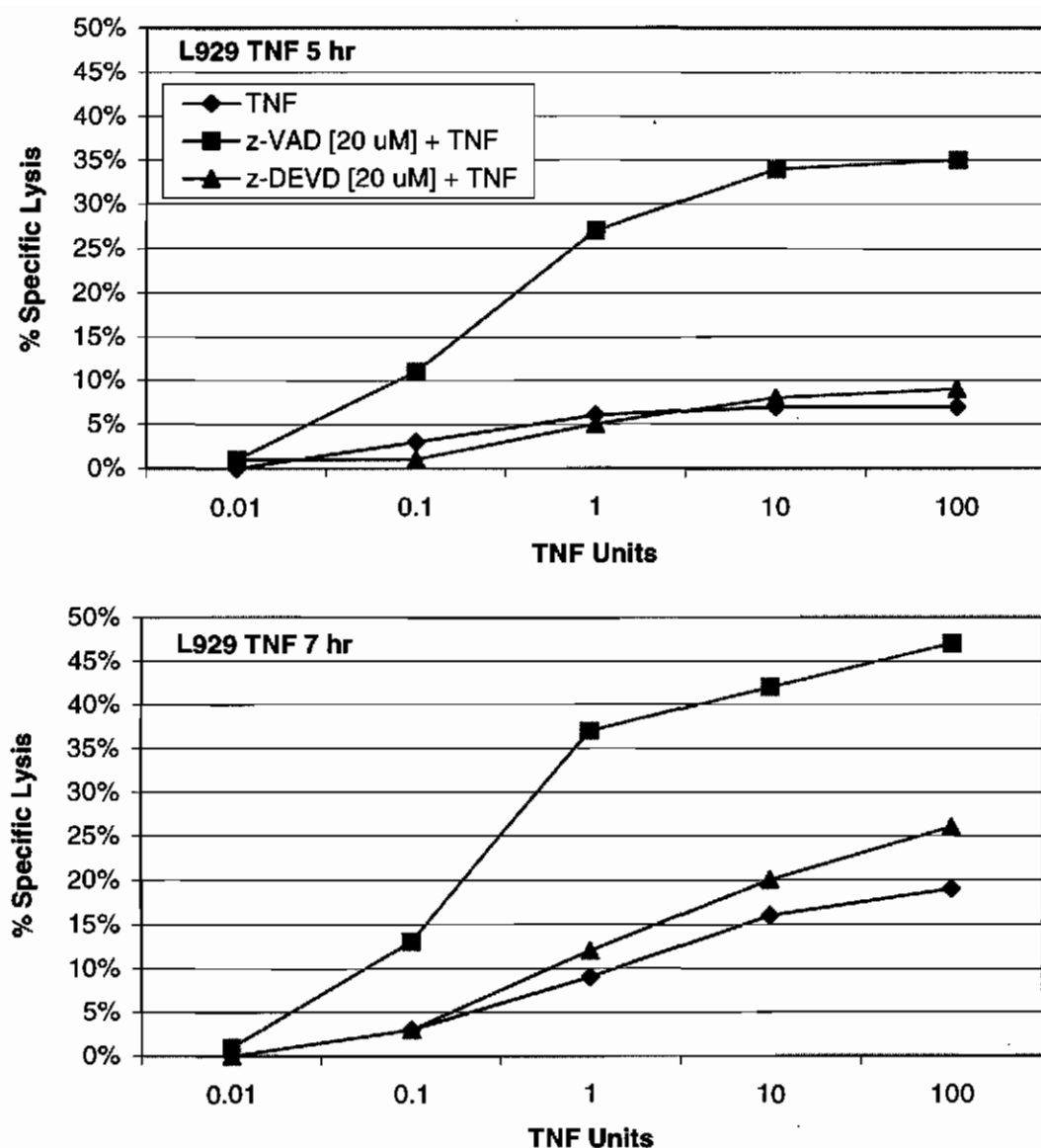


Figure 16 Preincubation with pan-caspase inhibitor z-VAD-fmk, but not with caspase-3, -7 and -8 inhibitor z-DEVD-fmk, caused increase in TNF-mediated cytolysis in L929. TNF-mediated cytolysis was measured by the chromium-release assay (see Chapter 2 materials and methods). Targets were preincubated with z-VAD-fmk 20  $\mu$ M or z-DEVD-fmk 20  $\mu$ M for one hour before TNF was added.

inhibitors, the chromium-release assays showed preincubation with z-VAD-fmk caused an increase in TNF-mediated cytotoxicity for B/C-N, 10ME, and L929, but showed a reduction in killing in L88.3 (Figures 8 – 11, 13 – 16) . Preincubation with z-DEVD-fmk showed only minimal or no significant changes in TNF-mediated cytotoxicity for B/C-N, 10ME, and L929, but showed reduction in killing in L88.3 (Figure 8 – 11, 13 – 16). The different effect caused by the two caspase inhibitors on TNF-mediated cytotoxicity in B/C-N, 10ME, and L929 suggest that caspases that are strongly inhibited by z-VAD-fmk, such as caspase-1, -5 and -9, may be involved in providing protection against TNF-mediated lysis. Failure of z-DEVD-fmk to block TNF-mediated killing may be due to a parallel activation of a caspase-independent lytic pathway in TNF-mediated cytotoxicity and/or an induced resistance mechanism that acts downstream of caspase-3 activation.

Caspase inhibitors data also suggest that caspase-8 was activated before caspase-9 in the four cell lines used in this study. Pan-caspase inhibitor z-VAD-fmk inhibits both caspase-8 and -9 relatively strongly; z-DEVD-fmk inhibits caspase-8, but not caspase-9 (Garcia-Calvo et al., 1998). Figure 12 showed that preincubation with z-DEVD-fmk inhibited caspase-8 and caspase-9 to a similar level, although it cannot directly interact with caspase-9. Thus, data indicated that caspase-8 inhibition leads to inhibition of caspase-9 and caspase-3. This suggested that activation of caspase-8 leads to activation of caspase-9, either through BID, caspase-2 pathway, or other yet unidentified mechanisms. (see Figure 1).

Involvement of caspases in an apoptotic death has been established through studies of *C. elegans*. Addition of caspase inhibitors have also been shown to block

apoptosis mediated by caspase activation. However, several researchers have shown recently that blocking the caspase cascade by the pan-caspase inhibitor z-VAD-fmk during drug-induced or TNF-induced apoptosis causes a switch from apoptotic death in the target cells to a necrotic form of death (Lemaire et al., 1998; Ruemmele et al., 1999). Others have also shown that caspases may be involved in protection against TNF-induced formation of excessive oxygen radicals leading to necrotic death (Vercammen et al., 1998; Khwaja and Tatton, 1999; Li and Beg., 2000).

Our caspase inhibitor data also suggested a protective role of caspases in TNF-mediated cytotoxicity in B/C-N, 10ME, L88.3 and L929, as well as an involvement of caspases in the lytic pathway of TNF. Our caspase inhibitor data provided some insight into possible caspase involvement in the TNF induced protein-synthesis-dependent resistance mechanism. In TNF-resistant cell line B/C-N, there are caspase dependent and caspase independent components in the protein-synthesis-dependent resistance mechanism. In another TNF-resistant cell line, L88.3, caspases are not involved in the protein-synthesis-dependent resistance mechanism. In TNF-sensitive cell lines 10ME and L929, there seems to be a caspase-dependent component in the protein-synthesis-dependent resistant mechanism.

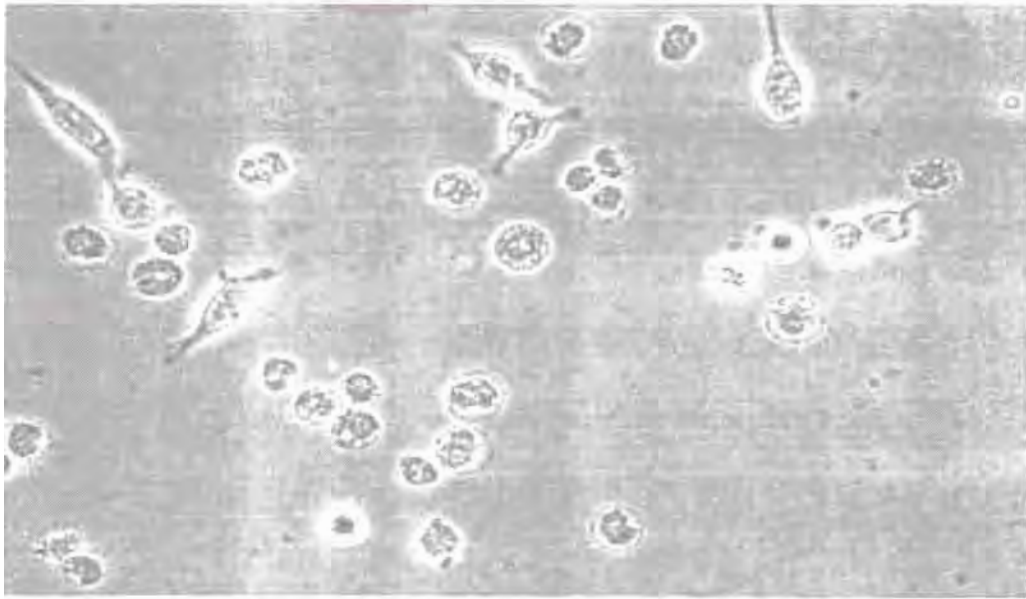
#### *3.4 For TNF-sensitive cell lines, preincubation with z-VAD-fmk caused TNF-mediated cytotoxicity to switch from apoptotic death to necrotic death.*

Lemaire et al. (1998) showed that, for B lymphocytes, inhibition of caspase activity by either of two pan-caspase inhibitors (z-Asp-cmk and z-VAD-fmk) caused a

switch from apoptotic death to necrotic death. Ruemmele et al. (1999) reported similarly that TNF induced apoptotic death in intestinal epithelia cells; however, complete inhibition of the caspase cascade, using high concentrations of z-VAD-fmk in the presence of TNF, induced necrotic death and not apoptotic death. We found that preincubation of L929 with z-VAD-fmk, prior to treatment with TNF, always resulted in swollen cells. This is indicative of necrosis. Most of these cells were lysed when centrifuged at relatively low speeds using a table-top microcentrifuge. This observation prompted us to perform microscopic observations of L929 cells treated with z-VAD-fmk alone, treated by preincubation with z-VAD-fmk for one hour followed by 5 hours of TNF treatment (100 units of TNF/dish), or treated for 5 hours with TNF alone. The morphology of the treated cells was observed, over the 6 hour period, using an inverted microscope at total magnification of 200x.

When L929 was preincubated with z-VAD-fmk for one hour followed with 5 hours of TNF treatment, cells were detached from the tissue culture plate and the majority of the L929 cells showed a necrotic phenotype (Figure 17). The cells, which are normally attached to the plate and spread in a spindle shape, had rounded up and were swollen, an indication of necrotic death. L929 cells incubated with z-VAD-fmk alone for 6 hours remained attached to the tissue culture plates, and showed the same morphology as the untreated controls (Figure 18). L929 cells treated with TNF for 5 hours were just starting to show some detached dying cells, but showed only a few blebbing and swollen cells (Figure 17). In fact, when L929 cells were preincubated with z-VAD-fmk for 1 hour followed by 3 hours of TNF treatment, many cells were detached from the plate,

A. L929 pretreated with z-VAD-fmk for 1 hour followed by TNF for 5 hours



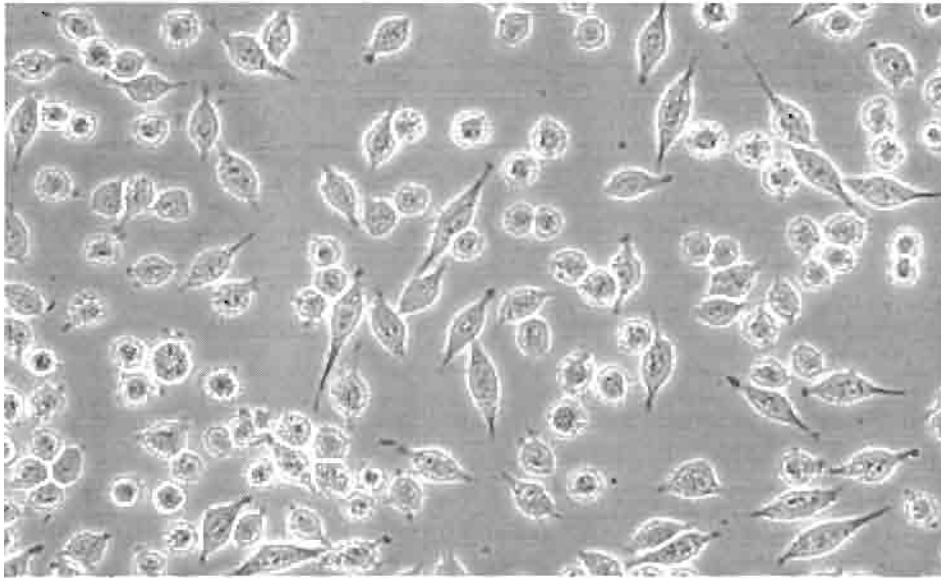
B. L929 treated with TNF for 5 hours



Figure 17 Preincubation with z-VAD-fmk caused necrotic death in L929 cell line. L929 cells were plated the night before, then incubated with no TNF (untreated control), TNF (100 units of TNF) or with z-VAD-fmk + TNF for an appropriate time. Pictures were obtained with a digital camera on an inverted microscope (20X objective).



A. L929 untreated control



B. L929 treated with z-VAD-fmk alone for 6 hours

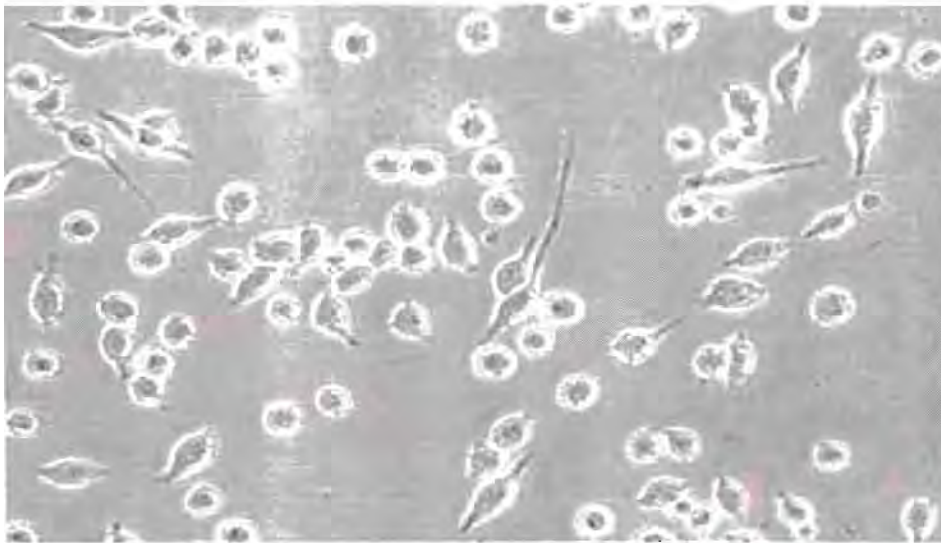
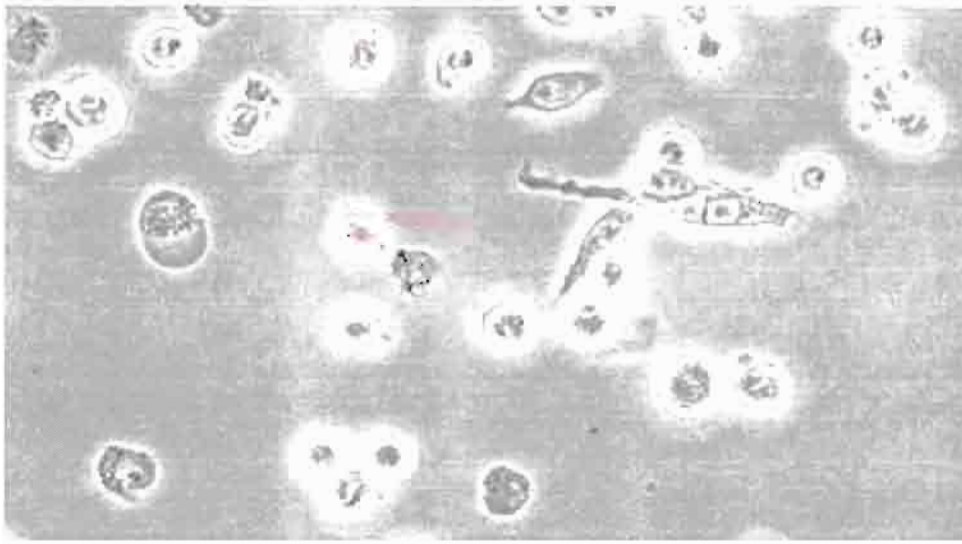


Figure 18 Preincubation with z-VAD-fmk alone caused no morphological changes in L929 cell line. L929 cells were plated the night before, then incubated with no TNF (untreated control), or TNF (100 units of TNF) for an appropriate time. Pictures were obtained with a digital camera on an inverted microscope (20X objective).

whereas TNF alone for 3 hours or z-VAD-fmk alone for 4 hours had little effect on cell attachment or morphology (data not shown). Therefore, in L929 cells, when caspase activities are blocked by z-VAD-fmk, the cells which normally die by apoptotic death, switch to necrotic death. Since necrotic death occurs much faster than apoptotic death, this also explains the much faster kinetics of killing seen in cells treated with z-VAD plus TNF in 5 and 7 hours chromium-release assay (Figure 16) when compared to cells treated with TNF alone.

These results prompted us to look at 10ME which also was sensitized to TNF-mediated cytolysis by z-VAD-fmk, although not to the same degree as was observed for L929. For 10ME, preincubation with z-VAD-fmk for one hour followed by 5 hours of TNF (100 units of TNF/dish) treatment resulted in a mixture of apoptotic and necrotic cells (Figure 19). In the same microscope field, one could observe some cells with blebbing (indicative of apoptotic cells) and some swollen cells (indicative of necrotic cells). The 10ME cells incubated with z-VAD-fmk alone for 6 hours showed no changes compared to the untreated control (Figure 20). However, 10ME cells treated with TNF for 5 hours clearly showed cell blebbing (Figure 19). Therefore, in 10ME cells, when caspase activities were blocked with z-VAD-fmk, TNF-mediated cytolysis seemed to switch from apoptosis to necrotic death, at least for some of the cells. It is not clear if the switch to necrotic death is a function of time and some cells did not have time to switch, or if there is a mixed population of cells such that some switch to necrosis and others remain committed to apoptosis.

A. 10ME pretreated with z-VAD-fmk for 1 hour followed by TNF for 5 hours



B. 10ME treated with TNF for 5 hours

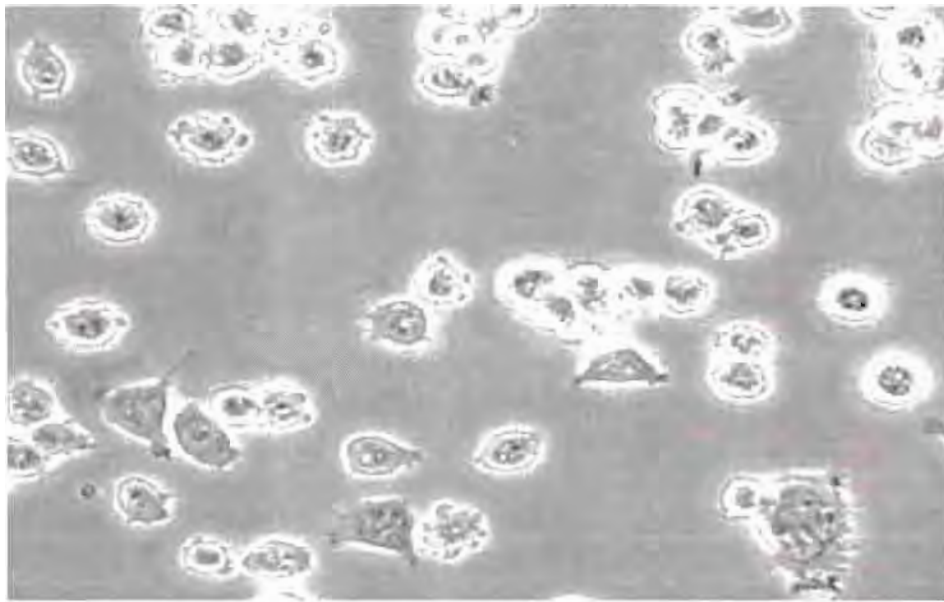
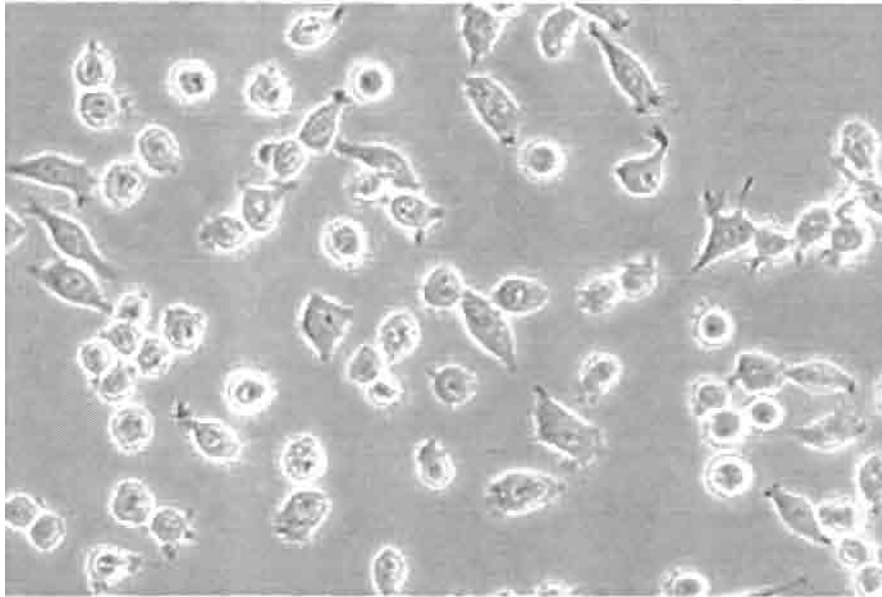


Figure 19 Preincubation with z-VAD-fmk caused switch from apoptotic to necrotic death in the 10ME cell line. 10ME cells were plated the night before, then incubated with no TNF (untreated control), TNF (equivalent to 100 units of TNF) or with z-VAD-fmk + TNF for an appropriate time. Pictures were obtained with a digital camera on an inverted microscope (20X objective).

A. 10ME untreated control



B. 10ME treated with z-VAD-fmk alone for 6 hours

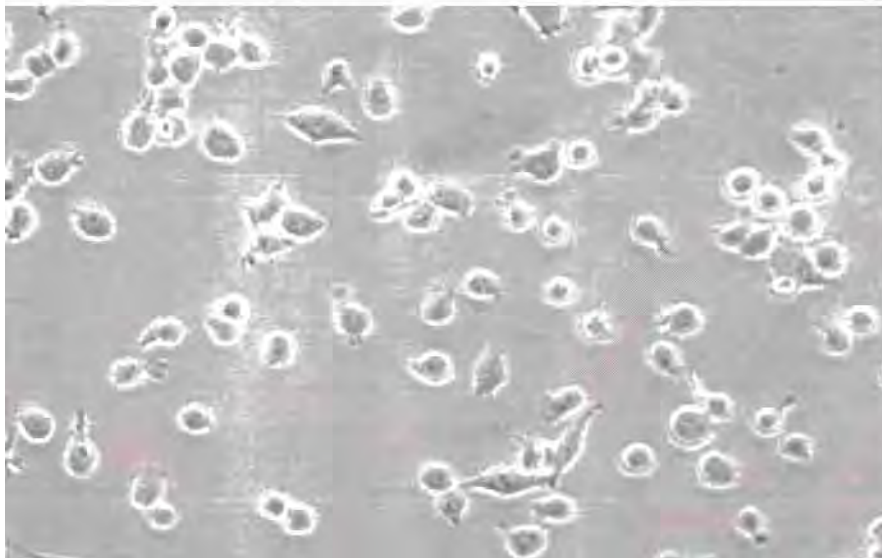


Figure 20 Preincubation with z-VAD-fmk alone caused no morphological changes in the 10ME cell line. 10ME cells were plated the night before, then incubated with no TNF (untreated control), or TNF (100 units of TNF) for an appropriate time. Pictures were obtained with a digital camera on an inverted microscope (20X objective).

Thus, these data showed TNF could cause apoptotic death in 10ME and L929 cell lines. However, when caspases activities were blocked by the pan-caspase inhibitor z-VAD-fmk, TNF-mediated cytolysis of two TNF-sensitive cell lines could switch from apoptotic to necrotic death.

### *3.5 Summary and discussion*

In TNF-resistant cell lines B/C-N and L88.3, TNF causes activation of the TNF-induced protein-synthesis-dependent resistance mechanism along with a protein-synthesis-independent lytic mechanism. Addition of the protein synthesis inhibitor CHX abrogates this protein-synthesis-dependent resistance mechanism, but leaves the protein-synthesis-independent lytic mechanism intact (Figure 3). The effects of CHX on TNF-mediated lysis of B/C-N were reported earlier by Patek et al. (1987). In TNF-sensitive cell lines 10ME and L929, TNF caused activation of a TNF-induced protein-synthesis-independent lytic mechanism (Figure 3). It was shown by Powell et al. (1995) that L929 possesses two TNF-mediated cytolytic pathways, one that is a protein-synthesis-dependent, and another one that is protein-synthesis-independent. Both lytic pathways caused DNA fragmentation, which is indicative of apoptotic death. For 10ME and L929 cell lines, addition of the protein synthesis inhibitor CHX caused a slight increase in TNF-mediated cytolysis (Figure 3). However, the level of increase in TNF-mediated cytolysis due to addition of CHX was higher in 10ME than L929. This suggested that in 10ME cells, there was an activation of some level of a protein-synthesis-dependent

resistant pathway. But in L929 cells, activation of a protein-synthesis-dependent resistance pathway was minimal compared to B/C-N, 10ME, and L88.3 cell lines.

TNF is known to induce apoptotic or necrotic death depending on the cell lines used (Laster et al., 1988). Even within the same cell line, it was shown that TNF could induce apoptotic or necrotic death depending on the phase of the cell cycle when the cells were treated with TNF (Faraco et al., 1999). Generally activation of the initiator caspases, caspase-2, -8 and -9, and activation of the effector caspases-3 and -7 were associated in TNF-induced apoptosis (Duan et al., 1996; Higuchi et al., 1997; Li H et al., 1997; Li P et al., 1997; Jaeschke et al., 1998; Kuida et al., 1998; Faraco et al., 1999; Khwaja and Tatton, 1999; Sun et al., 1999). Although Faraco et al. (1999) reported that , caspase-3 activity was detected in TNF induced necrosis, others have reported that in TNF-induced necrosis, neither active caspase-3 or -7 nor mitochondrial release of cytochrome c were detected (Denecker et al., 2001). Hence the activation of caspases is associated with TNF-induced apoptosis while no activation of caspases is associated with TNF-induced necrosis.

Our caspase activities data showed TNF treatment induced caspases-8, -9, and -3 activities in B/C-N, 10ME, L88.3, and L929 cell lines (Table 1 and Figures 4 – 7). This indicates that when TNF engages TNF-R1 in these four cell lines, it activates the caspase cascade through a TNF receptor of the apoptotic pathway as well as through the mitochondrial apoptotic pathway. Our caspase inhibitors data showed that caspase-8 was activated first and may be responsible for the activation of caspase-9 in all four cell lines (Figure 12). Z-DEVD-fmk inhibits caspase-8 only, while pan-caspase inhibitor z-VAD-

fmk inhibits both caspase-8 and -9 (Garcia-Calvo et al., 1998). Yet both inhibitors suppressed caspase-8,-9 and -3 activities to the same level. Thus, the data indicated that inhibition of caspase-8 alone could inhibit caspase-9 and caspase-3 activities in these cell lines. These data also implicated that the mitochondrial apoptotic pathway (activated by caspase-9) was activated as an amplification arm of the apoptosis induced via the death receptor apoptotic pathway (activated by caspase-8). Gross et al. (1999) reported that in TNF-induced apoptosis, caspase-8 can activate the mitochondrial pathway through activation of a proapoptotic Bcl-2 protein family protien, BID (Figure 1). Li H et al. (1997) showed that the mitochondrial pathway can be activated through activation of procaspase-2 by caspase-3 in TNF-induced apoptosis (Figure 1). Therefore, the link between caspase-8 activation and the mitochontrial pathway (i.e. activation of caspase-9) most likely would involve activation of BID or caspase-2 in our cell lines as well.

Activities in caspases-8, -9 and-3 increased in a time-dependent manner from 5 to 12 hours of TNF treatment (Table 1). Then, these caspase activity levels fell after 10 hours of TNF treatment in L88.3 cell line (data not shown). For cell lines B/C-N, 10ME, and L929, activity levels fell after 12 hours of TNF treatment (data not shown). We observed a correlation between the rise in caspases-8, -9, and -3 activity levels and the sensitivity to TNF-mediated cytolysis (Table 1 and Figures 4 – 7). The levels of caspase activities were higher in TNF-sensitive cell lines 10ME and L929 compared to the activities in the TNF-resistant cell lines B/C-N and L88.3. When protein-synthesis-dependent resistance mechanism was suppressed by addition of CHX, both B/C-N and L88.3 also showed significant rise in caspase-8, -9, and -3 activities (Figure 4 and 6).

Thus, protein-synthesis-dependent resistance mechanism seems to interfere with caspase activities in TNF-resistant cell lines. Between the two TNF-sensitive cell lines, 10ME and L929, 10ME showed a steady increase in caspase activities from 5 to 12 hours of TNF treatment, while L929 showed a dramatic increase in caspase activities between 7 and 12 hours of TNF treatment (Table 1). Thus, it seems that caspase activation takes longer in the L929 cell line compared to the 10ME cell line.

Our data also indicated that a large rise in caspase-3 activity alone was not sufficient to cause a large increase in TNF-mediated cytolysis in B/C-N, 10ME, and L88.3. There was some increase in TNF-mediated cytolysis with the rise in caspase-3 activity alone seen in the 5 hours TNF plus CHX treatment (Figure 4 – 7). But, TNF plus CHX data showed that activity levels of all three caspases (caspase-8, -9, and -3) have to be increased in order to see a large increase in TNF-mediated cytolysis (Figures 4 – 6). It seems that caspase-8 and -9 activities have to be at least 1.5-fold above baseline levels and caspase-3 to be at least above 2.5-fold to cause a large increase in TNF-mediated cytolysis in B/C-N, 10ME, and L88.3 cell lines. This suggests a threshold for caspase activity levels in order to activate the TNF-mediated lytic pathway or overcome the TNF-induced resistance mechanism in B/C-N, 10ME, and L88.3.

The large increase seen in caspase-3 activity without a corresponding large increase seen in caspase-8 or caspase-9 at 5 hours of TNF plus CHX (Figures 4 – 7) was most likely due to earlier rise in caspase-8 and caspase-9 activities for TNF plus CHX treatment. Khwaja and Tatton (1999) showed that in TNF-resistant human leukemic cells U937, preincubation with CHX showed marked increase in caspase-8 activity that



peaked 2 to 3 hours after TNF addition, while TNF alone resulted in a small increase in caspase-8 activity.

In the L929 cells, increases in TNF-mediated cytolysis at the early time points of 5 and 7 hours TNF treatment did not always correlate with increase in caspase activities (Figure 7). Earlier researches have shown that there are other lytic pathways such as generation of oxygen radicals and activation of phospholipase(s) are used in TNF-mediated cytolysis in L929 cells (Neale et al., 1988; Schulze-Osthoff et al., 1992; Goossens et al., 1995). The earlier increase in TNF-mediated cytolysis during 5 and 7 hours of TNF treatment are most likely due to these other lytic pathways in L929 cells, since caspases activities are low at these time points (Figure 7). The increase in TNF-mediated cytolysis in TNF plus CHX samples during 5 and 7 hours TNF treatment may be due to caspases activities, because there are corresponding increase in caspase-9 and caspase-3 activities. Caspases-mediated TNF cytolysis probably occurs after 7 hours of TNF treatment, since there is a dramatic increase in caspases activities after 7 hours of TNF treatment (Table 1).

These L929 data reinforced some earlier findings that showed chromatin condensation could be observed at 6 hours of TNF treatment in L929 cells, but DNA fragmentation and other nuclear changes occurred much later, between 12 hours and 18 hours of TNF treatment (Fady et al., 1995; Mirikina et al., 1996). Fady et al. (1995) showed that DNA fragmentation could be detected at 12 hours of TNF treatment only in non-adherent cells after TNF-mediated cytotoxicity (methylene blue cytotoxicity assay) had occurred. However, membrane permeability could be detected after 8 hours of TNF

treatment. These data could explain why some researchers argue that L929 cells die through necrosis or apoptosis in TNF-mediated cytotoxicity.

For B/C-N, 10ME, L88.3, and L929 cell lines, our caspase inhibitors data show that preincubation with the pan-caspase inhibitor z-VAD-fmk or caspase-3, -7 and -8 inhibitor z-DEVD-fmk did indeed suppress caspases-8, -9, and -3 activities at 5 hour and 7 hours of TNF treatment when compared to TNF alone (Figure 12). However the pan-caspase inhibitor z-VAD-fmk caused an increase in TNF-mediated killing in B/C-N, 10ME and L929 cell lines (Figures 8, 9, 11, 13, 15, and 16). The degree that z-VAD-fmk potentiated TNF-mediated killing in TNF-sensitive cell lines 10ME and L929 was as high as the samples treated with TNF and CHX. This was not the case for the B/C-N cell line. Caspase-3, -7 and -8 inhibitor z-DEVD-fmk either caused no difference or a slight decrease in TNF-mediated killing in B/C-N, 10ME and L929 (Figures 8, 9, 11, 13, 15, and 16). However, both caspase inhibitors caused a decrease in TNF-mediated killing of L88.3 cells (Figure 10). Pan-caspase inhibitor z-VAD-fmk showed a greater reduction in TNF-mediated cytotoxicity compared to z-DEVD-fmk.

These data suggest two things: 1) there is a caspase-independent lytic pathway activated during TNF-mediated cytotoxicity, even when caspase activities are inhibited, and 2) caspases are involved in protection against TNF-mediated cytotoxicity. It was shown that blocking the caspase cascade by a pan-caspase inhibitor z-VAD-fmk in a drug-induced or TNF-induced apoptosis causes a switch from apoptotic death in the target cells to a necrotic form of death (Lemaire et al., 1998; Ruemmele et al., 1999). Our data for TNF-sensitive 10ME and L929 cell lines show similar findings (Figures 17 – 20). When these

cells were incubated with TNF alone for 5 hours, they showed cell blebbing indicative of apoptosis (Figure 17 and 19). This morphology was more apparent in the 10ME cells (Figure 19) than in the L929 (Figure 17). This difference may be explained by the delayed rise in caspase activities in the L929 compared to the 10ME cells (Table 1). However, when cells were preincubated with z-VAD-fmk followed by 5 hours of TNF treatment, both 10ME and L929 cells showed necrotic phenotypes (Figure 18 and 20).

Other researchers have also shown that caspases may be involved in a protection against TNF-induced formation of an excessive concentration of oxygen radicals leading to necrotic cell death (Vercammen et al., 1998; Khwaja and Tatton, 1999; Li and Beg, 2000). In B/C-N, 10ME, and L929 cell lines, blocking of caspase activation caused an increase in TNF-mediated cytolysis, not a decrease. This suggests that caspases inhibited by z-VAD-fmk (e.g. caspase-1, 5, and -9) may have a protective role in TNF-mediated cytolysis in B/C-N, 10ME, and L929 cells, as a component of the protein-synthesis-dependent resistance mechanism. The effect of z-DEVD-fmk on TNF-mediated cytolysis may be obscured in B/C-N, 10ME, and L929 cell lines due to a parallel activation of caspase-independent lytic pathway induced by TNF as it was shown that inhibition of caspase activities lead to necrotic death (Lemaire et al., 1998; Ruemmele et al., 1999). Since preincubation with z-VAD-fmk or z-DEVD-fmk decreased TNF-mediated lysis of L88.3, caspases appear to be involved in TNF-mediated lytic pathway but not involved in protection. However, caspase activation is not the sole lytic pathway used in TNF cytolysis in L88.3, since z-VAD-fmk and z-

DEVD-fmk could only partially inhibit TNF-mediated cytolysis. This indicates there is also a caspase-independent lytic pathway in L88.3.

These data provided insight into the role of caspases in TNF-mediated cytolysis. Caspases are involved in TNF-mediated cytolysis, however, there seems to be a threshold for caspase activities in order to cause TNF-mediated cytolysis. It requires at least a 1.5 to 2-fold increase in both initiator caspases (caspases-8 and -9) and effector caspase (caspase-3). Large increases in caspase-3 alone do not contribute to a large increase in TNF-mediated cytolysis. TNF can also induce a caspase-independent lytic pathway. The inhibition of caspase activities causes a switch from TNF-induced apoptotic death to TNF induced necrotic death in the TNF-sensitive cell lines 10ME and L929. And lastly, some caspases may play a protective role in TNF-mediated cytolysis in B/C-N, 10ME, and L929, but not in L88.3.

## CHAPTER 4

### A SPECIFIC PHOSPHOLIPASE A<sub>2</sub> INHIBITOR, QUINACRINE, AND THE OXYGEN-RADICAL SCAVENGER, BUTYLATED HYDROXYANISOLE (BHA), REDUCED TNF-MEDIATED CYTOLYSIS

TNF-mediated lytic pathway involving generation of oxygen radicals and the activation of phospholipase(s) have been shown in the L929 cell line (Neale et al., 1988; Schulze-Osthoff et al., 1992; Goossens et al., 1995). Caspase inhibitors data showed that there is a caspase-independent lytic pathway activated by TNF in B/C-N, 10ME, L88.3 and L929 cell lines (Figures 8 – 11, and 13 – 16). In order to explore a caspase-independent lytic pathway in our cell lines, we investigated whether TNF-mediated cytotoxicity involves generation of oxygen radicals or activation of phospholipase A<sub>2</sub> in the cell lines B/C-N, 10ME, and L88.3.

#### *4.1 TNF-induced cytotoxicity involves activation of phospholipase A<sub>2</sub> in B/C-N, 10ME, and L88.3 cell lines.*

Several researchers have shown that activation of PLA<sub>2</sub> is involved in TNF-mediated cytotoxicity. In the TNF sensitive cell line 10ME, TNF causes activation of PLA<sub>2</sub> and release of arachidonic acid (Mutch et al., 1992). In TNF resistant cell lines, activation of PLA<sub>2</sub> is inhibited or it could be activated but there is a defect in arachidonic acid biosynthesis (Hayakawa et al., 1993; Hollenback et al., 1992; Reid et al., 1991). However, addition of transcription inhibitors or translation inhibitors can cause activation of PLA<sub>2</sub> in these TNF resistant cell lines, rendering them TNF sensitive (Hollenback et

al., 1992; Voelkel-Johnson et al., 1996). Thus B/C-N, 10ME, and L88.3 cell lines were used to investigate whether PLA<sub>2</sub> activation is involved in TNF-mediated cytotoxicity in the presence or absence of the translation inhibitor CHX. A specific PLA<sub>2</sub> inhibitor, quinacrine, was used to determine whether it could reduce TNF-mediated cytotoxicity in the 18-hour chromium-release assay. Quinacrine (1  $\mu$ M) was added immediately after target cells and TNF were mixed.

Mutch et al. (1992) showed that addition of  $5 \times 10^{-6}$  M quinacrine reduced TNF-mediated cytotoxicity in 10ME. Our data also showed that addition of 1  $\mu$ M quinacrine reduced TNF-mediated cytotoxicity of 10ME in the presence or absence of CHX (Figure 21). In the absence of CHX, addition of quinacrine reduced TNF-mediated cytotoxicity to one-half of the level seen when treated with TNF alone. In the presence of CHX, quinacrine also reduced TNF-mediated cytotoxicity; but a reduction was much smaller when compared to the set of data in the absence of CHX. Thus, the increase in TNF-mediated cytotoxicity due to addition of CHX was not due to increased PLA<sub>2</sub> activity in 10ME. These data clearly indicated that in the TNF-sensitive 10ME cell line, activation of PLA<sub>2</sub> does play a role in TNF-mediated cytotoxicity.

In the TNF-resistant cell line B/C-N, the effect of quinacrine could only be seen with addition of CHX because B/C-N is TNF resistant. However, in the presence of CHX, TNF-mediated cytotoxicity of B/C-N was reduced when quinacrine was added (Figure 21). Although the level of reduction in TNF-mediated cytotoxicity was smaller in B/C-N than 10ME, the level of reduction due to addition of quinacrine was still significant. Hence, in B/C-N, activation of PLA<sub>2</sub> was also involved in TNF-mediated

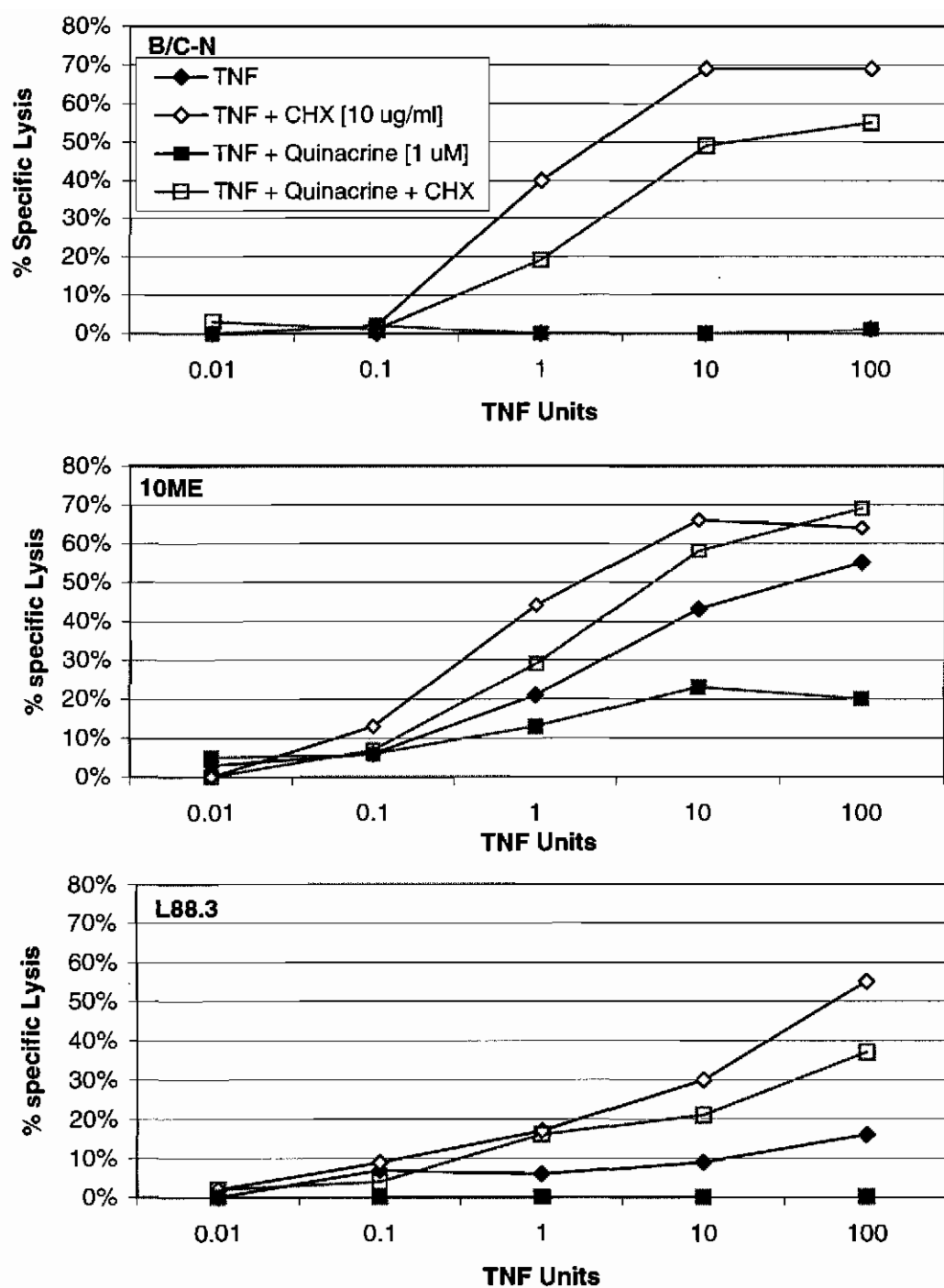


Figure 21 Addition of specific PLA<sub>2</sub> inhibitor quinacrine reduced TNF-mediated cytotoxicity in B/C-N, 10ME, and L88.3 cell lines. TNF-mediated cytotoxicity was measured by the 18-hour chromium-release assay (see Chapter 2 for detail). One  $\mu$ M of quinacrine was added immediately to wells after targets and TNF were mixed.

cytolysis. In another TNF-resistant cell line L88.3, addition of quinacrine resulted in a reduction in TNF-mediated cytolysis in the presence or absence of CHX (Figure 21). In the absence of CHX, addition of quinacrine completely inhibited TNF-mediated cytolysis in L88.3 cells. However, the increase in TNF-mediated cytolysis in the presence of CHX was not due to increased levels of PLA<sub>2</sub> activity, since the level of reduction seen by addition of quinacrine in the presence or absence of CHX was the same in L88.3.

Thus, for B/C-N, 10ME, and L88.3, inhibition of PLA<sub>2</sub> activation by quinacrine caused reduction in TNF-mediated cytolysis. These data indicated that activation of PLA<sub>2</sub> is a component of TNF-induced lytic pathway in all three cell lines.

#### *4.2 BHA reduces TNF-mediated cytolysis of TNF-sensitive cells*

Butylated hydroxyanisole (BHA), an oxygen-radical scavenger, has been shown to inhibit TNF-mediated cytotoxicity in the murine cell lines WEHI 164 clone 13 and L929 through inhibition of TNF-induced activation of phospholipase(s) and release of arachidonic acid (Brekke et al., 1992; Brekke et al., 1994). TNF-induced arachidonic acid metabolism causes generation of oxygen radicals (Matthews et al., 1987). BHA can significantly inhibit necrotic death due to generation of oxygen radicals as a result of mitochondrial damage. BHA has also been reported (Verhaegen, et al., 1995) to inhibit apoptotic death by reducing DNA fragmentation. Furthermore, BHA has been shown to block TNF-mediated necrotic death in L929 cells sensitized by the pan-caspase inhibitor z-VAD-fmk (Vercammen et al., 1998). BHA also significantly inhibits UV-irradiation and hydrogen peroxide induced apoptosis in human HL-60 leukemia cells (Verhaegen et



al., 1995). Therefore, the oxygen-radical scavenger BHA was used in an 18-hour chromium-release assay to investigate possible involvement of generation of oxygen radicals in TNF-mediated cytotoxicity of B/C-N, 10ME, and L88.3.

In TNF-sensitive cell lines such as 10ME and L929, BHA (100  $\mu$ M) added to the assays at the same time as TNF, caused greater reduction in TNF-mediated cytotoxicity than it did for the TNF-resistant cell lines B/C-N and L88.3 (Figure 22). When BHA was added to L929 cells at the same time as TNF there was a 50% reduction in TNF-mediated cytotoxicity (reduced from 69% to 36%) (Figure 22). In 10ME cells, reduction in TNF-mediated cytotoxicity due to BHA was almost 30% (from 62% to 44%). In B/C-N, the effect of BHA on TNF-mediated cytotoxicity was minimal. This is not surprising, since B/C-N is the most TNF-resistant cell line used in this study. As in B/C-N, BHA reduction of TNF-mediated cytotoxicity was also minimal for L88.3. For L88.3 lysis was reduced from 31% to 24% when BHA was added to the TNF cytotoxicity assay.

These results suggest that oxygen radicals generated through mitochondria or arachidonic acid metabolism are involved in the TNF-mediated lysis of 10ME and L929 cell lines. Perhaps, failure to generate oxygen radicals, or endogenous oxygen radical scavengers, such as manganous superoxide dismutase, plays key roles in B/C-N and L88.3 resistance to TNF lysis.

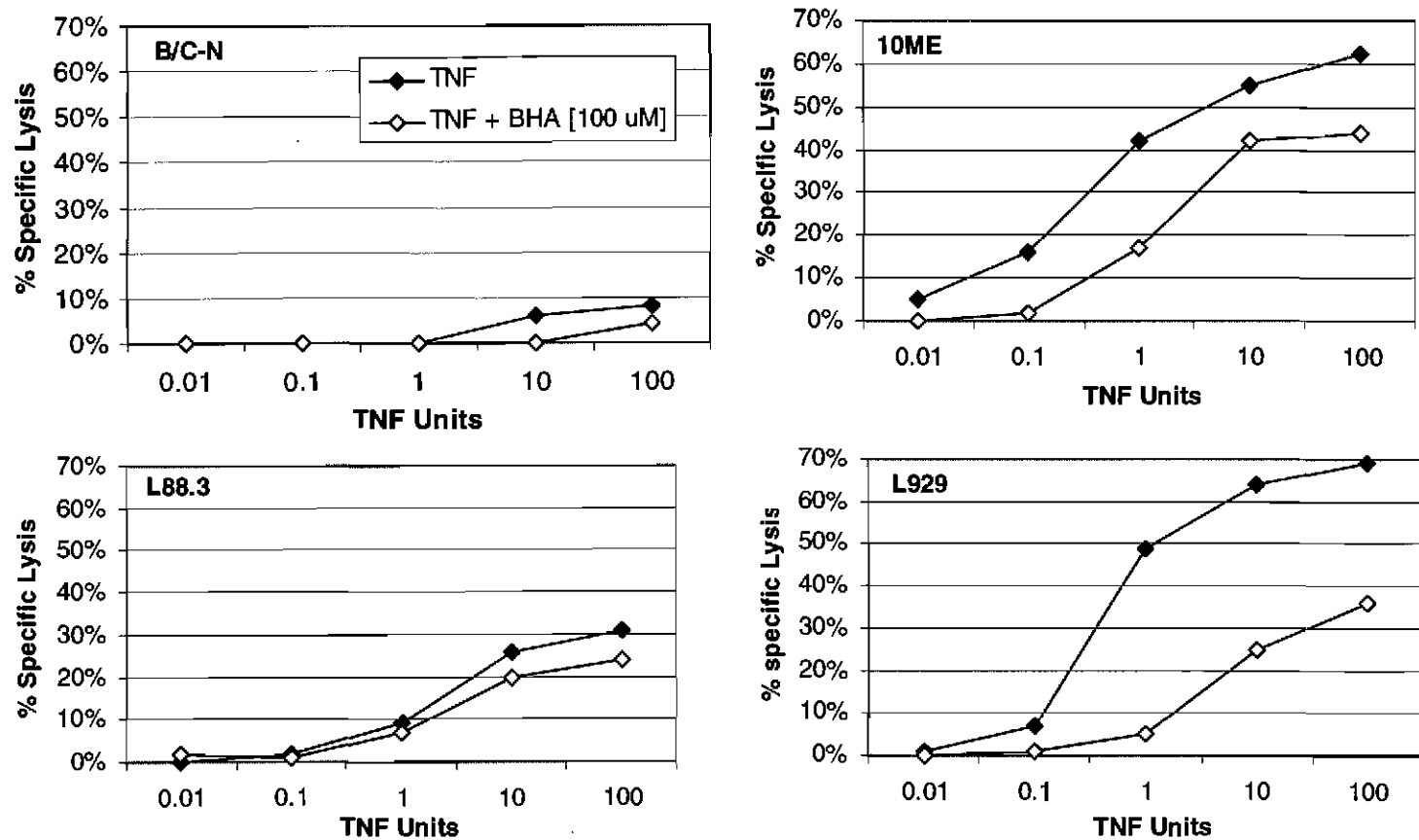


Figure 22 Addition of 100  $\mu$ M BHA reduced TNF-mediated cytotoxicity in TNF-sensitive cell lines 10ME and L929. TNF-mediated cytotoxicity was measured by an 18-hour chromium-release assay (see Chapter 2 for details). One hundred  $\mu$ M BHA was added right after targets and TNF were mixed.

#### *4.3 BHA reduces TNF-mediated cytotoxicity in cells sensitized to TNF by preincubation with the pan-caspase inhibitor z-VAD-fmk*

Preincubation of cells with caspase inhibitor z-VAD-fmk increased TNF-mediated cytotoxicity of B/C-N, 10ME, and L929 (see Chapter 3). To determine whether the potentiation of TNF-mediated lysis by the pan-caspase inhibitor z-VAD-fmk results from increased oxygen radical formation, we examined the effects of BHA on TNF-mediated lysis of cells treated with the caspase inhibitor z-VAD-fmk. TNF-mediated lysis was measured in an 18-hour chromium-release assay. Cells were either pretreated or not with the caspase inhibitor z-VAD-fmk (20  $\mu$ M), then TNF and BHA (100  $\mu$ M) were added.

Our results showed that BHA reduced z-VAD-fmk sensitized TNF-mediated cytotoxicity in B/C-N, 10ME, and L929 cell lines. In B/C-N, addition of BHA to cells pretreated with z-VAD-fmk (z-VAD-fmk + TNF + BHA) caused a reduction in TNF-mediated cytotoxicity, while addition of BHA to TNF (TNF + BHA) showed minimal reduction (Figure 23). Addition of BHA to z-VAD-fmk plus TNF (z-VAD + TNF + BHA) reduced TNF-mediated cytotoxicity to 1/3 (15 % to 4%) of z-VAD-fmk plus TNF (z-VAD + TNF). These results indicated that for B/C-N, an increase in TNF-mediated cytotoxicity due to preincubation with z-VAD-fmk caused generation of oxygen radicals, while TNF alone either did not induce generation of oxygen radicals or TNF induced production of endogenous oxygen-radical scavengers in large amounts.

In 10ME, BHA reduced killing by TNF alone, as well as TNF killing in the presence of z-VAD-fmk pretreated cells (Figure 24). The levels of BHA-induced

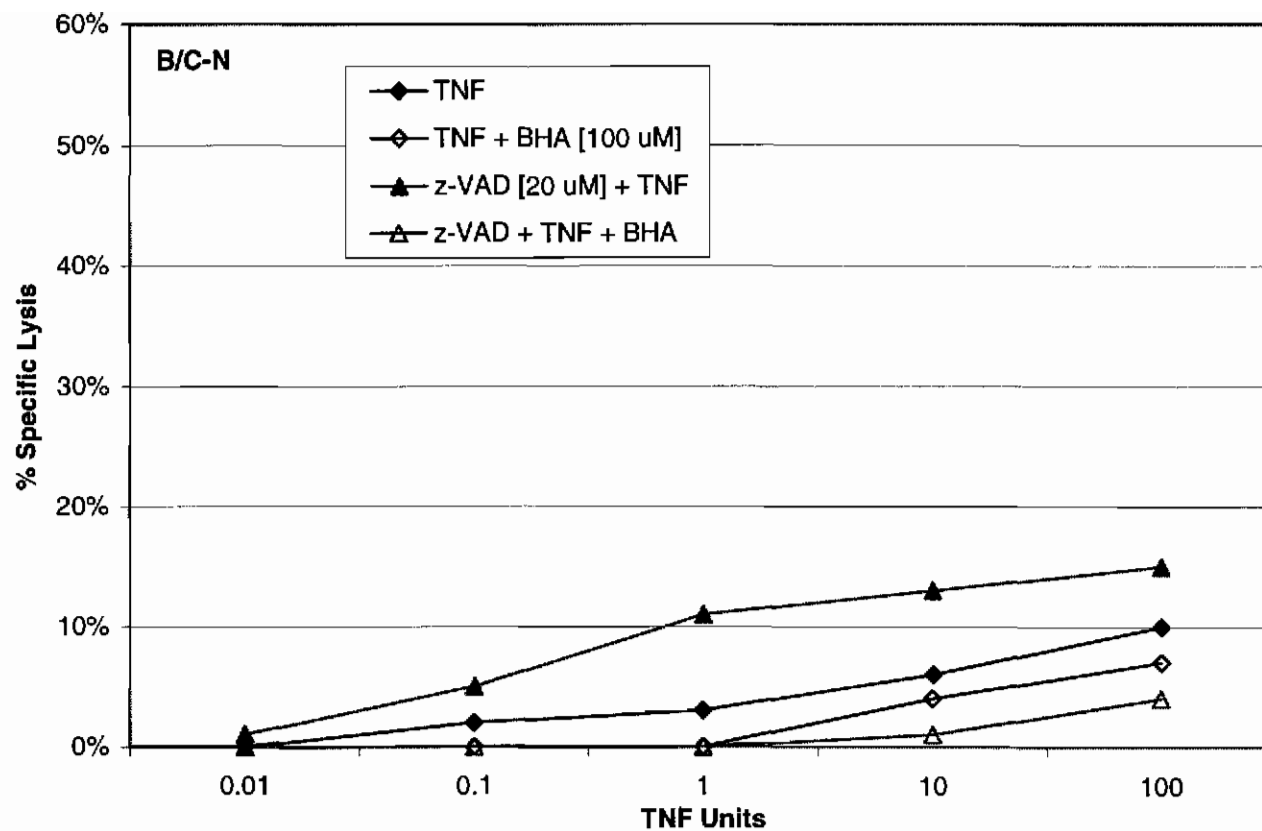


Figure 23 For B/C-N, the increase in TNF-mediated killing due to addition of the caspase inhibitor z-VAD-fmk can be blocked by BHA. Cells were pretreated with 20  $\mu$ M z-VAD-fmk for 2 hours then TNF were added. One hundred  $\mu$ M BHA was added right after targets and TNF were mixed. TNF-mediated cytotoxicity was measured using 18-hour chromium-release assay (see Chapter 2 for details).

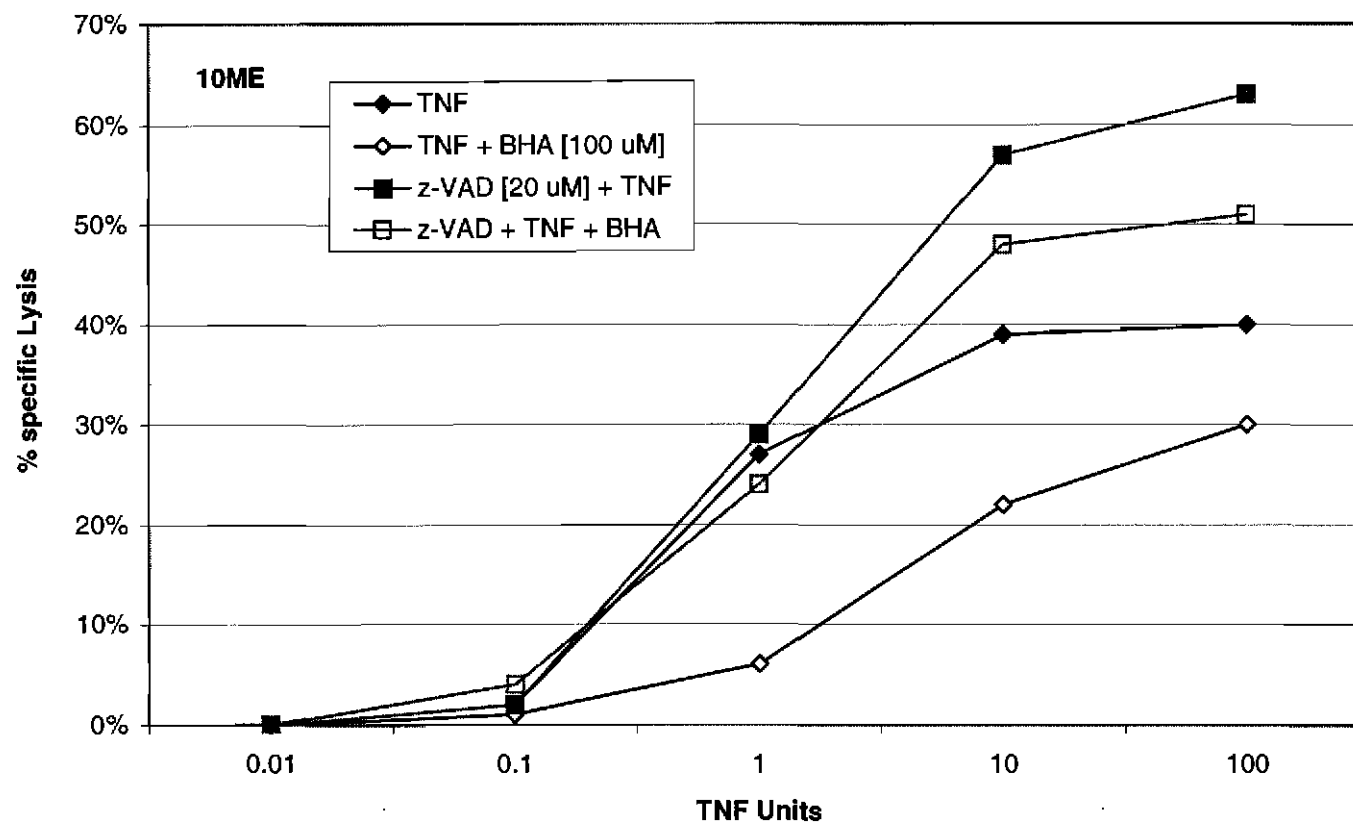


Figure 24 For 10ME, the increase in TNF-mediated killing due to addition of the caspase inhibitor z-VAD-fmk was not due to increase in generation of oxygen radicals. Cells were pretreated with 20  $\mu$ M z-VAD-fmk or 20  $\mu$ M z-DEVD-fmk for 2 hours then TNF were added. One hundred  $\mu$ M BHA was added right after targets and TNF were mixed. TNF-mediated cytolysis was measured using the 18-hour chromium-release assay (see Chapter 2 for details).

reduction in TNF-mediated killing seen for TNF alone (40% reduced to 30%) and reduction in TNF-mediated killing seen for TNF alone (40% reduced to 30%) and TNF on z-VAD-fmk pretreated cells (63% reduced to 51%) were about the same. These data indicated that for 10ME, the TNF-mediated lytic mechanism utilized the generation of oxygen radicals. However, the increase in TNF-mediated killing due to preincubation with z-VAD-fmk was not due to an increase in generation of oxygen radicals.

In L929, addition of BHA greatly reduced TNF-mediated killing. BHA also reduced lysis of L929 caused by TNF plus z-VAD-fmk, but the effect was not as great as in the absence of the caspase inhibitor (Fig. 25). Thus, TNF-mediated cytolysis was reduced from 52% in TNF alone to 24% in TNF + BHA. But in z-VAD-fmk pretreated L929, addition of BHA only reduced TNF-mediated cytolysis from 56% to 45%. Accordingly, like 10ME, these data indicated that for L929, TNF-mediated cytolysis utilized generation of oxygen radicals. However, increase in TNF-mediated cytolysis due to preincubation with z-VAD-fmk was not due to an increase in the generation of oxygen radicals.

Thus, the pan-caspase inhibitor z-VAD-fmk increased the susceptibility of B/C-N, 10ME, and L929 to TNF-mediated killing and BHA blocked that increase. However, for 10ME and L929, increases in TNF-mediated cytolysis due to preincubation with z-VAD-fmk were not due to increase in generation of oxygen radicals. B/C-N was the only cell line that showed correlation between the increase in generation of oxygen radicals and inhibition of caspase activities by z-VAD-fmk pretreatment. Accordingly, we speculate that the caspase inhibitor could work by stimulating, either directly or indirectly,

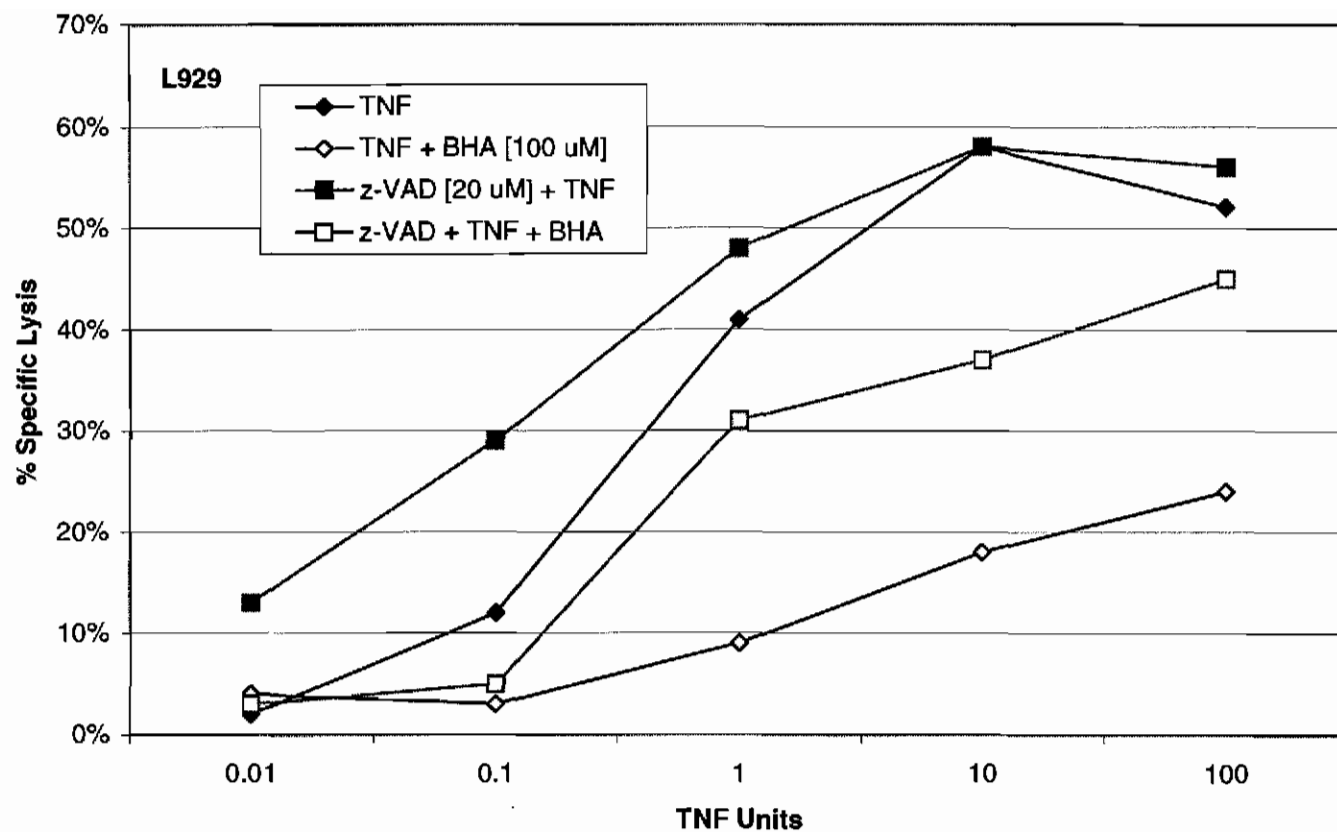


Figure 25 For L929, the increase in TNF-mediated killing due to addition of the caspase inhibitor z-VAD-fmk was not due to increase in generation of oxygen radicals. Cells were pretreated with 20  $\mu$ M z-VAD-fmk for 2 hours then TNF were added. One hundred  $\mu$ M BHA was added right after targets and TNF were mixed. TNF-mediated cytolysis was measured using 18-hour chromium-release assay (see Chapter 2 for details).

inhibition of production of oxygen-radical scavengers. This notion is supported by the work of Khwaja and Tatton (1999). They showed that U937 and NIH3T3 cells sensitized to TNF-mediated cytotoxicity by preincubation with z-VAD-fmk generated excessive amount of reactive oxygen species, leading to nonapoptotic form of death. They also showed that BHA inhibited z-VAD-fmk-induced increase in TNF-mediated cytotoxicity.

#### *4.4 BHA has no effect in reducing TNF-mediated cytotoxicity in L88.3*

As shown in Chapter 3, L88.3 was the only cell line that showed a decrease in TNF-mediated cytotoxicity when cells were preincubated with caspase inhibitors z-VAD-fmk or z-DEVD-fmk. Hence, it would be interesting to see whether addition of BHA can further reduce TNF-mediated cytotoxicity in combination with z-VAD-fmk treatment. Compared to B/C-N, 10ME or L929 cell lines, BHA did not significantly reduce TNF-mediated cytotoxicity in L88.3 whether pretreated with z-VAD-fmk or not (Figure 26). Pretreatment with z-VAD-fmk significantly reduced TNF-mediated lysis of L88.3 and BHA had only minor effects in further inhibiting this lysis. Thus, BHA had no effect in reducing TNF-mediated cytotoxicity in the L88.3 cell line, indicating that oxygen radicals do not play a central role in TNF-mediated lysis of L88.3 cells.

#### *4.5 Summary and discussion*

Reduction of TNF-mediated cytotoxicity by addition of a specific phospholipase A<sub>2</sub> inhibitor, quinacrine, showed that TNF activated PLA<sub>2</sub> pathway in B/C-N, 10ME, and L88.3 cell lines (Figure 21). However, in B/C-N, activation of PL A<sub>2</sub> pathway probably



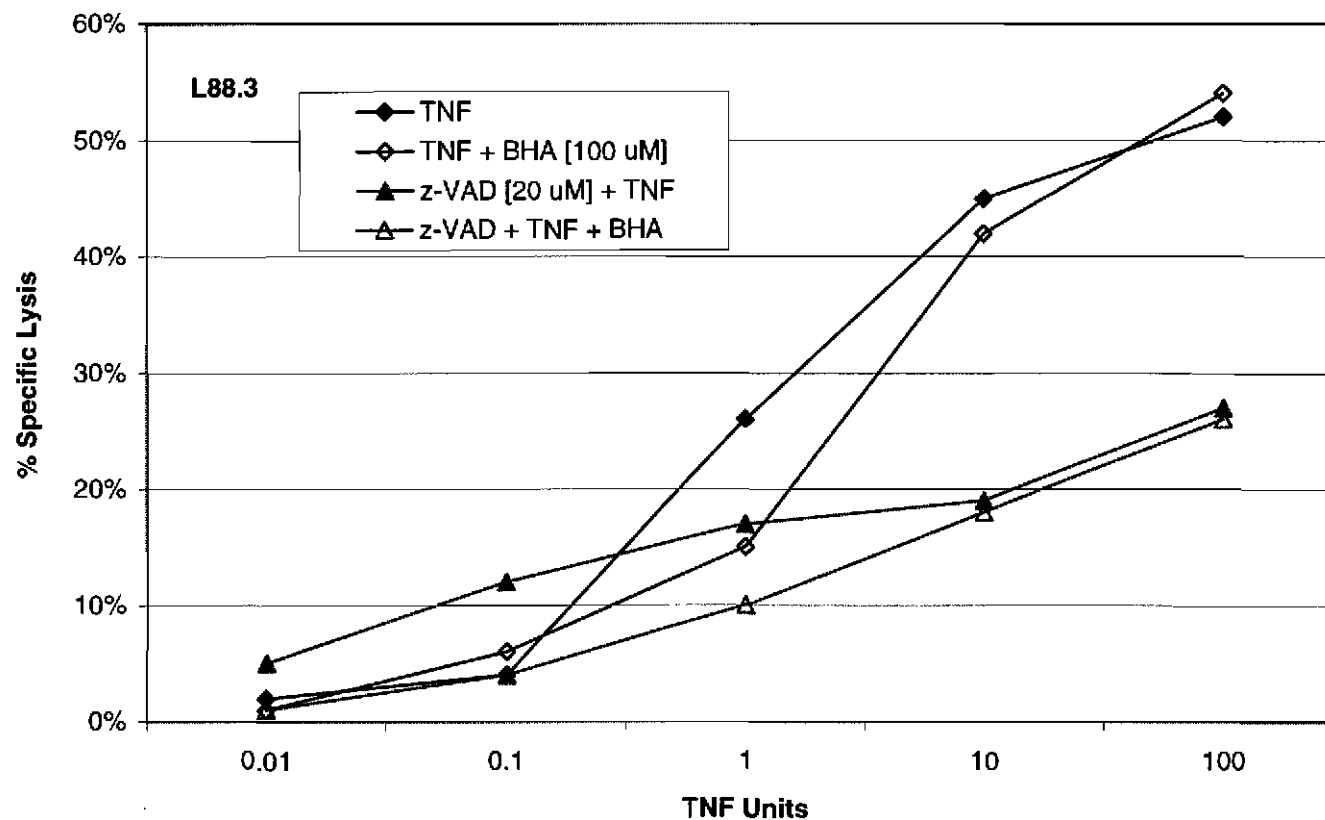


Figure 26 For the L88.3 cell line, BHA did not decrease TNF-mediated killing in the presence or absence of the caspase inhibitor z-VAD-fmk. Cells were pretreated with 20  $\mu$ M z-VAD-fmk for 2 hours prior to addition of TNF. One hundred  $\mu$ M BHA was added right after targets and TNF were mixed. TNF-mediated cytolysis was measured using 18- hour chromium-release assay (see Chapter 2 for details).

was not involved in TNF-mediated cytotoxicity unless a protein-synthesis-dependent resistance mechanism was blocked (Figure 21).

BHA, an oxygen-radical scavenger, reduced the TNF-mediated cytotoxicity of the cell lines 10ME and L929 (Figure 22). Moreover, BHA reduced the sensitizing effects of the pan-caspase inhibitor z-VAD-fmk (Figure 23 – 25). The sensitizing effect of the pan-caspase inhibitor z-VAD-fmk was due to increase in generation of oxygen radicals in B/C-N but not in 10ME and L929. In contrast, L88.3 cytotoxicity was inhibited by the caspase inhibitors, and BHA had no effect on TNF lysis of this cell line (Figure 26).

As shown in the chapter 3, activation of caspases appears to be involved in TNF-mediated cytotoxicity in all cell lines used. However, when caspase activation was blocked by the pan-caspase inhibitor z-VAD-fmk, TNF-mediated cytotoxicity was increased in B/C-N, 10ME, and L929 cell lines. This indicated that although caspases were involved in lysis of these cell lines, when the caspase-dependent lytic pathway was blocked, TNF-mediated cytotoxicity switched to a caspase-independent lytic pathway. As shown in Chapter 3, for 10ME and L929 cell lines, blocking of a caspase-dependent lytic pathway by a caspase inhibitor z-VAD-fmk, caused some cell death via a necrotic phenotype (Figure 17 – 20). Our data showed that the z-VAD-fmk-induced necrotic death in 10ME and L929 was not due to generation of oxygen radicals (Figures 24 and 25). Since the level of reduction by BHA for TNF alone and z-VAD-fmk plus TNF in 10ME was about the same, it appears that the increase in TNF-mediated killing seen in z-VAD-fmk pretreatment was not due to increase in generation of oxygen radicals (Figure 24). Thus in 10ME, necrotic death caused by inhibition of caspase activities was not due to increase

in generation of oxygen radicals. In L929, the effect of BHA on z-VAD-fmk plus TNF was smaller than the effect when the cells were treated with TNF alone (Figure 25). Therefore, as in 10ME, inhibition of caspase activities resulted in the increase in TNF-mediated necrotic death of L929, but the increase in killing was not caused by increase in generation of oxygen radicals. In B/C-N, TNF-mediated caspase-independent lytic pathway was blocked by BHA, suggesting oxygen radicals generated via mitochondria or arachidonic acid metabolism were involved (Figure 23).

For the L88.3 cell line, addition of z-VAD-fmk or z-DEVD-fmk caused a reduction in TNF-mediated cytolysis due to a blocked caspase-dependent lytic pathway (Figure 10). Unlike some other cell lines, these caspase inhibitors did not totally inhibit TNF-mediated cytolysis. Since it is not a complete inhibition, this suggests that the caspase-dependent lytic pathway is not the sole lytic pathway involved in TNF-mediated cytolysis of L88.3. Nonetheless, unlike the outcome in other cell lines tested, the caspase-independent lytic pathway resulted in only minor killing. The failure of BHA to further block the lysis indicates that oxygen radicals generated through mitochondria or arachidonic acid metabolic pathway are not involved. The L88.3 cell line either does not produce oxygen radicals or it produces very efficient or large amount of endogenous oxygen-radical scavengers.

Oxygen radical can be produced during both apoptotic and necrotic death. TNF can induce generation of oxygen radicals through two pathways: 1) damage to mitochondria (associated with necrosis), and 2) activation of arachidonic acid metabolism via phospholipase(s) activation. For the WEHI 164 and L929 cell lines, BHA has been

shown to inhibit TNF-induced cytotoxicity as well as TNF-induced release of arachidonic acid (Brekke et al., 1992 and Brekke et al., 1994); this suggests activation of phospholipase(s). BHA probably inhibits TNF-induced cytotoxicity through scavenging oxygen radicals generated by the phospholipase(s) lytic pathway, since oxygen radicals can be generated by arachidonic acid metabolism (Matthews et al., 1987). In L929 cells, BHA has also been shown to block TNF-induced necrotic death caused by reactive oxygen intermediates as well as blocking z-VAD-fmk-dependent sensitization to TNF cytotoxicity (Vercammet et al., 1998). Thus, partial reduction of TNF-mediated cytolysis of L929 and 10ME by BHA suggests that the generation of oxygen radicals, either by mitochondria or arachidonic acid metabolism, is most likely involved in their TNF-mediated cytolysis (Figures 22, 24, and 25). Our data (Fig. 21), as well as that of Mutch et al. (1992), showed that PLA<sub>2</sub> activation was involved in TNF-mediated cytolysis of 10ME cells. However, since neither release of arachidonic acid nor the generation of oxygen radicals through mitochondria damage caused by TNF-mediated cytolysis were directly measured in this study, we cannot pinpoint which mechanism is being partially blocked by BHA.

BHA reduced the TNF-mediated lysis of B/C-N cells sensitized with the pan-caspase inhibitor z-VAD-fmk (Figure 23). This indicates that some caspases are regulating oxygen radical production or degradation, and this accounts for the caspase inhibitor's ability to make the cells more sensitive to TNF. Khwaja and Tatton (1999) showed that in TNF-resistant cell lines, inhibition of caspase activity by z-VAD-fmk caused cells to die in a non-apoptotic mechanism. This non-apoptotic mechanism

involved increased production of ROS that preceded loss of the mitochondrial membrane potential. Thus, another possibility would be that, for B/C-N, z-VAD-fmk in combination with TNF induced oxygen radicals through mitochondrial damage.

In HL-60 cells, BHA inhibited apoptosis by reducing DNA fragmentation caused by UV irradiation or hydrogen peroxide (Verhaegen et al., 1995). Although BHA has been reported to reduce DNA fragmentation in some cell lines, we have shown that TNF did not cause significant DNA fragmentation in 10ME (Park et al., 2002) and this infers that DNA fragmentation may not be central in the death of the related cell lines, B/C-N and L88.3.

As for the L88.3 cell line, addition of BHA caused very little reduction in TNF-mediated cytotoxicity (Figures 22 and 26). As mentioned earlier, TNF either did not induce generation of oxygen radicals or TNF induced large amount of endogenous oxygen scavengers. These data also indirectly suggest there may be a defect in arachidonic acid metabolic pathway in L88.3 since TNF did induce PLA<sub>2</sub> activation in the presence or absence of CHX (Figure 21). Among the four cell lines used, L88.3 was the only cell line that showed partial reduction in TNF-mediated cytotoxicity when caspase inhibitors were added (Figure 10). Addition of BHA did not cause any further reduction in TNF-mediated cytotoxicity (Figure 26). This suggests a TNF-mediated caspase-independent lytic pathway in the L88.3 cell line does not involve generation of oxygen radicals. This is particularly interesting since L88.3 was derived from 10ME, and 10ME was derived from B/C-N. Since B/C-N is not tumorigenic and 10ME is tumorigenic in immune-deficient animals only, it appears that the carcinogen-induced transformation of B/C-N into 10ME

did not affect this TNF-induced pathway. However, when L88.3 was isolated from 10ME by its ability to form tumors in normal animals, it appears to have been selected for a TNF resistance mechanism that is unique, and expressed in neither of the parental cell lines, B/C-N and 10ME.

## CHAPTER 5

### THE EFFECT OF HEAT-SHOCK TREATMENT ON TNF-MEDIATED CYTOLYSIS AND CASPASES ACTIVITIES

Heat-shock treatment at 42 °C or above, has been used in cancer therapy over the past two decades. Mammalian cells exposed to a temperature above 42 °C are killed at a rate that is an exponential function of time. However, researchers have also shown that non-lethal heat-shock treatment of cells at 39 – 43 °C, or induction of chemical stress, can protect various tumorigenic cell lines from TNF-mediated lysis, and certain cell-mediated cytotoxic activities such as those mediated by cytotoxic T lymphocytes (CTLs) or activated monocytes (Jaattela et al., 1989; Gromkowski et al., 1989; Jaattela et al., 1990; Sugawara et al., 1990).

Induction of heat shock proteins (HSPs), especially induction of HSP 70 and HSP 27, were implicated in this resistance to TNF-mediated cytotoxicity (Kusher et al., 1990; Jaattela et al., 1992; Mehlen et al., 1995). Jaattela et al. (1998) showed that induction of HSP 70 protected cells from TNF-mediated cytotoxicity by interfering with metabolic pathways downstream of caspase-3 activity. Thus, one objective of this dissertation was to study the effects of heat-shock treatment on TNF-mediated cytolysis in B/C-N, 10ME, L88.3, and L929 cell lines.

*5.1 Heat-shock treatment at 42 °C for two hours prior to addition of TNF reduces TNF-mediated cytotoxicity of B/C-N, 10ME, and L88.3 cell lines, but not L929 cell line.*

The effect of heat-shock treatment on TNF-mediated cytotoxicity was assayed. Cells were incubated at 42 °C for two hours prior to addition of TNF then TNF-mediated cytotoxicity was measured in an 18-hour chromium-release assay. When B/C-N, 10ME, and L88.3 cells were treated at 42 °C for two hours, they looked elongated, spread out, and touched neighboring cells (data not shown) unlike non-heat shock controls. For L929 cells, treatment at 42 °C for two hours did not result in much difference in morphology between heat-shock treated and non-heat shock treated cells (data not shown). All four cell lines remained attached to the tissue-culture-treated plates following heat-shock treatment. Hence, heat-shock treatment at 42 °C for two hours did not appear to be lethal for B/C-N, 10ME, L88.3 or L929 cells.

Heat-shock treatment at 42 °C for two hours before addition of TNF in the 18-hour chromium-release assay reduced the cytotoxic effects of TNF to the cell lines B/C-N, 10ME, and L88.3 (Figure 27). Effect of heat-shock treatment was more prominent in 10ME cell line than in B/C-N and L88.3 cell lines. Although the level of reduction in TNF-mediated cytotoxicity seen in B/C-N and L88.3 cells due to heat-shock treatment was less than that seen for 10ME, heat-shock treatment consistently caused a reduction in TNF-mediated cytotoxicity in all three cell lines (Figure 27).

Heat-shock treatment of L929 cells generally did not cause a reduction in TNF-mediated cytotoxicity in the 18-hour chromium-release assays (Figure 27). Occasionally, L929 showed a reduction in TNF-mediated cytotoxicity with heat-shock treatment.



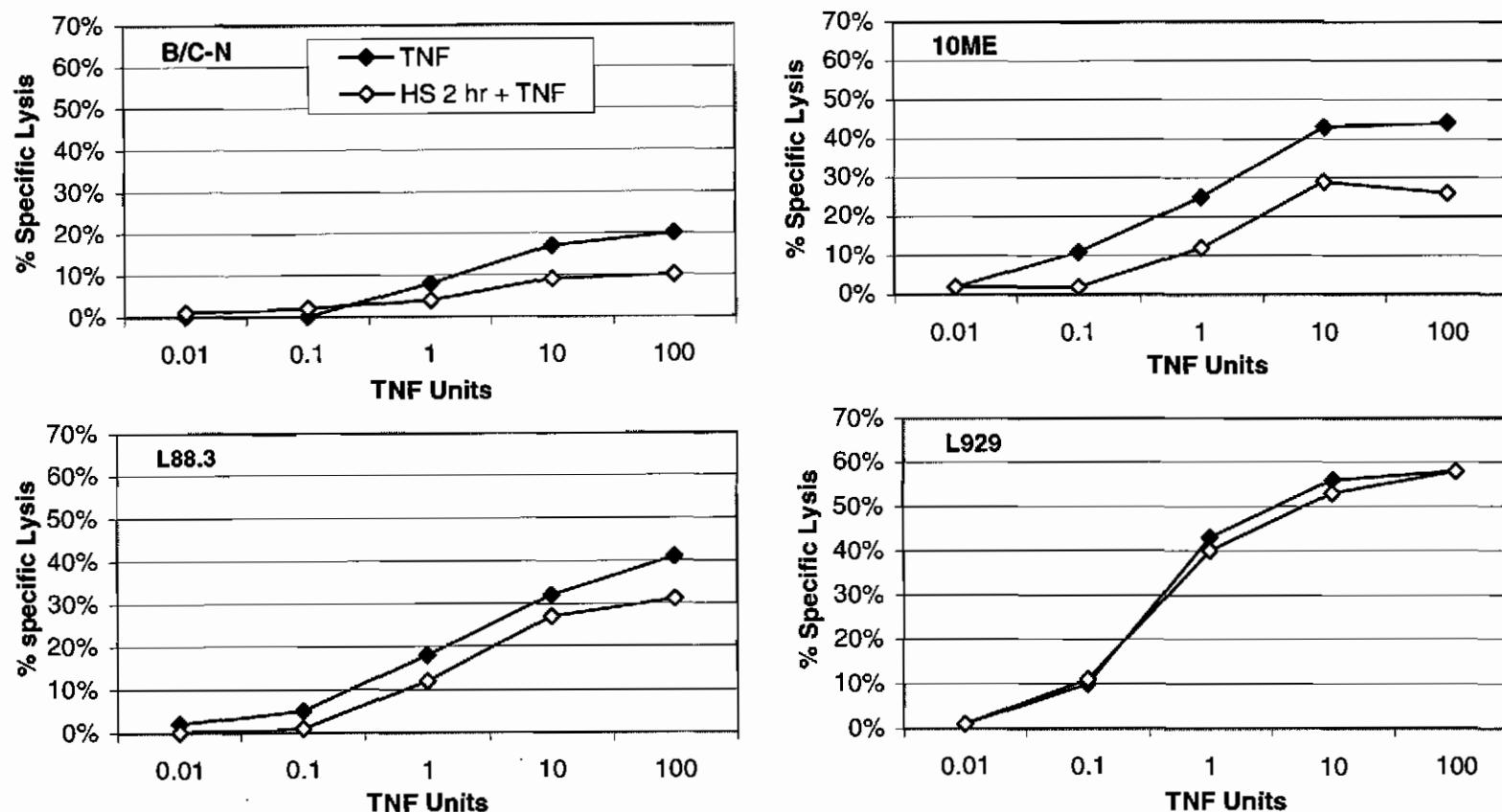


Figure 27 Prior heat-shock treatment at 42 °C for 2 hours before addition of TNF caused reduction in TNF-mediated cytolysis in B/C-N, 10ME, and L88.3 cell lines. TNF-mediated cytolysis was measured by the 18-hours chromium-release assay (see Chapter 2 material and methods).

However, most of the time, no reduction in killing, or even an increase in killing was observed (data not shown). This variability in the response of L929 cells was also seen in other assays. Similar effects on TNF-mediated killing due to two hours of heat shock were seen in 5 hours and 7 hours chromium-release assay in L929 (Figure 28). Thus, heat-shock treatment did not reduce TNF-mediated cytolysis of L929, and sometimes it caused an increase in TNF-mediated cytolysis.

### *5.2 Heat shock-induced reduction in TNF-mediated cytolysis was not due to suppression of caspases activities in B/C-N, 10ME, and L88.3.*

As shown in Figure 27, B/C-N, 10ME, and L88.3, but not in L929, showed a reduction in TNF-mediated cytolysis due to 2 hours heat-shock treatment at 42 °C prior to the addition of TNF. Thus B/C-N, 10ME and L88.3 were used to further study the TNF-mediated cytolysis-protection mechanism conferred by heat-shock treatment.

Some researchers have shown that induction of HSP 70 could protect targets from TNF-mediated apoptosis (Kim et al., 1997; Jaattela et al., 1998). Several studies have shown that heat shock proteins (HSP 27 and HSP 70) could block activation of caspase-9 and caspase-3 in drug-induced apoptosis (Garrido et al., 1999; Creagh et al., 2000). However, HSP 70 induction did not block activation of caspase-3 in TNF treated WEHI-S cell line (Jaattela et al., 1998). HSP 70 induction blocked post-caspase-3 activation event as evidenced by decrease in arachidonic acid release. Since TNF treatment resulted in increased caspase-8, -9, and -3 activities when compared to

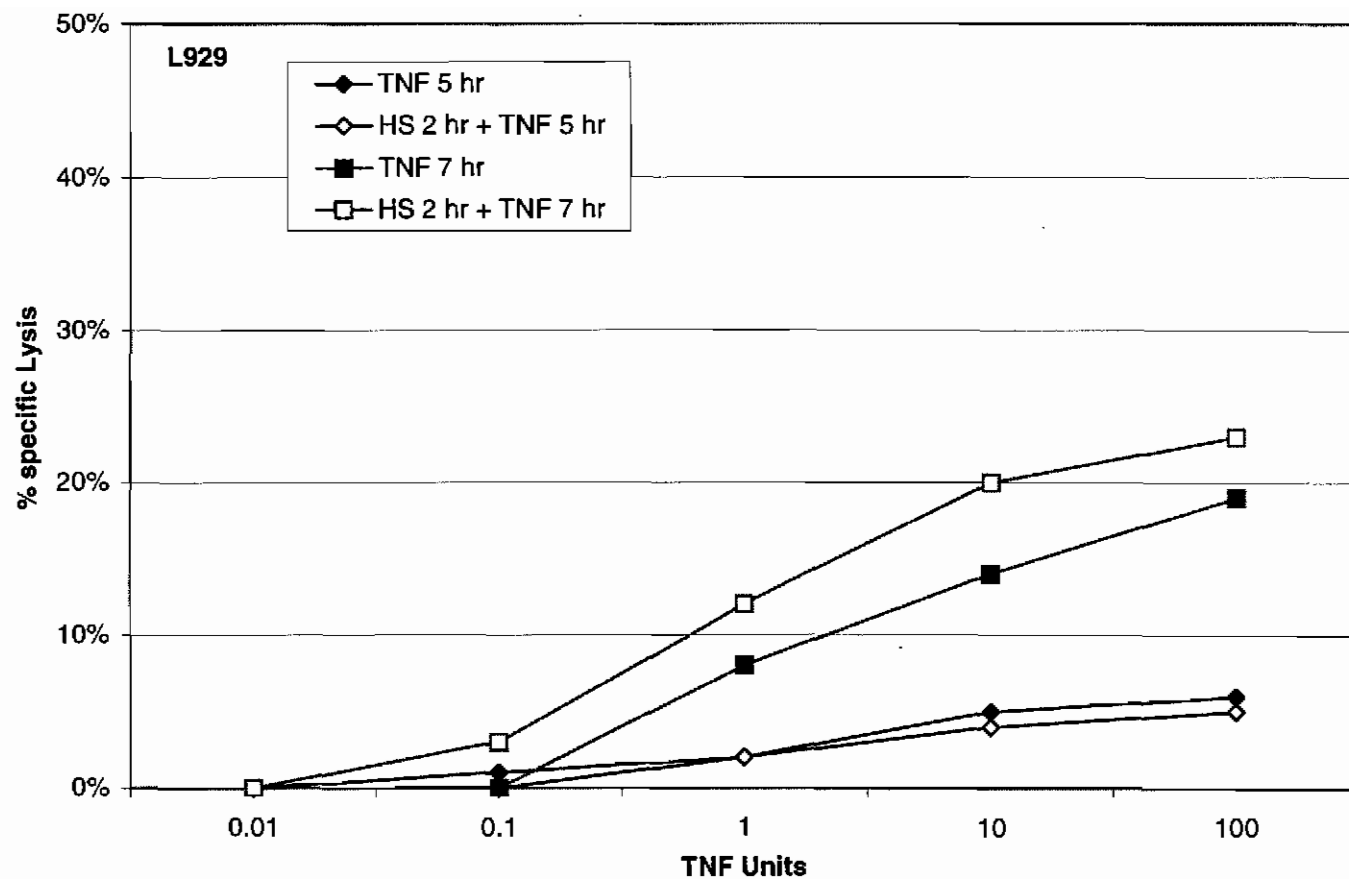


Figure 28 Heat-shock treatment of L292 at 42 °C for 2 hours prior to TNF treatment did not reduce TNF-mediated cytolysis, and could increase TNF-mediated cytolysis during the 5 hours and 7 hours chromium-release assays. TNF-mediated cytolysis was measured in the 5 hours and 7 hours chromium-release assays (see Chapter 2 materials and methods).

untreated controls in B/C-N, 10ME, and L88.3 (Table 1), we proceeded to investigate whether heat shock prior to TNF treatment would affect these caspases activities.

B/C-N, 10ME, and L88.3 cells were heat shocked at 42 °C for 2 hours prior to addition of TNF, then incubated with TNF (100 U TNF) for 5, 7 or 10 hours. Samples were then prepared for caspases activity assays as described in the Materials and Methods. Results of these caspase activity assays are shown in Figure 29. Our results with TNF showed similar findings to those of Jaattela et al. (1998) who also used TNF to induce apoptosis. For B/C-N, 10ME, and L88.3 cells, heat shock followed by 5 hours of TNF treatment (HS 2 hr + TNF 5 hr) showed increases in caspase-8, caspase-9, and caspase-3 activities when compared to the activities of these caspases after treatment with TNF alone (Figure 29). For B/C-N and 10ME cells, heat-shock followed by 7 hours of TNF treatment (HS 2 hr + TNF 7 hr) still showed higher caspase activities for all three caspases when compared to the activities of the caspases after treatment with TNF alone (Figure 29). But, for L88.3 cells which were heat-shocked and treated with TNF for 7 hours, we observed lower activities for all three caspases when compared to the activities of the caspases after treatment with TNF alone (Figure 29). At 10 hours of TNF treatment, B/C-N, 10ME, and L88.3 all showed lower activities of caspase-8, -9, and -3 in the heat shocked samples (HS 2 hr + TNF 10 hr) as compared to TNF alone (Figure 29).

Heat shock causes stress in cells. Increase in caspase-8, -9, and -3 activities in heat-shocked samples (Figure 29) may be due to heat-shock induced stress that enhances signaling from the mitochondrial pathway and is known to act through caspase-9

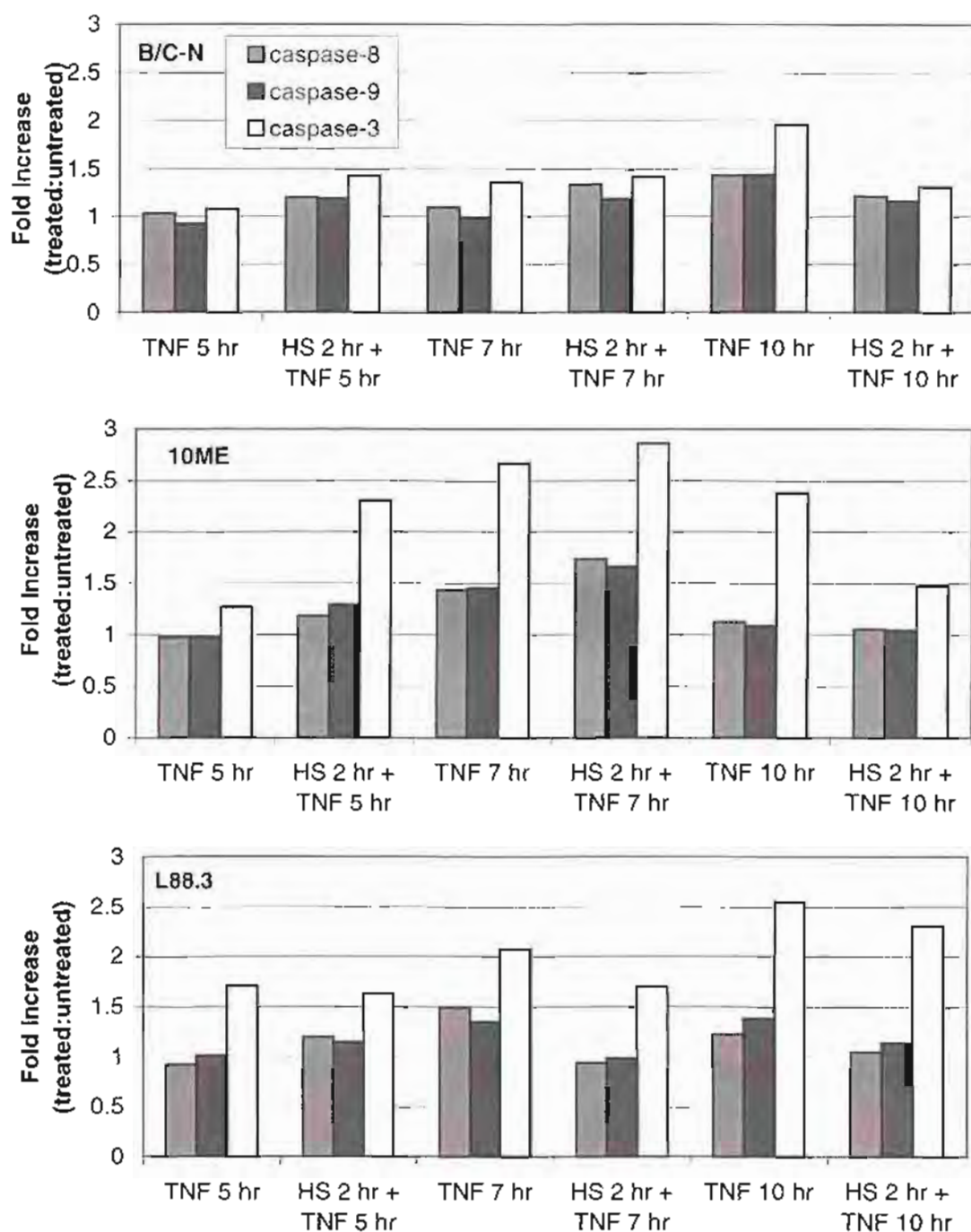


Figure 29 Prior heat-shock treatment cause increase in caspases activities at 5 hours and 7 hours of TNF treatment, but decrease in caspases activities at 10 hour of TNF treatment. Targets were either heat shocked at 42 °C for 2 hours or not heat shocked prior to addition of TNF. Caspase-activity assays were used to measure activities.

activation. It is also known that heat shock alone could cause cell death. There is a possibility that heat shock may quicken the time-course of TNF-mediated cytolysis. In order to investigate this possibility, we measured cell lysis, using the chromium-release assay, after only 5 and 7 hours of TNF treatment. Although the level of cell killing after only 5 hours of TNF treatment was low, results for these cytotoxic assays, using B/C-N, 10ME and L88.3 as targets, showed that heat shock followed by TNF treatment for either 5 or 7 hours (HS + TNF) suppressed TNF-mediated cytolysis compared to treatment with TNF alone (Figure 30). Thus, these data showed that a heat shock for 2 hours at 42 °C before addition of TNF does not block caspases-8, -9, and -3 activities. Heat shock actually caused an increase in caspases activities at the 5 hours time point. Yet, this increase in caspases activities due to prior heat-shock treatment did not translate into an increase in TNF-mediated cytolysis in heat-shocked cells. Therefore, reduction in TNF-mediated cytolysis due to heat-shock treatment is not due to a suppression of caspase activities and must be due to other as yet unknown mechanism.

### *5.3 Summary and Discussion*

Our cytotoxic assays showed that heat-shock treatment at 42 °C for 2 hours before the addition of TNF reduced TNF-mediated killing in B/C-N, 10ME, and L88.3, but not L929 (Figures 27, 28, and 30). In L929 cells, prior heat-shock treatment could increase TNF-mediated killing (Figure 28). Caspase activity assays showed that reduction in TNF-mediated cytolysis seen in chromium-release assays were not due to reductions in

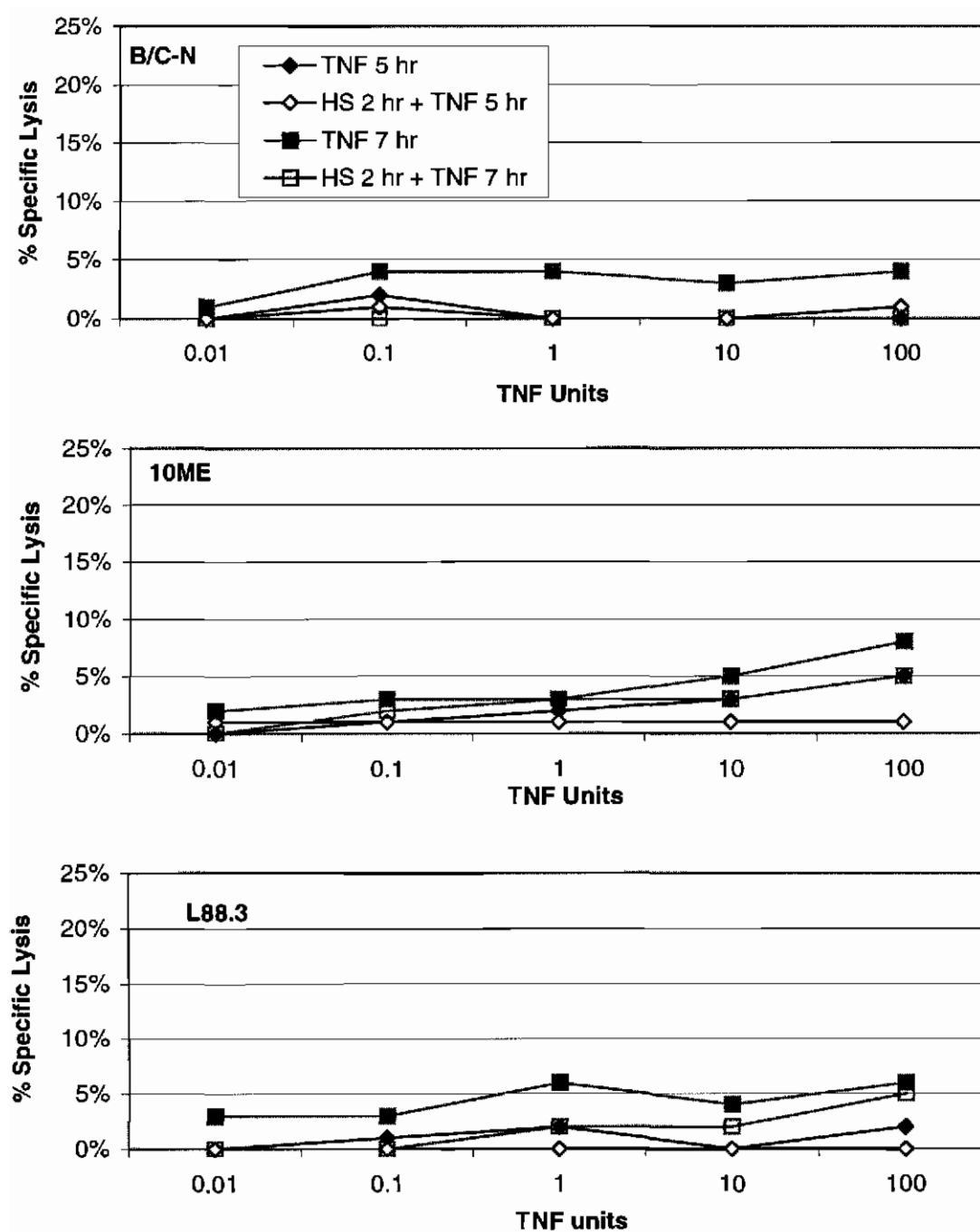


Figure 30 Prior heat-shock treatment reduced TNF-mediated cytotoxicity in B/C-N, 10ME, L88.3 at 5 hours and 7 hours time point. Targets were heat shocked at 42 °C for 2 hours prior to addition of TNF. TNF-mediated cytotoxicity was measured using the chromium-release assay (see Chapter 2 materials and methods).

caspase-8, -9, and -3 activities in B/C-N, 10ME, and L88.3 (Figure 29). Therefore, for our three cell lines, heat-shock treatment did not inhibit caspase-8, -9, and -3 activities. Maybe the reduction in TNF-mediated cytolysis was due to HSPs blocking lytic pathway after activation of caspase-3. Although induction of HSP synthesis due to heat-shock treatment was not investigated in this study, Sugawara et al. (1990) showed that 10ME became resistant to TNF-mediated cytolysis due to induction of GRP78 (HSP 70 family member in endoplasmic reticulum) by chemically induced stress. And GRP78-mediated resistance to TNF-mediated cytolysis could be reversed when GRP78 induction was suppressed by antisense *grp78* gene transfection of 10ME (Sugawara et al., 1993). Hence, it seems most likely that when 10ME was heat shocked for two hours there was induction of a HSP 70 family protein. Since 10ME was derived from B/C-N, and L88.3 was derived from 10ME, all three cell lines may induce a HSP 70 family protein when heat shocked.

The most plausible explanation for our heat-shock data would be the same reason presented by Jaattela et al. (1998); HSP 70 rescues cells from apoptosis late in the death signaling pathway beyond activation of caspase-3. It blocked release of arachidonic acid (AA) in these cells. Wissing et al. (1997) showed that in WEHI-S and MCF-7S1 cell lines, TNF-induced activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) was downstream of caspase-3 activation, and the activation of cPLA<sub>2</sub> could be blocked by Ac-DEVD-CHO, a caspase-3, -7 and -8 inhibitor, but activation is only partially blocked by a specific cPLA<sub>2</sub> enzyme activity inhibitor. Jaattela et al. (1998), using the same WEHI-S cell line used by Wissing et al., showed that cells transfected with a gene for HSP 70, such that there was



constitutive expression of high levels of HSP 70, were protected from TNF, staurosporin, and doxorubicin-induced apoptosis. However, these surviving HSP 70- expressing cells showed activation of stress-activated protein kinases, generation of free radicals, early disruption of mitochondrial transmembrane potential, release of cytochrome c from mitochondria, and activation of caspase-3-like protease. They also observed cleavage of caspase-3 substrates such as poly(ADP-ribose) polymerase (PARP), cytosolic PLA<sub>2</sub>, and protein kinase C  $\delta$  (PKC  $\delta$ ). However, HSP 70 expression reduced arachidonic acid release after cPLA<sub>2</sub> activation. Our data showed caspase-9 and caspase-3 activities in TNF and heat-shock treated B/C-N, 10ME, and L88.3. This is in concordance with their observation of release of cytochrome c from mitochondria, which leads to activation of caspase-9, and activation of caspase-3-like protease. However, other parameters were not investigated; hence, it may be premature to conclude that reduction in TNF-mediated cytolysis due to heat-shock treatment caused by blocking lysis at the very late stage of the lytic pathway, after activation of cPLA<sub>2</sub>.

As for activation of cPLA<sub>2</sub>, Mutch et al. (1992) has shown that PLA<sub>2</sub> activity is induced in TNF-mediated cytolysis of 10ME. TNF-mediated cytolysis of 10ME is reduced by addition of quinacrine, a PLA<sub>2</sub>-specific inhibitor. Our own results also showed that quinacrine reduced TNF-mediated cytolysis in 10ME (Figure 21). In TNF-resistant cell lines, B/C-N and L88.3, addition of quinacrine also showed some reduction in TNF-mediated cytolysis, although to a lesser extent than 10ME (Figure 21). Hence, if activation of PLA<sub>2</sub> is also downstream of caspase-3 activation in our cell lines B/C-N,

10ME, and L88.3, it would be plausible that heat-shock treatment may be blocking the TNF induced lytic pathway after activation of PLA<sub>2</sub>.

Ravagnan et al. (2001) showed that HSP 70 could bind to the apoptosis-inducing factor (AIF) directly and inhibit the activation of caspase-independent apoptosis. AIF is localized in the mitochondria and translocates into the nucleus when apoptosis is induced. Increased release of AIF from mitochondria was shown in HeLa cells treated with the PKC  $\delta$  inhibitor rottlerin and TNF (Basu et al., 2002). However, AIF activation has not been associated with apoptosis induced by TNF alone or TNF plus CHX. Hence, this mechanism is probably not used in our cell lines.

Thus, heat-shock induced reduction in TNF-mediated cytolysis in B/C-N, 10ME, and L88.3 was most likely due to inhibition of lytic pathway after caspase-3 activation.

## CHAPTER 6

### CONCLUSION

TNF activated caspase-8, caspase-9, and caspase-3 in the cell lines B/C-N, 10ME, L88.3, and L929 (Table 1, Figure 4 – 7). The data indicated that both TNF receptor-mediated and the mitochondrial-mediated apoptotic pathways were activated in these cell lines. The caspase inhibitors data (Figure 12) suggested that between the two initiator caspases (caspase-8 and -9), caspase-8 was activated before activation of caspase-9 in all four cell lines and it is conceivable that caspase-8 activity lead to activation of caspase-9. Activity levels of caspases correlated with TNF sensitivity of each cell line (Table 1). Additionally, increases in TNF-mediated cytolysis due to addition of the translation inhibitor CHX correlated with increase in caspase activities (Figures 4 – 7). However, caspase activities have to reach a certain threshold levels in order to cause increase in TNF-mediated cytolysis (Figures 4 – 7). Both initiator caspases and the effector caspase have to reach threshold levels of at least 1.5-fold to 2-fold increase above the baseline. These data also indicated that activation of a TNF resistance mechanism(s) affects the level of caspase activities in TNF-resistant cell lines B/C-N and L88.3.

When caspase activities were blocked by a pan-caspase inhibitor, z-VAD-fmk, or caspase-3, -7, and -8 inhibitor, z-DEVD-fmk, TNF induced a caspase-independent lytic pathway in B/C-N, 10ME, and L929 cell lines (Figures 8, 9, 11-13, 15, and 16). Both caspase inhibitors caused decrease in TNF-mediated cytolysis in L88.3 cell line (Figures 10 and 14). These data also revealed that some caspases that were strongly inhibited by z-VAD-fmk were involved in TNF-induced resistance mechanism in B/C-N, 10ME, and

L929 cell lines (Figures 8 – 9, & 11). TNF-induced resistance mechanism in B/C-N consisted of caspase-dependent and caspase-independent resistance mechanism. In TNF-sensitive 10ME and L929 cell lines, TNF-induced resistance mechanism only has a caspase-dependent component. In L88.3 cell line, caspases were not involved in TNF-induced resistance mechanism, but were involved in TNF-mediated lytic pathway only (Figure 10).

Microscopic observations showed that preincubation of 10ME and L929 with the pan-caspase inhibitor z-VAD-fmk caused some of the cells to switch from apoptotic death to necrotic death (Figures 17 – 20). Pan-caspase inhibitor z-VAD-fmk-induced necrotic death caused significant increase in TNF-mediated cytolysis seen at 5 and 7 hours of TNF treatment in 10ME and L929 cell lines (Figures 15 and 16).

When an oxygen radical scavenger, BHA, was added to 10ME and L929 undergoing TNF-mediated lysis, cytotoxicity was reduced. Thus, it appears that generation of oxygen radicals is a part of TNF-mediated lytic pathway in 10ME and L929. In similar experiments, the TNF-resistant cell lines B/C-N and L88.3 (Figure 22 – 26) showed no evidence of oxygen radical formation. These data suggested that TNF either did not induce generation of oxygen radicals in B/C-N and L88.3 cell lines, or TNF induced production of large amount of oxygen radical scavengers in these two cell lines. Increase in TNF-mediated cytolysis due to preincubation with the pan-caspase inhibitor z-VAD-fmk involved generation of oxygen radicals in B/C-N cell line (Figures 23), but not in 10ME and L929 cell lines (Figures 24 and 25). Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activation of arachidonic acid has been implicated as a late event in apoptosis. Our

analysis showed that quinacrine, an inhibitor of PLA<sub>2</sub> activation, significantly inhibits TNF-mediated lysis of B/C-N, 10ME, and L88.3. Thus, PLA<sub>2</sub> appears to be a component of TNF-mediated cytolysis of these three cell lines (Figure 21).

Heat-shock-induced protection to TNF-mediated cytolysis was observed in the cell lines B/C-N, 10ME, and L88.3, but not in L929 (Figure 27, 28, and 30). In L929 cell line, heat-shock treatment at 42 °C for 2 hours before addition of TNF could cause increase in TNF-mediated cytolysis (Figure 28 and data not shown). However, heat-shock treatment caused increase in TNF-mediated caspase activities in all four cell lines (Figure 29). Thus, heat shock induced decrease in TNF-mediated cytolysis for B/C-N, 10ME, and L88.3 was not due to decrease in caspase activities. The most plausible explanation for the seemingly conflicting results seen in B/C-N, 10ME, and L88.3 would be that heat-shock treatment inhibits very late steps in the lytic pathway, after activation of caspase-3.

Our findings about the lytic pathways and resistance pathways induced by TNF in the cell lines used are summarized in Table 2. These results suggest that 10ME, when it was derived from B/C-N, acquired a TNF-inducible lytic mechanisms involving generation of oxygen radicals. At the same time, 10ME lost a caspase-independent TNF-induced resistance mechanism. These changes in phenotype account for why 10ME is sensitive to TNF lysis whereas its parental cell line, B/C-N, is not. When L88.3 was derived from 10ME, it lost the oxygen radical dependent lytic mechanism and gained a caspase-independent resistance mechanism. These change in phenotype accounts for why L88.3 is resistant to TNF lysis whereas its parental cell line, 10ME, is not.

Table 2

Summary of lytic pathway and resistance pathway induced by TNF  
in B/C-N, 10ME, L88.3, and L929 cell lines

Characterstics	Cell lines			
	B/C-N	10ME	L88.3	L929
Highly sensitive to TNF cytolysis	No	Yes	No	Yes
Lytic mechanisms				
Caspase dependent	Yes*	Yes	Yes*	Yes
Oxygen radical dependent	No	Yes	No	Yes
Phospholipase A <sub>2</sub> dependent	Yes*	Yes	Yes	ND
Resistance mechanism				
Protein-synthesis-dependent	Yes	Yes	Yes	Yes
Caspase dependent	Yes	Yes	No	Yes
Caspase independent	Yes	No	Yes	No
Heat-shock dependent	Yes	Yes	Yes	No

ND: not done

\*: lytic mechanism that can be seen only with TNF plus CHX

## REFERENCES

- Aggarwal BB, Eessalu TE, and Hass P (1985) Characterization of receptors for human tumor necrosis factor and their regulation by gamma-interferon. *Nature* 318:665 -667
- Aggarwal BB (1992) Comparative analysis of the structure and function of TNF- $\alpha$  and TNF- $\beta$ . In *Tumor Necrosis Factor* (Aggarwal, B.B. and Vilcek, J., eds) pp. 61-78, Marcel Dekker, Inc., New York.
- Ahmad M., Virinivasula SM, Wang L, Talanian RV, Litwack G, Fernandes-Alnemri T, and Alnemri ES (1997) CRADD, a novel human apoptotic adaptor molecule for caspase-2 and FASL/tumor necrosis factor receptor-interacting protein RIP. *Cancer Res* 57:615 – 619.
- Armstrong RC, Aja T, Xiang J, Baur S, Krebs JF, Hoang K, Bai X, Korsmeyer SJ, Karanewsky DS, Fritz LC, and Tomaselli KJ (1996) Fas-induced activation of the cell death-related protease CPP32 is inhibited by Bcl-2 and by ICE family protease inhibitors. *J Biol Chem* 271:16850-16855.
- Baichwal VR, and Baeuerle PA (1997) Apoptosis: activate NF- $\kappa$ B or die? *Cur Biol* 7, R94-R96.
- Baeuerle PA, and Hendel T (1994) Function and activation of NF- $\kappa$ B in the immune system. *Annu Rev Immunol* 12:141-179
- Basu A, Johnson DE, Woolard MD (2002) Potentiation of tumor necrosis factor- $\alpha$ -induced cell death by rottlerin through a cytochrome-C-independent pathway. *Exp Cell Res* 278:209 – 214.
- Beg AA, and Baltimore D (1996) An essential role for NF- $\kappa$ B in preventing TNF- $\alpha$ -induced cell death. *Science* 274:782 - 784.
- Beidler DR, Tewari M, Friesen PD, Poirier G, and Dixit VM (1995) The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. *J Biol Chem* 270:16526 - 16528.
- Beyaert R and Fiers W (1994) Molecular mechanisms of tumor necrosis factor-induced cytotoxicity. What we do understand and what we do not. *FEBS Lett* 340:9 – 16.
- Binder C, Schultz M, Hiddemann W, and Oellerich M (1999) Caspas-activation and induction of inducible nitric oxide-synthase during TNF $\alpha$ -triggered apoptosis. *Anticancer Res* 19:1715 – 1720.

- Boldin MP, Goncharov TM, Goltsev YV, and Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 85:803 – 815.
- Brekke OL, Shalaby MR, Sundan A, Espevki T, Bjerve KS (1992) Butylated hydroxyanisole specifically inhibits tumor necrosis factor-induced cytotoxicity and growth enhancement. *Cytokine* 4:269 – 280.
- Brekke OL, Espevik T, and Bjerve KS (1994) Butylated hydroxyanisole inhibits tumor necrosis factor-induced cytotoxicity and arachidonic acid release. *Lipids* 29:91-102.
- Bump NJ, Hackett M, Hugunin M, Seshagiri S, Brady K, Chen P, Feren C, Franklin S, Ghayur T, Li P, Licari P, Mankovich J, shi L, Greenberg AH, Miller LK, and Wong WW (1995) Inhibition of ICE family protease by baculovirus antiapoptotic protein p35. *Science* 269:1885 – 1888.
- Butt AJ, Harvey NL, Parasivam G, Kumar S (1998) Dimerization and autoprocessing of the Nedd2 (caspase-2) precursor requires both the prodomain and the carboxyl-terminal regions. *J Biol Chem* 273:6763 – 6768.
- Buzzard KA, Giaccia AJ, Killender M, and Anderson RL (1998) Heat shock protein 72 modulates pathways of stress-induced apoptosis. *J Biol Chem* 273:17147 – 17153.
- Cain K, Inayat-Hussain SH, Couet C, and Cohen GM (1996) A cleavage-site-directed inhibitor of interleukin-1 $\beta$ -converting enzyme-like proteases inhibits apoptosis in primary cultures of rat hepatocytes. *Biochem J* 314:27 – 32.
- Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, and Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA* 72:3666 – 3670.
- Cheng E H-Y, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, and Hardwick JM (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* 278, 1966-1968.
- Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke I, Kischkel FC, Hellbardt S, Krammer PH, Peter ME, and Dixit VM (1996) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J of Biol Chem* 271:4961- 4965.



- Chou JJ, Matsuo H, Duan, H, Wagner G (1998) Solution structure of the RAIDD CARD and model for CARD/CARD interaction in caspase-2 and caspase-9 recruitment. *Cell* 94:171 – 180.
- Chu Z-L, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW (1997) Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF- $\kappa$ B control. *Proc Natl Acad Sci USA* 94:10057-10062.
- Creagh EM, Carmody RJ, and Cotter TG (2000) Heat shock protein 70 inhibits caspase-dependent and -independent apoptosis in Jurkat T cells. *Exp Cell Res* 257:58 – 66.
- Cryns VL, Bergeron L, Zhu H, Li H, and Yuan J (1996) Specific cleavage of  $\alpha$ -fodrin during Fas- and tumor necrosis factor-induced apoptosis is mediated by an interleukin-1 $\beta$ -converting enzyme/Ced-3 protease distinct from the Poly(ADP-ribose) polymerase protease. *J Biol Chem* 271, 31277-31282.
- Darmon Aj, Nicholson DW, and Bleackley RC (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* 377:446 – 448.
- Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prevost M-C, Leber B, Andrews D, Penninger J, and Kroemer G (2000) Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J* 14: 729 – 739.
- Dbaiibo GS, Obeid, LM, and Hannun YA (1992) Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) signal transduction through ceramide. *J Biol Chem* 268:17762 – 17766.
- Denecker G, Vercammen D, Steemans M, Vanden BT, Brouchaer G, Van Loo G, Zhivotovsky B, Fiers W, Grooten J, Declercq W, and Vandenabeele P (2001) Death receptor-induced apoptosis and necrotic cell death: differential role of caspases and mitochondria. *Cell Death Differ* 8:829 – 840.
- De Valck D, Beyaert R, Van Roy F, and Fiers W (1993) Tumor necrosis factor cytotoxicity is associated with phospholipase D activation. *Eur J Biochem* 212: 491 - 497.
- Deveraux QL, Takahashi R, Salvesen GS, and Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 388:300 – 304.

- Deveraux QL, Stennicke RN, Van Arsedale T, Zhou Q, Srinivasul SM, Alnemri ES, Salvesen GS, and Reed JC (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 17:2215 – 2223.
- Devin A, Lin Y, Yamaoka S, Li Z, Karin M, and Liu Z-G (2001) The  $\alpha$  and  $\beta$  subunits of I $\kappa$ B kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. *Mol Cell Biol* 21:3986 – 3994.
- Dolle, R. E., Hoyer, D., Prasad, C. V. C., Schmidt, S. J., Helaszek, C. T., Miller, R. E., and Ator, M. A. (1994) P1 aspartate-based peptide  $\alpha$ -((2,6-Dichlorobenzoyl)oxy)methyl ketones as potent time-dependent inhibitors of interleukin-1 $\beta$ -converting enzyme. *J Med Chem* 37:563-564.
- Duan H, Chinnaiyan A M, Hudson PL, Wing JP, He W-W, and Dixit VM (1996) ICE-LAP3, a novel mammalian homologue of the *Caenorhabditis elegans* cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. *J Biol Chem* 271: 1621 - 1625.
- Duan H and Dixit VM (1997) RAIDD is a new 'death' adaptor molecule. *Nature* 385: 86 – 89.
- Dudley E, Hornung F, Zheng L, Scherer D, Ballard D, Lenardo M (1999) NF- $\kappa$ B regulates Fas-APO-1/CD95- and TCR- mediated apoptosis of T lymphocytes. *Eur J Immunol* 29, 878-886.
- Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong, WW, Kamen R, Weichselbaum R, and Kufe D (1995) Proteolytic activation of protein kinase C  $\delta$  by an ICE-like protease in apoptotic cells. *EMBO J* 14:6148-6156.
- Enari M, Hug H, Hayakawa M, Ito F, Nishimura Y, and Nagata S (1996) Different apoptotic pathways mediated by Fas and the tumor-necrosis-factor receptor: cytosolic phospholipase A<sub>2</sub> is not involved in Fas-mediated apoptosis. *Eur J Biochem* 236:533 – 538.
- Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, and Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391:43 – 50.
- Erl W, Hansson GK, de Martin R, Draude G, Weber KSC, Weber C (1999) Nuclear Factor- $\kappa$ B regulates induction of apoptosis and inhibitor of apoptosis protein-1 expression in vascular smooth muscle cells. *Circ Res* 84:668-677.

- Fady C, Gardner A, Jacogy F, Briskin K, Tu Y, Schmid I, and Lichtenstein A (1995) Atypical apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. *J Interferon Cytokine Res* 15:71 – 80.
- Faraco PR, legerwood, EC, Vandenabeele P, Prins, JB, and Bradley JR (1999) Tumor necrosis factor induces distinct patterns of caspase activation in WEHI-164 cells associated with apoptosis or necrosis depending on cell cycle state. *Biochem Biophys Res Comm* 261:385-392.
- Garcia-Calvo M, Ptersen, EP, Leiting B, Ruel R, Nichoson EW, and Thornberry NA (1998) Inhibition of human caspases by peptide-based and maacromolecular inhibitors. *J Biol Chem* 273: 32608 – 32613.
- Garrido C, Bruey, JM, Fromentin A, Hammann A, Arrigo AP and Solary E (1999) Hsp27 inhibits cytochromec-dependent activation of procaspase-9. *FASEB J* 13: 2061 – 2070.
- Garrido C, Gurbuxani S, Ravagnan, L, and Kroemer G (2001) Heat shock proteins: Endogenous modulators of apoptotic cell death. *Biochem Biophys Res Comm* 286:433 – 442.
- Gearing AJH., Beckett P, Chrostodoulou M, Churcill M, Clements J, Davidson AH, Drummond AJ, Galloway WA, Gilbert R, Gordon JL, et al. 1994. Processing of tumor necrosis factor- $\alpha$  precursor by metalloproteinases. *Nature* 370:555 – 557.
- Ghayur T, Hugunin M, Talanian RV, Ratnofsky S, Quinlan C, Emoto Y, Pandey P, Datta R, Huang Y, Kharbanda S, Allen H, Kamen R, Wong,W, and Kufe D. (1996) Proteolytic activation of protein kinase C  $\delta$  by an ICE/CED 3-like protease induces characteristics of apoptosis. *J Exp. Med.* 184:2399-2404.
- Goossens V, Grooten J, De Vos K, Fiers W (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc. Natl. Acad. Sci. USA* 92:8115 – 8119.
- Gorman AM, Heavey B, Creagh E, Cotter TG, and Samali,A (1999) Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. *FEBS Lett* 445: 98 – 102.
- Greenblatt MS and Elias L (1992) The Type B receptor for tumor necrosis factor-a mediates DNA fragmentation in HL-60 and U937 cells and differentiation in HL-60 cells. *Blood* 80:1339 – 1346.

- Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, Georgopoulos S, Lessauer W, Kollias G, Pfizenmaier K, and Scheurich, P (1995) The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83:793 – 802.
- Gromkowski SH, Yagi J, and Janeway Jr. CA (1989) Elevated temperature regulates tumor necrosis factor-mediated immune killing. *Eur J Immunol* 18:1709 – 1714.
- Gross A, Yin X-M, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, and Korsmeyer SJ (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while Bcl-X<sub>L</sub> prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem* 274:1156 – 1163.
- Hahn, G.M. and G.C. Li. (1990) Thermotolerance, thermoresistance, and thermosensitization. In *Stress Proteins in biology and Medicine* (R.I. Morimoto, A. Tissieres, and C. Georgopoulos, eds) pp. 117 – 129, Cold Spring Harbor Laboratory Press, New York.
- Han Z, Hendrickson EA, Bremner TA, and Wyche JH (1997) A sequential two-step mechanism for the production of the mature p17:p12 form of caspase-3 *in vitro*. *J Biol Chem* 272:13432 – 13426.
- Hayakawa M, Ishida, N, Takeuchi K, Shibamoto S, Hori T, Oku N, Ito F, and Tsujimoto M (1993) Arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor. *J Biol Chem* 268:11290 – 11295.
- He KL, and Ting AT (2002) A20 inhibits tumor necrosis factor (TNF) alpha-induced apoptosis by disrupting recruitment of TRADD and RIP to the TNF receptor 1 complex in Jurkat T cells. *Mol Cell Biol* 22: 6034 – 6045.
- Higuchi M and Aggarwal BB (1994) Differential roles of two types of the TNF receptor in TNF-induced cytotoxicity, DNA fragmentation, and differentiation. *J Immunol* 152:4017 – 4025.
- Higuchi M, Aggarwal BB, and Yeh ETH (1997) Activation of CPP-32 like protease in tumor necrosis factor-induced apoptosis is dependent on mitochondrial function. *J Clin Invest* 99:1751 – 1758.
- Hofmann K, Bucher P and Tschopp J (1997) The CARD domain: A new apoptotic signaling motif. *Trends Biochem Sci* 22:155 – 156.
- Hollenback PW, Zilli DL and Laster SM (1992) Inhibitors of transcription and translation act synergistically with tumor necrosis factor to cause the activation of phospholipase A2. *J Biol Chem* 267:39-42.

- Hotamisligil GS, Shargill NS, and Spiegelman BM (1993) Adipose expression of tumor necrosis factor- $\alpha$ : direct role in obesity-linked insulin resistance. *Science*. 259:87 – 91.
- Hsu H, Xiong J, Goeddel DV (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 81:495 – 504.
- Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV (1996a) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity* 4:387 – 396.
- Hsu H, Shu H-B, Pan M-G, and Goeddel DV (1996b) TRADD-TRAF2 and TRADD- FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84:299 – 308.
- Irmeler M, Thome, M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer J-L, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschoep J (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* 388:190 – 195.
- Jaattela M, Saksela K, and Saksela E (1989) Heat shock protects WEHI-164 target Cells from the cytotoxicity by tumor necrosis factor  $\alpha$  and  $\beta$ . *Eur J Immunol* 19: 1413 – 1417.
- Jaattela M (1990) Effects of heat shock on cytotoxicity mediated by NK cells, LAK cells, activated monocytes and TNFs  $\alpha$  and  $\beta$ . *Scand J of Immunol* 31:175 – 182.
- Jaattela M, Wissing D, Bauer, PA, and Li GC (1992) Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J* 11:3507 – 3512.
- Jaattela M (1993) Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A<sub>2</sub>. *J Immunol* 151:4286 – 4294.
- Jaattela M, Wissing D, Kokholm K, Kallunki T, and Egeblad M (1998) Hsp70 exerts its anti-apoptotic function down stream of caspase-3 like proteases. *EMBO J* 17: 6124 – 6134.
- Jaeschke, H., Fisher, M.A., Lawson, J.A., Simmons, C.A., Farhood, A., and Jones, D.A. (1998) Activation of caspase 3 (CPP32)-like proteases is essential for TNF- $\alpha$  induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. *J Immunol* 160:3480 – 3486.

- Jiang Y, Woronicz JD, Liu W, Goeddel DV (1999) Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. *Science* 283:543 – 546.
- Kamradt, M, Chen F, and Cryns VL (2001) The small heat shock protein  $\alpha$ B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem* 276:16059 – 16063.
- Khwaja A, and Tatton L (1999) Resistance to the cytotoxic effects of tumor necrosis  $\alpha$  can be overcome by inhibition of a FADD/caspase-dependent signaling pathway. *J Biol Chem*. 274: 36817 – 36823.
- Kim Y-M, de Vera MIE, Watkins SC, and Billiar T (1997) Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor- $\alpha$ -induced apoptosis by inducing heat shock protein 70 expression. *J Biol Chem* 272:1402.
- Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner, MW, Kothe KR, Kwiatkowski DJ, and Williams LT (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278:294- 298.
- Kuida, K, Haydar TF, Kuan C-Y, Gu Y, Taya C, Karasuyama H, Su MS-S, Rakic P, and Flavell RA (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* 94:325 – 337.
- Kusher DI, Ware, CF, and Gooding LR. (1990) Induction of the heat shock response protects cells from lysis by tumor necrosis factor. *J Immunol* 145: 2925 – 2931.
- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371:346 – 347.
- Lassus P, Opitz-Araya X, Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297:1352 – 1354.
- Laster SM, Wood JG, and Gooding LR (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J Immunol* 141:2629 – 2634.
- Lemaire C, Amdreau K, Souvannavong V, Adam A (1998) Inhibition of caspase activity induces a switch from apoptosis to necrosis. *FEBS Lett* 425:266 – 270.

- Li C-Y, Lee J-S, Ko Y-G, Kim J-I, and Seo J-S (2000) Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *J Biol Chem* 275:256665-25671.
- Li GC and Hahn GM (1987) Influence of temperature on the development and decay of thermotolerance and heat shock proteins. *Radiation Res* 112:517 – 524.
- Li H, Bergeron L, Cryns V, Pasternack MS, Zhu H, Shi L, Greenberg A, and Yuan J. (1997) Activation of caspase-2 in apoptosis. *J Biol Chem* 272:21010 – 21017.
- Li H, Zhu H, Xu CJ, and Yuan J (1998) Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94:491 – 501.
- Li M and Beg, AA (2000) Induction of necrotic-like cell death by tumor necrosis factor alpha and caspase inhibitors: novel mechanism for killing virus-infected cells. *J Virol* 74:7470 – 7477.
- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, and Wang X (1997) Cytochrome c and dATP-Dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479 - 489.
- Li WX, Chen CH, Ling CC, and Li GC (1996) Apoptosis in heat-induced cell killing: the protective role of hsp-70 and the sensitization effect of the c-myc gene. *Radiation Res* 145:324 – 330.
- Lin Y, Devin A, Rodriguez Y, and Liu Z-G (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Gene & Dev* 13:2514-2526.
- Liu X, Zou H, Cain M, sun X-N, Alnemri ES, and Cohen GM (1997) DFF a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 89:175 – 184.
- Liu Z-G, Hsu H, Goeddel DV, Karin M (1996) Dissection of TNF receptor I effector functions: JNK activation is not linked to apoptosis while NF- $\kappa$ B activation prevents cell death. *Cell* 87:565- 576.
- Matthews N, Neale ML, Jackson SK, and Stark JM (1987) Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology* 62:153 – 155.

- Mattson MP, Goodman Y, Luo H, Fu W, Furukawa K (1997) Activation of NF- $\kappa$ B protects hippocampal neurons against oxidative stress-induced apoptosis: evidence for induction of manganese superoxide dismutase and suppression of peroxynitrite production and protein tyrosine nitration. *J Neurosci Res* 49:681-697.
- Mayo MW, Wang C-Y, Cogswell PC, Rogers-Graham KS, Lowe SW, Der CJ, and Badlwin AS Jr. (1997) Requirement of NF- $\kappa$ B activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* 278:1812-1815.
- Mehlen, P, Preville, X, Chareyron, P, Briolay, J, Klemenz, R, and Arrigo A-P (1995) Constitutive expression of human hsp27, Drosophila hsp27, or human  $\alpha$ B-crystallin confers resistance to TNF- and oxidative stress-induced cytotoxicity in stably transfected murine L929 fibroblasts. *J Immunol* 154:363 – 374.
- Mirkin II, mernenko OA, Satpaev DK, karelin, AA, and Blishchenko E Y (1996) Cytolytic processes induced by TNF in L929 and K562 differ in DNA fragmentation mechanisms. *Immunology* 52: 105 – 108.
- Mizushima N, Koike R, Kohsaka H, Kushi Y, Handa S, Yagita H, Miyasaka N (1996) Ceramide induces apoptosis via CPP32 activation. *FEBS Lett* 395:267 – 271.
- Mohler K M., Torance D S, Smith, C A, Goodwin R G, Stremmler K E., Fung V P, Madani H, and Widmer MB (1993) Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. *J of Immunol* 151:1548 – 1561.
- Monney L, Olivier R, Otter I, Jansen, B, Poireir GG, and Borner C (1998) Role of an acidic compartment in tumor-necrosis-factor- $\alpha$ -induced production of ceramide, activation of caspase-3 and apoptosis. *Eur J Biochem* 251:295 – 303.
- Morimoto RI, Tissieres A, and Georgopoulos C (1994) Progress and perspectives on the Biology of Heat Shock Protein and Molecular chaperones. In *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto RI, Tissieres A, Georgopoulos C, eds) pp. 1 –30, Cold Spring Harbor Laboratory Press, New York.
- Morishima N, Nakanish K, Takenouchi H, Shibata T, Yasuhiko Y (2002) An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 277:34287 – 34294.



- Mutch DG, Powell CB, Kao M-S, and Collins JL (1992) Resistance to cytolysis by tumor necrosis factor  $\alpha$  in malignant gynecological cell lines is associated with the expression of protein(s) that prevent the activation of phospholipase A<sub>2</sub> by tumor necrosis factor  $\alpha$ . *Cancer Res* 52:866 – 872.
- Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, and Dixit VM (1998) An induced proximity model for caspase-8 activation. *J Biol Chem* 273:2926-2930.
- Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J (2000) Caspase-12 mediated endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403:98 – 103.
- Natoli G, Costanzo A, Moretti F, Fulco M, Balsano C, and Levrero M (1997) Tumor necrosis factor (TNF) receptor 1 signaling downstream of TNF receptor associated factor 2. *J Biol Chem* 272:26079 – 26082.
- Neale ML, Fiers RA, Matthews N (1988) Involvement of phospholipase A<sub>2</sub> activation in tumour cell killing by tumour necrosis factor. *Immunology* 64:81 – 85.
- Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik UA, Munday NA, Raju SM, Smulson ME, Yamin T-T, Yu VL and Miller DK (1995) Identification and inhibition of ICE/DEC-3 protease necessary for mammalian apoptosis. *Nature* 376:37 – 43.
- Nicholson DW, and Thornberry NA (1997) Caspases: killer proteases. *TIBS* 22:299 – 306.
- Orth K, O'Rourke K, Salvesen GS, and Dixit VM (1996) Molecular order of apoptotic mammalian CED3/ICE-like proteases. *J Biol Chem* 271:20977 – 20980.
- Pandey P, Saleh A, Nakazawa A, Kumar S, Srinivasula S M., Kumar V, Weichselbaum R, Nalin C, Alnemri ES, Kufe D, and Kharbanda S (2000) Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *EMBO J* 19:4310 – 4322.
- Park DJ, Chang WC, Patek PQ. (2002) Protein kinase C modulates tumor necrosis factor-alpha internucleosomal DNA fragmentation. *J Interferon Cytokine Res*, submitted.
- Paroni G, Henderson C, Schneider C, and Brancolini C (2001) Caspase-2-induced apoptosis is dependent on caspase-9, but its processing during UV- or tumor necrosis factor-dependent cell death requires caspase-3. *J Biol Chem* 276:21907 – 21915.

- Patek, PQ, Collins, JL, and Cohn M (1978) Transformed cell lines susceptible or resistant to *in vivo* surveillance against tumorigenesis. *Nature* 276:510 – 511.
- Patek PQ, Lin Y, and Collins JL (1987) Natural cytotoxic cells and tumor necrosis factor activate similar lytic mechanisms. *J Immunol* 138:1641 – 1646.
- Powell CB, Herzog TJ, Scott JH, and Collins JL (1995) Evidence for a protein synthesis-dependent and –independent TNF $\alpha$  cytolytic mechanism. *Gynecol Oncol* 58:327 – 335.
- Pronk, G. J., Ramer, K., Amiri, P., Williams, L. T. (1996) Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. *Science* 271, 808-810.
- Rao RV, Hermel E, Castro-Obregon S, del Rio G, Ellerby LM, Ellerby HM, Bredesen DE (2001) Coupling endoplasmic reticulum stress to the cell death program mechanism of caspase activation. *J Biol Chem* 276:33869 – 33874.
- Ravagnan L, Gurbuxani S, Susin, S A, Maise C, Daugas E, Zamzami N, Mak T, Jaattela M, Penninger JM, Garrido C, and Kroemer G (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* 3:839 – 843.
- Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, and Rothe M (1997) Identification and characterization of an I $\kappa$ B kinase. *Cell* 90:373 – 383.
- Reid T, Ramesha CS, and Ringold GM (1991) Resistance to killing by tumor necrosis factor in an adipocyte cell line caused by a defect in arachidonic acid biosynthesis. *J Biol Chem* 266:16580 – 16586.
- Reuther JY, and Baldwin AS. Jr. (1999) Apoptosis promotes a caspase-induced amino-terminal truncation of I $\kappa$ B $\alpha$  that function as a stable inhibitor of NF- $\kappa$ B. *J Biol Chem* 274:20664 – 20670.
- Ritossa, F (1962) A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia* 18:571-573.
- Roy S, Bayly CI, Gareau Y, Houtzaer VM, Kargman S, Keen SLC, Rowland K, Seiden IM, Thornberry NA, and Nicholson DW (2001) Maintenance of caspase-3 proenzyme dormancy by an intrinsic “safety catch” regulatory tripeptide. *Proc. Natl. Acad. Sci. USA* 98:6132 – 6137.

- Rudel, T., and Bokoch, G. M (1997) Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science* 276:1571-1574.
- Ruemmele FM, Dionne S, Levy E, and Seidman E G (1999) TNF $\alpha$ -induced IEC-6 cell apoptosis required activation of ICE caspases whereas complete inhibition of the caspase cascade leads to necrotic cell death. *Biochem Biophys Res Comm* 260: 159 – 166.
- Sakahira H, Anari M, and Nagata, S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 319:96 – 99.
- Salvesen GS, and Dixit VM (1997) Caspases: intracellular signaling by proteolysis. *Cell* 91:443 – 446.
- Samali A, and Cotter TG (1996) Heat shock proteins increase resistance to apoptosis. *Exp Cell Res* 223:163 – 170.
- Samali A, Cai , Zhivotovsky B, Jone DP, and Orrenius S (1999) Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. *EMBO J* 18: 2024 – 2048.
- Sanford 1948
- Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers W (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radial generation. *J Biol Chem* 267:5317 – 5323.
- Schutze S, Machleidt T, and Kronke M (1994) The role of diacylglycerol and ceramide in tumor necrosis factor and interleukin-1 signal transduction. *J Leukocyte Biol* 56:533 – 541.
- Sen. R, and Baltimore D (1986a) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46:705 – 716.
- Sen R, and Baltimore D (1986b) Inducibility of  $\kappa$  immunoglobulin enhancer-binding protein NF- $\kappa$ B by a posttranslational mechanism. *Cell* 47:921 – 928.
- Shi C-S, and Kehrl JH (1997) Activation of stress-activated protein kinase/c-Jun N-terminal kinase, but not NF- $\kappa$ B, by the tumor necrosis factor (TNF) receptor 1 through a TNF receptor-associated factor 2-and germinal center kinase related-dependent pathway. *J Biol Chem* 272:32102 – 32107.

- Shoji Y, Uedono Y, Ishikura H, Takeyama N, and Tanaka T (1995) DNA damage induced by tumour necrosis factor- $\alpha$  in L929 cells is mediated by mitochondrial oxygen radical formation. *Immunology* 84:543 – 548.
- Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang H-G, Reed JC, Nicholson DW, Alnemri ES, Green DR, and Maring SJ (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspase-2, -3, -6, -7, -8, and -10 in caspase 9-dependent manner. *J Cell Biol* 144:281-292.
- Srinivasula SM, Fernandes-Alnemri T, Zehngrilli J, Robertson N, Armstrong RC, Wang L, Trapani JA, Tomaselli KJ, Litwack G, and Alnemri ES (1996) The Ced-3 /Interleukin 1 $\beta$  converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2 $\alpha$  are substrates for the apoptotic mediator CPP32. *J Biol Chem* 271:27099-27106.
- Stennicke HR, Jurgensmeier JM, Shin H, Deveraux Q, Wold BB, Yang X, Zhou Q, Ellerby HM, Ellerby LM, Bredesen D, Green DR, Reed JC, Forielich CJ, and Salvesen GS (1998) Pro-caspase-3 is a major physiologic target of caspase-8. *J Biol Chem* 273:27084 – 27090.
- Stennicke HR, Deveraux QL, Humke EW, Reed JC, Dixit VM, and Salvesen GS (1999) Caspase-9 can be activated without proteolytic processing. *J Biol Chem* 274: 8359 – 8362.
- Sugarman BJ, Aggarwal BB, Hass PH, Figari IS, Palladino MA Jr., and Shepard H.M (1985) Recombinant human tumor necrosis factor- $\alpha$ : effects on proliferation of normal and transformed cells in vitro. *Science* 230:943 – 945.
- Sugawara S, Nowicki M, Xie S, Song H-J, and Dennert G (1990) Effects of stress on lysis of tumor targets by cytotoxic T cells and tumor necrosis factor. *J Immunol.* 145:1991 – 1998.
- Sugawara S, Takeda K, Lee A, and Dennert G (1993) Suppression of Stress Protein GRP78 induction in tumor B/C10ME eliminates resistance to cell mediated cytotoxicity. *Cancer Res* 53:6001 – 6005.
- Sun X-M, MacFarlane M, Zhuang J, Wolf BB, Green DR, and Cohen GM (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J Biol Chem* 274:5053 – 5060.

- Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow B E, Brothers GM, Mangion J, Jacotot E, Constantini P, Loeffler M, Larochette N, Goodlett, D R, Aebersold R, Siderovski DP, Penninger JM, and Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397:441 – 446.
- Takahashi A, Alnemri ES, Lazebnik YA, Fernandes-Alnemri T, Litwack G, Moir RD, Goldman RD, Poirier GG, Kaufmann SH, and Earnshaw WC (1996) Cleavage of lamin A by Mch2 $\alpha$  but not CPP32: multiple interleukin 1 $\beta$ -converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc Natl Acad Sci USA* 93:8395-8400.
- Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino Jr., MA, and Goeddel DV (1991) The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci USA* 88:9292 - 9296.
- Tartaglia LA, and Goeddel DV (1992) Two TNF receptors. *Immunol Today* 13: 151 – 153.
- Tartaglia LA, Roth M, Hu Y-F, and Goeddel DV (1993) Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. *Cell* 73:213 – 216.
- Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvensen GS, and Dixit VM (1995) Yama/CPP32b, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81:801 - 809.
- Thevenod F, Friedmann JM, Katsen AD, and Hauser IA (2000) Up-regulation of multidrug resistance P-glycoprotein via nuclear factor-kB activation protects kidney proximal tubule cells from cadmium- and reactive oxygen species-induced apoptosis. *J Biol Chem* 275:1887 - 1896.
- Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Elliston KO, Ayala JM, Casano FJ, Chin J, Ding GJ-F, Egger LA., Gaffney EP, Limjuco G, Palyha OC, Faju SM, Rolando AM., Sallye JP, Yamin T-T, Lee TD, Shively JE, MacCross M, Mumford RA, Schmidt JA., and Tocci MJ (1992) A novel heterodimeric cysteine protease is required for interleukin-1 $\beta$  processing monocytes. *Nature* 356:768-774.
- Thornberry NA (1997). The caspase family of cysteine proteases. *British Med Bull* 53: 478-490.
- Thornberry NA, and Lazebnik Y (1998) Caspases: enemies within. *Science*. 281: 1312 – 1316.

- Ting AT, Pimentel-Munoz FX, and Seed B (1996) RIP mediates tumor necrosis factor receptor-1 activation of NF- $\kappa$ B but not Fas/APO-1-initiated apoptosis. *EMBO J* 15:6189 – 6196.
- Totpal K, Agarwal S, and Aggarwal BB (1992) Phosphatase inhibitors modulate the growth-regulatory effects of human tumor necrosis factor on tumor and normal cells. *Cancer Res* 52:2557 – 2562.
- Tracey KJ, and Cerami A (1993) Tumor necrosis factor, other cytokines and disease. *Annu Rev Cell Biol* 9:317 – 343.
- Tsujimoto M, Yip YK, and Vilcek J (1985) Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. *Proc Natl Acad Sci USA* 82: 626 – 630.
- Uren AG, Pakusch M, Hawkins CJ, Puls KL, and Vaux DL (1996) Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proc Natl Acad Sci USA* 93: 4947 – 4948.
- van Antwerp DJ, Martin SJ, Kafri TAL, Green DR., and Verma IM (1996) Suppression of TNF- $\alpha$ -induced apoptosis by NF- $\kappa$ B. *Science*. 274:787 – 789.
- van Antwerp DJ, Martin SJ, Verma IM, Green DR (1998) Inhibition of TNF-induced apoptosis by NF- $\kappa$ B. *Trends in Cell Biol* 8:107-111.
- VanArsdale TL, and Ware CF (1994) TNF receptor signal transduction: ligand-dependent stimulation of a serine protein kinase activity associated with (CD120a) TNFR<sub>60</sub>. *J Immunol* 153:3043 – 3050.
- Van Lint J, Agostinis P, Vandevoorde V, Haegeman G, Fiers W, Merlevede W, and Vandenheede JR (1992) Tumor necrosis factor stimulates multiple serine/threonine protein kinases in Swiss 3T3 and L929 cells. *J Biol Chem* 267: 25916 – 25921.
- Van Zee KJ, Kohno T, Fischer E, Rock, C S, Moldawer L L, and Lowry S F (1992) Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor a in vitro and in vivo. *Proc Natl Acad Sci USA* 89:4845 – 4849.
- Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grootein J, Fiers W, and Vandenabeele P (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med* 187:1477 – 1485.

- Verhaegen S, McGowan AJ, Brophy AR, Fernandes RS, and Cotter TG (1995) Inhibition of apoptosis by antioxidants in the human HL-60 Leukemia cell line. *Biochem Pharm* 50:1021 – 1029.
- Voelkel-Johnson C, Thorne TE, Iuster SM (1996) Susceptibility of TNF in the presence of inhibitors of transcription or translation is dependent on the activity of cytosolic phospholipase A2 in human melanoma tumor cells. *J Immunol* 156: 201 – 207.
- Wang C-Y, Mayo MW, and Baldwin Jr., AS (1996) TNF-and Cancer Therapy-induced apoptosis: potentiation by inhibition of NF- $\kappa$ B. *Science* 274:784 – 787.
- Wang C-Y, Mayo MW, Korneluk RG, Goeddel DV, and Baldwin Jr., AS (1998) NF- $\kappa$ B antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science*. 281:1680 – 1683.
- Wen L-P, Farni JA, Troie S, Guan J-L, Orth K, and Rosen GD (1997) Cleavage of focal adhesion kinase by caspases during apoptosis. *J Biol Chem* 272:26056-26061.
- Wiegmann K, Schutze S, Kampen E, Himmler A, Machleidt T, and Kronke M (1992) Human 55-kDa receptor for tumor necrosis factor coupled to signal transduction cascades. *J Biol Chem* 267:17997-18001.
- Wiegmann K, Schutze S, Machleidt T, Witte D, and Kronke M (1994) Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell* 78:1005 – 1015.
- Wissing D, Mouritzen H, Egeblad M, Poirier GG, and Jaattela M (1997) Involvement of caspase-dependent activation of cytosolic phospholipase A2 in tumor necrosis factor-induced apoptosis. *Proc Natl Acad Sci USA* 94:5073 – 5077.
- Woronica JD, Gao X, Shu H-B, Cao Z, Rothe M, and Goeddel DV (1997) I $\kappa$ B kinase- $\beta$ : NF- $\kappa$ B activation and complex formation with I $\kappa$ B kinase- $\alpha$  and NIK. *Science* 278:886 – 880.
- Xanthoudakis S, Roy S, Rasper D, Hennessey T, Aubin Y, Cassady R, Tawa P, Ruel R, Rosen A, and Nicholson DW (1999) Hsp60 accelerated the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. *EMBO J* 18:2049 – 2056.
- Xue D, and Horvitz H R (1995) Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature* 377: 248 – 251.

- Yamashita K, Takahashi A, Kobayashi S, Hirata H, Mesner Jr., PW, Kaufmann SH., Yonehara S, Yamamoto K, Uchiyama T, and Sasada M (1999) Caspases mediate tumor necrosis factor- $\alpha$ -induced neutrophil apoptosis and downregulation of reactive oxygen production. *Blood* 93:674 – 685.
- Yanaga F, Abe M, Koga T, and Hirata M (1992) Signal transduction by tumor necrosis factor  $\alpha$  is mediated through a guanine nucleotide-binding protein in osteoblast-like cell line, MC353-E1. *J Biol Chem.* 267:5114 – 5121.
- Yang X, Chang HY, and Baltimore D (1998a) Essential role of DED-4 oligomerization in CED-3 activation and apoptosis. *Science* 281:1355 – 1357.
- Yang X, Chang HY, and Baltimore D (1998b) Autoproteolytic activation of procaspases by oligomerization. *Mol Cell* 1:319 – 325.
- Zhou P, Chou J, Clea RS, Uan, J, and Wagner G (1999) Solution structure of Apaf-1 CARD and its interaction with caspase-9 CARD: A structural basis for specific adaptor/caspase interaction. *Proc. Natl Acad. Sci USA* 96:11265 – 11270.
- Zhou Q, Snipas S, Orth K, Muzio M, Dixit VM, and Salvesen GS (1997) Target protease specificity of the Viral Serpin CrmA. *J Biol Chem* 272:7797 – 7800.
- Zou H, Henzel WJ, Liu X, Lutschg A, and Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90:405 – 413.