

**Therapeutic Interventions Against Symptoms or Disease caused by the Ribotoxic
Stressors: Shiga Toxin & Doxorubicin**

By

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TABLE OF CONTENTS

Abstract	iii
CHAPTER 1: Thesis Introduction	
MAP kinases and their roles in homeostatis and disease	1
Regulation of protein synthesis and ribotoxic stressors	2
Definition/discovery of ribotoxic stressors	3
Figure 1 Secondary structure of the region of the 28S rRNA targeted by Anisomycin and by the ribotoxin enzymes ricin A chain, shiga toxin, and α -sarcin.	7
The role of ZAK in mediating the ribotoxic stress response	9
Figure 2 Ribotoxic stressor signaling cascade.	10
Ribotoxic stressor-mediated apoptosis	11
Figure 3. Extrinsic and intrinsic apoptosis	13
Biological significance of ribotoxic stress	14
Shiga toxin	14
Ultraviolet radiation	15
Palytoxin	15
Doxorubicin	16
Potential inhibitors of the ribotoxic stressors doxorubicin & stx	17
(1) Stx2 antibodies in animal models	17
(2) MAPK inhibitors in vitro	17
(3) Multi-kinase inhibitors with high binding affinity for ZAK	18
Summary	18
CHAPTER 2: Mouse Model of Hemolytic-Uremic Syndrome Caused by Endotoxin-Free Shiga Toxin (Stx2) and Protection from Lethal Outcome by Anti-Stx2 Antibody	
Abstract	21
Introduction	22
Materials and Methods	25
Results	31
Figure 1	37
Figure 2	39
Figure 3	40
Figure 4	41
Figure 5	43
Figure 6	44
Figure 7	46
Discussion	47

CHAPTER 3: ZAK is required for doxorubicin, a novel ribotoxic stressor, to induce SAPK activation and apoptosis in HaCaT cells.

Abstract	53
Introduction	54
Materials and Methods	57
Results	60
Figure 1	68
Figure 2	69
Figure 3	71
Figure 4	73
Figure 5	75
Discussion	77

CHAPTER 4: Doxorubicin and daunorubicin activate the NALP3 inflammasome in primary mouse bone marrow derived macrophages.

Abstract	84
Introduction	85
Materials and Methods	86
Results	89
Figure 1	93
Figure 2	94
Figure 3	95
Figure 4	96
Figure 5	97
Discussion	98

CHAPTER 5: Summary and Conclusions

A mouse model of HUS	100
Therapeutic intervention against two ribotoxic stressors	102
Stx	102
Doxorubicin	103

REFERENCES 107

ABSTRACT

Ribotoxic stressors can be defined as chemical, biological, or physical agents that: (i) inhibit translation by means of interactions with, and/or damage to, the ribosome and (ii) in doing so, provoke productive cellular responses that result in the active expression of gene products (e.g. cytokines or growth factors) that modulate the organismal response to the stressor. The responses best characterized to date involve potentiation of the JNK and p38 families of SAPKs. SAPKs are activated by upstream SAP kinase kinases (SAPKKs), which, in turn, are subject to phosphorylation and activation by SAPKKKs. ZAK is a pivotal MAP3K mediating ribotoxic stress response. For millennia, humans have been exposed to ribotoxic stressors through accidental ingestion or inhalation, or, in recent times, as occasional agents of bioterrorism. Among the known ribotoxic stressors, of greatest importance to human health are probably the Shiga toxins. Hemolytic-uremic syndrome (HUS) results from an infection by Shiga toxin (Stx)-producing *Escherichia coli* (STEC), most commonly serotype O157:H7, and is the most common cause of acute renal failure in children. In this thesis, I demonstrate that doxorubicin, an anthracycline drug that is one of the most effective and widely used anticancer agents for the treatment of both hematologic and solid tumors, is a novel ribotoxic stressor that inhibits protein synthesis and requires actively translating ribosomes to activate SAPKs. Numerous ribotoxic stressors play significant roles in the development of various human diseases. However, if a person is infected with a ribotoxic stressor, such as Stx, or has side effects from treatment with a ribotoxic stressor, such as doxorubicin, then care is primarily supportive without any actual directed therapies. There are experiments demonstrating that some potential inhibitors of ribotoxic stressors, such as monoclonal antibodies

against Stx2 or MAPK inhibitors may be beneficial in preventing the severe inflammation and apoptosis after exposure to Stx or doxorubicin. In this thesis, I demonstrate that a monoclonal antibody against Stx2 can prevent HUS-associated lethality. In addition, I provide evidence that sorafenib and nilotinib, kinase inhibitors that have a high affinity for ZAK, prevent doxorubicin-induced proinflammatory and apoptotic responses in a normal keratinocyte cell line, but not in a cancerous cell line. Another potential therapeutic approach for blocking doxorubicin-mediated inflammation would be to interfere with IL-1 β signaling by blocking its processing, release, or binding to IL-1 receptors. In this thesis, I demonstrate that doxorubicin activates the NALP3 inflammasome in murine BMDM leading to processing and secretion of mature IL-1 β and an area for potential therapeutic intervention. In conclusion, a better understanding of which antibiotics, cancer chemotherapeutics, and toxins are ribotoxic stressors, as well as how ribotoxic stressors inhibit protein translation, signal to MAPKs, and cause apoptosis could lead to novel therapeutic interventions to treat ribotoxic stressor-mediated human disease.

CHAPTER 1

Thesis Introduction

MAP kinases and their roles in homeostasis and disease. The mitogen-activated protein kinases (MAPKs) are involved in fundamental biological processes, such as cell proliferation, differentiation, migration, and death, as well as in various aspects of embryonic development, morphogenesis, inflammation, wound healing, and cellular responses to stress [1-11]. The mammalian MAPK superfamily encompasses the extracellular signal-regulated kinases (ERK1 and ERK2), the p38 MAPKs (p38 α , β , γ , and δ), the big MAP kinase (BMK1/ERK5), and the cJun NH₂-terminal kinases (JNK1, JNK2, and JNK3) [1-7]. The JNK family of kinases is of particular importance for cellular responses to stress [8-11]. P38 MAPKs occupy a central role in mediating inflammatory processes [12-15], for instance in response to bacterial lipopolysaccharide [16]. The JNK and p38 families of MAPKs are also referred to as “stress-activated protein kinases” (SAPKs). SAPKs are, in turn, activated by upstream MAP kinase kinases (MKKs or MAP2Ks) called SAPKKs, which, in turn, are subject to phosphorylation and activation by MKKKs/MAP3Ks/SAPKKKs [12, 16-18]. The main SAPKKs upstream of JNK are MKK4 and MKK7, whereas the activation of p38 MAPK is effected largely by MKK3 and MKK6 (Figure 2) [8-10, 19, 20]. Upon phosphorylation by SAPKKs, JNK and p38 MAPK translocate into the nucleus where they phosphorylate target proteins, including other kinases (e.g. MAPKAP2) or

transcription factors (c-Fos, c-Jun, and ATFs 1-4) [21, 22]. Transcription factors are important trans-activators of a number of genes that encode inflammatory cytokines such as IL-1 and TNF- α [22].

In contrast to JNK and p38 MAPK, the extracellular signal-regulated kinases (p44 ERK1 and p42 ERK2, hereafter referred to as ERKs) have been mainly associated with mediating growth signals through receptor tyrosine kinases such as epidermal growth factor receptor (EGFR). It is generally accepted that mitogen-induced sustained activation of ERKs mediates cell cycle progression through G1 and S-phase entry by regulating the expression of cyclin D1 [23-28].

Regulation of Protein Synthesis and Ribotoxic Stressors. All living organisms and cells depend critically on the process of protein synthesis (hereafter also referred to, interchangeably, as protein translation). Translation is as fundamental to, and characteristic of, life on Earth as the other two processes of macromolecular biosynthesis, i.e. genome replication and genome transcription. Protein synthesis is the final and irreversible step in the transformation of nucleic acid-encoded genetic information into function.

As a fundamental biological process, translation is subject to bioregulation. Metabolic state and nutrient availability are major physiological regulators of protein synthesis [29-31]. Among the pathological regulators of translation, a very important one is viral infection. Several cellular mechanisms have evolved to detect the presence of viral genomes or their products (e.g. double-stranded RNA) in infected eukaryotic cells and to trigger reactive suppression of global protein synthesis [32-34]. These mechanisms attempt to prevent or diminish the expression of viral genomes and production of new

virus particles, often at the price of the eventual demise of the infected cell [32-34].

Important as they are, however, the physiological and viral modes of regulation of translation are beyond the scope of this thesis. Instead, my research is concerned, in general, with the biological significance of the regulation of protein synthesis by *cellular stress* and, in particular, with the cellular mechanisms of response to an expanding group of inhibitors of protein synthesis termed ribotoxic stressors.

In a broader sense, **ribotoxic stressors** [35-39] can be defined as such: *chemical, biological, or physical agents that:*

- (i) *inhibit translation by means of interactions with, and/or damage to, the ribosome (i.e. they act as ‘ribosomal toxins’ or ‘ribotoxins’);*
- (ii) *in doing so, provoke productive (i.e. biosynthetic) cellular responses that result in the active expression of gene products (e.g. cytokines or growth factors) that modulate the organismal response to the stressor. The responses best characterized to date involve potentiation of the JNK and p38 families of SAPKs;*

At first sight, these two characteristic features of ribotoxic stressors appear seemingly contradictory. How can an agent inhibit protein synthesis and, at the same time, increase gene expression? Wouldn't the increase in gene expression at the level of mRNA abundance be cancelled out by the inhibition of protein synthesis, and thus, the inability of this mRNA to be translated? Prior to the work presented here, the most compelling answer to this apparent contradiction was the theoretical argument of ‘magnitude of response’. If global protein synthesis is decreased by 90% in response to ribotoxic stress, and, yet, the transcriptional expression of the mRNA encoding a particular gene product

with ‘alarmone’ functions (e.g. a chemokine) is increased a 100-fold (which is at the lower end of the range of mRNA increases observed in animal studies using ricin [40]), one could speculate that the protein product of this gene might be expressed at ~10 times the basal level (i.e. 100-fold increase in mRNA translated with 10% efficiency). As shown in Chapter 4 of my thesis, I have identified an additional mechanism to resolve the seeming contradiction, namely, the ability of ribotoxic stressors to dramatically increase the production of mature interleukin 1 β (IL-1 β) at the level of proteolytic maturation of preexisting pro-IL-1 β protein.

An important distinction with mechanistic implications made by Iordanov et al. in their original definition of ribotoxic stress [35] is that not every translational inhibitor is also a ribotoxic stressor. Using a line of normal rat embryonic fibroblasts (Rat-1), Iordanov et al. tested the ability of eight bacterial or fungal metabolites with known activities as translational inhibitors (anisomycin, puromycin, pactamycin, cycloheximide, emetine, T-2 toxin, gougerotin, and blasticidin S) to cause the activation of JNK1 SAPK. All inhibitors were carefully titrated to ensure equal degree of inhibition of protein synthesis [35]. Somewhat surprisingly, these researchers observed that only anisomycin, gougerotin, and blasticidin S appeared to be potent activators of JNK activity, whereas the other inhibitors proved inefficient in activating JNK. A common feature of anisomycin, gougerotin, and blasticidin S (not shared with the other 5 compounds) is their ability to bind specifically to the peptidyl-transferase center located in the 3’-end of the 28S rRNA in the 60S ribosomal subunit ([35] and references therein; Figure 1). Iordanov et al. hypothesized, therefore, that the 3’-end of the 28S rRNA might be involved in the generation of a signal transduction cascade from the ribosome to JNK.

To directly test this hypothesis, they took advantage of the fact that two ribotoxic enzymes, ricin A chain and α -sarcin, catalyze highly specific RNA damage within a conserved loop, the α -sarcin/ricin (S/R) loop, of the 3'-end of 28S rRNA, located near the peptidyl-transferase center (Figure 1). Ricin A chain is an RNA N-glycosidase that depurinates a single adenosine at position A4324 of the rat 28S rRNA ([35] and references therein). α -Sarcin is an RNA endonuclease from *Aspergillus giganteus* that cleaves selectively the phosphodiester bond on the 3'-side of position G4325, adjacent to the A4324 that is depurinated by the ricin A chain ([35] and references therein).

Iordanov et al. asked whether ricin A chain and α -sarcin could activate JNK and found that both toxins are potent activators of JNK. In contrast, two equally potent proteinaceous inhibitors of translation, *Pseudomonas* exotoxin A and diphtheria toxin, that act by mechanisms not involving interactions with the 28S rRNA, were unable to cause the activation of JNK.

Based on these results, Iordanov et al. concluded that the activation of SAPK by ribotoxic stress is not directly caused by the inhibition of protein synthesis *per se*. Rather, they argued, the interaction of the ribotoxic stressor with specific sites in the ribosome, located at or near the 3'-end of the 28S rRNA, is the likely apical event triggering the productive biosynthetic response to ribotoxic stress. This conclusion was further strengthened by the experimental finding that pre-blocking actively translating ribosomes by exposure to a translational inhibitor that did not, by itself, trigger the ribotoxic stress response (such as the fast-acting inhibitor emetine), completely and specifically abolished the activation of JNK by all known ribotoxic stressors [35-39]. In contrast, emetine had no effect on the activation of JNK by other activators and stressors, such as pro-inflammatory cytokines,

oxidants, the DNA-damaging agent diepoxybutane, or hyperosmotic shock [35-39]. This requirement for actively-translating ribosomes at the moment of cellular encounter with ribotoxic stress became an important test to discover novel ribotoxic stressors and it was employed here to identify the ribotoxic nature of the cancer therapeutic doxorubicin (Chapter 3; [41]).

anisomycin-resistant mutants of *Tetrahymena* 28S rRNA and *Halobacterium* 23S rRNA [42,Sweeney, 1991 #529] are denoted by open squares. Open circles represent conserved nucleotides protected by anisomycin from chemical modifications *in vitro* [43]. From Iordanov, et al [35].

The role of ZAK in mediating the ribotoxic stress response. The signal transduction cascade(s) initiated by ribotoxic stress and leading to the activation of JNK and p38 MAPK is still poorly understood. MAP kinase kinases (MAPKKs), such as MKK3, MKK4, MKK6, and MKK7 are phosphorylated, in various cell types, by exposure to ribotoxic stressors [35-39], indicating that (an) upstream MAP3K(s) must be activated as well. For almost a decade since the original discovery of the ribotoxic stress response, the identity of such MAP3K(s) eluded researchers, until Wang et al. [44] reported that siRNA-mediated knock-down of the MAP3K ‘Mixed-Lineage Kinase 7’ (MLK7), also known as ‘leucine Zipper- and sterile Alpha motif-containing Kinase’ (ZAK), suppressed potently and specifically the activation of JNK and p38 MAPK by anisomycin, while exerting no effect on the activation of the same kinases by pro-inflammatory cytokines. ZAK has two different isoforms, ZAK- α and ZAK- β . ZAK- α has an apparent molecular weight of 91 kDa. The smaller species of ZAK, ZAK- β (51 kDa), is generated by alternative splicing and has a distinct C-terminus. The two isoforms share identical protein kinase domains, including the ATP binding site, and separate functions for the two have not been defined [45]. Jandhyala et al. reported subsequently that ZAK mediates the induction of pro-inflammatory cytokine expression by ricin A chain and Shiga toxin, an enzymatically-identical ribotoxin typically produced by *E. coli* of serotype O157:H7, and the etiological cause of pediatric hemolytic uremic syndrome (described below; for reviews, see refs. [46-49]). Thus, ZAK is likely a pivotal MAP3K mediating ribotoxic stress response in general (Figure 2). In my thesis, I demonstrate that ZAK is an essential regulator of the activation of JNK and p38 MAPK by doxorubicin, a widely used chemotherapeutic and a novel ribotoxic stressor (Chapter 3; [41]).

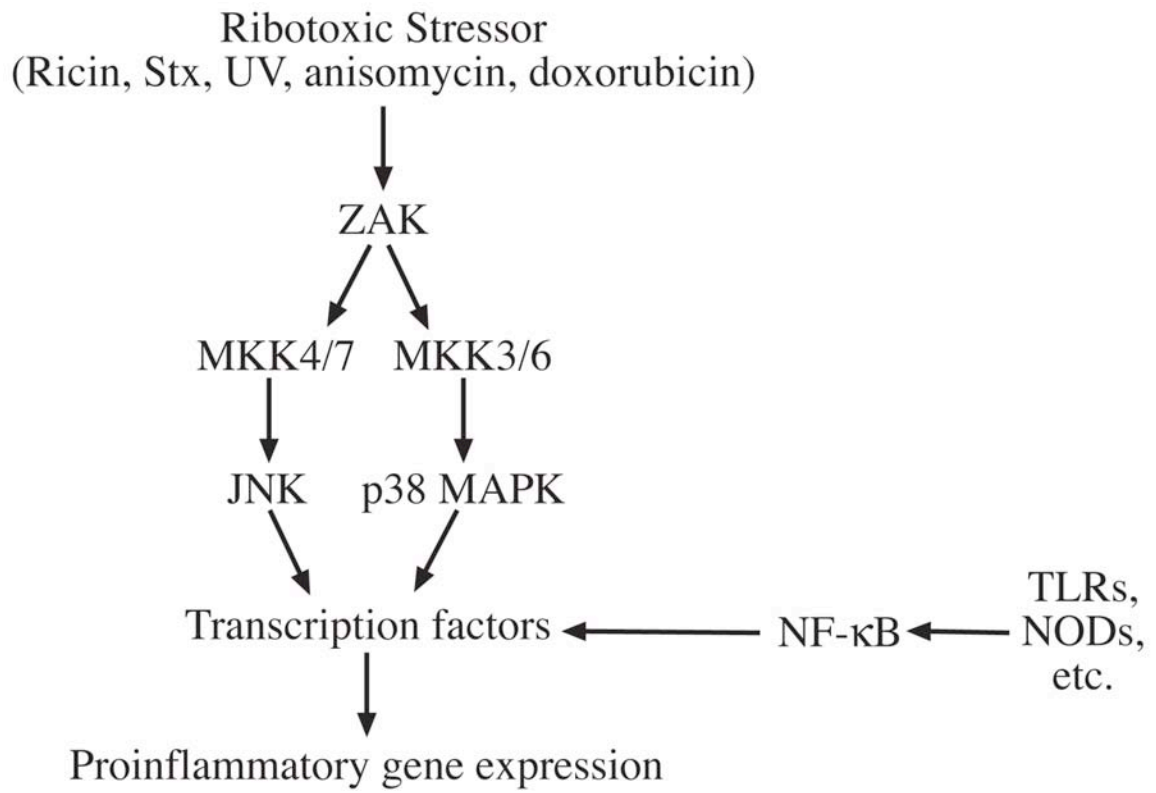


Figure 2 *Ribotoxic stressor signaling cascade.* Schematic diagram of the known ribotoxic stressor signaling through ZAK (a SAPKKK), MKK4/7 and MKK 3/6 (SAPKKs), and JNK and p38 MAPK (SAPKs) leading to proinflammatory gene expression. Adapted from [44].

Ribotoxic stressor-mediated apoptosis. In response to chemical toxicity, cells in target tissues undergo numerous changes including alterations in the expression of genes associated with proliferation and differentiation, and/or apoptosis. These processes are controlled by cues received from circulating growth factors and cytokines. The induction of cytokines is known to occur via SAPK signaling cascades [50]. Ribotoxic stressors, in addition to activation of SAPKs, are also known to cause apoptosis, which can be initiated by both intrinsic and extrinsic signaling pathways (Figure 3; reviewed in refs [51-54]). In the extrinsic pathway, extracellular death ligands (FasL, TRAIL, or TNF- α) bind to death receptors (reviewed in ref [55]). The cytoplasmic portions of the death receptors contain death domains that interact with death domains of adaptor proteins such as FADD or TRADD. These adaptor proteins contain death effector domains (DEDs) that recruit additional proteins like procaspase 8 [56-58]. The death receptors, adaptor proteins, and procaspase 8 are all part of a complex known as DISC. This allows for spontaneous autocatalytic cleavage and activation of procaspase 8 due to proximity [57]. The intrinsic apoptotic pathway begins when various stimuli (such as cytokine deprivation or DNA damage) converge and through downstream signaling allow the mitochondria to release cytochrome c (reviewed in refs. [59-61]) Once released from the mitochondria into the cytosol, cytochrome c becomes a positive regulator of the apoptosome [62], a complex that contains procaspase 9 [63]. Like procaspase 8, the proximity of procaspase 9 triggers its own autocatalytic processing and activation [63]. Caspase 8 and 9 predominantly activate downstream caspases referred to as effector caspases, such as caspases 3, 6, and 7 [64]. All procaspases undergo activation via two

consecutive proteolytic events [64, 65]. Effector caspases dismantle the apoptotic cell by systematically cleaving substrates, thus interfering with virtually every cellular function.

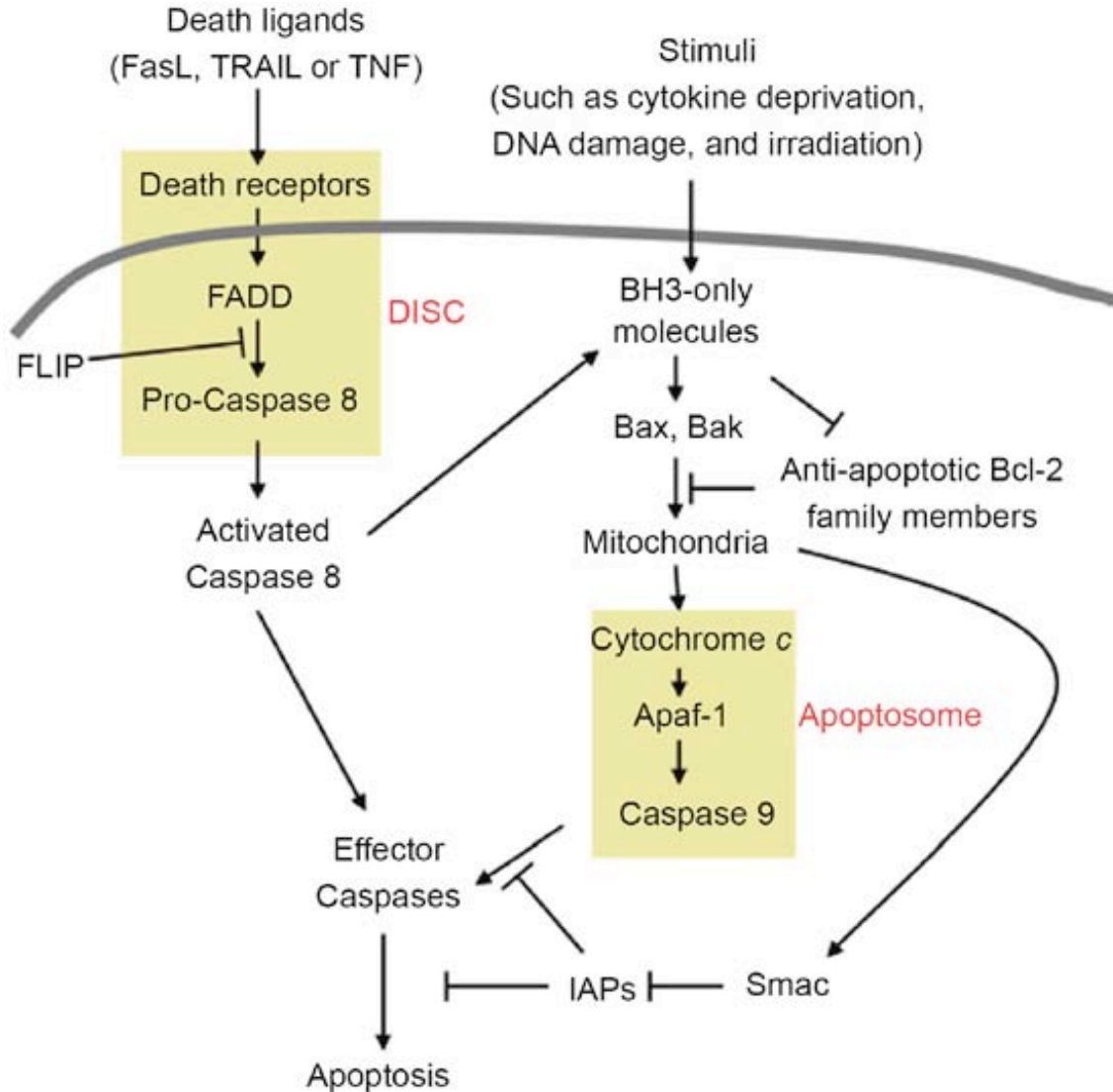


Figure 3. *Extrinsic and intrinsic apoptosis.* Schematic diagram of apoptosis pathways.

The left side is the extrinsic pathway while the right is intrinsic. The two pathways converge at the activation of effector caspases. DISC and apoptosome are highlighted in yellow. From [66].

Biological significance of ribotoxic stress. Ribotoxins are frontline participants of the ‘biological warfare’ between taxa in nature. A far from complete list of documented ribotoxic stressors includes bacterial and fungal small molecule metabolites (e.g. anisomycin, aminohexose pyrimidine nucleoside antibiotics, or trichothecene mycotoxins), as well as various bacterial (e.g. Shiga toxins, α -sarcin) and plant-derived ribosome-inactivating proteins (e.g. ricin, abrin, saporin, gelonin). For millennia, humans have been exposed to them through accidental ingestion or inhalation, or, in recent times, as occasional agents of bioterrorism. Among the known ribotoxic stressors, of greatest importance to human health are probably the Shiga toxins.

Hemolytic-uremic syndrome (HUS) results from an infection by **Shiga toxin** (Stx)-producing *Escherichia coli* (STEC), most commonly serotype O157:H7, and is the most common cause of acute renal failure in children (for reviews, see refs. [46-49]). STEC expresses either Stx1 or Stx2, which are usually encoded by bacteriophages. Each Shiga holotoxin consists of one A and five B subunits. The B subunit binds to cells via glycosphingolipid receptors such as globotriaosyl ceramide (Gb3), while the A subunit contains N-glycosidase activity [67, 68]. Following endocytosis and retrograde transfer through the Golgi apparatus, the A subunit enters the cytosol. There, it depurinates a single adenine (A4256 in mice) in a conserved region of the 28S ribosomal-RNA, thereby inhibiting protein synthesis [69-72] and simultaneously activating the SAPKs, JNK and p38 [73-75]. Stxs and ricin, a related ribotoxin, induce the release of proinflammatory cytokines and activate the transcription of genes that encode them [47, 76, 77].

Activation of SAPKs by Stx and ricin has been tied to their proinflammatory effects [73].

One unexpected ribotoxic stressor of significant relevance to human health appeared to be not a chemical molecule, but a physical agent, namely **ultraviolet radiation** (specifically, UV-B and UV-C, but not UV-A, [37-39]). In addition to being a mutagen and a complete carcinogen, the UV-B and UV-C components of the solar spectrum are potent inducers of gene expression in the skin, a phenomenon known as the 'UV response' [78-80]. Iordanov et al. demonstrated that the UV response displayed, surprisingly, characteristics of a ribotoxic stress response, inasmuch as: (i) the activation of JNK and gene expression in response to UV required the presence of active ribosomes at the moment of irradiation; (ii) UV irradiation inhibited protein synthesis; and (iii) irradiation of cells with UV causes specific damage to the 3'-end of the 28 S rRNA [37]. In contrast, the activation of SAPKs by hyperosmolarity, by the DNA-cross-linking agent diepoxybutane, by growth factors and cytokines, or by oxidative stress did not depend on the presence of active ribosomes [37-39]. These results identified UV as a potent ribotoxic stressor and supported the notion that some of the cellular signaling cascades in response to UV might be generated in the ribosome, possibly triggered by damage to rRNA.

Palytoxin is a non-peptide water-soluble marine toxin that is a potent tumor promoter in the mouse skin carcinogenesis model. Palytoxin has been found to activate JNK and p38 MAPK [36]. In search of possible mechanisms for both the cytotoxicity and the activation of stress kinases by palytoxin, Iordanov et al. found that palytoxin is a potent inhibitor of cellular protein synthesis, making it a potential ribotoxic stressor [36]. Indeed, palytoxin acted as a ribotoxic stressor, inasmuch as it required actively translating ribosomes at the time of exposure to activate SAPKs. However, the inhibition of

translation by palytoxin did not result from its direct binding to the translational apparatus. Palytoxin augments ion fluxes by binding to the Na^+/K^+ -ATPase in the plasma membrane of cells. Using specifically designed tissue culture media, in which a major portion of the Na^+ was replaced by either K^+ or by choline⁺, Iordanov et al. demonstrated that palytoxin-induced efflux of cellular K^+ mimics ribotoxic stress by provoking both translational inhibition and activation of protein kinases associated with cellular defense against stress [36].

Doxorubicin is an anthracycline drug that is one of the most effective and widely used anticancer agents for the treatment of both hematologic and solid tumors [81]. The SAPKs, p38 MAPK and JNK, are frequently activated by a number of cancer chemotherapeutics [82]. When phosphorylated, the SAPKs initiate a cascade that leads to the production of proinflammatory cytokines. Doxorubicin is known to induce the activation of SAPKs in a number of normal cell types. Patients receiving doxorubicin frequently present with acute side effects such as fatigue, nausea/vomiting, pain, sleep disturbances, cachexia, and depression [82] that may be due to the increased inflammatory condition. In Chapter 3, I demonstrate that doxorubicin is a novel ribotoxic stressor that inhibits protein synthesis and requires actively translating ribosomes to activate SAPKs [41].

Numerous ribotoxic stressors play significant roles in the development of various human diseases. However, none of the resulting diseases have treatment options other than supportive care. It would be beneficial to develop therapeutics to intervene in ribotoxic stressor-mediated disease.

Potential inhibitors of the ribotoxic stressors doxorubicin & Stx: (1) Stx2 antibodies in animal models. Some experiments demonstrate that Stx2 specific antibodies can rescue Stx2-mediated lethality in mice. An Stx2 A subunit-specific human monoclonal antibody (HuMAb) 6C3 protected 100% of mice against Stx2 challenge at 50 µg/mouse dose [83]. A DNA vaccine encoding the enterohemorrhagic E. coli Stx2 A2 and B subunits conferred protective immunity to Stx challenge in a murine model [84]. However, it would be more beneficial to have an Stx2 antibody that could be administered after infection with Stx-producing E. coli occurred. A human antibody against Stx2 administered to piglets after the onset of diarrhea due to E. coli O157:H7 prevented fatal systemic complications [85]. Stx2-neutralizing antibodies, administered passively or generated by immunization with an Stx2 toxoid, reduced the E. coli O157:H7 burden in infected mice and, as anticipated, also protected them from weight loss and death [86]. (2) MAPK inhibitors in vitro.

Doxorubicin: Inhibitors of p38 MAPK and JNK have been employed to determine whether activation of SAPKs contributes either to the efficacy or to the undesirable side effects of doxorubicin. Inhibitors of p38 MAPK have been effective in blocking apoptosis of cardiomyocytes following treatment by doxorubicin or daunorubicin, a related anthracycline [87, 88]. Inhibitors of p38 MAPK reduce the proinflammatory actions of doxorubicin in macrophages but do not reduce the anti-proliferative actions of doxorubicin in a cancer cell line [89]. **Stx:** Inhibitors of p38 MAPK and JNK have been employed to demonstrate that SAPKs contribute to Stx-mediated cell death, transport of Stx, and Stx-receptor synthesis and expression. Blocking Stx1-induced p38 and JNK activation with the inhibitor SB202190 prevented cell death and diminished Stx1-associated caspase 3 cleavage in an intestinal epithelial cell line [75]. P38 MAPK

inhibitors partially inhibited the Stx-induced cell death in Vero cells [90]. Walchli et al demonstrated that transport of Stx to the golgi apparatus is dependent on p38 MAPK. When cells were treated with chemical inhibitors or siRNA targeting p38, Stx transport was inhibited and Stx toxicity was reduced [91]. Additionally, inhibition of p38 MAPK reduced inflammatory cytokine up-regulation of Stx-receptor synthesis and cell-surface expression, thereby decreasing Stx cytotoxicity [92]. (3) Multi-kinase inhibitors with high binding affinity for ZAK. In a comprehensive effort to identify the target of 38 small molecule kinase inhibitors, Karaman et al determined the dissociation constants of a panel of 287 distinct protein kinases, including ZAK [93]. Sorafenib (Bayer), a multi-kinase inhibitor that has been employed in the treatment of renal cell carcinoma and hepatocellular carcinoma, was found to have a very high binding affinity for ZAK ($K_d=6.3$ nM) [93]. Another small molecule kinase inhibitor with a high binding affinity for ZAK is nilotinib (Novartis, $K_d=3$ nM), which also inhibits BCR-ABL and is currently in clinical use for treatment of chronic myelogenous leukemia [94]. Although the binding affinities of sorafenib and nilotinib for ZAK have been reported, neither agent has been tested for their ability to inhibit ZAK activity *in vitro* or *in vivo*.

Summary

Many different ribotoxic stressors have now been identified and their molecular mechanisms defined. A few ribotoxic stressors are known to cause human disease that is fatal in some cases. However, if a person is infected with a ribotoxic stressor, such as Stx, or has side effects from treatment with a ribotoxic stressor, such as doxorubicin, then care is primarily supportive without any actual directed therapies. However, there are

experiments demonstrating that some potential inhibitors of ribotoxic stressors, such as monoclonal antibodies against Stx2 or MAPK inhibitors may be beneficial in preventing the severe inflammation and apoptosis after exposure to Stx or doxorubicin. Therefore, my hypothesis is that identifying the most efficient type of inhibitor against each ribotoxic stressor--Stx and doxorubicin--could lead to a reduction in inflammation and apoptosis after exposure, and in the case of Stx2, prevent lethality in a mouse model.

CHAPTER 2

Mouse Model of Hemolytic-Uremic Syndrome Caused by Endotoxin-Free Shiga Toxin (Stx2) and Protection from Lethal Outcome by Anti-Stx2 Antibody

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Contributions: Figure 3-organs were processed for electron microscopy and pictures were taken by Kay Larkin

ABSTRACT

Hemolytic uremic syndrome (HUS) results from infection by Shiga toxin (Stx)-producing *Escherichia coli* and is the most common cause of acute renal failure in children. We have developed a mouse model of HUS by administering endotoxin-free Stx2 in multiple doses over 7-8 days. At sacrifice, moribund animals demonstrated symptoms of HUS: increased BUN and serum creatinine, proteinuria, deposition of fibrin, glomerular endothelial damage, hemolysis, leukocytopenia, and neutrophilia. Increased expression of proinflammatory chemokines and cytokines in the sera of Stx2-treated mice indicated a systemic inflammatory response. Currently, specific therapeutics for HUS are lacking, and therapy for patients is primarily supportive. Mice that received 11E10, a monoclonal anti-Stx2 antibody, four days after initiating injections of Stx2 recovered fully, displaying normal renal function and levels of neutrophils and lymphocytes. In addition, these mice showed decreased fibrin deposition and expression of proinflammatory mediators when compared to Stx2-treated mice in the absence of antibody. These results indicate that, when administered during progression of HUS, passive immunization of mice with anti-Stx2 antibody prevented the lethal consequences of Stx2.

INTRODUCTION

Hemolytic-uremic syndrome (HUS) results from an infection by Shiga toxin (Stx)-producing *Escherichia coli* (STEC), most commonly serotype O157:H7, and is the most common cause of acute renal failure in children (for reviews, see refs. [46-49]).

Accumulated evidence points to endothelial cell damage as the hallmark of the HUS-associated thrombotic microangiopathy, which is characterized by fibrin(ogen) deposition within small vessels, swelling of glomerular endothelial cells, and thrombotic occlusion of capillaries [95]. Severe cases of HUS exhibit renal cortical necrosis, pervasive inflammatory cell infiltrates in the kidney, and apoptosis of renal and cortical glomerular and tubular cells [96-99].

STEC expresses either Stx1 or Stx2, which are usually encoded by bacteriophages. Each Shiga holotoxin consists of one A and five B subunits. The B subunit binds to cells via glycosphingolipid receptors such as globotriaosyl ceramide (Gb3), while the A subunit contains N-glycosidase activity [67, 68]. Following endocytosis and retrograde transfer through the Golgi apparatus, the A subunit enters the cytosol. There, it depurinates a single adenine (A4256 in mice) in a conserved region of the 28S ribosomal-RNA, thereby inhibiting protein synthesis [69-72] and simultaneously activating the SAPKs, JNK and p38 [73-75]. Stxs and ricin, a related ribotoxin, induce the release of proinflammatory cytokines and activate the transcription of genes that encode them [73, 88, 89].

Activation of SAPKs by Stx and ricin has been tied to their proinflammatory effects [73].

Although administration of intravenous Stx to primates has been able to reproduce the features of HUS [100], the development of an HUS model in small animals has been less successful [101, 102]. The inability of Stx to reproduce glomerulopathy in animal models

may be due to the variable distribution of receptors for Stx among species. In view of the availability of mice containing null mutations in a variety of proinflammatory and regulatory genes, a mouse model of HUS using Stx alone that reproduces the manifestations of human disease would be valuable. The primary impediment in developing a murine model of HUS has been the inability of investigators to produce glomerular thrombotic microangiopathy (TMA), which is a hallmark of human HUS. Bacterial endotoxin, or lipopolysaccharide (LPS), has been employed in combination with Stx2 to reproduce the signs of HUS. However, LPS has been shown to either reduce or enhance Stx toxicity, depending on the time and dose of administration [103]. For example, pretreatment with LPS protects animals from the effects of Stx, whereas LPS administered 8 or 24h but not 0 or 72h after challenge with Stx enhances the toxicity [104]. Mortality rates and cytokine production in mice remained unchanged by administration of various concentrations of Stx in combination with sublethal doses of LPS at various times [105]. Ikeda, et al. found that LPS, when administered at the appropriate time, was essential for induction of HUS; however, this model lacked the typical hemolytic anemia. [106]. Keepers et al. developed another murine model using Stx and LPS; however some of the signs, such as lymphocytopenia and neutrophilia were transient, lasting only a few hours [107].

Currently, specific therapeutics for HUS are lacking, and therapy for HUS patients is primarily supportive. Although diagnostic reagents have recently been developed for early detection of Stx [108], and antibodies (chimeric, humanized, and fully human) have been developed for potential passive immunization [109-113], it is unclear whether administration of anti-Stx therapeutics would be effective when administered after signs

have developed in humans, though these Abs are protective after infection with STEC in a mouse model of infection. Stx that is bound to polymorphonuclear leukocytes was detected for up to 1 week after the diagnosis in the circulation of patients who had developed HUS. This suggests that delayed delivery of toxin to the microvasculature, over an extended time, may contribute to the clinical signs of HUS[114]. For these reasons, passive immunization with anti-Stx2 Ab following the appearance of initial signs may block the development of clinical signs and alleviate disease in patients who have been diagnosed with HUS [115].

In the present studies, we have developed a model of HUS in mice by administering multiple sub-lethal doses of Stx2, in the absence of LPS, over a period of 7-8 days. Administration of a mouse monoclonal Ab (11E10) directed against Stx2 was able to halt the lethality and reverse the manifestations of HUS when Ab was delivered as late as 4 days after the initial exposure to Stx2. These data suggest that, in this model of murine HUS, Stx-2 mediated renal failure required extended exposure and that administration of anti-Stx2 Ab midway through the course of administration interfered with an otherwise lethal outcome.

MATERIALS AND METHODS

Mice. C57BL/6J were purchased from The Jackson Laboratory, Bar Harbor, ME. Male mice aged 8 to 10 weeks of age and weighing 18 to 24 g were used throughout the experiments. Mice were housed under 12-hour light-dark cycle and fed with standard diet *ad libitum*. Mice received intraperitoneal injection of: a) 50mL of saline on days 0, 3, and 6; b) 5ng Stx2/20g bwt in 50mL of saline on day 0; c) 1ng Stx2/20g bwt in 50mL of saline on days 0, 3, and 6; or d) 1ng Stx2/20g bwt in 50mL of saline on days 0, 3, and 6; 30ng 11E10 Ab/20g bwt in 50mL of saline was administered on day 4. Mice were weighed on day 0 and every day thereafter. To collect 24-hour urine, mice were housed for 24 to 48 hours in “diuresis metabolic cages” (model M-D-METAB, Nalgene, Braintree, MA) provided with ground standard diet *ad libitum*. Before experimental procedures, mice were anesthetized intraperitoneally with 80mg/kg ketamine and 10mg/kg xylazine. All of the animal procedures were performed according to protocols that have been approved by the Institutional Animal Care and Use Committee at Oregon Health and Sciences University (OHSU), Portland, OR.

Reagents and antibodies. The mouse monoclonal anti-Stx2 antibody (11E10) has been previously described [116]. Antibody against fibrin/fibrinogen (YNGMFbg7S) was purchased from Accurate Chemical & Scientific (Westbury, NY). Stx2 was produced in *E. coli* DH5 using the pLPSH3 plasmid and purified by immunoaffinity chromatography as previously described [117]. Levels of endotoxin were undetectable, as determined by Limulus amoebocyte assay (Cambrex).

Mouse blood studies. All blood analyses were preformed by IDEXX Laboratories (Portland, OR). Mice were euthanized and blood was collected. Whole blood for cell-count analysis and cell-type determination was collected in hematology tubes with tripotassium ethylenediaminetetraacetic acid (EDTA) (Microtainer; Becton Dickinson, Franklin Lakes, NJ). In addition, total blood was left to coagulate at room temperature for 10 minutes, after which samples were centrifuged at 8,000rpm, 4°C, for 10 min, and serum was collected. Biochemical determinations of blood urea nitrogen and serum creatinine were performed.

Proteinuria studies. Mice were housed in “diuresis metabolic cages” for 24 to 48 hours during which time urine was collected. Equal amounts of urine from each mouse were further concentrated through 30-kDa Micropore concentration tubes. Urine samples were mixed 1:2 with 4x SDS-PAGE loading buffer and boiled at 95°C for 5 min. Samples from each group of three mice were pooled and separated via 10% SDS-PAGE. Gels were stained with Gelcode Blue staining solution. Bovine serum albumin was loaded on the same gel as a positive control.

Spectrophometric determination of hemoglobin in serum. Total blood was left to coagulate at room temperature for 10 minutes, after which samples were centrifuged at 8,000rpm, 4°C, for 10 min. Two µL of serum were used to obtain an absorption spectrum in a Nano Drop spectrophotometer (Nano Drop, Wilmington, DE).

Immunohistochemistry. Mice were anesthetized and sacrificed by cervical dislocation. The kidneys were dissected, fixed in Carnoy solution for 2 hours and transferred to 70% ethanol. The organs were embedded in paraffin blocks and sectioned into 5- μ m sections. After blocking in serum, the slides were incubated with primary antibodies overnight at 4°C at appropriate dilutions. Slides were further processed using the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendation using 3,3'-diaminobenzidine as substrate. Photomicrographs of immunohistochemical preparations in Figures 2A and 7A were taken at 1000X magnification at identical exposure times.

Detection of Tissue Fibrin. Fibrin deposition in kidneys was detected as described previously [118]. Frozen kidneys were homogenized in 10mM sodium phosphate buffer (pH 7.5) containing 5mM EDTA, 100mM ϵ -aminocaproic acid, 10U/ml heparin, and 2mM PMSF. The homogenate was incubated for 18h on a top-over-top rotor at 4°C. After centrifugation (10,000rpm, 4°C, 10 min), the pellet was resuspended in the same 10mM sodium phosphate buffer as above, without PMSF, and recentrifuged. Pellets were resuspended in 3M urea, agitated for 2h at 37°C, and centrifuged at 14,000rpm at 4°C for 15 min. The pellets were resuspended in reducing SDS sample buffer (10mM Tris (pH 7.5), 5% glycerol, 2% SDS, 5% β -mercaptoethanol, and .4 mg/ml bromophenol blue) and dissolved at 65°C for 90 min with vortexing every 15 min. Samples were subject to SDS-Page (7.5%; 5% stacking gel) and transferred to a PVDF membrane. Fibrin chains were detected with a fibrin antibody and a chemiluminescence system. Fibrin standards were prepared by clotting a known amount of rat fibrinogen (Sigma)

with an excess of human thrombin (Sigma) at 37°C for 10 min. After adding 2X SDS sample buffer, the standards were incubated at 65°C for 90 min.

Electron Microscopy. After dissection of the kidneys, tissue was fixed and processed using standard procedures, including immediate fixation in 2.5% glutaraldehyde in sodium phosphate buffer for two hours, post-fixation in osmium tetroxide, and embedding in plastic resin. Thick sections (1 mm) were stained with toluidine blue, and ultrathin sections were stained with uranyl acetate and lead citrate. Photomicrographs were taken at 11,000 X magnification.

RNA isolation. After dissection of the kidneys, the tissue was immediately frozen and ground in liquid nitrogen. RNA was extracted using TRIzol reagent in accordance to the manufacturer instructions and was further digested with DNase. Both reagents were purchased from Invitrogen Life Technologies, Carlsbad, CA.

Real time PCR analysis. Two µg of RNA were reverse-transcribed in the presence of SuperScript II and oligo-dT primers (both reagents were purchased from Invitrogen Life Technologies). The amplification of the cDNA was accomplished in an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) in the presence of the commercially available SYBR Green PCR Master Mix (Applied Biosystems) and 20µmol/L of the corresponding sense and antisense RT-PCR primers for 120-bp amplicons in a 40-cycle PCR. Each sample was analyzed in triplicate. Fold induction in gene expression was measured using absolute quantitation of a standard

curve in arbitrary units. The denaturing, annealing, and extension conditions of each PCR cycle were 95° for 15 s, 55° for 30 s, and 72° for 30 s, respectively. The SDS software was used for normalization of the raw data. The nucleotide sequences of primers used in this study have been previously published [40].

Reverse transcription of rRNA by primer extension. Reverse transcription of rRNA was performed as described by Iordanov and colleagues [35]. The oligonucleotide primer 5'-CACATACACCAAATGTC-3' (Invitrogen Life Technologies) was end labeled with T4 polynucleotide kinase (Life Technologies, Inc). A 10µl mixture of 2µg total RNA and 1.0 pmol primer in 50mmol/L Tris-HCl (pH 8.3), 75mmol/L KCl, 3mmol/L MgCl₂ was incubated at 90°C for 3 minutes and then placed on ice for 5 minutes, followed by an incubation at room temperature for 5 minutes. The reverse transcription was initiated by the addition of 10µl of a mixture of 2mmol/L deoxynucleoside triphosphates and 30U SuperScript (Life Technologies, Inc.) in 50mmol/L Tris-HCl (pH 8.3), 75mmol/L KCl, 3mmol/L MgCl₂, 10mmol/L dithiothreitol, followed by an incubation at 48°C for 15 minutes, when the reactions were stopped by the addition of 5mmol/L EDTA. Reaction products were precipitated in ethanol, resuspended in formamide gel loading buffer, heat denatured, and eletrophoresed in 8% acrylamide sequencing gel, which was subsequently dried and exposed to a PhosphorImager screen.

Multiplex Cytokine Detection. Serum samples were analyzed using a customized Multiplex Mouse Cytokine Kit from Linco Research (St. Charles, MO) and detected on

the LiquiChip Workstation (Qiagen, Valencia, CA). Each sample was analyzed in duplicate.

Statistical analysis. Individual groups were compared using unpaired *t*-test analysis. To estimate P values, all statistical analyses were interpreted in a two-tailed manner. P values < 0.05 were considered statistically significant.

RESULTS

To examine the consequences of Stx2 administered intraperitoneally to mice, we first determined the toxicity of Stx2 when administered as a single dose. Dose-response experiments determined that the 50% lethal dose (LD_{50}) was 3ng Stx2/20g body weight (bwt, data not shown). In initial experiments, administration of a lethal dose of Stx2 (a single dose of 5ng Stx2/20g bwt; hereafter referred to as "1x5") resulted in weight loss detected 24h later, which continued for 3 days (Figure 1A). At that time, animals were moribund, having lost at least 20% of their body weight and displaying tremors and ataxia. For ethical reasons, these mice were euthanized on the third day following injection of Stx2. Blood urea nitrogen (BUN) from these animals increased from 31 ± 6.3 to 103.8 ± 36.6 mg/dL ($p < 0.05$), but creatinine levels failed to differ significantly (Figure 1B). Urine from these animals failed to show signs of increased protein content, an indicator of renal failure, as determined by polyacrylamide gel electrophoresis (Figure 1C). Presence of hemoglobin in serum was evaluated by determining the absorbance spectrum of serum. Mice receiving 1x5 Stx2 showed an increased spectral absorbance profile characteristic of free hemoglobin [119] (Figure 1D). Animals receiving 1x5 Stx2 failed to show an increase in circulating neutrophils, although they exhibited a significant decrease in circulating lymphocytes (Figure 1E). From these data, we concluded that administration of 1x5 Stx2 triggered some hemolysis and a modest impairment of renal function, as evidenced by increased BUN levels, but failed to display other significant signs of renal dysfunction, such as increased creatinine and proteinuria.

Symptoms of HUS in humans usually do not appear earlier than one week following exposure to STEC [115]. We considered, therefore, that mice may require a extended

time interval between initial exposure to Stx2 and the development of symptoms of HUS. First, we investigated whether lower doses of Stx2 that allowed mice to survive for at least one week would permit the animals to develop more severe manifestations of HUS, including renal failure. However, single doses of Stx2 lower than 5ng/20g bwt that permitted animals to survive more than 3 days were ineffective in inducing either toxicity or symptoms of renal failure (not shown). We then tested whether administration of successive, lower, doses of Stx2 over an extended time course would permit the animals to develop symptoms characteristic of HUS in humans. When we administered two injections of 1ng Stx2/20g bwt (at days 0 and 3), mice showed minor symptoms of toxicity and renal failure when sacrificed 8 days later (data not shown). We then injected animals with three doses of 1ng Stx2/20g bwt at day 0, day 3, and day 6 (hereafter referred to as “3x1”). The mice receiving 3x1 Stx2 did not begin to lose significant bwt until day 5 (Figure 1A). By day 7 or 8, these mice had lost a total of 20 to 25% of their initial bwt and were euthanized. Unlike mice receiving 1x5 Stx2, these animals did not exhibit neurological symptoms at time of sacrifice. BUN levels increased nearly 6-fold ($p<0.001$) in the 3x1 Stx2 mice, while creatinine levels increased 2.5-fold ($p<0.01$) when compared to saline-injected control mice (Figure 1B). The urine of the 3x1 Stx2 mice displayed increased protein content with a major protein band corresponding in size to serum albumin and additional bands of proteins with higher molecular weight (Figure 1C). The increased serum levels of BUN and creatinine and the appearance of proteinuria suggested that administration of 3x1 Stx2 resulted in compromised renal function. Furthermore, spectrophotometric analysis of serum from these mice revealed hemolysis, as evidenced by levels of hemoglobin in the serum that were greater than that

seen in 1x5 animals (Figure 1D). Unlike the 1x5 mice, the 3x1 mice exposed for 7-8 days developed increased numbers of circulating neutrophils (from 738.8 ± 83.1 cells/ μ l in control mice to 1047.6 ± 223.8 cells/ml in Stx2-treated mice ($p < 0.05$)) and decreased numbers of lymphocytes (from 3779.6 ± 767.1 cells/ml in control mice to 566 ± 327.9 cells/ml in Stx2-treated mice ($p < 0.001$)) (Figure 1E).

Glomerular thrombotic microangiopathy (TMA) is a distinguishing feature of human HUS that has been difficult to recapitulate in animal models. Compared with animals treated with saline, kidneys from mice receiving 1x5 Stx2 showed a small amount of fibrin deposition in glomerular vessels, as demonstrated by both anti-fibrin immunohistochemistry (Figure 2A) and immunoblotting (Figure 2B). However, mice injected with 3x1 Stx2 demonstrated abundant deposition of fibrin in the glomerular capillary loops, with some capillaries appearing to be completely occluded (Figure 2A). The deposition of increased fibrin in kidneys from these mice was confirmed by immunoblotting (Figure 2B). Transmission electron microscopy of kidneys from 3x1 Stx2 animals revealed abundant focal detachment of glomerular endothelial cells, swelling and fragmentation of endothelial cells, and enlargement of subendothelial spaces (asterisk, Figure 3B). Kidneys from 1x5 Stx2-treated animals showed similar, but less pronounced, alterations in morphology of glomeruli (data not shown).

Inflammatory responses

We next analyzed RNA from mouse kidneys by real-time RT-PCR, to determine the effects of Stx2 on the expression of mRNA transcripts that encode proteins implicated in inflammatory responses. Groups of five mice injected with 1x5 Stx2 or 3x1 Stx2 were sacrificed on day 3 or day 7, respectively. Animals receiving saline alone were sacrificed

on day 7. The transcripts evaluated included cytokines (TNF- α , IL-1 α , IL-1 β , IL-6), chemokines (CCL2/MCP-1, CXCL1/Gro- α), transcription factors (c-Jun, c-Fos, EGR1), and a surface adhesion protein (ICAM1). The results were expressed as fold increase in RNA expression in Stx2-treated animals compared with saline controls, using glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as a control to standardize the samples (Figure 4A). The administration of either 1x5 or 3x1 Stx2 significantly elevated the expression of the majority of transcripts investigated. The expression of two gene transcripts, CXCL1/Gro- α and IL-6, were significantly greater in the 3x1 Stx2-treated mice compared to the 1x5 Stx2-treated mice.

We employed a customized Multiplex Mouse Cytokine Kit to measure the expression of several proinflammatory cytokines and chemokines in the serum of mice treated with saline or Stx2. Administration of either 1x5 or 3x1 Stx2 significantly elevated the levels of all proinflammatory proteins investigated, compared to saline-injected control mice (Figure 4B). However, levels of these proteins in sera from 1x5 and 3x1 mice did not differ significantly from each other (Figure 4B). The data shown in Figure 4B suggest that the Stx2-induced increase in expression of transcripts encoding proinflammatory cytokines and chemokines was associated with an increased level of corresponding proteins in the serum. Importantly, these data indicated that the induced expression of proinflammatory mRNAs associated with Stx2 intoxication resulted in the synthesis of proteins directed by those mRNAs, even in the face of potentially decreased levels of protein synthesis that may occur following Stx-2-induced damage to 28S rRNA.

Stx2-mediated lesions in 28S rRNA .

Stx2 may impair kidney function by acting directly on cells of the kidney and/or indirectly by inducing the release of inflammatory mediators into the systemic circulation from body tissues. To determine the existence of damage to renal 28S rRNA specifically caused by Stx2, we applied primer-extension analysis, a technique that produces truncated radiolabeled transcripts at the site of depurination (A4256) in 28S rRNA [35]. RNA extracted from the kidneys of mice exposed to 1x5 Stx2 displayed the strongest lesion-specific signals (Figure 5). Lesions in A4256 were also observed in the 3x1 Stx2 mice, but were diminished in amount compared with lesions produced by 1x5 Stx2. These results indicated that a single dose of 5ng Stx2 produced more lesions in renal 28S rRNA than three successive injections of 1ng Stx2.

Passive immunization by anti-Stx2 antibody

To determine whether administration of anti-Stx2 antibody could prevent progression of HUS in our model, we administered 11E10, a monoclonal antibody, to mice exposed to 3x1 Stx2. To mice receiving three injections of 1ng Stx2/20g bwt (on days 0, 3, and 6), we injected a single bolus of 30ng 11E10 Ab/20g bwt at different times prior to sacrifice at day 7-8. All mice that received antibody on day 4 did not develop subsequent weight loss and appeared normal at time of sacrifice on day 8 (Figure 6A). However, when antibody was administered to mice on day 5, rescue of some, but not all, mice was achieved (data not shown). Administration of antibody on day 6 failed to rescue any mice (data not shown). To determine whether mice receiving antibody on day 4 would develop impaired kidney function, we evaluated levels of BUN and serum creatinine at day 8 (two days after receiving the final dose of 1ng Stx2/20g bwt). Levels of BUN and

serum creatinine in these mice were not different from saline-injected control animals (Figure 6B). In addition, the level of free hemoglobin appearing in sera of 3x1 mice was not increased in the sera of 3x1 Stx2 mice receiving 11E10 Ab on day 4 (Figure 6C), suggesting that administration of anti-Stx2 antibody to the 3x1 Stx2 mice prevented intravascular hemolysis. The neutrophilia and lymphocytopenia normally present in 3x1 Stx2 mice was not present in 3x1 mice receiving anti-Stx2 antibody on day 4 (Figure 6D). In addition, the administration of 3x1 Stx2 and 11E10 Ab reduced the deposition of fibrin in kidneys harvested at 8 days, as determined both by immunohistochemistry and immunoblotting (Figure 7A,B).

The administration of 11E10 Ab in concert with 3x1 Stx2 significantly decreased the expression of the majority of proinflammatory mRNA transcripts that were investigated in the kidneys. Expression of most transcripts decreased by more than 50% compared to kidneys from 3x1 mice that did not receive 11E10 Ab (Figure 4A). There was also a coordinate decrease in abundance of circulating proinflammatory proteins in sera from these mice compared with mice that had received 3x1 Stx2 alone (Figure 4B). 3x1 mice receiving 11E10 antibody remained healthy, and displayed normal renal function at the time of sacrifice four weeks later (data not shown).

Figure 1

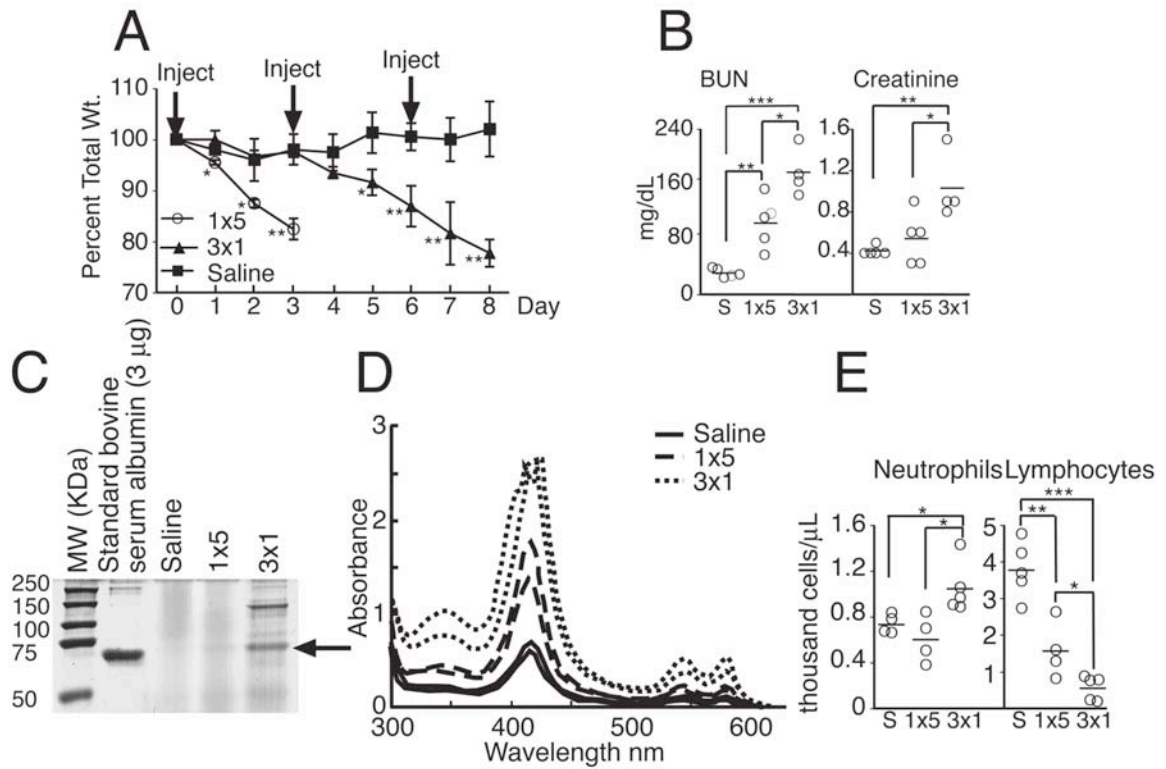


Figure 1. Analysis of body weight, blood parameters, and urine after administration of Stx2 to mice.

Groups of mice were injected with Stx2 or saline as follows: a) 1x5: a single injection of 5ng Stx2/20g bwt at t=0; b) 3x1: three injections of 1ng Stx2/20g bwt at t=0, t=3 days, and t=6 days; c) saline (S): three injections of 50ml saline at t=0, t=3 days, and t=6 days.

A: Percent total body weight loss in mice injected with Stx2 or saline. Mice weight at day 0 was set at 100%. Data reflect 5 mice per group $\pm$ SD. *: $p < .05$; **: $p < .01$; ***: $p < .001$. **B:** Measurement of BUN and creatinine in sera. Each circle represents measurement of BUN or creatinine from one mouse; black bars represent the mean of

samples in each group. Brackets indicate level of significance between groups. *: $p < .05$; **: $p < .01$; ***: $p < .001$. C: *Presence of protein in urine of mice following administration of Stx2 or saline*. Equal amounts of pooled urine from groups of three saline, 1x5, and 3x1-injected mice were separated by SDS-PAGE. Bovine serum albumin (3 μ g) was used as a standard for albumin. D: *Measurement of hemoglobin by absorption spectrophotometry of sera*. Absorption spectrophotometry of sera from two saline, 1x5, and 3x1-injected mice demonstrate absorbance maxima at 430nm and 575nm, characteristic of hemoglobin. E: *Measurement of neutrophil and lymphocyte numbers*. Each circle represents measurement of neutrophil or lymphocyte numbers from one mouse; black bars represent the mean of samples in each group. Brackets indicate level of significance between groups. *: $p < .05$; **: $p < .01$; ***: $p < .001$.

Figure 2

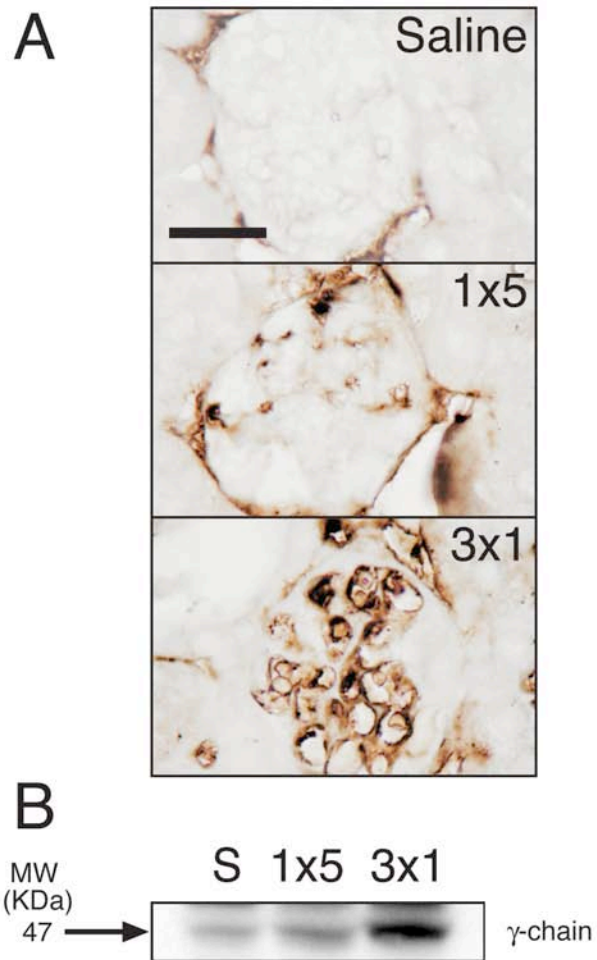


Figure 2. Detection of fibrin/fibrinogen by immunohistochemistry and immunoblotting.

Groups of mice were injected with Stx2 or saline as described in Figure 1. A: Immunohistochemical detection of fibrin/fibrinogen. Size bar indicates 20 mM. B: Detection of fibrin/fibrinogen by immunoblotting.

Figure 3

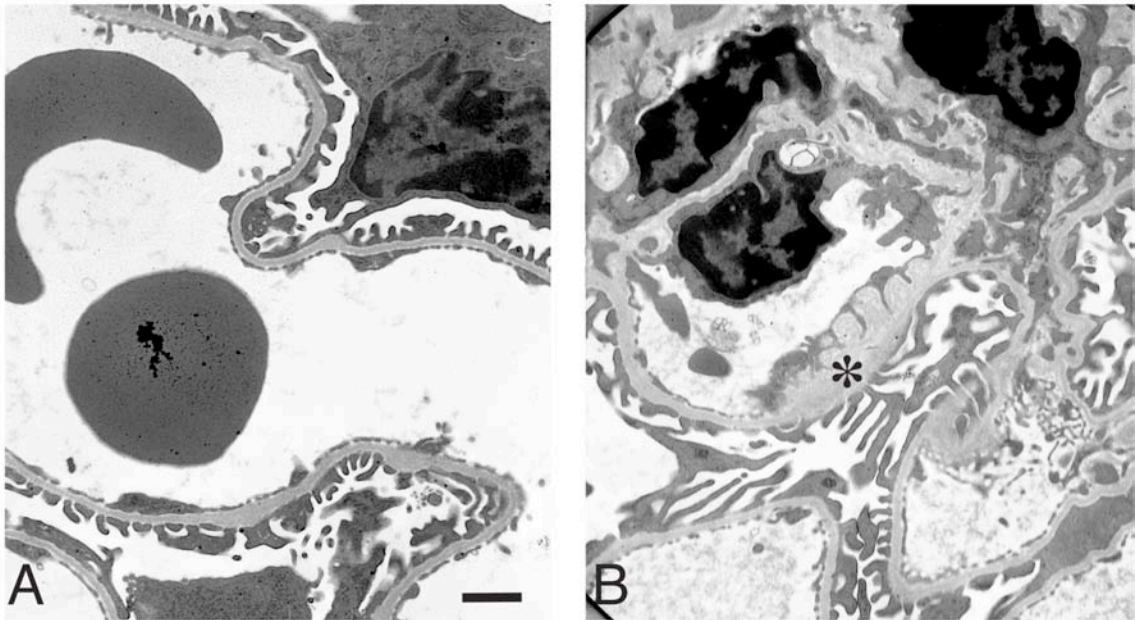


Figure 3. *Transmission electron microscopy of glomeruli from saline (A) or 3x1 (B) mouse. Groups of mice were injected with Stx2 or saline as described in Figure 1. Asterisk in B indicates enlarged subendothelial space. Size bar in A indicates 1 mM.*

Figure 4

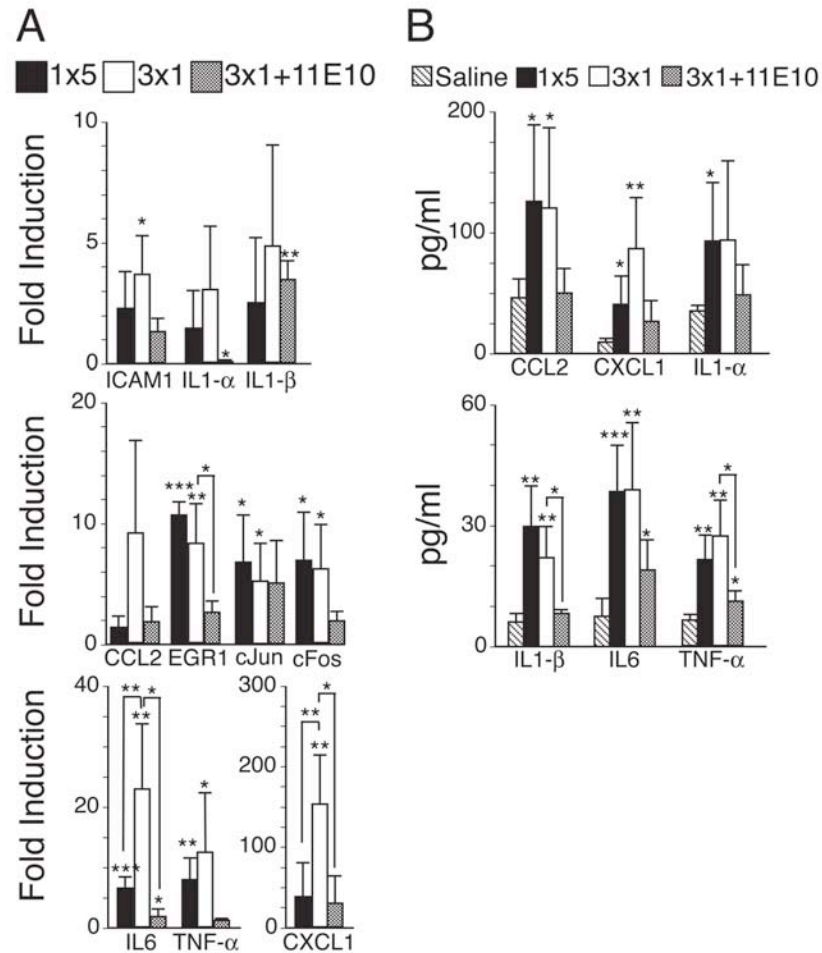


Figure 4. Measurement of proinflammatory gene products in mouse kidneys. Groups of mice were injected with Stx2 or saline as described in Figure 1. In addition, a group of 3x1 mice was injected with 11E10 on day 4 (grey bars). A: Measurement of RNA transcripts by quantitative real-time RT-PCR. Kidney RNA was analyzed from five animals per group (Saline, 1x5, 3x1, or 3x1+11E10). Each bar represents the mean fold increase in RNA expression $\pm$ S.E.M, compared with values obtained from control saline-

injected mice. Brackets indicate level of significance between groups. *: $p < .05$; **: $p < .01$; ***: $p < .001$. B: *Measurement of cytokines and chemokines in sera from groups of three mice*. Sera was analyzed from three animals per group (Saline, 1x5, 3x1, or 3x1+11E10). Each bar represents the mean value of each protein $\pm$ SD. Brackets indicate level of significance between groups. *: $p < .05$; **: $p < .01$; ***: $p < .001$.

Figure 5

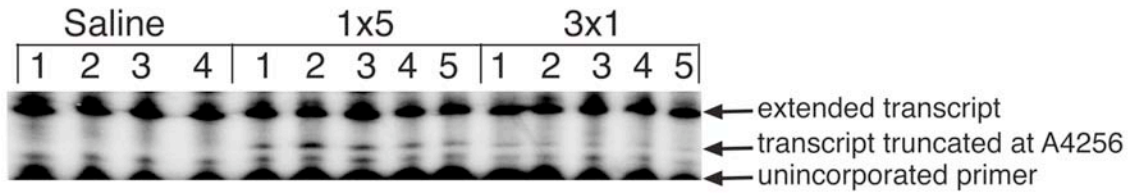


Figure 5. *Detection of lesions at A4256 in 28S rRNA by primer-extension.* Groups of mice were injected with Stx2 or saline as described in Figure 1. Total RNA was purified from kidneys of five mice per group. Truncated transcripts at A4256 were detected from all Stx2-treated kidneys (middle arrow).

Figure 6

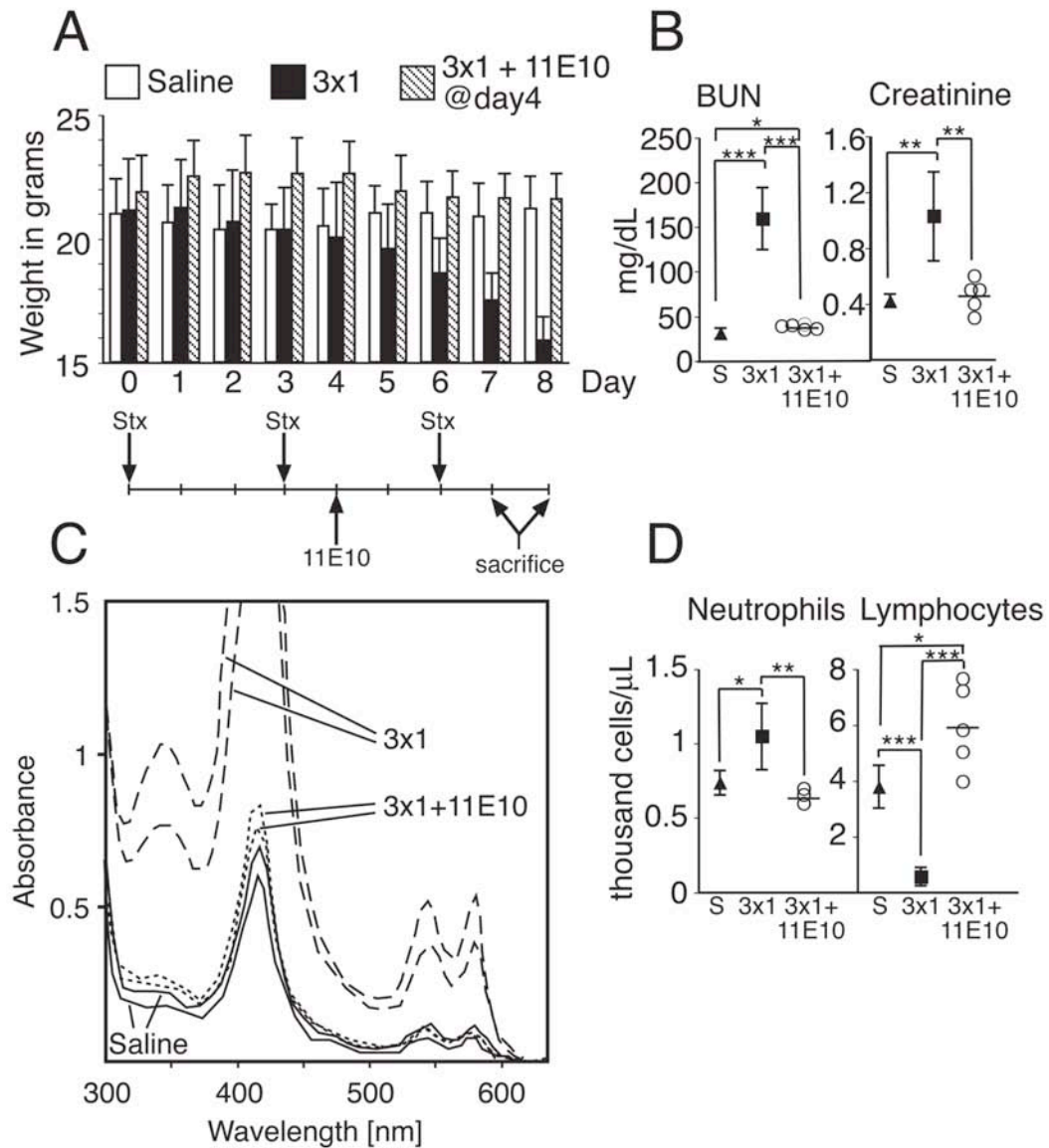


Figure 6. Analysis of body weight and blood parameters after administration of Stx2 ± 11E10. Mice received saline, 3x1, or 3x1+11E10 (mouse monoclonal antibody against Stx2) on day 4. A: Weight loss in mice injected with Stx2 ± 11E10. Each bar represents mean from 5 mice ± SD. B: Measurement of BUN and creatinine in sera. Data for S and 3x1 has been shown in Figure 1B; these are included here for comparison with

3x1+11E10. Data are the average of 5 mice $\pm$ SD. Each circle for 3x1+11E10 represents one mouse; black bars indicate the mean of samples for the group. Brackets indicate level of significance between groups. *: $P < .05$; **: $P < .01$; ***: $P < .001$. C: *Measurement of hemoglobin by absorption spectrophotometry of sera.* Absorption spectrophotometry of sera from two saline, 3x1, and 3x1+11E10-injected mice. D: *Measurement of neutrophil and lymphocyte numbers.* Data for S and 3x1 has been shown in Figure 1E; these are included here for comparison with 3x1+11E10. Data values represent the average of 5 mice $\pm$ SD. Each circle for 3x1+11E10 represents one mouse; black bars indicate the mean of samples for the group. Brackets indicate level of significance between groups. *: $P < .05$; **: $P < .01$; ***: $P < .001$.

Figure 7

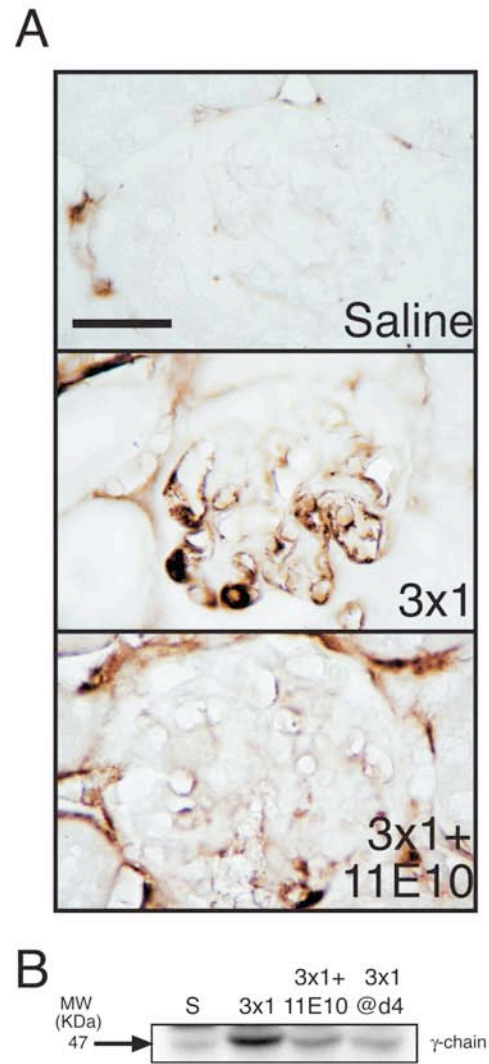


Figure 7. Detection of fibrin/fibrinogen by immunohistochemistry and immunoblotting after administration of *Stx2* $\pm$ 11E10. Groups of mice were injected as described in Figure 6. A: Immunohistochemical detection of fibrin/fibrinogen. Size bar represents 20 mM. B: Detection of fibrin/fibrinogen by immunoblotting.

DISCUSSION

Stx is the primary etiologic factor in the pathogenesis of HUS [47]. Although the administration of Stx alone to primates recapitulates symptoms of HUS [100], Stx alone has not been able to produce the majority of HUS symptoms in small animal models. The development of a murine model of HUS would provide an economic advantage for therapeutic experimentation and, in view of the ready availability of mouse strains that are deficient in key mediators of inflammatory or coagulatory processes, would further offer the benefit of employing the power of mouse genetics. Although the co-administration of LPS and Stx has been able to reproduce many of the symptoms of HUS in a murine model [107] these data are controversial because LPS is not required to produce HUS in a primate model [100] and because the required presence of LPS in humans with HUS remains undefined. Furthermore, LPS has been shown to either reduce or enhance Stx toxicity in murine models, depending on the time and dose of administration [103]. In this study, we have sought to develop a mouse model of HUS by administering endotoxin-free Stx2.

Administration of a single lethal dose of Stx2 to mice induced hemolysis, leukocytopenia, and modest impairment of renal function, as evidenced by increased BUN levels at 72h. Although the lethal dose of Stx2 produced modest renal dysfunction, it was not able to generate many of the significant indicators of renal failure, such as increased creatinine and proteinuria. Mice exposed to a single bolus (5 ng/20g bwt) of Stx2 developed tremors and ataxia prior to death, indicating that the early demise of these mice may have resulted from toxicity to the nervous system. Stx binds to Gb3 receptors in neurons of the central nervous system of mice [120] and has been found to produce brain damage in

mice [121]. Neurological damage in humans is a frequent complication in HUS [122-124] and in experimental animals (rabbits) exposed to Stx [125, 126]. We reasoned that administration of a lower dose of Stx2 may allow the mice to survive for a longer period of time, permitting them to develop a symptomatic course that would more completely recapitulate the characteristics of HUS. Although administration of low doses of Stx2 in a single or double injection were ineffective in producing the array of symptoms that characterizes HUS, the administration of three successive injections of 1ng Stx2/20g bwt at 72h intervals resulted in the development of many of the manifestations of HUS that develop in humans. The 3x1 mice began losing weight 4 days after the initial injection and, at the time of sacrifice (7-8 days), manifested increased BUN, serum creatinine, and proteinuria, all of which are indicators of compromised renal function. In addition, these mice developed hemolysis, leukocytopenia and neutrophilia compared with mice that received saline alone. Injury of the endothelium contributes to the development of microvascular thrombosis, a common feature of HUS that has been tied to development of renal failure in human patients {Paton, 1998 #73;Proulx, 2001 #75;Ray, 2001 #87}. Endothelial cell injury and thrombosis have been difficult to reproduce in Stx-induced animal models [103, 127-129]. The degree of fibrin deposition in glomerular capillaries was greater in 3x1 than in 1x5 mice; some capillaries in the 3x1 mice appeared to be completely occluded (Figure 2). Furthermore, examination of kidneys from multiple Stx2-injected mice by electron microscopy revealed focal detachment, swelling, fragmentation of glomerular endothelial cells, and enlargement of subendothelial spaces. These characteristics are commonly observed in HUS in humans.

Evidence suggests that HUS involves an acute inflammatory response. Patients with HUS display elevated circulating proinflammatory cytokines and chemokines [47, 49, 76, 130, 131]. In addition, excretion of urinary TNF- α and IL-6 is elevated during the acute phase of HUS in patients [132]. Increased expression of proinflammatory transcripts and the presence of proinflammatory chemokines and cytokines in the sera of Stx2-treated mice provided evidence that these animals developed an abundant inflammatory response. At the time of death, expression of circulating proinflammatory mediators was similar in singly- and multiply-injected mice, suggesting that the development of multiple manifestations of HUS may require an extended exposure to these circulating mediators.

Depurination of a single adenine (A4256 in mice) in a conserved region of the 28S ribosomal-RNA by Stx is similar to ricin, a related toxin [133], and is the single known mechanism by which the toxins transduce their signals following entrance into cells [35]. This event not only leads to inhibition of protein translation, but also to the rapid activation of stress-activated protein kinases (SAPKs), p38 and JNK, by activating kinases situated upstream in the activating cascade [35, 75]. Activation of SAPKs and the subsequent increased production of proinflammatory transcripts occur in the presence of only partial translational inhibition [40]. As a consequence, increased levels of transcribed proinflammatory mRNA molecules are capable of being translated into functional proteins [40]. Importantly, the present study shows that, similar to ricin, the Stx2-induced expression of proinflammatory mRNAs resulted in the synthesis of proinflammatory proteins, despite potentially decreased levels of protein synthesis.

Detection of lesions in ribosomal-RNA by primer extension analysis showed that the accumulated damage in 1x5 mice at 72h was greater than 3x1 mice, suggesting that the

development of Stx2-mediated renal failure may require an extended time to permit the animals to develop symptoms characteristic of HUS. In human disease, 10-15% of infected individuals progress to HUS one week after the initial onset of symptoms [134]. Our results suggest that the administration of small, multiple, injections of Stx2 may recapitulate the development of HUS that occurs in humans over an extended period of time.

To determine whether administration of an anti-Stx2 antibody would prevent progression of HUS in our mouse model, we administered 11E10, a specific monoclonal antibody, that recognizes the A subunit of Stx2, at various times after the second dose of 1ng Stx2/20g bwt. Mice receiving 11E10 on day four showed normal renal function, normal neutrophil and lymphocyte levels, and lacked hemolysis at the time of sacrifice. In addition, these mice showed decreased fibrin deposition when compared to Stx-treated mice in the absence of antibody. The inflammatory response, as determined by measurement of proinflammatory transcripts in the kidney and demonstration of protein in the sera, was reduced by more than 50% in proinflammatory mRNAs and proteins that were examined. Taken together, these results indicate that passive immunization with anti-Stx2 antibody in mice was capable of preventing the lethality of the toxin, even after initial exposure to Stx and in the continued presence of circulating Stx.

Current treatment of HUS in patients is supportive, and effective therapeutic intervention has not been successful [115, 134]. Our results suggest that passive immunization against Stx may be therapeutically effective in humans at risk of developing HUS, if antibody is able to be administered at an early time after development of symptoms and before the accumulated effects of HUS have resulted in irreversible structural damage.

CHAPTER 3

**ZAK is required for doxorubicin, a novel ribotoxic stressor, to induce SAPK
activation and apoptosis in HaCaT cells.**

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Contributions: Figure 4A-this experiment was performed by Eli Magun

ABSTRACT

Doxorubicin is an anthracycline drug that is one of the most effective and widely used anticancer agents for the treatment of both hematologic and solid tumors. The stress-activated protein kinases (SAPKs) are frequently activated by a number of cancer chemotherapeutics. When phosphorylated, the SAPKs initiate a cascade that leads to the production of proinflammatory cytokines. Some inhibitors of protein synthesis, known as ribotoxic stressors, coordinately activate SAPKs and lead to apoptotic cell death. We demonstrate that doxorubicin effectively inhibits protein synthesis, activates SAPKs, and causes apoptosis. Ribotoxic stressors share a common mechanism in that they require ZAK, an upstream MAP3K, to activate the pro-apoptotic and proinflammatory signaling pathways that lie downstream of SAPKs. By employing siRNA mediated knockdown of ZAK or administration of sorafenib and nilotinib, kinase inhibitors that have a high affinity for ZAK, we provide evidence that ZAK is required for doxorubicin-induced proinflammatory and apoptotic responses in HaCaT cells, a pseudo-normal keratinocyte cell line, but not in HeLa cells, a cancerous cell line. ZAK has two different isoforms, ZAK- α (91 kDa) and ZAK- β (51 kDa). HaCaT or HeLa cells treated with doxorubicin and immunoblotted for ZAK displayed a progressive decrease in the ZAK- α band and the appearance of ZAK- β bands of larger size. Abrogation of these changes after exposure of cells to sorafenib and nilotinib suggests that these alterations occur following stimulation of ZAK. We suggest that ZAK inhibitors such as sorafenib or nilotinib may be effective when combined with doxorubicin to treat cancer patients.

INTRODUCTION

Doxorubicin (dox, adriamycin) is an anthracycline drug that is one of the most effective and widely used anticancer agents for the treatment of both hematologic and solid tumors [81]. Several mechanisms for the chemotherapeutic actions of doxorubicin have been proposed, including: a) intercalation into DNA, leading to inhibition of macromolecular synthesis; b) generation of reactive oxygen species (ROS), leading to DNA damage or lipid peroxidation; and c) inhibition of topoisomerase II, followed by DNA damage. Doxorubicin-mediated apoptotic cell death is likely a response to one or more of these upstream actions [81, 135, 136]. The clinical efficacy of doxorubicin is limited by both acute and chronic complications. Patients receiving doxorubicin frequently present with acute side effects such as fatigue, nausea/vomiting, pain, sleep disturbances, cachexia, and depression [82]. In addition, patients may develop cardiomyopathy, leading to life-threatening congestive heart failure. Cardiomyopathy frequently correlates with the total amount of administered drug [136]. Production of oxygen radicals has been proposed for doxorubicin-mediated cardiotoxicity, whereas the inhibition of both topoisomerase enzyme and DNA synthesis is thought to underlie doxorubicin-induced death of tumor cells [136, 137]. Identifying the mechanism(s) by which normal and healthy cells respond differentially to doxorubicin may present opportunities to decrease the toxicity of doxorubicin on normal tissues while maintaining the efficacy of doxorubicin as an anti-cancer drug.

The SAPKs, p38 MAPK and JNK, are frequently activated by a number of cancer chemotherapeutics [82]. When phosphorylated, the SAPKs initiate a cascade that leads to the production of proinflammatory cytokines. Doxorubicin is known to induce the

activation of SAPKs in a number of normal cell types, including hepatocytes [138], primary mouse macrophages [89] and cardiomyocytes [87, 88]. Inhibitors of p38 MAPK and JNK have been employed to determine whether activation of SAPKs contributes either to the efficacy or to the undesirable side effects of doxorubicin. Inhibitors of p38 MAPK have been effective in blocking apoptosis of cardiomyocytes following treatment by doxorubicin or daunorubicin, a related anthracycline [87, 88]. Inhibitors of p38 MAPK reduce the proinflammatory actions of doxorubicin in macrophages but do not reduce the anti-proliferative actions of doxorubicin in a cancer cell line [89].

The intercalation of doxorubicin into DNA is thought to comprise a major mode of action in producing cell death. In addition to its effects on DNA, doxorubicin also causes RNA damage [139] and inhibits the synthesis of DNA, RNA, and proteins [140-143]. Some inhibitors of protein synthesis, known as ribotoxic stressors, activate SAPKs. Well-known ribotoxic stressors include anisomycin, blasticidin, ricin, Shiga toxin, sarcin and ultra-violet radiation. Here we demonstrate that doxorubicin effectively inhibits protein synthesis, activates JNK and p38 MAPK, and causes apoptosis.

Ribotoxic stressors share a common mechanism in that they require ZAK, an upstream MAP3K, to activate the pro-apoptotic and proinflammatory signaling pathways that lie downstream of p38 MAPK and JNK [44, 45]. SiRNA-mediated knockdown of ZAK or administration of a small molecule inhibitor (DHP-2) of ZAK, suppress anisomycin- and UV-induced apoptosis and the phosphorylation of p38 MAPK and JNK [44]. Inhibition of ZAK also suppresses Shiga toxin- and ricin-induced SAPK activation and improves cell viability [45]. By employing siRNA-mediated knockdown of ZAK or administration of sorafenib or nilotinib, kinase inhibitors that have a high affinity for ZAK, we provide

evidence that ZAK is required for doxorubicin-induced proinflammatory and apoptotic responses in HaCaT cells, a pseudo-normal human keratinocyte cell line, but not in HeLa cells.

MATERIALS AND METHODS

Antibodies and other reagents. Antibodies against the phosphorylated forms of ERK, JNK, p38, and against active caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against PARP (H-250), JNK1 (FL), and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Doxorubicin, daunorubicin, CdCl₂, and emetine were purchased from Sigma (St. Louis, MO). SB 203580, SP 600125, MG-132, and zVAD-fmk were purchased from EMD Chemicals (Gibbstown, NJ). ZAK siRNA oligonucleotides (sequence from Wang et al [44]) were synthesized by the Research Core Facility at Oregon Health & Science University, Portland, OR. Sorafenib and nilotinib were purchased from LC Laboratories (Woburn, MA). [³H]-leucine was purchased from PerkinElmer (Waltham, MA). ZAK antibody was kindly provided by Cheleste M. Thorpe (Tufts-New England Medical Center).

siRNA knockdown of ZAK. HaCaT or HeLa cells were grown to 70% confluency and treated in Dulbecco modified Eagle medium /10%FBS. ZAK or scrambled (scr) control siRNA were transfected at 50 nM siRNA per well using DharmaFECT 4 (Dharmacon, Lafayette, CO) according to the manufacturer's instructions. After 72h (HaCaT) or 48h (HeLa), complete medium was removed and the cells were serum deprived for 1h prior to addition of doxorubicin for 24h, at the indicated doses, prior to the harvest of cell lysates. Cell lysates were subjected to immunoblotting.

Measurement of protein synthesis via [³H]-leucine incorporation. HaCaT cells were

grown in 12-well tissue culture plates. Treatments were performed in leucine-free/serum-free DMEM, for the indicated times and doses of doxorubicin. For the final 30 minutes of doxorubicin exposure prior to harvesting, cells were pulse-labeled with 1 μ Ci [^{3} H]-leucine in 1ml DMEM. Ten percent trichloroacetic acid was added to terminate incorporation. Following thorough rinsing with water, 88% formic acid was added to solubilize the trichloroacetic acid-insoluble proteins and the samples were counted in a liquid scintillation counter. In each experiment, three wells were used per experimental point.

Immunoblotting. Equal numbers of HaCaT or HeLa cells were plated, serum deprived for 30 minutes, treated, and lysed in 2XESB lysis buffer in preparation for immunoblotting. Equal volumes of the cell lysates were separated on a 10% or 13% (for caspase-3 antibody) denaturing polyacrylamide gel in the presence of sodium dodecyl sulfate and were transferred onto polyvinylidene difluoride membranes according to standard laboratory procedures. Membranes were incubated with the indicated antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies; signals were detected by using enhanced chemiluminescence.

Experiments with emetine. HaCaT cells were serum deprived and then pretreated for 0.5h with 100 μ g/ml emetine or vehicle, followed by treatment with 0.5mM doxorubicin or 100 μ M CdCl₂ for the indicated times. Cells were harvested at various times and subjected to immunoblotting.

Treatment with inhibitors. Serum-deprived HaCaT or HeLa cells were pretreated for 0.5

hours with sorafenib (1 μ M), nilotinib (1 μ M), SB 203580 (10 μ M), SP 600125 (20 μ M), or zVAD-fmk (25 μ M). At 12h an additional dose of equal amount was added to each well. Cells were treated with the indicated doses of doxorubicin (10 or 25 μ M) or daunorubicin (2.5, 10, or 25 μ M) for 24h, harvested, and subjected to immunoblotting.

Doxorubicin time course. HaCaT cells were serum deprived and treated with 25 μ M of doxorubicin in four-hour increments from 4-24h. Cell lysates were subjected to immunoblotting.

Treatment with MG-132, a proteasome inhibitor. HaCaT cells were serum deprived and treated with 25 μ M of doxorubicin. Eight hours after addition of doxorubicin, 5 μ M MG-132 was added to the corresponding wells. Treatment continued for 12 hours for a total of 20 hours of doxorubicin treatment then cells were harvested and subjected to immunoblotting.

RESULTS

Doxorubicin-induced MAPK activation and apoptosis are decreased after siRNA-mediated knockdown of ZAK.

Ribotoxic stressors are characterized by their ability to activate JNK and p38 MAPK through inhibition of protein synthesis [35]. In many cell types, ZAK, a MAP3K, is required for ribotoxic stressors to be capable of activating the MAPKs, through which these kinases modulate the transcription of downstream genes [44, 45]. In order to determine whether doxorubicin activates JNK and p38 MAPK, we exposed HaCaT cells to varying concentrations of doxorubicin and detected the increased phosphorylation of JNK and p38 MAPK by immunoblotting against an antibody specific for the phosphorylated form of JNK and p38 MAPK. Phosphorylation of these MAPKs was detected 24h after addition of doxorubicin (5 to 50 μ M; Figure 1A). To determine whether ZAK is required for doxorubicin to induce the phosphorylation of JNK and p38 MAPK, we employed siRNA directed against the 5' portion of ZAK mRNA. SiRNA-mediated knockdown of ZAK strongly suppressed the phosphorylation of JNK and p38 MAPK, determined in cells that harvested 24h after the addition of doxorubicin (Figure 1A).

Similar to other cancer chemotherapeutic agents, doxorubicin induces apoptosis both *in vivo* [144-147] and *in vitro* [87, 88, 135]. To determine whether the pro-apoptotic effects of doxorubicin are regulated through activation of ZAK, we examined the cleavage of PARP (from 116 kDa to 89 kDa) and caspase-3 (from procaspase to p20/p19 and p17 subunits), which are frequently employed as indicators of apoptosis. Knockdown of ZAK suppressed the doxorubicin-induced cleavage of both PARP and caspase-3 in

HaCaT cells (Figure 1A).

Doxorubicin-induced inhibition of protein translation measured by incorporation of [³H]-leucine.

An invariant feature of ribotoxic stressors is their ability to inhibit protein translation [35]. To determine if doxorubicin inhibits protein synthesis, we exposed HaCaT cells to doxorubicin (1 to 25 μ M) for varying times (6, 12, or 24h), at which times cells were exposed to [³H]-leucine for 30 min. Exposure to doxorubicin at concentrations of 2.5 μ M or greater resulted in a progressive decrease in the incorporation of [³H]-leucine (Figure 1B). Cells treated with 2.5 μ M doxorubicin decreased incorporation of [³H]-leucine to approximately 35% by the end of 24h; treatment with 10 and 25 μ M reduced levels of [³H]-leucine incorporation to below 10% at 24h.

Emetine blocks MAPK activation after a high dose of doxorubicin.

Transduction by ribotoxic stressors of signals that lead to activation of SAPKs requires that the ribosomes be involved in protein synthesis at the time that cells are exposed to the stressor [35]. Blockade of protein synthesis by fast-acting inhibitors such as emetine, prior to the exposure of cells to ribotoxic stressors, prevents transduction of the signal(s) that lead to activation of JNK and p38 MAPK [35]. To determine whether prior treatment of HaCaT cells with emetine would block the activation of JNK and p38 MAPK, cells were exposed to emetine or vehicle prior to the addition of doxorubicin. We employed a high concentration of doxorubicin (0.5 mM) to induce the rapid phosphorylation of JNK and p38 MAPK. Doxorubicin induced the phosphorylation of

JNK and p38 MAPK at 2h, but not at 1h or earlier (Figure 2A). Addition of emetine prior to the exposure to doxorubicin completely blocked the phosphorylation of JNK and p38 MAPK (Figure 2A). Doxorubicin suppressed the incorporation of [³H]-leucine by 50% at 1h and completely at 2h. We performed a similar experiment using CdCl₂, which is not a ribotoxic stressor [38] and leads to the activation of JNK and p38 MAPK through other mechanisms. In contrast to doxorubicin, the phosphorylation of JNK and p38 MAPK was not suppressed by emetine (Figure 2C).

Inhibitors of ZAK block doxorubicin-induced apoptosis and MAPK activation in HaCaT cells.

An important goal in cancer chemotherapy is to reduce collateral damage in normal tissues and organs. The administration of effective doses of doxorubicin to cancer patients is frequently limited by the potential for development of cardiotoxicity and other adverse responses [136]. Identification of agents that could selectively suppress the destruction of normal tissue by doxorubicin may permit the administration of larger or more frequent doses of doxorubicin to cancer patients. Previous studies have demonstrated that inhibition of ZAK by an experimental small molecule inhibitor (DHP-2; Eli Lilly) reduces ribotoxic stressor-induced cell death [44, 45]. However, DHP-2 is no longer produced by Eli Lilly and is unavailable. Karaman et al determined the dissociation constants of a panel of 287 distinct protein kinases, including ZAK [93]. Sorafenib (Bayer) was found to have a very high binding affinity for ZAK (K_d=6.3 nM) [93]. In one trial for hepatocellular carcinoma, patients who received sorafenib and doxorubicin together had significantly longer median durations of overall survival and

progression-free survival than patients receiving doxorubicin alone [148]. Another small molecule kinase inhibitor with a high binding affinity for ZAK is nilotinib (Novartis, $K_d=3\text{nM}$)[94]. Although the binding affinities of sorafenib and nilotinib for ZAK have been reported, neither agent has been tested for their ability to inhibit ZAK activity. To determine whether sorafenib or nilotinib would inhibit downstream actions of ZAK, we administered these agents ($1\mu\text{M}$) to HaCaT cells 30 min prior to treatment with doxorubicin ($10\mu\text{M}$ or $25\mu\text{M}$) for 24h. The presence of either inhibitor strongly suppressed doxorubicin-induced phosphorylation of JNK and p38 MAPK (Figure 3A). Sorafenib and nilotinib also reduced the cleavage of PARP and caspase-3, suggesting that doxorubicin-mediated apoptosis was also suppressed.

ZAK inhibitors block daunorubicin-induced apoptosis and MAPK activation in HaCaT cells.

Daunorubicin is an anthracycline that is considered to act by similar mechanisms as doxorubicin but shows less potent antitumor activity [136]. To determine whether the inhibition of ZAK effects daunorubicin-induced apoptosis and MAPK activation, we pretreated HaCaT cells with sorafenib or nilotinib ($1\mu\text{M}$) followed by daunorubicin (2.5, 10, $25\mu\text{M}$) for 24h. Similar to the experiments with doxorubicin, the presence of either inhibitor strongly suppressed daunorubicin-induced phosphorylation of JNK and p38 MAPK (Figure 3B). Sorafenib and nilotinib also reduced the cleavage of PARP and caspase-3, suggesting that daunorubicin-mediated apoptosis was also suppressed.

Inhibitors of JNK or p38 partially block doxorubicin-induced apoptosis in HaCaT cells.

ZAK is a MAP3K that has been shown to induce the phosphorylation of p38 MAPK and JNK. To determine whether suppression of JNK or p38 MAPK would inhibit doxorubicin-induced apoptosis, we administered SB 203580 (10 μ M), SP 600125 (20 μ M), or both in combination to HaCaT cells 30 min prior to treatment with 25 μ M doxorubicin for 24h. The presence of either inhibitor or a combination of resulted in diminished cleavage of PARP and caspase-3, suggesting that JNK and p38-MAPK participated to an extent in doxorubicin-mediated apoptosis. In the presence of a pancaspase inhibitor, zVAD-fmk, doxorubicin-induced apoptosis was completely inhibited (Figure 3C).

ZAK inhibitors and ZAK siRNA do not block doxorubicin-induced apoptosis in HeLa cells.

To test whether ZAK inhibitors would reduce cell death in a cancerous cell line we pretreated HeLa cells with sorafenib or nilotinib (1 μ M) followed by doxorubicin (10 μ M or 25 μ M) for 24h. In contrast to their ability to suppress PARP and caspase-3 cleavage in HaCaT cells, sorafenib and nilotinib failed to reduce PARP or caspase-3 cleavage in HeLa cells (Figure 4A). In HeLa cells, doxorubicin failed to increase the phosphorylation of JNK and p38 MAPK, perhaps because the basal levels of these phosphorylated SAPKs were already elevated in the absence of an inducer.

Nevertheless, the phosphorylation of SAPKs was suppressed by sorafenib and nilotinib, suggesting that the inhibitors were capable of suppressing ZAK in these cells. These data suggest that the elevated endogenous activity of ZAK in HeLa cells may be responsible for the increased basal phosphorylation of JNK and p38 MAPK.

To test whether ZAK siRNA would reduce doxorubicin-mediated apoptosis in HeLa cells, we employed ZAK-targeting siRNA. SiRNA-mediated knockdown of ZAK slightly reduced doxorubicin-mediated cleavage of PARP and caspase-3 in HeLa cells (Figure 4B), indicating that the pro-apoptotic actions of doxorubicin in these cells was mediated in part through activation of ZAK.

Doxorubicin-induced alterations of ZAK protein.

ZAK has two different isoforms, ZAK- α and ZAK- β . ZAK- α has an apparent molecular weight of 91 kDa. ZAK- β is a shorter species of ZAK (51 kDa) [45]. When HaCaT or HeLa cells were treated with doxorubicin and immunoblotted for ZAK, we noticed that the ZAK- α band decreased in intensity (Figure 1A and Figure 4B). In addition, bands of slightly higher molecular weight appeared above the 51 kDa ZAK- β band (Figure 1A * and Figure 4B *).

To determine the kinetics of the disappearance of the ZAK- α band and the appearance of slightly higher molecular weight ZAK- β bands, we added 25 μ M of doxorubicin to HaCaT cells and harvested at 4-hour intervals up to 24 hours for immunoblotting with ZAK Ab. The higher molecular weight ZAK- β bands appeared 8 hours after doxorubicin treatment and increased in intensity thereafter. The disappearance of the 91 kDa ZAK- α began 16 hours after doxorubicin treatment (Figure 5A).

To determine if the doxorubicin-induced disappearance of the ZAK- α band and the appearance of the higher molecular weight ZAK- β bands were due to phosphorylation, we exposed lysates to calf intestinal phosphatase (CIP). The presence of CIP did not alter the disappearance or appearance of the ZAK bands, indicating that neither was a result

phosphorylation (data not shown). Immunoblotting with phospho-p38 confirmed the efficacy of the phosphatase treatment (data not shown).

To determine if the doxorubicin-induced changes in the two ZAK isoforms could result from ubiquitin-mediated proteolysis, we utilized MG-132, an inhibitor of proteasomal degradation. The presence of the MG-132 compound did not affect the disappearance of the 91 kDa ZAK- α band, suggesting that its disappearance was not proteasome dependent (Figure 5B). By contrast, the higher molecular weight ZAK- β bands increased in intensity in the presence of the MG-132 compound, suggesting that these bands undergo proteasome-mediated degradation after doxorubicin treatment.

To determine if the multi-kinase inhibitors, sorafenib and nilotinib, could prevent the doxorubicin-induced changes in ZAK, we pretreated HaCaT cells with sorafenib or nilotinib (1 μ M) followed by doxorubicin (10 or 25 μ M) for 24h. The presence of either inhibitor prevented both the disappearance of ZAK- α and the appearance of the higher molecular weight ZAK- β bands, suggesting that the degradation of ZAK- α and the appearance of higher molecular weight ZAK- β bands are coupled to the activation of ZAK (Figure 5C). To determine whether suppressing the phosphorylation of JNK or p38 MAPK would inhibit doxorubicin-induced degradation or modification of the ZAK isoforms, we administered SB 203580 (10 μ M), SP 600125 (20 μ M), or a combination of the two to HaCaT cells 30 min prior to treatment with 25 μ M doxorubicin for 24h. The presence of either inhibitor or a combination of both did not prevent the disappearance of ZAK- α or the appearance of the higher molecular weight ZAK- β bands (Figure 5D), suggesting that the ZAK-mediated activation of JNK or p38 MAPK did not act retrogradely to cause the disappearance of ZAK- α or the appearance of the higher

molecular weight ZAK- β bands.

Figure 1

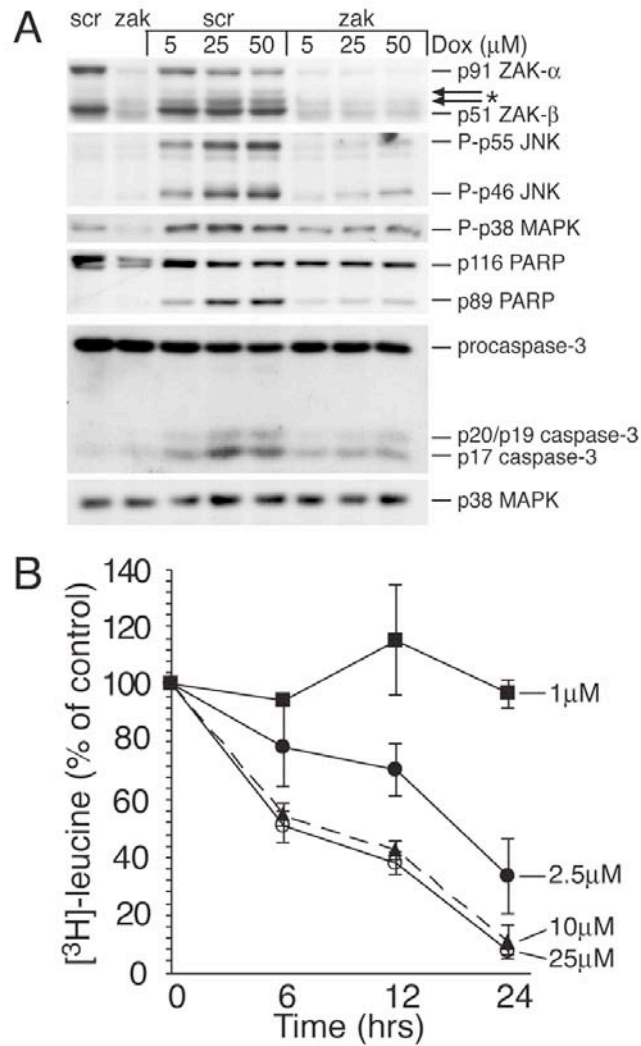


Fig. 1. Effect of ZAK knockdown on doxorubicin-induced apoptosis, activation of SAPKs, and inhibition of protein synthesis. (A) HaCaT cells were exposed to ZAK-specific siRNA (zak) or scrambled siRNA (scr) prior to treatment for 24h with vehicle or doxorubicin (5, 25, or 50 μM), as described in *Materials and Methods*. Lysates were collected and subjected to SDS-PAGE followed by Western blot analysis with the antibodies indicated. (B) HaCaT cells were treated in triplicate for 6, 12, or 24 hours with either vehicle or 1, 2.5, 10, or 25 μM of doxorubicin. Cells were pulse-labeled with [³H]-leucine for the final 30 minutes of treatment.

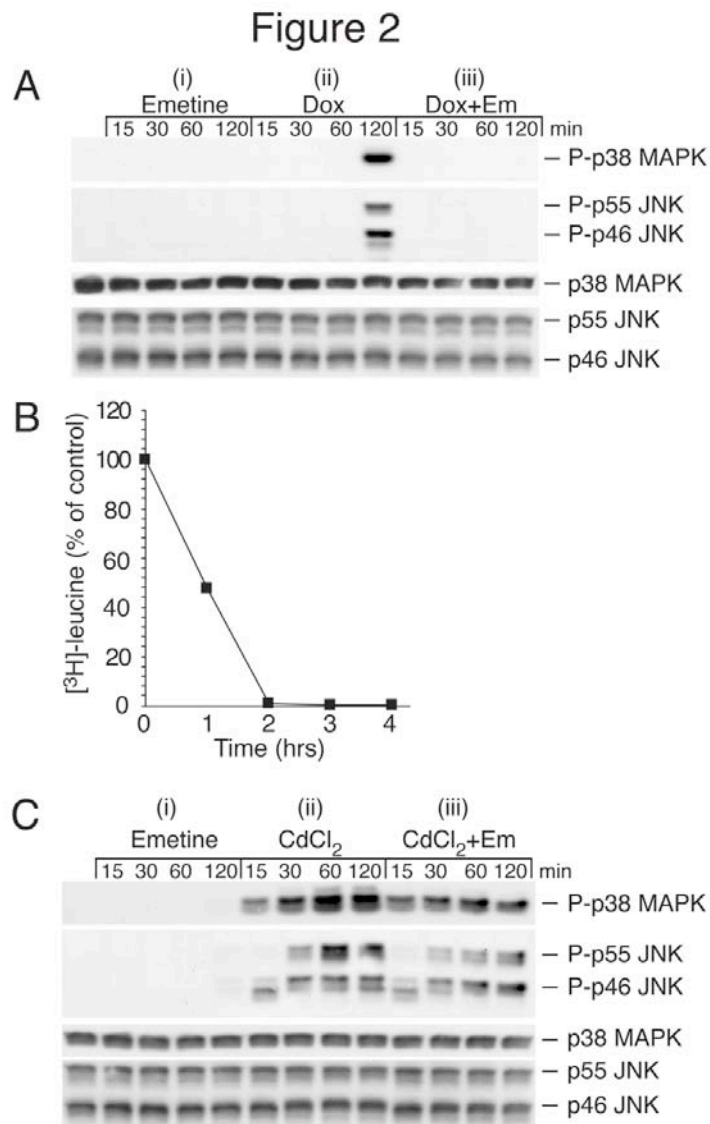


Fig. 2. Inhibition of doxorubicin-induced activation of SAPKs by pretreatment with emetine. (A) HaCaT cells were pretreated with vehicle (ii) or emetine (100 μ g/mL; i and iii) for 30 minutes prior to stimulation with doxorubicin (0.5mM; ii and iii) for 15, 30, 60, or 120 min. Lysates were collected and submitted to SDS-PAGE, followed by Western blot analysis with the antibodies as indicated. (B) HaCaT cells were treated in triplicate for 1, 2, 3, or 4 hours with vehicle or 0.5mM doxorubicin. Cells were pulse-labeled with [³H]-leucine for the final 30 minutes of treatment. (C) HaCaT cells were pretreated with vehicle (ii) or emetine (100 μ g/mL; i and iii) for 30 minutes prior to stimulation as

indicated with CdCl₂ (100μM; ii and iii) for 15, 30, 60, or 120 min. Lysates were collected and subjected to SDS-PAGE, followed by Western blot analysis with the antibodies as indicated.

Figure 3

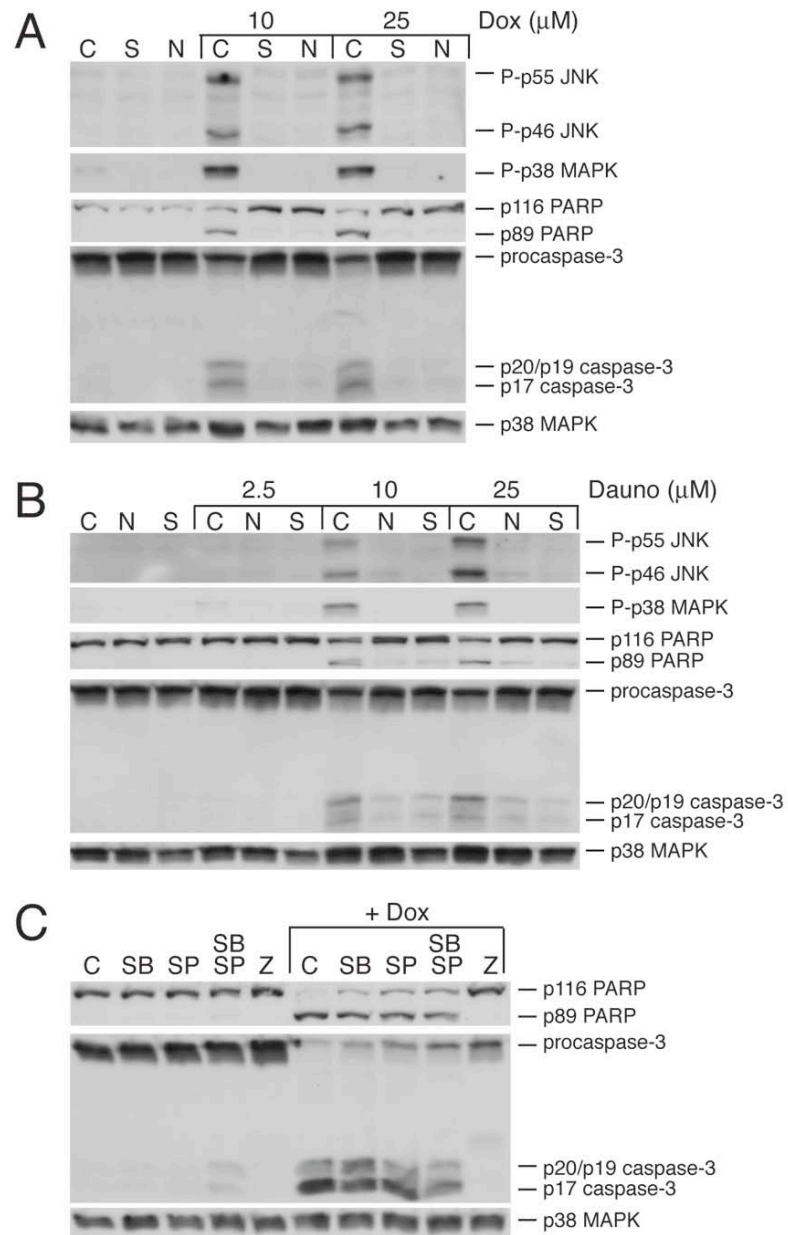


Fig. 3. Effect of inhibitors on doxorubicin- and daunorubicin-induced apoptosis and activation of SAPKs. HaCaT cells were pretreated for 30 minutes with vehicle or inhibitors. Twelve hours later an additional dose of inhibitor was added to each well. Twenty-four hours after the addition of doxorubicin or daunorubicin, lysates were collected and subjected to SDS-PAGE followed by Western blot analysis with the

antibodies as indicated. (A) HaCaT cells were pretreated with vehicle (C), sorafenib (S; 1 μ M), or nilotinib (N; 1 μ M) for 30 minutes, followed by treatment with doxorubicin (10 or 25 μ M) for 24h. (B) HaCaT cells were pretreated with vehicle (C), sorafenib (S; 1 μ M), or nilotinib (N; 1 μ M) for 30 minutes, followed by treatment with daunorubicin (2.5, 10, or 25 μ M) for 24h. (C) HaCaT cells were pretreated with vehicle (C), SB 203580 (SB; 10 μ M), SP 600125 (SP; 20 μ M), both (SB SP), or zVAD-fmk (Z; 25 μ M) for 30 minutes, followed by treatment with doxorubicin (25 μ M) for 24h.

Figure 4

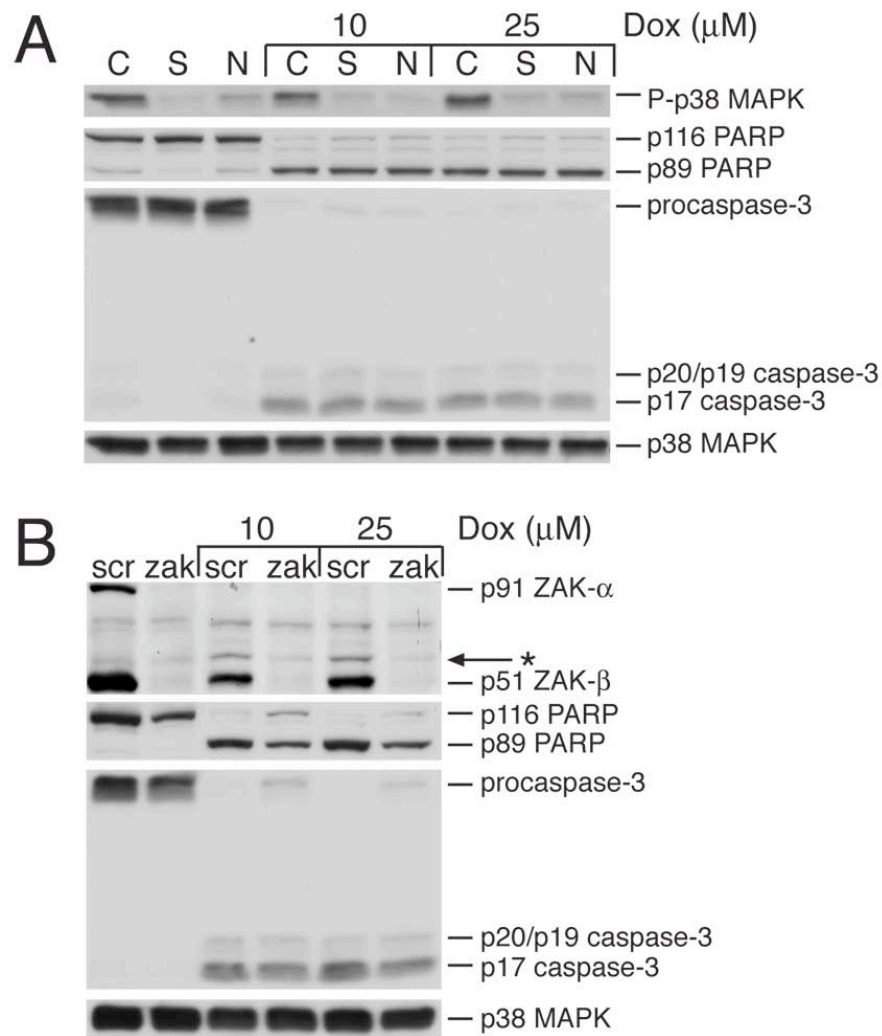


Fig. 4. Effect of ZAK knockdown, sorafenib, or nilotinib on doxorubicin-induced apoptosis in HeLa cells. Following exposure to 10 or 25 μ M doxorubicin, HeLa cell lysates were collected and subjected to SDS-PAGE, followed by Western blot analysis with the antibodies as indicated. (A) Cells were pretreated with vehicle (C), sorafenib (S; 1 μ M), or nilotinib (N; 1 μ M) for 30 minutes, followed by treatment with doxorubicin for 24h. At 12h an additional dose of vehicle or inhibitors was added to each well. (B) Cells

were exposed to scrambled siRNA (scr) or ZAK-specific siRNA (zak) as described in *Materials and Methods*, followed by treatment for 24h with vehicle or doxorubicin.

Figure 5

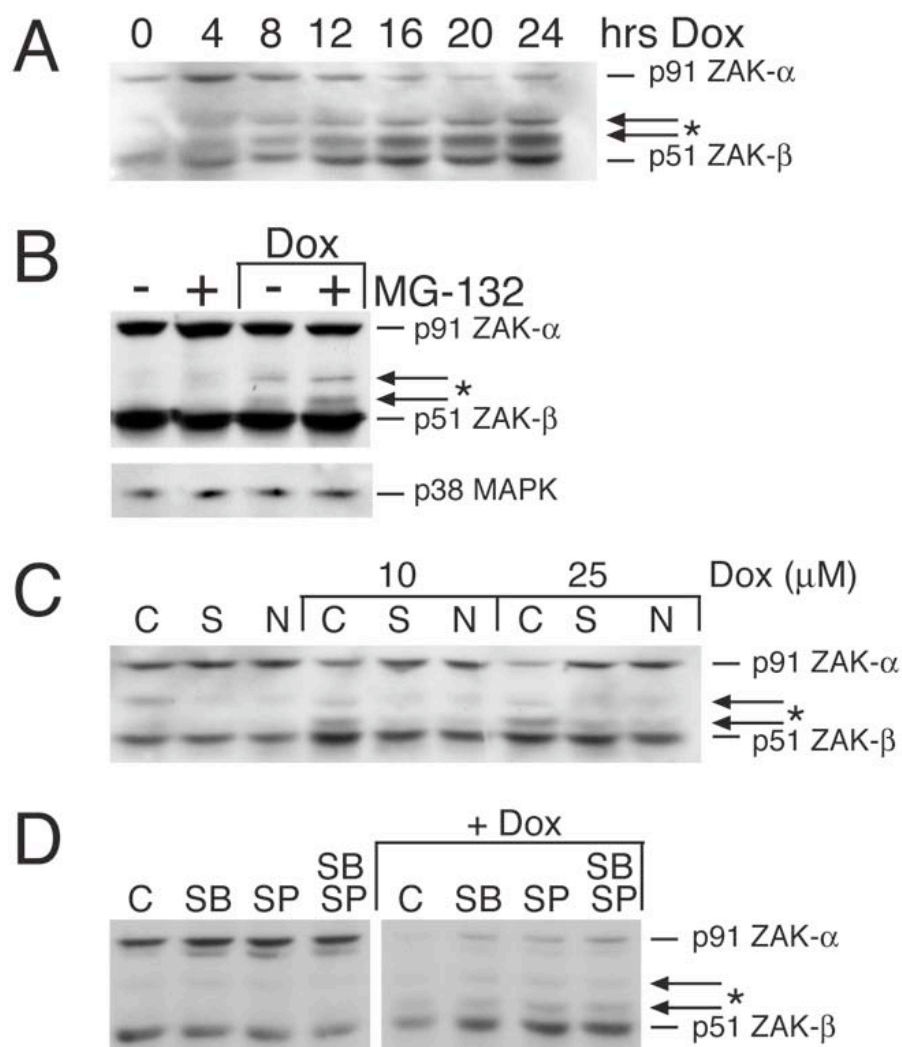


Fig. 5. Effect of inhibition of ZAK, p38 MAPK, or JNK on doxorubicin-induced degradation or modification of ZAK. (A) HaCaT cells were exposed to 25 μ M doxorubicin were harvested at 4h intervals. Lysates were subjected to SDS-PAGE, followed by Western blot analysis with the antibodies as indicated. (B) HaCaT cells were exposed to 25 μ M doxorubicin. Eight hours later, 5 μ M MG-132 or vehicle was added to the corresponding wells. Treatment continued for an additional 12 hours, at which time cells were harvested. Lysates were subjected to SDS-PAGE, followed by Western blot

analysis with the antibodies as indicated. (C) Western blot analysis with ZAK antibody on cell lysates from Figure 3A. (D) Western blot analysis with ZAK antibody on cell lysates from Figure 3C.

DISCUSSION

In an effort to identify initial cellular targets of stressors, we uncovered a novel stress signaling pathway termed “ribotoxic stress”, which results from the inhibition of protein synthesis due to interaction of the translational apparatus with disparate compounds such as antibiotics (anisomycin, blasticidin S, gougerotin), toxins (ricin, Shiga toxin, sarcin, palytoxin, tricothecene mycotoxins) and ultraviolet radiation. Transduction of signals that lead to activation of SAPKs occurs immediately upon exposure to these stressors and requires that the ribosome be actively engaged in protein synthesis at the time of exposure [35-39].

Employing siRNA knockdown and chemical inhibition of ZAK, a MAP3K, Jandhyala et al demonstrated that ZAK was required for ricin and Shiga toxin to mediate the activation of SAPKs and proinflammatory gene expression [45]. ZAK is one of 7 known mixed lineage kinases (MLKs) whose actions have been shown to mediate the activation of JNK and p38 MAPK [149]. An earlier study had demonstrated that siRNA-mediated knockdown of ZAK suppressed the activation of JNK and p38 MAPK by anisomycin and ultraviolet radiation, two other ribotoxic stressors [44]. Taken together, these studies suggest that ZAK uniquely communicates signals between ribosomes and the SAPKs. The intercalation of doxorubicin and daunorubicin into DNA could comprise a major mode of anthracycline-induced cell death induced by these chemotherapeutics. Because doxorubicin also causes RNA damage [139] and inhibits DNA and RNA synthesis [140, 141], it is not unexpected that doxorubicin would also inhibit the synthesis of proteins [142, 143]. In addition to inhibition of protein translation, doxorubicin induces the activation of SAPKs in a number of normal cell types, including hepatocytes [138],

primary mouse macrophages [89] and cardiomyocytes [88], [87]. Our work presented here demonstrates that doxorubicin inhibits protein synthesis (Figure 1B) and activates SAPKs (Figure 1A), which suggests that doxorubicin, may act as a ribotoxic stressor and transmit signals through activation of ZAK.

We have employed clinically relevant doses of doxorubicin, ranging from 1-10 μ M.

HaCaT cells exposed to doxorubicin concentrations that are 2.5 μ M or greater resulted in a progressive decrease in the incorporation of [³H]-leucine over 24h, suggesting that doxorubicin causes inhibition of translation (Figure 1B). Cells treated with higher concentrations (10 and 25 μ M) of doxorubicin responded with decreased levels of [³H]-leucine incorporation to less than 10%, 24h later. Doxorubicin also induced the phosphorylation of p38 MAPK and JNK when examined 24h after addition of 5 to 50 μ M doxorubicin. Knockdown of ZAK with siRNA abrogated the doxorubicin-induced phosphorylation of JNK and p38 MAPK (Figure 1A), suggesting that ZAK was required for doxorubicin-induced activation of SAPKs. Taken together, these results demonstrated that doxorubicin behaves as a characteristic ribotoxic stressor by activating p38 MAPK and JNK through the upstream activation of ZAK.

SAPKs and NF- κ B participate together in the increased expression of proinflammatory cytokines [150-155]. Patients undergoing cancer chemotherapy display many of the classic symptoms caused by the increased expression of cytokines, including IL-1 β , TNF- α , and IL-6. Some of the acute side effects that accompany administration of chemotherapeutics include fatigue, nausea/vomiting, pain, sleep disturbances, cachexia, and depression [82]. A life-threatening adverse reaction to doxorubicin treatment is cardiotoxicity, which is a serious limiting factor in the clinical use of doxorubicin [136].

Preclinical studies indicate that inflammatory responses may be involved in doxorubicin-induced apoptosis of cardiomyocytes [156]. For example, treatment with soluble Fas, an inhibitor of Fas/Fas ligand interaction that can lead to apoptosis, prevents doxorubicin-induced cardiotoxicity and concurrently attenuates the inflammation in cardiomyocytes [157].

Pretreatment with statins can attenuate doxorubicin-induced cardiotoxicity via anti-inflammatory effects [158]. Olson et al reported a novel anthracycline analog, DIDOX, which was structurally modified from doxorubicin. DIDOX inhibits the production of the pro-inflammatory cytokines, TNF- α and IL-2. Studies in animal models show that, compared to doxorubicin, DIDOX inhibits inflammation and reduces cardiotoxicity [159]. Identification of agents that could selectively suppress the doxorubicin-induced inflammation in heart and other organs may prevent the death of non-tumor cells permitting the administration of larger doses of doxorubicin to cancer patients.

Inhibitors of p38 MAPK have been effective in blocking apoptosis of cardiomyocytes following treatment by doxorubicin or daunorubicin [87, 88]. Inhibitors of p38 MAPK reduce the proinflammatory actions of doxorubicin in macrophages but do not reduce the anti-proliferative actions of doxorubicin in a cancer cell line [89]. Employing inhibitors of p38 MAPK, JNK, or ZAK we have asked whether activation of SAPKs would contribute to the doxorubicin-induced inflammation and apoptosis of non-tumor cells. Our findings that siRNA-mediated knockdown of ZAK suppressed the doxorubicin-induced apoptosis in HaCaT cells, as demonstrated by the reduction in cleavage of PARP and caspase-3 (Figure 1A), is consistent with the role of ZAK acting through JNK and p38 MAPK to induce apoptotic death.

Previous studies have demonstrated that inhibition of ZAK by an experimental small molecule inhibitor (DHP-2; Eli Lilly) reduces ribotoxic stressor-induced cell death [44, 45]. To further demonstrate the role of ZAK in doxorubicin-induced apoptosis of normal cells we employed two multi-kinase inhibitors with high affinity for ZAK, sorafenib and nilotinib [93, 94]. We demonstrated that sorafenib and nilotinib were each as effective as siRNA-mediated ZAK knockdown (Figure 3), suggesting that these inhibitors can suppress the signaling pathway initiated by ZAK. In HaCaT cells, a pseudo-normal cell line derived from keratinocytes, sorafenib and nilotinib blocked doxorubicin- and duanorubicin-induced apoptosis and the phosphorylation of SAPKs (Figure 3A & B). The suppression of JNK or p38-MAPK by the kinase inhibitors SP 600125 and/or SB 203580 showed partial protection against doxorubicin-induced apoptosis. However, the inhibition of apoptosis by these inhibitors was not as complete as sorafenib or nilotinib (Figure 3C).

HeLa cells were more sensitive than HaCaT cells to the pro-apoptotic effects of doxorubicin. In contrast to the results in HaCaT cells, both sorafenib and nilotinib were unable to block doxorubicin-induced apoptosis (Figure 4A). We confirmed the role of ZAK in cytotoxicity following doxorubicin treatment by employing siRNA knockdown of ZAK (Figure 4B). The inability of ZAK inhibition to suppress the pro-apoptotic actions of doxorubicin in HeLa cells, in contrast to HaCaT cells, suggests that pathways other than ZAK may play a role in cytotoxicity, in these cells, after doxorubicin treatment. The differential sensitivity of normal and cancer cells to the pro-apoptotic actions of doxorubicin suggest that inhibitors of ZAK may be effective in protection of

normal cells against the cytotoxic activities of doxorubicin. However, this possibility must await further studies in an animal model.

ZAK has two different isoforms, ZAK- α (91 kDa) and ZAK- β (51 kDa). The two isoforms have identical protein kinase domains, including the ATP binding site, and separate functions for the two have not been defined [45]. HaCaT or HeLa cells treated with doxorubicin and immunoblotted for ZAK displayed a progressive decrease in the ZAK- α band and the appearance of ZAK- β bands of larger size (Figure 1A and Figure 4B). Abrogation of these changes after exposure of the cells to sorafenib and nilotinib suggests that these changes occur following stimulation of ZAK by upstream signaling pathways. Degradation of ZAK following its activation suggests a homeostatic mechanism to suppress the continued activation of SAPKs by ZAK. Pretreatment of cells with the p38 MAPK inhibitor SB 203580, the JNK inhibitor SP 600125, or a combination of the two failed to prevent the doxorubicin-induced protein changes in ZAK, suggesting that activation of p38 MAPK or JNK are not involved in targeting ZAK for degradation. We utilized MG-132, an inhibitor of proteasomal degradation, to determine if the doxorubicin-induced alterations in the two ZAK isoforms could result from ubiquitin-mediated proteolysis. The disappearance of the 91 kDa ZAK- α band was not prevented by the presence of MG-132, suggesting that it was not proteasome dependent. By contrast, the higher molecular weight isoforms of ZAK- β accumulated in the presence of the MG-132 compound, suggesting that these bands may represent ubiquitinated forms of ZAK- β .

Sorafenib and nilotinib are in clinical use and exhibit very few side effects in patients.

We suggest that these inhibitors could be employed in combination with doxorubicin to

treat cancer patients because our data suggests that sorafenib or nilotinib might be able to reduce doxorubicin-induced apoptosis and SAPK phosphorylation in normal tissues. However, it is unknown if the presence of sorafenib or nilotinib in combination with doxorubicin could effect the antitumor activity of doxorubicin. Further experiments in animal models would determine the efficacy of treatment with doxorubicin and either sorafenib or nilotinib.

CHAPTER 4

Doxorubicin and daunorubicin activate the NALP3 inflammasome in murine primary bone marrow derived macrophages.

Manuscript in Preparation

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Contribution: BMDM cells from Wt, Asc-, Caspase-1-, and NALP3- mice, in Figure 4, were made by Meghan Lindauer.

ABSTRACT

IL-1 has been shown to be required for many xenobiotic compounds and pathogens to initiate proinflammatory responses. IL-1 β is synthesized as an inactive precursor, pro-IL-1 β , which is processed into the mature 17 kDa IL-1 β by a multiprotein complex called the inflammasome. The inflammasome consists of a member of the NALP protein family, the adaptor protein ASC, and the IL-1 β -converting enzyme caspase-1. Patients undergoing cancer chemotherapy display many of the classic symptoms of sickness behavior caused by the increased expression of cytokines, including IL-1 β , TNF- α , and IL-6. The anthracycline drugs doxorubicin and daunorubicin are two of the most effective and widely used anticancer agents for the treatment of both hematologic and solid tumors. In the current studies we demonstrate that both doxorubicin and daunorubicin activate the inflammasome in murine bone marrow derived macrophages (BMDM), leading to secretion of mature IL-1 β . BMDM from mice deficient in ASC, caspase-1, or NALP3 all show a significant decrease in the doxorubicin-mediated release of mature IL-1 β , indicating that these proteins are involved in doxorubicin-induced inflammasome activation. In addition, the use of BMDM from mice deficient in NALP3 demonstrates that doxorubicin specifically activates the NALP3 inflammasome.

INTRODUCTION

In recent years, IL-1 (an apical cytokine) has been shown to be required for many xenobiotic compounds and pathogens to initiate proinflammatory responses. IL-1 is produced by activated macrophages and epithelial cells and it induces the production of chemokines, matrix metalloproteases and promotes neutrophil recruitment and fibroblast proliferation [160-162]. IL-1 β is synthesized as an inactive precursor, pro-IL-1 β , and requires two distinct signals for synthesis, processing, and secretion of IL-1 β . The first signal, which induces the expression of the 35 kDa pro-IL-1 β , is mediated by the activation of NF- κ B and is usually conveyed in macrophages by activation of TLRs. The second signal induces the processing of the pro-IL-1 β protein into the mature 17 kDa IL-1 β by a multiprotein complex called the inflammasome [163-166]. The inflammasome is fundamental for microbial detection [167] and sensing stress or endogenous danger signals such as extracellular ATP, hypotonic stress, or toxins associated with cell injury [168, 169]. Upon sensing a danger signal, the inflammasome complex is assembled by a member of the NALP protein family of NOD-like receptors, such as NALP1, NALP2, or NALP3, and the adaptor protein ASC that forms a scaffold connecting the NALP with the IL-1 β -converting enzyme (ICE), or caspase-1 (Figure 3) [170, 171]. In the studies presented here, we suggest a novel mechanism for doxorubicin-induced inflammation by activation of the NALP3 inflammasome.

MATERIALS AND METHODS

Reagents and Antibodies. Doxorubicin, daunorubicin, LPS (L-2630), N-acetyl cysteine (NAC), diphenyleneiodonium chloride (DPI) and insulin (I6634) were purchased from Sigma-Aldrich (St. Louis, MO). Trichloroacetic acid (TCA) was purchased from Fisher Scientific (Pittsburgh, PA). Antibody against IL-1 β was purchased from Abcam (Cambridge, MA) and antibody against p38 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse IL-1 β enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go was purchased from eBioscience (San Diego, CA).

Animals and Animal Procedures. All animal procedures were performed according to protocols that have been approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University, Portland, Oregon. C57BL/6J and caspase-1 deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). ASC- and NALP3-deficient mice were kindly provided by Genetech (San Francisco, CA). Male mice, 8 to 10 weeks of age, were used throughout the experiments. Before experimental procedures, mice were anesthetized intraperitoneally with 80 mg/kg of ketamine and 10 mg/kg of xylazine.

Isolation of Bone Marrow- Derived Macrophages (BMDM). Cells were prepared from C57BL/6J, ASC-, Caspase-1-, and NALP3-deficient mice. Marrow was flushed from femurs and tibias with PBS and cultured in alpha-Minimum Essential Medium (α MEM, Cellgro, Herndon, VA), supplied with 10% Fetal Bovine Serum (FBS, Cellgro, Herndon,

VA), 50 µg/ml gentamicin, and 100 ng/ml recombinant mouse Colony Stimulating Factor 1 (CSF-1, R&D Systems, Minneapolis, MN) for 72 hrs on non-tissue culture treated 10-cm Petri dishes. BMDM were passaged and cultured for an additional 72h. One confluent 10-cm dish was plated into one 6-well or one 12-well tissue culture plate (Sarstedt, Newton, NC) for an additional 24 hrs before experiments.

Treating BMDM. Cells were serum deprived in α -MEM for 30 minutes followed by treatment with 50ng/mL LPS for 4 hours. Cells were rinsed and fresh media was added followed by exposure to the indicated doses of doxorubicin or daunorubicin for 8hrs prior to harvest. In experiments involving co-treatment with doxorubicin/daunorubicin, NAC (30mM) or DPI (25µM) were added after LPS pretreatment.

Immunoblotting. BMDM cells were lysed in 2XESB lysis buffer in preparation for immunoblotting. Equal volumes of the cell lysates were separated on a 10% denaturing polyacrylamide gel in the presence of sodium dodecyl sulfate and were transferred onto polyvinylidene difluoride membranes according to standard laboratory procedures. Proteins from BMDM media supernatants were precipitated using TCA and run on 13% gels. Briefly, samples were incubated at 4°C overnight with 200µg insulin carrier and 400µL ice cold 100% TCA prior to centrifugation at 12,000 rpm for 5 min. Pellets were air dried and resuspended in 2X ESB lysis buffer followed by separation on a 13% denaturing polyacrylamide gel. Membranes were incubated with the indicated antibodies and the corresponding horseradish peroxidase-conjugated secondary antibodies; signals were detected by using enhanced chemiluminescence.

ELISA. Media from BMDM were collected and run in triplicate according to the manufacturer's protocol.

RESULTS

Doxorubicin-induced IL-1 β release from primed bone marrow derived macrophages (BMDM).

IL-1 is a key component for the *in vivo* proinflammatory actions of anthracyclines.

Addition of anthracyclines alone to unprimed macrophages *in vitro* fails to elicit the expression of IL-1 β . The inability to induce expression of IL-1 β *in vitro* is shared by several other proinflammatory agents such as asbestos, silicates, and uric acid crystals, all of which exert their proinflammatory actions by inducing the processing of IL-1 β through activation of the inflammasome [169, 172, 173]. Although these agents are unable to induce the transcriptional expression of IL-1 β in unprimed macrophages, when added to macrophages that have been primed by LPS to express pro-IL-1 β , the pro-IL-1 β is processed by caspase 1 and secreted as active IL-1 β .

To determine whether doxorubicin would activate the inflammasome, BMDM were primed for 4 hours with LPS and then exposed to doxorubicin (2.5, 10, 25, or 100 μ M) for 8 hours. Lysates and media were analyzed for both pro-IL-1 β and mature IL-1 β respectively. In unprimed macrophages, doxorubicin failed to induce pro-IL-1 β in the cells (Figure 1A, upper panel) or to secrete IL-1 β into the medium (Figure 1B). When primed macrophages were exposed to doxorubicin there was a dose dependent increase in pro-IL-1 β (Figure 1A, upper panel). Primed macrophages exposed to doxorubicin showed increased secretion of mature IL-1 β by immunoblot, which was maximal at 10 μ M doxorubicin (Figure 1B). Secreted IL-1 β from LPS primed macrophages was maximal (~700pg/mL) after exposure to 10 μ M of doxorubicin and decreased to 510 and

410pg/mL in BMDM exposed to 25 μ M and 100 μ M doxorubicin, respectively (Figure 1A, lower panel).

Daunorubicin-induced IL-1 β release from primed BMDM.

Daunorubicin is an anthracycline that is considered to act by similar mechanisms as doxorubicin but shows less potent antitumor activity [136]. To determine whether daunorubicin would activate the inflammasome, LPS primed BMDM were exposed to daunorubicin (.1, .25, 1, or 2.5 μ M) for 8 hours. Lysates and media were analyzed for both pro-IL-1 β and mature IL-1 β respectively. In unprimed macrophages, daunorubicin failed to induce pro-IL-1 β in the cells (Figure 2A, upper panel) or to secrete IL-1 β into the medium (Figure 2B). When LPS primed macrophages were exposed to doses of daunorubicin greater than .25 μ M there was an increase in pro-IL-1 β (Figure 2A, upper panel). Secreted IL-1 β from LPS primed macrophages was maximal (230 pg/mL) after exposure to 1 μ M or 2.5 μ M of daunorubicin (Figure 2A, lower panel).

Doxorubicin-induced IL-1 β release requires ASC, caspase-1 and NALP3.

The processing of pro-IL-1 β into mature IL-1 β is mediated by a multiprotein complex called the inflammasome [163-166], which consists of the adaptor protein ASC that forms a scaffold connecting NALP with the IL-1 β -converting enzyme (ICE), or caspase-1 [170, 171]. There are numerous members of the NALP protein family of NOD-like receptors, such as NALP1, NALP2, or NALP3 [170, 171] that vary depending on the agent activating the inflammasome (Figure 3). To determine if BMDM respond to doxorubicin through the NALP3 inflammasome, we compared the doxorubicin-induced

IL-1 β release from wildtype cells to the IL-1 β release from BMDM deficient in ASC, caspase-1, or NALP3. BMDM were primed for 4 hours with LPS and exposed to doxorubicin (10 or 25 μ M) for 8 hours, after which media were analyzed for mature IL-1 β . In all four strains of unprimed BMDM, doxorubicin failed to induce secretion of IL-1 β into the medium (Figure 4A&B). IL-1 β secretion in LPS primed wildtype BMDM was maximal (~325pg/mL) when cells were exposed to either 10 or 25 μ M doxorubicin. Secreted IL-1 β from LPS primed macrophages exposed to doxorubicin was reduced by more than 13-fold in ASC-, caspase-1-, and NALP3-deficient BMDM compared to wild type BMDM (Figure 4A).

ROS inhibitors reduce doxorubicin- and daunorubicin-induced secretion of IL-1 β from BMDM.

If doxorubicin- and daunorubicin-mediated release of IL-1 β acts through the NALP3 inflammasome then agents shown to inhibit the activity of the NALP3 inflammasome should block the appearance of IL-1 β after exposure to doxorubicin or daunorubicin. ROS generation has been implicated in the activation of NALP3 inflammasome [173, 174] and ROS inhibitors like N-acetyl-cysteine (NAC) and diphenyliodonium (DPI) have been reported to block NALP3 inflammasome activation by a poorly understood mechanism [175]. BMDM were primed for 4 hours with LPS and then co-treated with NAC or DPI and doxorubicin (10 μ M) or daunorubicin (2.5 μ M) for 8 hours. Primed macrophages exposed to doxorubicin or daunorubicin demonstrated an increase in secreted IL-1 β to 275pg/mL and 250pg/mL, respectively. Primed BMDM co-treated with doxorubicin and DPI or NAC caused an 11- or 2.75-fold decrease in mature IL-1 β ,

respectively. Primed BMDM co-treated with daunorubicin and DPI or NAC caused a 10- or 2-fold decrease in mature IL-1 β , respectively (Figure 5).

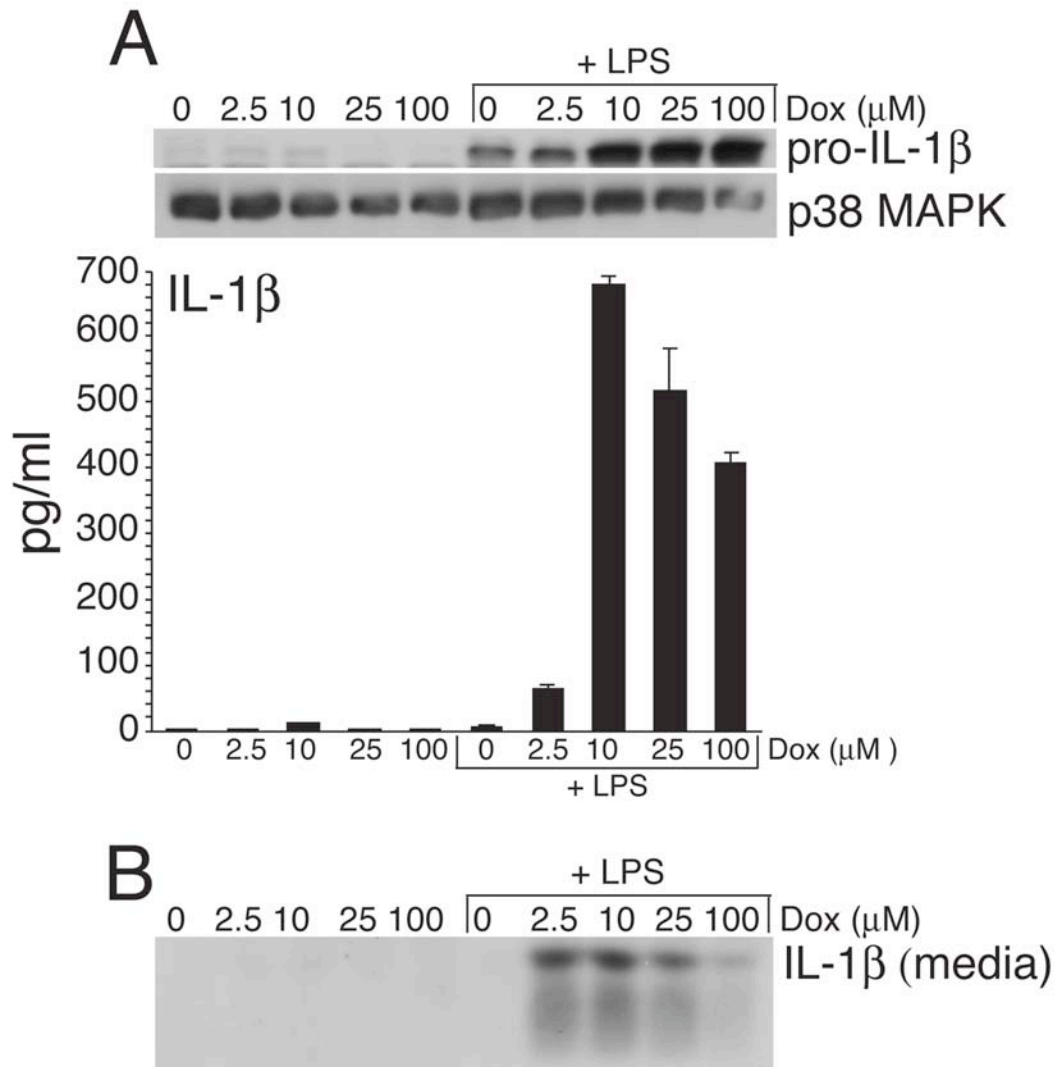


Figure 1. Doxorubicin-induced IL-1 β secretion from BMDM. BMDM were pretreated with LPS (50ng/ml) or control vehicle for 4h, after which they were exposed to doxorubicin (2.5, 10, 25, or 100 μ M) or control vehicle for 8h. (A) Upper panel: Immunoblotted protein lysates show pro-IL-1 β expression in primed cells. Lower panel: Determination of IL-1 β released into the culture medium at time of harvest by ELISA. (B) Immunoblotted media show mature IL-1 β secretion from primed cells treated with doxorubicin.

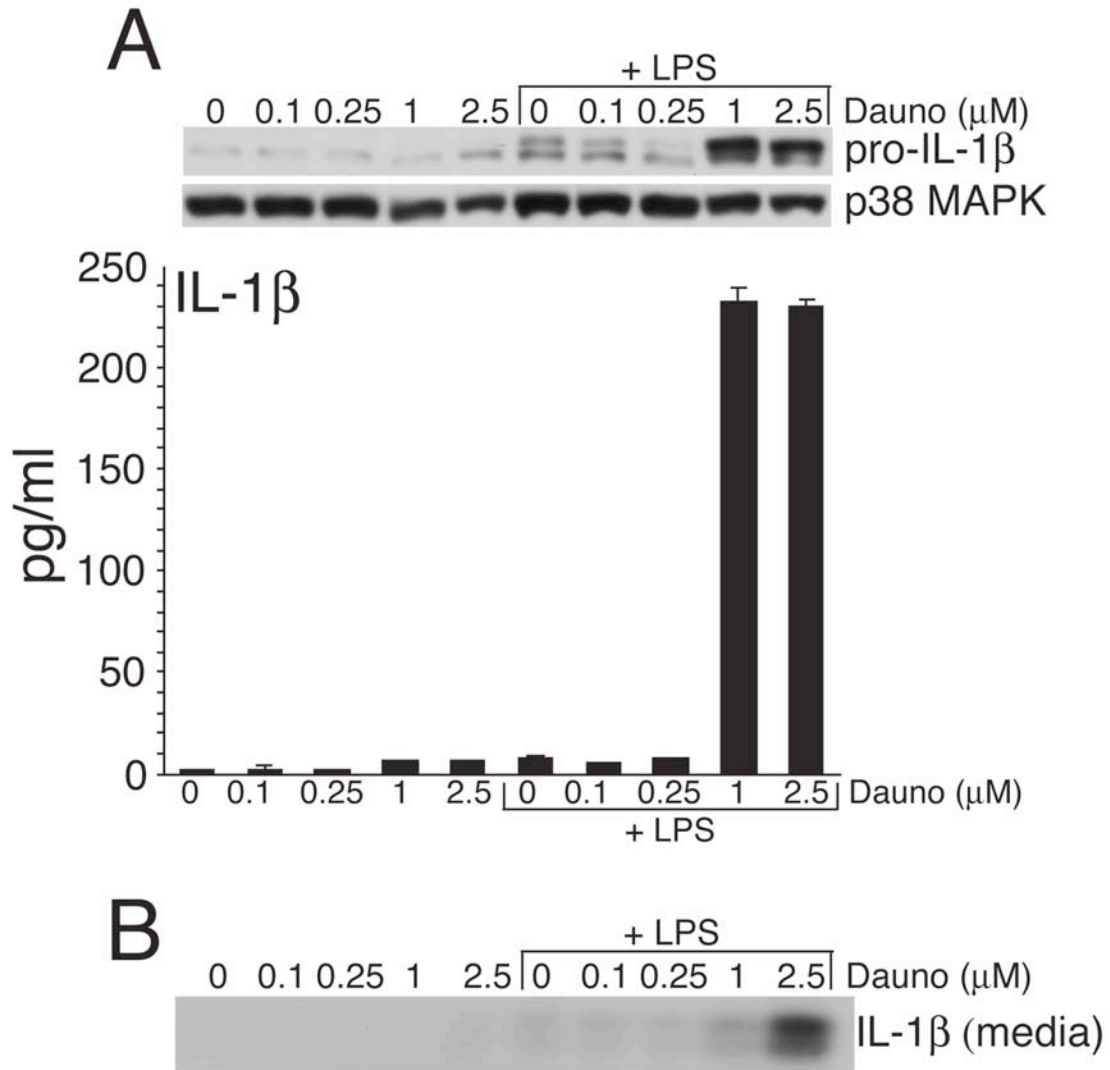


Figure 2. Daunorubicin-induced IL-1 β secretion from BMDM. BMDM were pretreated with LPS (50ng/ml) or control vehicle for 4h, after which they were exposed to daunorubicin (0.1, 0.25, 1 or 2.5 μ M) or control vehicle for 8h. (A) Upper panel: Immunoblotted protein lysates show pro-IL-1 β expression in primed cells. Lower panel: Determination of IL-1 β released into the culture medium at time of harvest by ELISA. (B) Immunoblotted media show mature IL-1 β secretion from primed cells treated with doxorubicin.

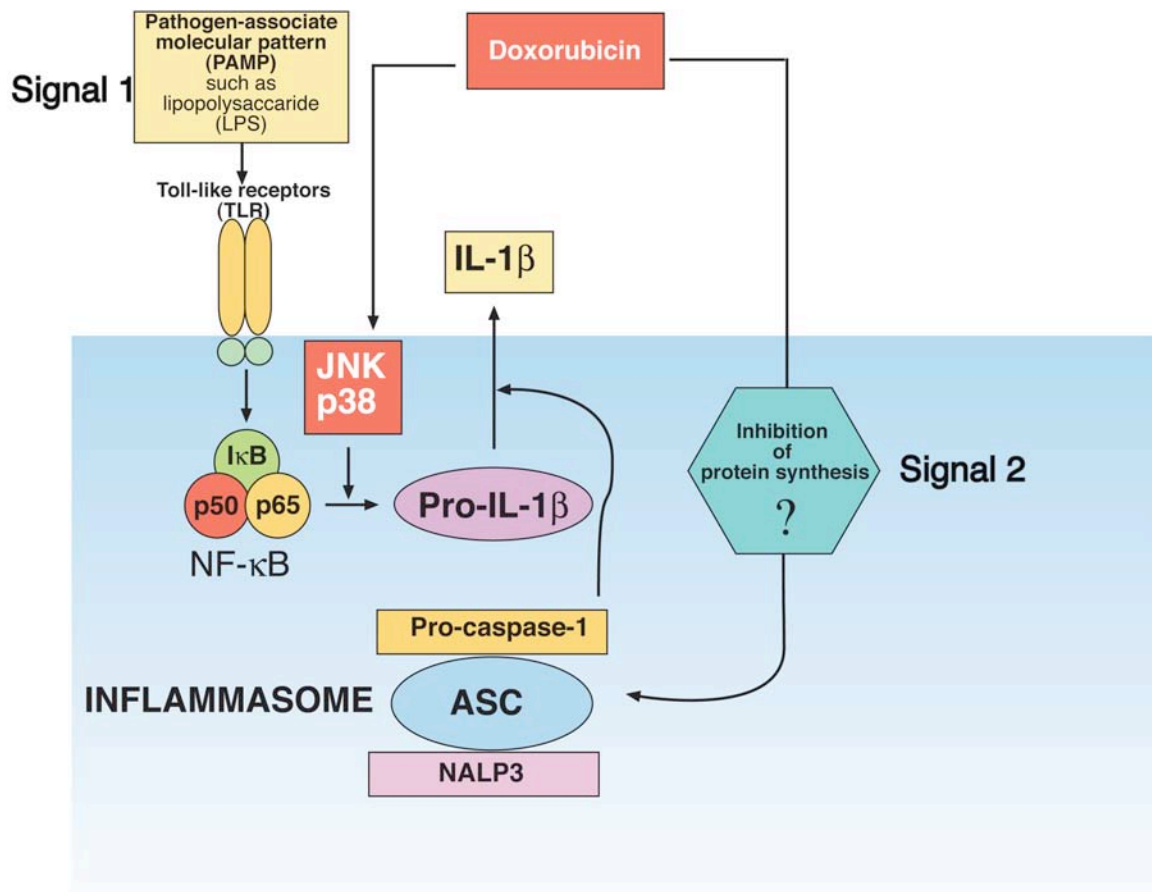


Figure 3. Activation of the NALP3 Inflammasome. Signal 1: After the recognition and binding of PAMPs to TLRs, these receptors mediate the activation of NF-κB. Activation of NF-κB and SAPKs leads to the increased transcription of pro-IL-1β. Signal 2: Perhaps by inhibiting protein synthesis, doxorubicin mediates the release of IL-1β protein by inducing the assembly of the inflammasome, which activates caspase-1 and cleaves pro-IL-1β.

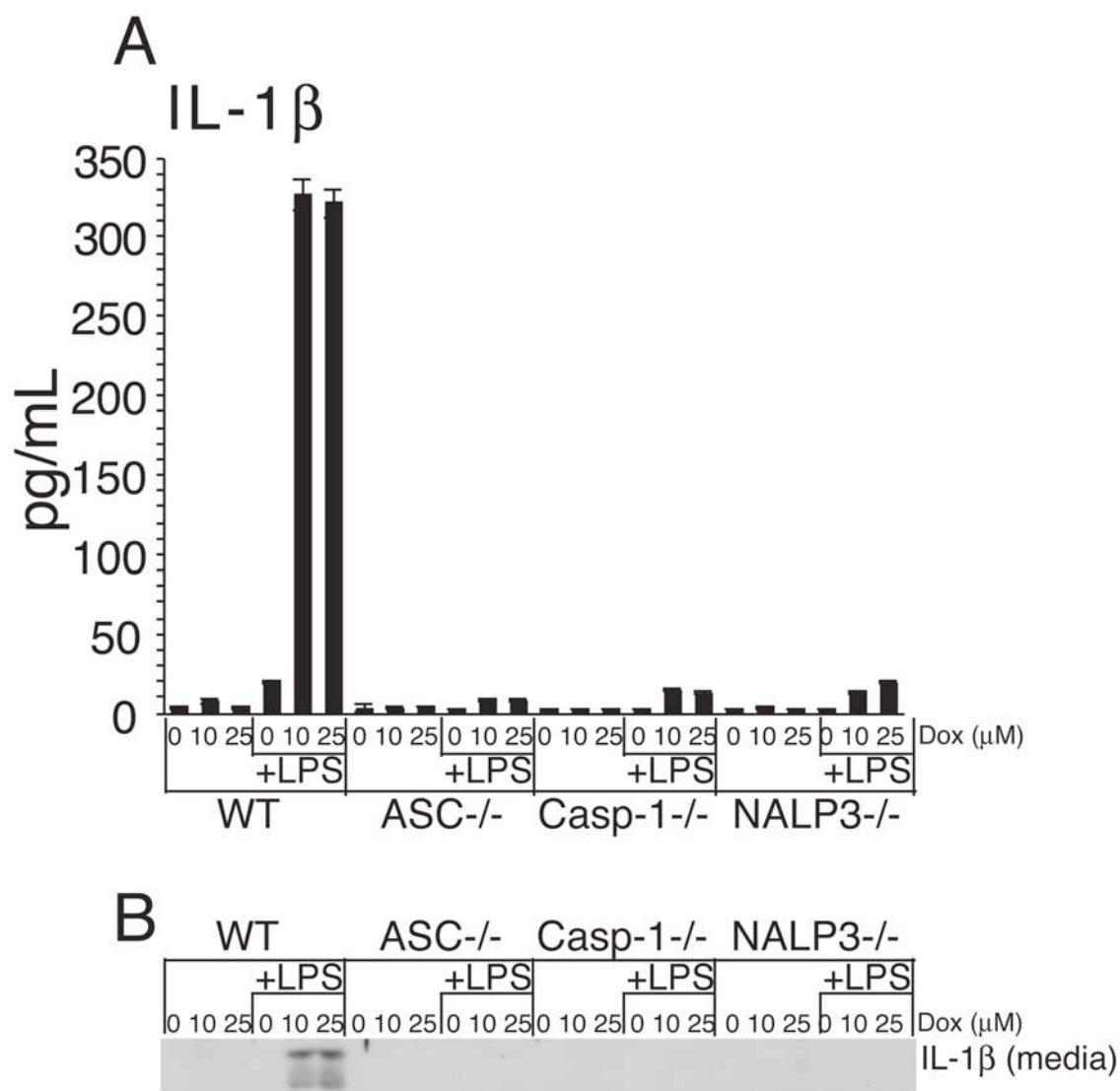


Figure 4. Doxorubicin-induced IL-1 β release requires ASC, caspase-1 and NALP3.

BMDM were pretreated with LPS (50ng/ml) or control vehicle for 4h, after which they were exposed to doxorubicin (10 or 25 μ M) or control vehicle for 8h. (A) Determination of IL-1 β released into the culture medium at time of harvest by ELISA. (B)

Immunoblotted media show mature IL-1 β secretion from primed wildtype cells treated with doxorubicin.

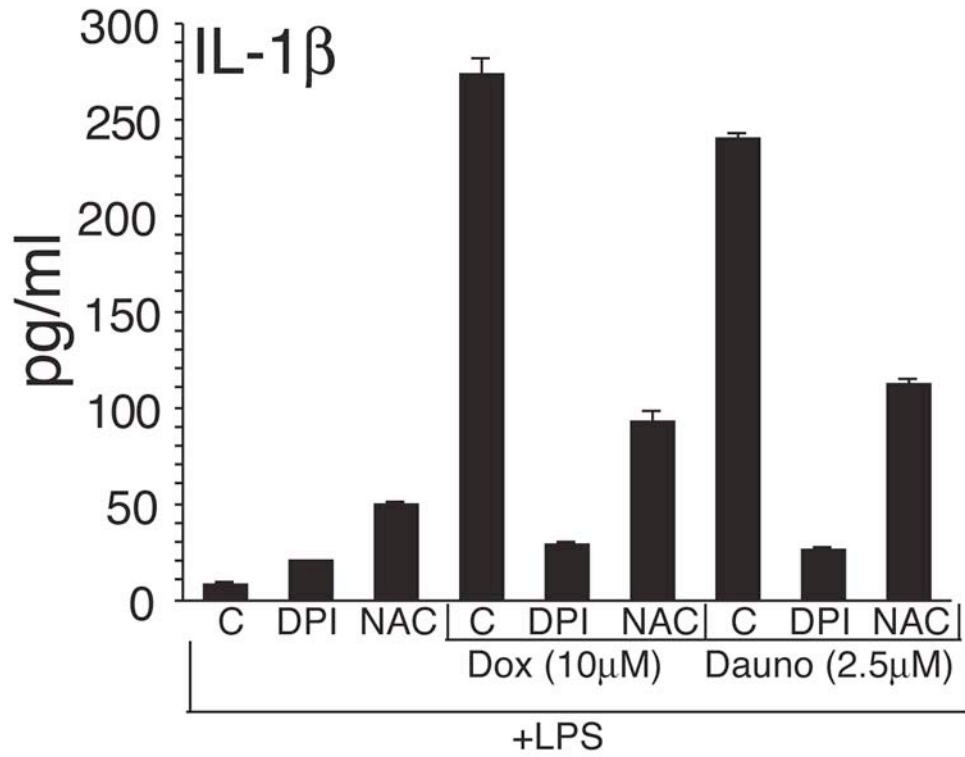


Figure 5. ROS inhibitors reduce doxorubicin- and daunorubicin-induced secretion of IL-1 β from BMDM. BMDM were pretreated with LPS (50ng/ml) for 4h, after which they were exposed to DPI (25 μ M), NAC (30mM), doxorubicin (10 μ M), dox+DPI, dox+NAC, daunorubicin (2.5 μ M), dauno+DPI, or dauno+NAC for 8h. Determination of IL-1 β released into the culture medium at time of harvest by ELISA.

DISCUSSION

As mentioned previously, patients who are undergoing cancer chemotherapy display many side effects caused by the increased expression of cytokines, such as IL-1 β , TNF- α , and IL-6. A specific example of the effect of increased cytokines is patients with non-Hodgkin lymphoma, who have a negative prognostic factor if they have inflammatory or B symptoms (fever >38°C, weight loss >5%, or night sweats), which are associated with increased plasma levels of inflammatory cytokines [176]. Elucidating the molecular basis by which doxorubicin communicates its signals that result in inflammation, which might not be related to doxorubicin's efficacy as an anti-cancer drug, may present opportunities to decrease the inflammation in patients treated with doxorubicin.

Our data suggest a novel mechanism for doxorubicin- and daunorubicin-mediated inflammatory signaling through the NALP3 inflammasome. We demonstrate that doxorubicin and daunorubicin activate caspase-1, resulting from inflammasome assembly, which leads to subsequent conversion of pro-IL-1 β to IL-1 β , followed by secretion of mature IL-1 β (Figures 1&2). The composition of the various inflammasomes differs according to the signals that induce their assembly. Identifying the components of the doxorubicin-induced inflammasome could facilitate utilizing methods to reduce doxorubicin-mediated inflammation. The data in Figure 3 demonstrates that BMDM require the 3 components (ASC, caspase-1, and NALP3) of the NALP3 inflammasome in order to process and release mature IL-1 β following exposure to doxorubicin. ROS production is a common event that occurs during NALP3 inflammasome activation by all known activators, but is insufficient to activate the NALP3 inflammasome on its own [175]. Two different ROS inhibitors, DPI or NAC, were able to reduce IL-1 β secretion in

primed BMDM exposed to either doxorubicin or daunorubicin (Figure 4). This is consistent with the notion that intracellular ROS may be permissive for activation of the NALP3 inflammasome.

Taken together, these results indicate a novel mechanism for doxorubicin-mediated inflammation. Further studies in whole animal models are necessary to confirm the importance of the individual proteins of the inflammasome complex. If future studies can confirm that doxorubicin acts through the NALP3 inflammasome, *in vivo*, then drugs that dampen doxorubicin-triggered inflammasome activation may complement conventional chemotherapy drugs to reduce inflammation in non-cancerous cells and healthy tissues.

CHAPTER 5

Summary and Conclusions

A Mouse Model of HUS

Stx is the ribotoxic stressor responsible for HUS. Therapeutic experimentation and an in-depth understanding of the key players in the development of the symptoms of HUS require an animal model of human HUS. Although the administration of Stx alone to primates recapitulates symptoms of HUS [100], the development of an HUS model in small animals has been less successful [101, 102]. Although the co-administration of LPS and Stx has been able to reproduce many of the symptoms of HUS in a murine model [107], these data are controversial because LPS is not required to produce HUS in a primate model [100] and because the required presence of LPS in humans with HUS has not been substantiated.

In chapter 2, we developed a mouse model of HUS by administering only endotoxin-free Stx2 in multiple doses over 7 days. Our model is unique from other mouse models for a number of reasons. First, we were able to administer Stx2, without LPS, over a longer time period. This paradigm is similar to exposure times in humans who come into contact with STEC. Second, our model demonstrated glomerular thrombotic microangiopathy (TMA), which is a hallmark of human HUS. The lack of glomerular TMA has been a primary impediment in developing a murine model of HUS.

The development of a murine model of HUS provides an economic advantage for therapeutic experimentation and, in view of the ready availability of mouse strains that are deficient in key mediators of inflammatory processes. Furthermore, a mouse model

offers the benefit of employing the power of mouse genetics to elucidate the molecular basis by which Stx communicates its signals during HUS, resulting in kidney failure. There are numerous opportunities for further studies that could define Stx signaling during HUS using our mouse model. For example, in addition to the work presented in chapter 2, we attempted to use this mouse model to examine the importance of TNF- α , IL-1, and macrophages in the development of HUS. HUS is viewed as an inflammatory disease, in which the apical cytokines, TNF- α and IL-1 β , appear to play a significant role in the human disease. In data not shown, we determined that mice deficient in TNFR1, IL-1R1, or a combination of TNFR1 and IL-1R1 did not show a significant decrease in the appearance of the symptoms of HUS, suggesting that these cytokines may not play a role in orchestrating the complex phenotype that characterizes HUS. Macrophages are thought to play a central role in the development of glomerular lesions in HUS. Palermo et al. demonstrated that depletion of macrophages by administration of liposome-encapsulated clodronate resulted in a 50% increase in survival of mice following injection of Stx2 [127], suggesting a potential role for macrophages in development of HUS. To test the potential role of macrophages in HUS in our model, we employed transgenic (MAFIA) mice that express an inducible gene driven by the c-fms promoter for FAS-mediated apoptosis of macrophages upon injection of a synthetic dimerizer, AP20187. After the five day depletion of macrophages, the mice were administered Stx2 in multiple doses over 7 days. In data not shown, these mice demonstrated less severe symptoms of HUS, specifically a reduction in the indicators of kidney failure. However, when these mice were examined in more detail, we determined that after the 5 days of injection of AP20187, and before beginning Stx2 injections, the mice exhibited very high

levels of circulating cytokines. Palermo et al. demonstrated that when high doses of IL-1 β were injected in vivo, Stx2 lethality was decreased [103]. Therefore, it was difficult for us to determine whether the reduction in kidney failure was due to the absence of macrophages or whether it was due instead to the increased pro-inflammatory condition caused by the depletion of macrophages.

Therapeutic Intervention Against Two Ribotoxins

Stx

Currently, specific therapeutics for HUS are lacking, and therapy for patients is primarily supportive. Antibodies (chimeric, humanized, and fully human) have been developed for potential passive immunization [109-113]. However, it is unclear whether administration of anti-Stx therapeutics would be effective when administered after signs have developed in humans. Some investigators have demonstrated that passive immunization may be a viable option in the treatment of signs of HUS in animal models. A human antibody against Stx2 administered to piglets after the onset of diarrhea due to *E. coli* O157:H7 prevented fatal systemic complications [85]. Stx2-neutralizing antibodies, administered passively, reduced the *E. coli* O157:H7 burden in infected mice and, as anticipated, also protected them from weight loss and death [86]. However, the piglet and oral infection mouse model lack many of the signs of HUS.

Our mouse model that demonstrates a more complete array of signs common to human HUS provides a superior model for testing the efficacy of antibody therapy. Our results indicate that, when administered during progression of HUS, passive immunization of mice with anti-Stx2 antibody prevents the lethal consequences of Stx2 even when

administered several days after initial exposure to Stx and in the continued presence of circulating Stx. Mice that received 11E10, a monoclonal anti-Stx2 antibody, four days after initiating injections of Stx2 recovered fully, displaying normal renal function. Our results suggest that passive immunization against Stx may be therapeutically effective in humans at risk of developing HUS if antibody is able to be administered at an early time after development of symptoms and before the accumulated effects of HUS have resulted in irreversible structural damage.

Dowling et al created a chimeric antibody called α Stx2, in which the variable regions of the Stx2-neutralizing murine monoclonal antibody 11E10 are genetically fused directly to the human kappa light chain constant domain sequence and to the human immunoglobulin G1 (IgG1) heavy chain constant domain sequence [109].

Approximately 87% of the antibody sequences are human based. In a phase I study, intravenous α Stx2 was generally well tolerated at doses from 0.1 mg/kg to 10 mg/kg. The next step of testing α Stx2 as a prophylactic agent against HUS would be better accomplished during an outbreak, because exposed individuals would be more easily identified. Obviously, this type of study would require careful planning for feasibility and approval by regulatory agencies

Doxorubicin

Doxorubicin is known to induce the activation of SAPKs in a number of normal cell types. When phosphorylated, the SAPKs initiate a cascade that leads to the production of proinflammatory cytokines. Patients receiving doxorubicin frequently present with acute side effects such as fatigue, nausea/vomiting, pain, sleep disturbances, cachexia, and depression [82], which may be due to the increased inflammatory condition. Currently,

there is no method of reducing acute side effects without affecting the ability of doxorubicin to destroy cancer cells.

Inhibitors of p38 MAPK and JNK have been employed to determine whether activation of SAPKs contributes either to the efficacy or to the undesirable side effects of doxorubicin. Inhibitors of p38 MAPK have been effective in blocking apoptosis of cardiomyocytes following treatment by doxorubicin or daunorubicin, a related anthracycline [87, 88]. Inhibitors of p38 MAPK reduce the proinflammatory actions of doxorubicin in macrophages but do not reduce the anti-proliferative actions of doxorubicin in a cancer cell line [89]. Our goal has been to employ kinase inhibitors to block ZAK signaling farther upstream of p38 MAPK and JNK. By employing siRNA mediated knockdown of ZAK or administration of sorafenib and nilotinib, kinase inhibitors that have a high affinity for ZAK, we provide evidence that ZAK is required for doxorubicin-induced proinflammatory and apoptotic responses in HaCaT cells, a pseudo-normal keratinocyte cell line, but not in HeLa cells, a cancerous cell line. The differential sensitivity of normal and cancer cells to the pro-apoptotic actions of doxorubicin suggest that inhibitors of ZAK may be effective in protection of normal cells against the cytotoxic activities of doxorubicin. Because our data suggests that sorafenib or nilotinib may be able to reduce doxorubicin-induced apoptosis and SAPK phosphorylation in normal cell types, we propose that these inhibitors may be effective in reducing inflammatory responses when employed in combination with doxorubicin to treat cancer patients. Further experiments in animal models of cancer would determine the efficacy of treatment with doxorubicin and either sorafenib or nilotinib. To pursue this goal, we have formed a collaboration with Dr. Lisa Wood, an Assistant

Professor in the School of Nursing and the Cancer Institute. The long-range goal of her research is to effectively treat and manage fatigue, which is the most common symptom experienced by cancer patients undergoing chemotherapy, radiation therapy, or both. With her help, we plan to treat mice with doxorubicin in combination with sorafenib or nilotinib and to examine circulating cytokines. These experiments should determine if one or both of these multi-kinase inhibitors could prevent doxorubicin-mediated inflammation *in vivo*. If these inhibitors could prevent doxorubicin-induced inflammation, further experiments would be designed to ensure that sorafenib and/or nilotinib would not diminish the chemotherapeutic effects of doxorubicin *in vivo*. Another potential therapeutic approach for blocking doxorubicin-mediated inflammation would be to interfere with IL-1 β signaling by blocking its processing, release, or binding to IL-1 receptors. In chapter 4, we demonstrate that both doxorubicin and daunorubicin activate the inflammasome in murine BMDM leading to processing and secretion of mature IL-1 β . IL-1 β is an apical cytokine that leads to increased expression of other proinflammatory mediators. BMDM from mice deficient in ASC, caspase-1, or NALP3 all show a drastic decrease in the doxorubicin-mediated release of mature IL-1 β , indicating that these proteins are involved in doxorubicin-induced inflammasome activation. Therefore, blocking IL-1 β after doxorubicin treatment may be beneficial in reducing inflammation in patients who are being treated with doxorubicin. To determine if IL-1 is necessary for doxorubicin-mediated inflammation, Lisa Wood is currently examining the effect of doxorubicin in mediating expression of circulating cytokines in mice deficient in the receptor for IL-1. If IL-1R1 $-/-$ mice show a decreased inflammatory response after exposure to doxorubicin, when compared to wildtype, then

we would test whether the IL1 receptor antagonist, anakinra, given in co-treatment with doxorubicin could reduce doxorubicin-mediated inflammation.

A better understanding of which antibiotics, cancer chemotherapeutics, and toxins are ribotoxic stressors, as well as how ribotoxic stressors inhibit protein translation, signal to MAPKs, and cause apoptosis could lead to novel therapeutic interventions to treat ribotoxic stressor-mediated human disease.

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