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Simultaneous Induction of Apoptotic and Survival Signaling Pathways in Macrophage-Like THP-1 Cells by Shiga Toxin 1[▽]

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Shiga toxins have been shown to induce apoptosis in many cell types. However, Shiga toxin 1 (Stx1) induced only limited apoptosis of macrophage-like THP-1 cells in vitro. The mechanisms regulating macrophage death or survival following toxin challenge are unknown. Differentiated THP-1 cells expressed tumor necrosis factor receptors and membrane-associated tumor necrosis factor alpha (TNF-α) and produced soluble TNF-α after exposure to Stx1. However, the cells were refractory to apoptosis induced by TNF-α, although the cytokine modestly increased apoptosis in the presence of Stx1. Despite the partial resistance of macrophage-like THP-1 cells to Stx1-mediated killing, treatment of these cells with Stx1 activated a broad array of caspases, disrupted the mitochondrial membrane potential ($\Delta\Psi_m$), and released cytochrome *c* into the cytoplasm. The $\Delta\Psi_m$ values were greatest in cells that had detached from plastic surfaces. Specific caspase inhibitors revealed that caspase-3, caspase-6, caspase-8, and caspase-9 were primarily involved in apoptosis induction. The antiapoptotic factors involved in macrophage survival following toxin challenge include inhibitors of apoptosis proteins and X-linked inhibitor of apoptosis protein. NF-κB and JNK mitogen-activated protein kinases (MAPKs) appeared to activate survival pathways, while p38 MAPK was involved in proapoptotic signaling. The JNK and p38 MAPKs were shown to be upstream signaling pathways which may regulate caspase activation. Finally, the protein synthesis inhibitors Stx1 and anisomycin triggered limited apoptosis and prolonged JNK and p38 MAPK activation, while macrophage-like cells treated with cycloheximide remained viable and showed transient activation of MAPKs. Collectively, these data suggest that Stx1 activates both apoptotic and cell survival signaling pathways in macrophage-like THP-1 cells.

Shiga toxins (Stxs) are structurally and functionally related protein toxins expressed by *Shigella dysenteriae* serotype 1 and certain serotypes of *Escherichia coli*. These bacteria are a serious public health concern because they are capable of causing widespread outbreaks of bloody diarrhea in which a fraction of patients subsequently develop life-threatening complications, such as acute renal failure and neurological abnormalities (41). The histopathological hallmark of disease caused by Stxs is the destruction of blood vessels serving the colon, kidneys (especially renal glomeruli), and central nervous system (27). The toxins consist of a single enzymatic A subunit in noncovalent association with a pentameric ring of glycolipid-binding B subunits. Stxs which cause disease in humans bind the neutral glycolipid globotriaosylceramide (Gb3). Once bound to Gb3, holotoxin molecules are internalized and trafficked to the endoplasmic reticulum through the Golgi apparatus. In the transport process, the A subunit is cleaved, and the A1 fragment is translocated into the cytosol. The A1 fragment mediates protein synthesis inhibition through cleavage of a single adenine residue in the 28S rRNA component of eukaryotic ribosomes (26). *S. dysenteriae* expresses the prototypical Shiga toxin, while toxin-producing *E. coli* may express one or more toxins designated Shiga toxin type 1 (Stx1) or Stx2 based on their antigenic similarity to Shiga toxin (39).

Stxs kill epithelial cell lines (e.g., Vero cells) in vitro in pg/ml quantities, and a concept that Stxs may directly target epithelial and vascular endothelial cells for damage has emerged. However, the role of the toxins in pathogenesis may be more complex. Stxs elicit the expression of proinflammatory cytokines and chemokines from epithelial cells (11, 43) and macrophages (31, 46). The cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1β sensitize endothelial cells to the cytotoxic action of the toxins in vitro (32, 45) by upregulating expression of enzymes involved in Gb3 biosynthesis (38). The production of chemokines may account for the influx of inflammatory cells into sites where there is vascular damage and the activation of platelets leading to the deposition of microthrombi (6). Stxs also induce apoptosis in human epithelial, endothelial, and myelomonocytic cell lines in vitro and in human and murine renal epithelial cells in vivo (2, 15). The toxins may not activate a common apoptotic signaling pathway in all cell types. Jones et al. (13) showed that Stxs induced apoptosis in HEp-2 cells which was associated with increased expression of the proapoptotic Bax protein and was blocked by a caspase-3 inhibitor. Fujii et al. (5) showed that Stxs induced apoptosis in HeLa cells that required the rapid activation of caspase-6 and caspase-8, the cleavage of Bid, and the release of cytochrome *c* from mitochondrial membranes. Stxs also appeared to upregulate expression of the antiapoptotic protein X-linked inhibitor of apoptosis protein (XIAP) in HeLa cells, leading to inhibition of caspase-9 function. Induced expression of the transfected *stx1B* gene in HeLa cells led to apoptosis,

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characterized by caspase-1 and -3 activation and DNA fragmentation, while expression of the transfected *stx1A* gene triggered necrotic cell death characterized by increased lactate dehydrogenase release and a lack of DNA laddering (25). In Burkitt's lymphoma cell lines, purified Stx1 or anti-Gb3 monoclonal antibody was capable of triggering apoptotic cell death through mechanisms involving rapid activation of caspase-8, followed by caspase-3 and caspase-7 activation (16). Finally, TNF-α is a well-characterized apoptosis inducer. It is unclear whether Stxs directly activate apoptosis or indirectly activate apoptosis through induction of TNF-α expression.

We have demonstrated that there are differences in apoptosis induction in the myelogenous leukemia cell line THP-1 that are dependent on the state of cell maturation. Treatment of undifferentiated, monocyte-like THP-1 cells with purified Stx1 led to the rapid onset of apoptosis in the absence of cytokine expression, resulting in approximately 85% cell death in 12 h (7). Signaling for apoptosis in monocytic cells involved the rapid activation of caspase-3, caspase-8, caspase-6, and caspase-9, the cleavage of Bid, the disruption of mitochondrial membrane potential, and the release of cytochrome *c* into the cytosol. Apoptosis induction in monocytes required toxin enzymatic activity and was TNF-α and tumor necrosis factor receptor (TNFR) independent (17). Differentiation of THP-1 cells to the macrophage-like state was accompanied by the ability to express soluble cytokines after treatment with Stxs and a loss of sensitivity to Stx1-mediated apoptosis induction, with only ~11% cell death at 12 h and cell death peaking at 36% at 48 h (7). Furthermore, only in macrophage-like THP-1 cells was the transcriptional activator NF-κB translocated to the nucleus, and the JNK and p38 mitogen-activated protein kinase (MAPK) pathways were activated for a prolonged time after Stx1 stimulation (3, 34). Stxs and other protein synthesis inhibitors that act on the peptidyltransferase center of eukaryotic ribosomes induce the ribotoxic stress response through activation of JNK and p38 MAPKs (3, 12, 37). It is known that activation of NF-κB induces expression of many antiapoptotic proteins (14). Recent studies have demonstrated that p38 MAPK signaling induces a proapoptotic response, while activation of JNK induces either anti- or proapoptotic signaling depending on the cell type (22, 37). The purpose of this study was to better understand the different responses of monocytic and macrophage-like THP-1 cells to Stxs. We found that in contrast to the effect on toxin-sensitive monocytic cells, in macrophage-like THP-1 cells Stx1 triggered NF-κB- and JNK MAPK-mediated antiapoptotic signals, resulting in prolonged expression of inhibitor of apoptosis proteins (IAPs). Furthermore, Stx1 triggered the ribotoxic stress response only in macrophage-like cells. Thus, the toxin appears to simultaneously activate pro- and antiapoptotic signaling mechanisms in macrophages.

MATERIALS AND METHODS

Cells. The human myelogenous leukemia cell line THP-1 (44) was purchased from the American Type Culture Collection (Manassas, VA) and was maintained in RPMI 1640 medium (GibcoBRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), penicillin (50 μg/ml), and streptomycin (50 μg/ml) at 37°C in the presence of 5% CO_2 in a humidified incubator. For differentiation to the macrophage-like state, THP-1 cells (1×10^6 cells/ml) were treated with phorbol 12-myristate 13-acetate (Sigma Chemical Co., St. Louis, MO) at a concentration of 50 ng/ml for 48 h. Adherent, differentiated cells were washed with cold, sterile phosphate-buffered saline (PBS) (Sigma) and incubated with medium lacking phorbol 12-myristate 13-acetate. The medium was changed every 24 h for 3 days. Cells were stimulated with Stx1 at a concentration of 400 ng/ml, a concentration previously demonstrated to induce JNK and p38 phosphorylation in differentiated THP-1 cells (3).

Reagents. Purified Stx1 was prepared by expression of the toxin operon from recombinant strain *E. coli* DH5α(pCKS112) (42). Crude bacterial lysates were subjected to sequential ion-exchange, chromatofocusing, and immunoaffinity chromatography procedures. Toxin purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining and by Western blot analysis using bovine polyclonal antibodies directed against Stx1. Purified Stx1 holotoxin containing a double mutation (E167Q, R170L) in the A subunit which inactivates enzymatic activity (28) was a kind gift from Yoshifumi Takeda, Cine-Science Laboratories, Tokyo, Japan. Purified toxin preparations were determined to contain <0.1 ng of endotoxin per ml by the *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Inc., East Falmouth, MA). Recombinant human TNF-α (rhTNF-α), nonfluoresceinated monoclonal antibodies directed against human membrane-bound TNF-α (mTNF-α) and TNFR1, and fluorescein-conjugated antibodies directed against mTNF-α, TNFR1, and TNFR2 (for fluorescence-activating cell sorting [FACS] analysis) were purchased from R&D Systems Inc., Minneapolis, MN. Murine immunoglobulin (IgG) was obtained from Sigma. The caspase-1 inhibitor Z-Tyr-Val-Ala-Asp-fmk, the caspase-2 inhibitor Z-Val-Asp-Val-Ala-Asp-fmk, the caspase-3 inhibitor Z-Asp-Glu-Val-Asp-fmk, the caspase-6 inhibitor Z-Val-Glu-Ile-Asp-fmk, the caspase-8 inhibitor Z-Ile-Glu-Thr-Asp-fmk, the caspase-9 inhibitor Z-Leu-Glu-His-Asp-fmk, and the general caspase inhibitor *N*-benzyl-oxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl-ketone (ZVAD-fmk) were purchased from Calbiochem, San Diego, CA. The concentrations of caspase inhibitors that optimally inhibited specific caspase activities in the experiments were determined in previous studies (17). Antibodies directed against human caspase-3, caspase-8, and caspase-9 were obtained from Cell Signaling Technology, Beverly, MA. The NF-κB inhibitor pyrrolidine dithiocarbamate, the JNK inhibitor SP600125, and the p38 MAPK inhibitor SB203580 were purchased from Calbiochem. Anisomycin and cycloheximide (CHX) were purchased from Sigma.

Analysis of cell surface receptor expression. Immunofluorescence staining for TNFR1, TNFR2, and mTNF-α was performed as previously described (17). Briefly, macrophage-like THP-1 cells (5×10^6 cells/ml) were incubated with or without Stx1 for 4 h. Following this treatment, cells were washed twice with cold PBS, 3.0 ml of trypsin was added, and the preparations were incubated for 2 min at 37°C in the presence of 5% CO_2. Trypsinization was stopped by adding an equal volume of medium, and supernatants were removed after centrifugation at $260 \times g$ for 5 min. Cells were fixed in 4% paraformaldehyde for 15 min on ice, washed with PBS containing 0.5% bovine serum albumin, and incubated with fluorescein-conjugated antibodies directed against human mTNF-α, TNFR1, or TNFR2 for 30 to 45 min on ice. After washing, membrane fluorescence was measured by flow cytometry (FACSCalibur; Becton Dickinson, Palo Alto, CA); 1×10^4 cells were counted for each treatment. Untreated cells and cells treated with fluorescein-conjugated mouse IgG were used as controls.

Analysis of apoptosis by the MTT assay. Differentiated THP-1 cells (1×10^5 cells/well) were plated in 96-well microtiter plates. Before Stx1 treatment, NF-κB (50 μM), JNK (50 μM), and p38 (20 μM) inhibitors were added for 1 h prior to stimulation with Stx1 and incubation at 37°C in the presence of 5% CO_2. In a separate set of experiments, cells were treated with Stx1 for 24 h in RPMI 1640 medium containing 0.5% FBS in the presence or absence of anti-human TNF-α antibody (0.01 μg/ml), anti-human TNFR1 antibody (5.0 μg/ml), or rhTNF-α (40 ng/ml). To measure cytotoxicity, 25 μl of a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution was added to each well and incubated at 37°C in the presence of 5% CO_2 for 2 h. At the end of the incubation period, the microtiter plates were centrifuged at $260 \times g$ for 5 min. The supernatants were removed, and 100 μl of lysis buffer (20% SDS, 50% 2,2 dimethylformamide [pH 4.7]) was added to each well. The plates were incubated at 37°C without CO_2 for 3 h. Cell lysis was measured by determining the optical density at 570 nm (OD_{570}) with an automated microtiter plate reader (Dynatech MR5000; Molecular Dynamics, Chantilly, VA). The percentage of apoptosis was determined using the following equation: percentage of apoptosis = [(average OD_{570} of treated cells − average OD_{570} of control cells)/average OD_{570} of control cells] × 100.

In previous studies we used the MTT assay, annexin V/propidium iodide staining, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining to show that the results were closely correlated; that is, the majority of Stx1-induced cell death detected by MTT is apoptotic cell death (7).

Analysis of apoptosis by annexin V and propidium iodide staining. Apoptosis of nonadherent monocytic THP-1 cells was evaluated by measuring the percentage of annexin V-positive (annexin V^+) propidium iodide-positive (PI^+) cells. Monocytic THP-1 cells were pretreated with each inhibitor for 1 h, and Stx1 in RPMI 1640 medium containing 0.5% FBS was added for 6 h. Cells were washed with cold PBS, stained with annexin V-fluorescein isothiocyanate, and then incubated with propidium iodide for 15 min at room temperature using an annexin V-FLUOS staining kit (Roche Diagnostics Corp., Indianapolis, IN). Cells were centrifuged, washed twice in incubation buffer, and suspended in 0.5 ml of incubation buffer. Cells were analyzed by flow cytometry with gating for annexin V^+ and PI^+ cells. Total apoptosis was expressed as follows: (% of annexin V^+ cells + % of annexin V^+ PI^+ cells) − the background fluorescence.

Analysis of apoptosis by the TUNEL assay. To detect the presence of apoptotic adherent macrophage-like THP-1 cells, the TUNEL assay was performed using an in situ cell death detection kit with fluorescein (Roche). TUNEL staining was performed in accordance with the protocol provided by the manufacturer. Briefly, macrophage-like THP-1 cells (2×10^5 cells/well) were plated on 16-well Lab-Tek chamber slides (Nalge-Nunc International, Naperville, IL). Cells were treated with Stx1 in RPMI 1640 medium containing 0.5% FBS in the presence or absence of caspase-1, -2, -3, -6, -8, or -9 inhibitors (all at a concentration of 40 μM) for 24 h. Following the inhibitor treatments, media were removed and cells were fixed in freshly prepared 4% paraformaldehyde for 1 h at room temperature. Cells were rinsed with PBS and incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing, the labeling reaction (TUNEL reaction mixture and a 1:10 mixture of enzyme solution to label solution) was performed for 1 h in a humidified atmosphere at 37°C in the dark. After 1 h, slides were washed with PBS, and labeled DNA fragments were visualized using fluorescence microscopy. The percentage of apoptosis was calculated by using the following equation: percentage of apoptosis = (number of TUNEL-positive cells treated with Stx1 plus inhibitor/number of TUNEL-positive Stx1-treated cells) × 100.

Preparation of cellular lysates and Western blot analysis. Prior to Stx1 stimulation, THP-1 cells (5×10^6 cells/well) were washed twice in cold PBS, and RPMI 1640 medium with 0.5% FBS was added to reduce endogenous kinase activity. Cells were pretreated with caspase, NF-κB, JNK, or p38 inhibitors for 1 h and stimulated with Stx1. After various times, cells were washed with PBS and suspended with modified radioimmunoprecipitation assay buffer (1.0% Nonidet P-40, 1.0% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.25 mM sodium pyrophosphate, 2.0 mM sodium vanadate, 2.0 mM sodium fluoride, 10 μg/ml aprotinin, 1.0 μg/ml leupeptin, 1.0 μg/ml pepstatin, 200 mM phenylmethylsulfonyl fluoride) at 4°C. Cellular lysates were centrifuged at 10,000 × *g* for 10 min, and supernatants were collected for the Western blot assay. To measure cytochrome *c* release from mitochondria, cytosolic and mitochondrial fractions were collected using a mitochondrion isolation kit for tissues (Pierce, Rockford, IL). Equal amounts of proteins (60 to 80 μg protein per gel lane) were separated by 8% or 12% Tris-glycine SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk prepared with TBST (20 mM Tris [pH 7.6], 137 mM NaCl, 0.1% Tween 20). The membranes were incubated with primary antibodies for 24 h at 4°C. After washing, the membranes were incubated with secondary antibodies (horseradish peroxidase-labeled anti-rabbit or anti-mouse antibodies) for 2 h at room temperature. Bands were visualized using the Western Lightning chemiluminescence system (NEN-Perkins Elmer, Boston, MA). The data shown below are the data from at least two independent experiments.

Measurement of changes in the mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\Psi_m$) detection kits (Stratagene, La Jolla, CA) were used to detect alterations in the transmembrane potential. Differentiated THP-1 cells (5×10^6 cells/well) were treated with Stx1 for 24 h. After stimulation, cells were detached by treatment with Accutase (Innovative Cell Technologies Inc., San Diego, CA) for 10 min. After centrifugation at 260 × *g* for 5 min, supernatants were removed, and cells were washed in ice-cold PBS and resuspended in 0.5 ml JC-1 assay buffer containing the reagent 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenz-imidazolocarbocyanine iodine (JC-1). Cells were incubated for 15 min at 37°C in the presence of 5% CO_2, centrifuged at 400 × *g* for 5 min, and washed twice in JC-1 assay buffer. JC-1 fluorescence associated with mitochondrial membranes was detected using flow cytometry.

Measurement of caspase activity. Caspase activation was measured with the colorimetric substrates for caspase-1 (YVAD-*p*-nitroanilide), caspase-2 (Ac-VDQQD-*p*-nitroanilide), caspase-3 (Ac-DEVD-*p*-nitroanilide), caspase-6 (VEID-*p*-nitroanilide; Biovision, Mountain View, CA), caspase-8 (Ac-IETD-*p*-nitroanilide), and caspase-9 (Ac-LEHD-*p*-nitroanilide; Chemicon, Temecula, CA) used according to the manufacturers' recommendations. Briefly, differentiated THP-1 cells (5×10^6 cells/well) were stimulated with Stx1 in the presence or absence of inhibitors for various times. Cell lysates were prepared, and protein concentrations in the supernatants were determined using a Bio-Rad D_c protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were incubated for 2 h at 37°C with the colorimetric substrates for caspase-1, caspase-2, caspase-3, caspse-6, caspase-8, and caspase-9. The results were determined spectrophotometrically at 405 nm with a microtiter plate reader. Caspase activity was expressed as picomoles of *p*-nitroanilide liberated per minute per microgram of protein.

Statistical analysis. Significance was determined by Student's *t* test (Excel; Microsoft Corp., Redmond, WA).

RESULTS

Role of TNFRs and TNF-α in Stx1-mediated killing of macrophage-like THP-1 cells. We previously showed that undifferentiated, monocyte-like THP-1 cells express the toxin-binding glycolipid Gb3 and are sensitive to apoptosis induction by purified Stx1. In the undifferentiated state, the cells did not express mTNF-α, TNFR1, or TNFR2 and were refractory to apoptosis induction by exogenously added rhTNF-α (17). These data suggested that Stxs induce apoptosis in monocytic cells via a TNFR/TNF-α-independent mechanism. In contrast, differentiated macrophage-like THP-1 cells expressed lower levels of membrane Gb3 and were less sensitive to apoptosis induction but secreted soluble TNF-α when they were stimulated with Stx1. To investigate the role of TNF-α in apoptosis induction of macrophage-like cells, we examined the expression of TNFR1, TNFR2, and mTNF-α by FACS analysis (Fig. 1A). Differentiated THP-1 cells expressed both TNFRs and mTNF-α. Treatment of the cells with purified Stx1 did not alter TNFR or mTNF-α expression (data not shown). Following exposure to Stx1 (400 ng/ml) for 24 h, approximately 30% of THP-1 cells died (Fig. 1B). Treatment of cells with an equivalent dose of an enzymatically inactive Stx1 toxoid ($Stx1A^-$) failed to trigger cell death. We have shown previously that purified Stx1 B subunits failed to induce apoptosis in differentiated THP-1 cells (7). Together, these data suggest that binding and intracellular routing of functional toxin are necessary for signaling leading to cell death in macrophage-like cells. Despite expression of TNFRs, addition of rhTNF-α (40 ng/ml) to the cells did not result in apoptosis at 24 h. Prior to use of rhTNF-α in these experiments, we verified the cytokine biological activity using the actinomycin D-treated L929 cytotoxicity assay. However, if the cells were simultaneously treated with Stx1 and exogenous rhTNF-α for 24 h, apoptosis was significantly increased compared to the apoptosis observed after treatment with Stx1 alone ($P < 0.01$). To investigate whether TNF-α elicited from Stx1-treated cells enhanced apoptosis, we added neutralizing antibodies directed against TNFR1 or TNF-α to macrophage-like THP-1 cells for 1 h prior to a 24-h treatment with purified Stx1. Neither TNFR1- nor TNF-α-neutralizing antibodies inhibited Stx1-induced apoptosis (Fig. 1B). Prior to use of the antibodies in these experiments, we verified the neutralizing activity using rhTNF-α in the actinomycin D-treated L929 cytotoxicity assay. Treatment of cells with TNFR1- or TNF-α-neutralizing antibodies alone did not affect cell viability. These data suggest that even though macrophage-like THP-1 cells express TNFRs and mTNF-α and Stx1 induces soluble TNF-α synthesis or release from the cells (34), apoptosis following exposure to purified Stx1 occurs through a mechanism independent of TNFR/TNF-α interaction. However, macrophage-like cells respond differently to

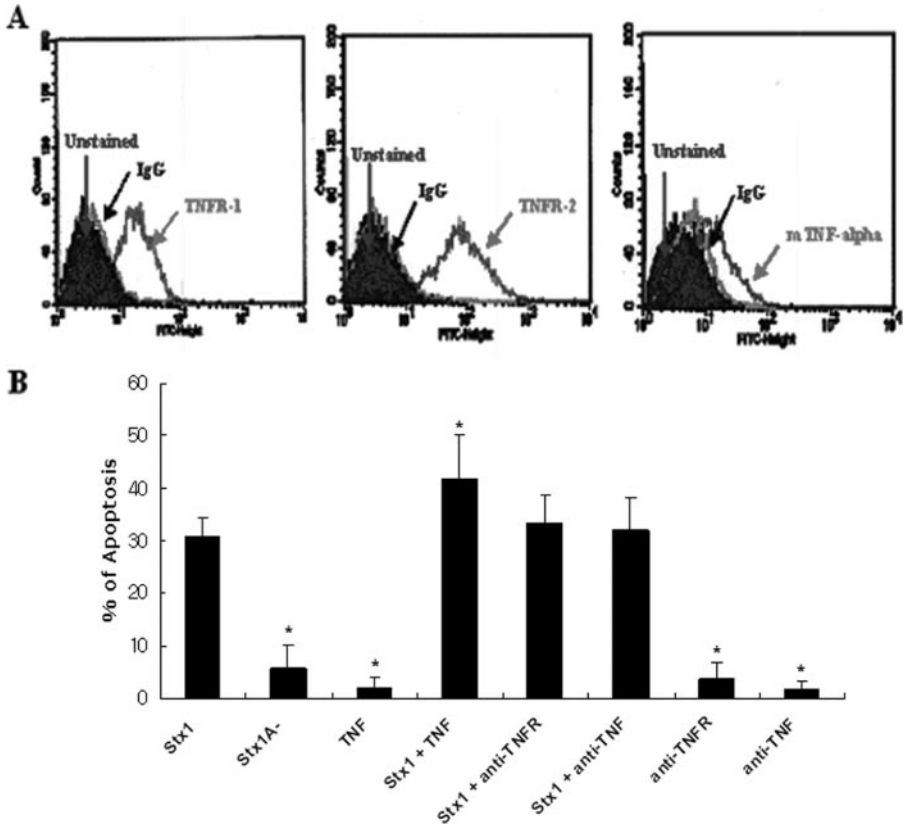

FIG. 1. Role of TNF-α and TNFR in Stx1-induced apoptosis of macrophage-like THP-1 cells. (A) After differentiation, cells were fixed and incubated with fluorescein-conjugated anti-TNFR1, anti-TNFR2, anti-mTNF-α, or control mouse IgG antibody. Fluorescence was measured by flow cytometry. The data from at least two independent experiments are shown in representative histograms. (B) Macrophage-like THP-1 cells were treated with or without neutralizing anti-TNFR1 or anti-TNF-α antibodies for 1 h and then treated with Stx1 (400 ng/ml), Stx $1A^-$ enzymatic mutant (400 ng/ml), rhTNF-α (40 ng/ml), or Stx1 plus rhTNF-α for 24 h. Apoptosis was measured by the MTT assay. The percentage of apoptosis was calculated based on the formula described in Materials and Methods. The data are means ± standard errors of the means from three independent experiments. An asterisk indicates a significant difference ($P < 0.01$) compared to Stx1-treated cells.

simultaneous challenge with two potentially proapoptotic molecules (Stx1 and TNF-α) than to challenge with either stimulant alone.

Stx1 triggers caspase activation in macrophage-like THP-1 cells. Activated cysteine-dependent aspartate-specific proteases (caspases) are key mediators of programmed cell death. In previous studies, we showed that Stx1-induced apoptosis of macrophage-like THP-1 cells was blocked by the general caspase inhibitor ZVAD-fmk (7). To further investigate caspase activation, we examined the extent and kinetics of activation of caspase-1 (also known as interleukin-1β-converting enzyme), initiator caspases (caspase-2, caspase-8, and caspase-9), and executioner caspases (caspase-3 and caspase-6) isolated from THP-1 cells treated with Stx1 for 8 h. With the exception of caspase-1, increases in all caspase activities were detected, with caspase-3 and caspase-6 showing the highest activity and maximal induction (Table 1). We also used the TUNEL assay to show that pretreatment of cells with individual specific caspase inhibitors (caspase-1, -2, -3, -6, -8, and -9 inhibitors) for 1 h before Stx1 treatment for 24 h blocked Stx1-induced apoptosis in macrophage-like THP-1 cells to some extent, with the caspase-3 and -9 inhibitors resulting in the greatest percentage of reduction in apoptosis compared to control cells (Table 1). We detected minimal caspase-1 activation by Stx1, and blocking caspase-1 activity resulted in modest (29.9%) inhibition of Stx1-induced apoptosis. Stx1 induced a twofold increase in caspase-2 activity, but a caspase-2 inhibitor also failed to significantly reduce toxin-mediated cell killing. To correlate caspase functional activity with activation, we

TABLE 1. Measurement of caspase activities and effect of caspase inhibitors on Stx1-induced apoptosis of macrophage-like THP-1 cells

Caspase	Caspase activity (pmol/min/μg protein)		% Inhibition of apoptosis in the presence of specific caspase inhibitor[c]
	Control[a]	Stx1 treated (8 h)[b]	
Caspase-1	2.5 ± 0.1	3.0 ± 0.3 (1.2)	29.9 ± 8.8
Caspase-2	4.4 ± 0.2	8.7 ± 0.3 (2.0)	30.9 ± 4.7
Caspase-3	5.4 ± 0.1	13.2 ± 0.4 (2.4)	46.7 ± 11.6
Caspase-6	4.5 ± 0.4	10.9 ± 0.5 (2.4)	41.4 ± 16
Caspase-8	4.9 ± 0.4	8.3 ± 0.8 (1.7)	41.0 ± 10.5
Caspase-9	3.9 ± 0.4	7.0 ± 1.0 (1.8)	51.2 ± 12.9

[a] The control was not treated with Stx1.
[b] The numbers in parentheses indicate fold induction.
[c] Determined by the TUNEL assay. The level of apoptosis in the absence of inhibitor was 100%.

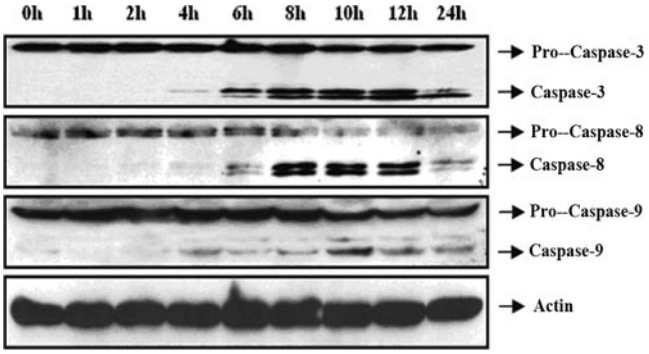

FIG. 2. Caspase cleavage in macrophage-like THP-1 cells treated with Stx1. Macrophage-like THP-1 cells were treated with Stx1 (400 ng/ml) for various times. Cells were collected, washed with PBS, and lysed. Total protein concentrations were determined, and equal amounts of total protein (60 to 80 μg) were separated by 12% Tris-glycine SDS-PAGE. Caspase-3, -8, and -9 cleavage was detected using caspase-specific antibodies. Actin was included as a control for protein loading. The immunoblots are representative immunoblots from three independent experiments.

examined the cleavage of procaspase-3, procaspase-8, and procaspase-9 by Western blot analysis (Fig. 2). Cleavage of procaspases was first observed 4 h after toxin treatment. The maximal caspase-3 and -8 levels occurred 8 h after Stx1 treatment, while the caspase-9 levels peaked 10 h after Stx1 treatment. These data suggest that in both monocytes (17) and macrophage-like cells, caspase-1 and caspase-2 are not major signaling molecules in Stx1-induced apoptosis. Stx1 appears to activate similar patterns of caspases in monocytic and macrophage-like THP-1 cells. Inhibitor studies suggested that in monocytic cells, caspase-3 and caspase-8 play major roles in apoptosis (17), while caspase-3, caspase-6, caspase-8, and caspase-9 appear to contribute to macrophage apoptosis.

Stx1 activates the mitochondrial pathway of apoptosis in a fraction of macrophage-like THP-1 cells. The intrinsic pathway of apoptosis is induced by the proapoptotic Bcl-2 family of proteins. Active Bcl-2 proteins translocate to mitochondrial membranes and release cytochrome *c* from mitochondria into the cytoplasm by disruption of the mitochondrial membrane potential (8). Following cytochrome *c* release, the apoptosome is formed, leading to recruitment and activation of caspase-9 (18). We previously showed that treatment of monocytic THP-1 cells with Stx1 resulted in cleavage of Bid, a proapoptotic BH3 domain-only member of the Bcl-2 family, within 4 h of toxin treatment, followed by the release of cytochrome *c* (17). To investigate whether apoptosis of a fraction of macrophage-like THP-1 cells was triggered through the intrinsic pathway, we examined Bid cleavage, $\Delta\Psi_m$, and cytochrome *c* release over time following exposure to Stx1. We detected late activation of Bid in cytosolic fractions, which was first noted after 12 h of toxin treatment in macrophage-like THP-1 cells (Fig. 3A). To ensure that truncated Bid was associated with mitochondria, we showed that after 24 h of incubation with Stx1, the cytosolic Bid levels were significantly decreased, while we detected increased concentrations of Bid in mitochondrial fractions (Fig. 3A). Release of mitochondrial stores of cytochrome *c* lagged behind Bid activation, and only trace quantities of cytochrome *c* were detected in the cytosol 12 h after Stx1

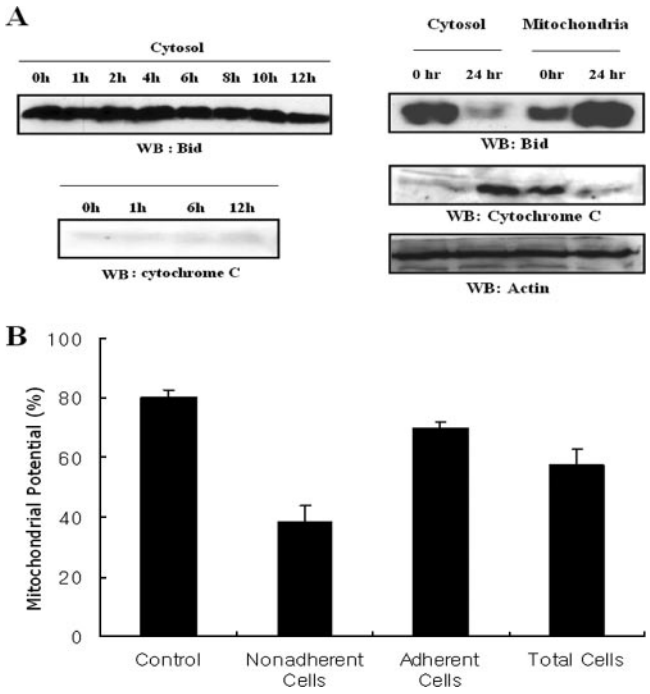

FIG. 3. Bid translocation, cytochrome *c* release, and loss of $\Delta\Psi_m$ in Stx1-treated THP-1 cells. (A) Macrophage-like THP-1 cells were incubated for different times with Stx1 (400 ng/ml). Cytosolic and mitochondrial fractions were prepared as described in Materials and Methods. Bid and cytochrome *c* were visualized by Western blotting (WB) using Bid- or cytochrome *c*-specific antibodies. The immunoblots are representative immunoblots from at least two separate experiments. (B) THP-1 cells were treated with Stx1 (400 ng/ml) for 24 h. After incubation, total, adherent, or nonadherent cells were collected, and JC-1 was used to measure $\Delta\Psi_m$ by flow cytometry. Control cells were maintained in medium for 24 h. The data are the means ± standard errors of three independent experiments.

treatment (Fig. 3A). However, Stx1 significantly increased cytochrome *c* release from mitochondria to the cytoplasm compared to the release in untreated control cells after 24 h of toxin treatment, as shown by an increase in the cytosolic levels along with a concomitant reduction in the concentrations of cytochrome *c* in mitochondrial fractions. To measure $\Delta\Psi_m$, THP-1 cells were incubated for 24 h with Stx1, separated into nonadherent, adherent, and total (nonadherent plus adherent) fractions, and stained with the fluorescent mitochondrial membrane binding agent JC-1 (Fig. 3B). We predicted that apoptotic cells would detach from plastic surfaces and become suspended, while live cells would remain adherent. As predicted, in the nonadherent fraction, $\Delta\Psi_m$ (determined from the percentage of cells maintaining mitochondrial membrane-associated fluorescence) was significantly decreased (only 38.2% ± 5.4% of the cells maintained the mitochondrial membrane potential) compared to the results for control untreated cells (80.2% ± 2.5%), while a modest $\Delta\Psi_m$ value was obtained for the adherent fraction (69.8% ± 2.1%). The percentage of cells maintaining a normal mitochondrial membrane potential in the total fraction of Stx1-treated cells was intermediate (57.6% ± 5.4%) between the values for the nonadherent and adherent fractions. Taken together, these data suggest that Stx1 triggers apoptosis by the intrinsic pathway in a fraction of macrophage-

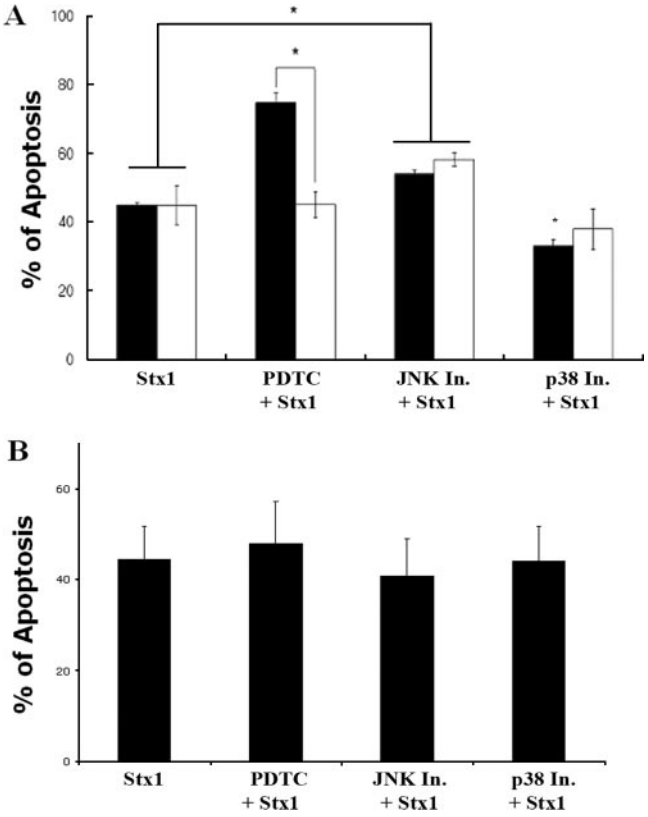

FIG. 4. Comparison of Stx1-induced apoptosis and survival signaling factors in monocytic and macrophage-like THP-1 cells. (A) Macrophage-like THP-1 cells were treated with Stx1 alone (400 ng/ml) or with inhibitors of NF-κB (50 μM PDTC), JNK (50 μM SP600125) (JNK In.), and p38 (20 μM SB203580) (p38 In.) 1 h before (solid bars) or 4 h after (open bars) Stx1 treatment. After inhibitor treatment, cells were incubated for 24 h, and apoptosis was measured by the MTT assay. The data are means ± standard errors of the means from three independent experiments. An asterisk indicates a significant difference ($P < 0.01$). (B) Monocytic THP-1 cells were pretreated with NF-κB (50 μM PDTC), JNK (50 μM SP600125) (JNK In.), and p38 (20 μM SB203580) (p38 In.) inhibitors for 1 h prior to Stx1 (400 ng/ml) treatment. Monocytic THP-1 cells were incubated with Stx1 for 6 h, stained with annexin V and propidium iodide, and analyzed by flow cytometry to determine the relative percentages of apoptosis.

like THP-1 cells through Bid activation, disruption of the mitochondrial membrane potential, and cytochrome *c* release. The kinetics of intrinsic pathway activation in macrophage-like cells are slower than the kinetics in Stx1-sensitive monocytic THP-1 cells (17).

Roles of NF-κB and MAPKs in Stx1-induced apoptosis and cell survival. Stx1 rapidly activates the transcriptional factor NF-κB in human peripheral blood monocytes and macrophage-like cells (34). NF-κB has been shown to be an important antiapoptotic signaling molecule in response to various stimuli. To determine if NF-κB activation is involved in cell survival, macrophage-like cells were pretreated with the NF-κB inhibitor PDTC for 1 h prior to treatment with Stx1 for 24 h. As shown in Fig. 4A, apoptosis in response to toxin treatment was significantly increased (up to 70%) in the face of NF-κB inhibition. Treatment of macrophage-like cells with Stx1 also resulted in activation of the JNK and p38 MAPK cascades (3). To examine the role of these pathways in cell death or survival, we blocked JNK and p38 activation 1 h before toxin treatment using specific inhibitors and measured apoptosis following exposure of cells to Stx1. Macrophage apoptosis was modestly but significantly ($P < 0.01$) increased in the face of JNK inhibition, while blocking p38 phosphorylation resulted in decreased apoptosis ($P < 0.01$) (Fig. 4A). Stx1 rapidly induced nuclear localization of NF-κB and p38 activation in macrophage-like THP-1 cells, starting at 30 min and 2 h, respectively, after toxin stimulation (3, 34). Based on these data, we hypothesized that rapid NF-κB and MAPK activation are necessary for cell survival and apoptotic signaling. To address this hypothesis, macrophage-like THP-1 cells were treated with NF-κB, JNK, or p38 inhibitors 4 h after Stx1 treatment, and after 24 h of incubation, apoptosis was measured. Compared to cells treated with toxin alone, we did not detect significant differences in the percentages of apoptosis for cells treated with PDTC or the p38 inhibitor 4 h after toxin treatment (Fig. 4A), suggesting that NF-κB and p38 MAPK must be activated within a 4-h time frame to have their protective and proapoptotic effects, respectively. In contrast, the levels of apoptosis were significantly increased compared to the levels with toxin treatment alone ($P < 0.01$) in cells pre- or posttreated with the JNK inhibitor, suggesting that long-term activation of the JNK MAPK cascade may be necessary for antiapoptotic signaling.

In contrast to the results obtained with macrophage-like cells, NF-κB and JNK and p38 MAPKs were not extensively activated by Stx1 treatment of monocytic THP-1 cells (data not shown), and NF-κB, JNK, and p38 inhibitor pretreatment did not significantly alter monocyte apoptosis in response to Stx1 (Fig. 4B). Collectively, these data suggest that NF-κB and JNK trigger survival pathways in macrophages, while the p38 MAPK cascade may be involved in signaling for apoptosis. Neither NF-κB nor the MAPKs appear to be involved in Stx1-induced apoptosis of undifferentiated THP-1 cells.

Effect of caspase inhibitors on Stx1-induced NF-κB and MAPK activation. Having shown that caspases are activated in Stx1-treated macrophage-like cells, we tested whether JNK and p38 MAPK pathways are upstream regulators of caspase activation. Cells were pretreated with inhibitors specific for caspase-3, caspase-8, and caspase-9 and the general caspase inhibitor ZVAD-fmk for 1 h, followed by incubation with purified Stx1 for 4 h. Cells were then harvested and lysates were prepared for examination of JNK and p38 activation. As shown in Fig. 5, JNK phosphorylation occurred in the presence of caspase-3, -8, and -9 inhibitors, but surprisingly, pretreatment of cells with ZVAD-fmk resulted in a substantial decrease in JNK activation in response to Stx1 exposure. Activation of p38 was not affected by any of the caspase inhibitors. These data suggest that JNK and p38 are upstream signaling pathways involved in the regulation of Stx1-induced caspase activation.

Effect of NF-κB and MAPK inhibition on Stx1-induced caspase activation. Since Stx1 triggers NF-κB, MAPK, and caspase activation in macrophage-like THP-1 cells, we examined the effects of NF-κB, JNK, and p38 inhibitors on caspase activation. Cells were pretreated with inhibitors for 1 h and then incubated with Stx1 for an additional 8 h. We observed significantly increased ($P < 0.01$) caspase-3 and -8 activities in cells pretreated with PDTC or the JNK inhibitor (Fig. 6). In

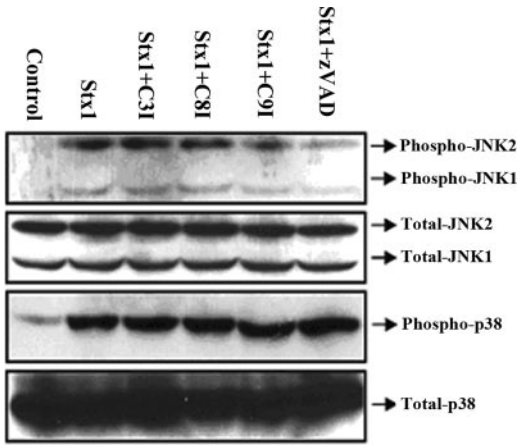

FIG. 5. Effects of caspase inhibitors on activation of JNK and p38. Macrophage-like THP-1 cells were treated with specific caspase-3 (C3I), caspase-8 (C8I), and caspase-9 (C9I) inhibitors (all at a concentration of 40 μM) or the general caspase inhibitor ZVAD-fmk (zVAD) (50 μM) for 1 h. After inhibitor treatment, cells were treated with Stx1 (400 ng/ml) for 4 h. Cells were then collected and lysed. Cell lysates were subjected to Western blotting using phospho-JNK or phospho-p38 specific antibodies. Membranes were stripped and reprobed with antibodies recognizing both activated and nonactivated JNK and p38 to verify equal protein loading. The control results are the results for no inhibitor and Stx1 treatment. The immunoblot shown is representative of the results of two independent experiments.

contrast, inhibition of p38 signaling resulted in slight, but statistically insignificant, decreases in caspase-3 and -8 activation in response to Stx1 treatment. We did not detect significant differences in caspase-9 activity in cells treated with the NF-κB or JNK inhibitor and Stx1, whereas cells pretreated with the p38 inhibitor showed significant decreases in caspase-9 activity ($P < 0.02$). Cells treated with inhibitors alone showed basal (unstimulated control) levels of caspase activities. These data suggest that in response to Stx1 treatment, NF-κB and JNK may be involved in the negative regulation of caspase-3 and -8 activation, while the p38 MAPK cascade may mediate proapoptotic signaling through regulating increased caspase-9 activity.

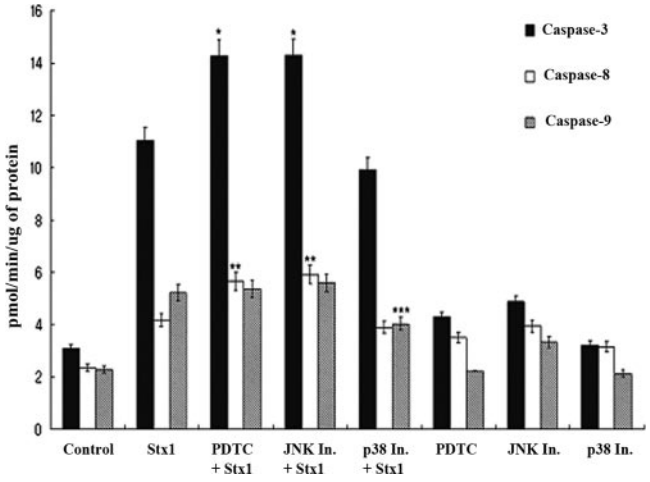

FIG. 6. Stx1 induced caspase activation in cells pretreated with NF-κB or MAPK inhibitors. Macrophage-like THP-1 cells were treated with NF-κB (50 μM PDTC), JNK (50 μM SP600125) (JNK In.), and p38 (20 μM SB203580) (p38 In.) inhibitors prior to Stx1 (400 ng/ml) treatment. Cells were incubated for 8 h, and caspase-3, -8, and -9 activities were analyzed using specific colorimetric substrates as described in Materials and Methods. The control data are the data for no inhibitor and Stx1 treatment. The data are means ± standard errors of the means from three independent experiments. One, two, and three asterisks indicate significant differences ($P < 0.001$, $P < 0.01$, and $P < 0.02$, respectively) for comparisons with Stx1-treated cells.

Role of IAPs in Stx1-induced apoptosis and survival signaling pathways. IAPs share conserved polypeptide domains, and their ectopic expression protects recipient cells from apoptosis induced by viral infection, TNF-α, DNA-damaging agents, or growth factor withdrawal (4). We observed different patterns of IAP expression and degradation in monocytic and macrophage-like THP-1 cells treated with Stx1. In macrophage-like cells, the IAP-1 levels rapidly increased after toxin stimulation, peaking at a 1.5-fold increase 4 h after toxin treatment (Fig. 7A). IAP-2 was constitutively expressed in Stx1-treated macrophage-like cells (Fig. 7B). In contrast, IAP-1 and IAP-2 were almost completely degraded at 12 and 24 h, respectively, in Stx1-treated monocytic THP-1 cells. In macrophage-like cells, XIAP expression increased beginning 4 h after toxin treatment and peaked at 6 h with a 1.7-fold increase (Fig. 7C). XIAP appeared to be degraded 24 h after toxin treatment. In monocytic cells, XIAP degradation began 6 h after toxin treatment. Thus, in macrophage-like cells, Stx1 simultaneously triggers apoptotic signaling through caspase activation (Table 1) and transient activation or constitutive expression of proteins involved in cell survival. In monocytic cells, which are highly sensitive to killing by Stxs, caspases are activated while IAPs are degraded.

Role of protein synthesis inhibition and the ribotoxic stress response in cell death and survival. We previously showed that Stx1 and anisomycin treatment of differentiated THP-1 cells caused a transient increase in total protein synthesis, followed by a decline (3). To ensure that CHX also mediates protein synthesis inhibition in macrophage-like cells, we monitored the incorporation of ^{3}[H]leucine into nascent polypeptides after CHX treatment. We did not detect a transient increase in total protein synthesis in CHX-treated cells; rather, protein synthesis rapidly decreased 1 h after CHX treatment to 8 to 9% of the control levels and for 10 h remained at 3 to 4% of control values (data not shown). We treated THP-1 cells with the three protein synthesis inhibitors for 24 h, and cell killing was measured by the MTT assay. As shown in Fig. 8A, treatment with CHX at concentrations of 10 and 100 μM resulted in significantly less cell death than Stx1 treatment (19.6% ± 3.7% and 29.2% ± 5.5%, respectively, compared with 49.2% ± 15%; $P < 0.01$), while anisomycin (0.07, 1.0, and 10 μg/ml) effectively induced cell death (51.6% ± 3.1%, 86.3% ± 0.5%, and 87.2% ± 0.1%, respectively). We compared the time courses of JNK and p38 activation in response to treatment with the three protein synthesis inhibitors. As shown in Fig. 8B, JNK activation was induced at 1 h and rapidly declined after 2 h of treatment with CHX (10 μM). Similar to the JNK activation results, phospho-p38 was detected after 1 h of CHX treatment, and the level slowly decreased thereafter. In marked contrast, anisomycin and Stx1 triggered prolonged activation of JNK and p38 MAPK cascades. These data suggest that the simultaneous activation of apoptotic and survival signaling in mac-

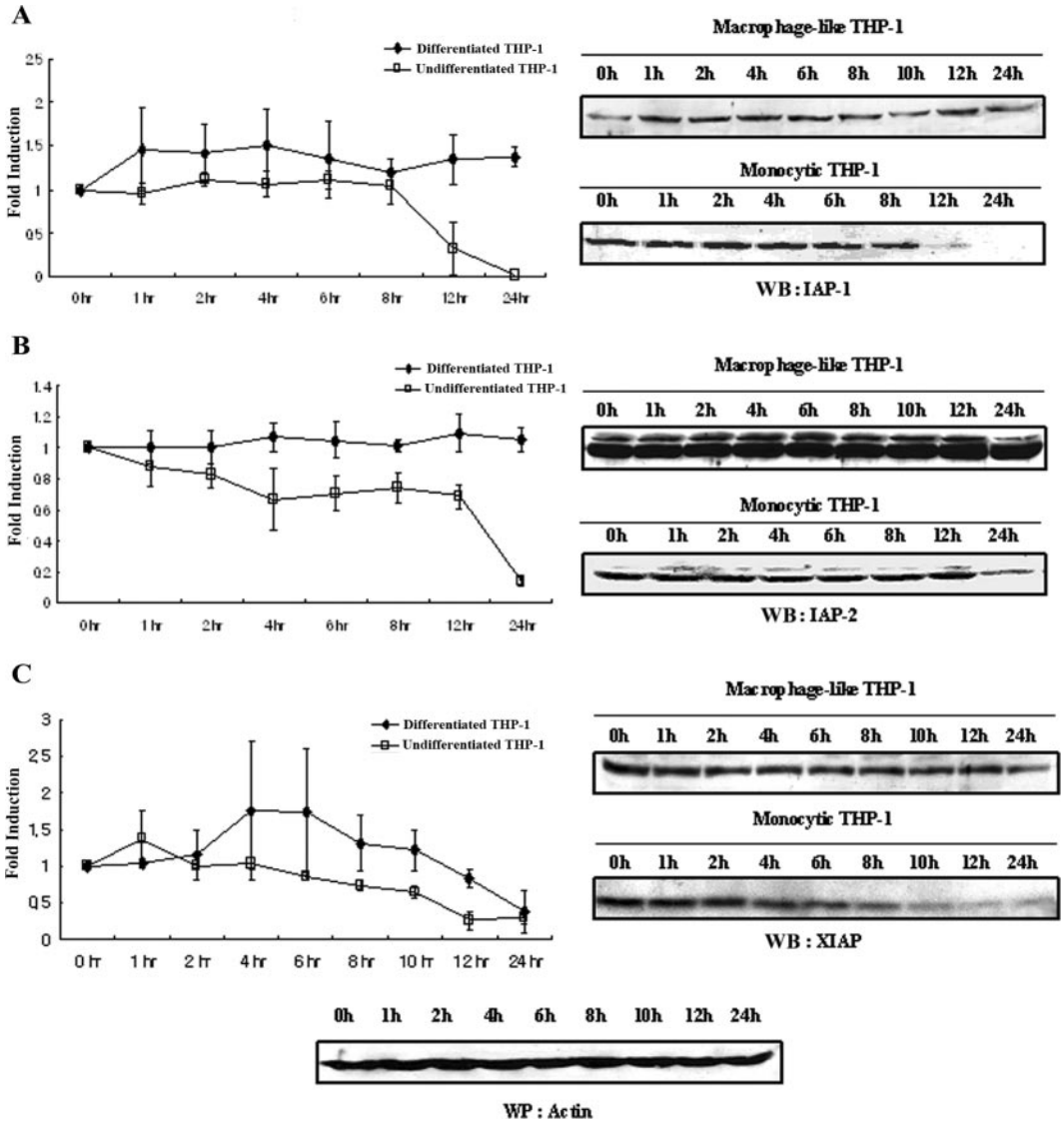

FIG. 7. Comparison of IAP-1, IAP-2, and XIAP expression in Stx1-treated macrophage-like and monocytic THP-1 cells. Both macrophage-like and monocytic THP-1 cells were treated for different times with Stx1 (400 ng/ml). After stimulation, cells were lysed, and equal amounts of total proteins were separated by 8% Tris-glycine SDS-PAGE. For detection of IAP-1 (A), IAP-2 (B), and XIAP (C) by Western blotting (WB), IAP-specific antibodies were used. Based on the relative mobility of IAP-2 in HeLa cell lysates, IAP-2 is the lower band in panel B. The levels of IAP-1, IAP-2, and XIAP were determined by densitometric scanning and compared to the control levels (left panels). The right panels show representative immunoblots from at least two separate experiments. A representative immunoblot for actin is shown at the bottom as a control for protein loading.

rophage-like cells is not associated with protein synthesis inhibition per se but is correlated with prolonged activation of JNK and p38 MAPKs.

DISCUSSION

In this study, we investigated the bases for the cell maturation-dependent differences in THP-1 cell responses to Stx1 (Table 2). Monocytic THP-1 cells grow in suspension, replicate with a characteristic doubling time, express membrane Gb3, and are sensitive to killing by Stx1 so that 83.7% of cells undergo apoptosis within 12 h of toxin exposure. We failed to detect TNFR and mTNF-α expression by monocytic cells using FACS analysis (17). Thus, our data suggested that Stx1-induced apoptosis of monocytic THP-1 cells does not require TNF-α/TNFR1 ligation. Differentiation to the mature, macrophage-like state is accompanied by a cessation of replication, adherence to plastic with the development of pseudopodia, increased phagocytic activity, modest increases in membrane CD14 expression, and decreases in Gb3 expression. The reduction in toxin receptor expression that we have observed in differentiated THP-1 cells may contribute to the relative resistance to Stx1-mediated apoptosis since fewer toxin molecules may be internalized by the cells. In addition, both cell survival and apoptotic signaling pathways may be activated by the toxin in macrophages. As was the case with undifferentiated cells,

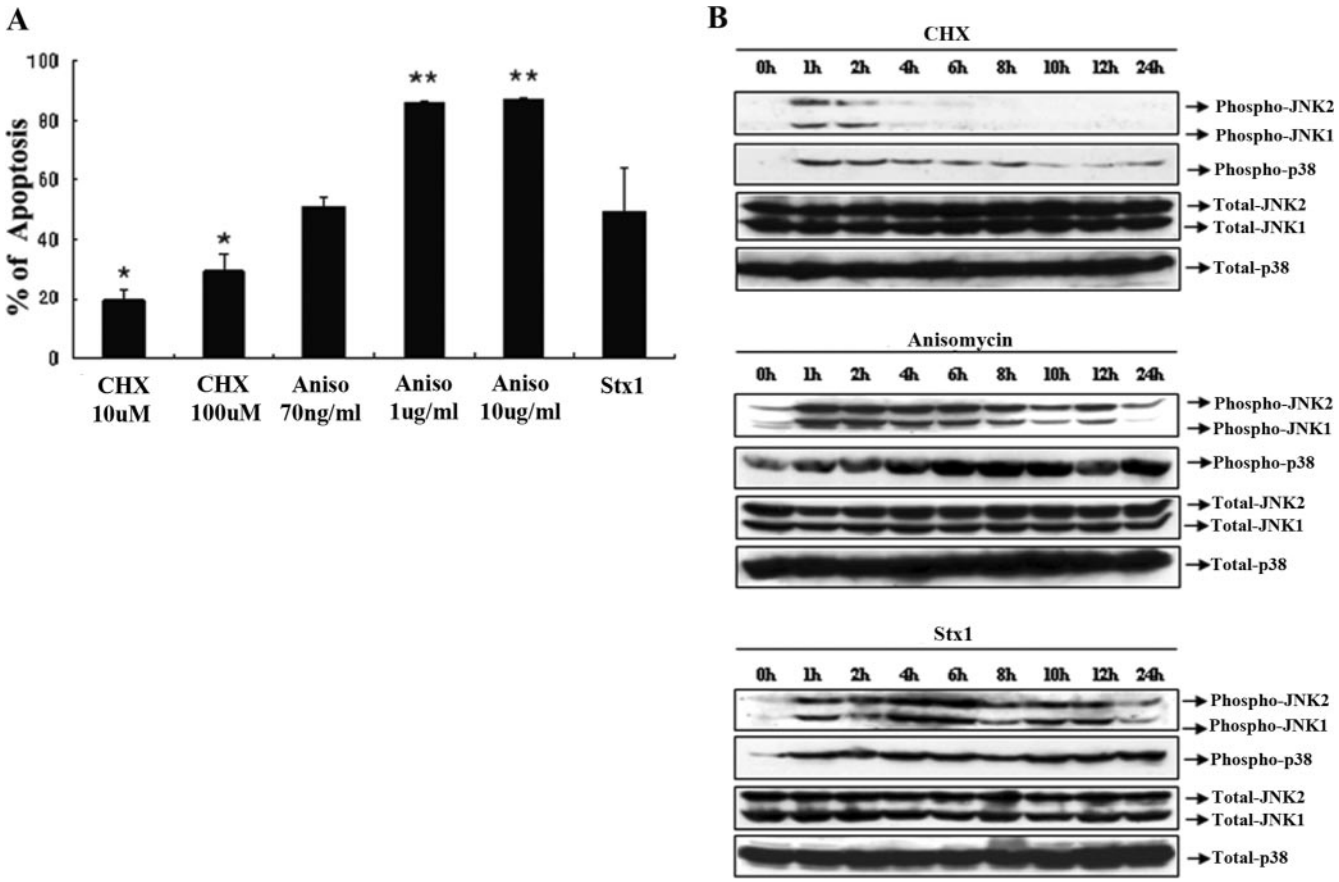

FIG. 8. Anisomycin and Stx1 trigger prolonged activation of JNK and p38 MAPKs in macrophage-like THP-1 cells. (A) Cells were incubated with cycloheximide (10 and 100 μM), anisomycin (Aniso) (70 ng/ml, 1.0 μg/ml, and 10 μg/ml), or Stx1 (400 ng/ml) for 24 h. Cell death was measured by the MTT assay. The data are means ± standard errors of the means from three independent experiments. One and two asterisks indicate significant ($P < 0.01$) decreases and increases, respectively, compared to Stx1-treated cells. (B) Cells were incubated with CHX (10 μM), anisomycin (1.0 μg/ml), or Stx1 (400 ng/ml) for different times. After cells were lysed, equal amounts of total proteins were separated by 8% SDS–PAGE and subjected to Western blotting with antibodies specific for phospho-JNK or phospho-p38. Blots were stripped and reprobed with antibodies recognizing total JNK or p38. The results are representative of two independent experiments.

neither purified Stx1 B subunits (7) nor an enzymatically inactive Stx1 toxoid was capable of triggering cell death in differentiated THP-1 cells. Macrophage-like THP-1 cells become competent to rapidly express or release proinflammatory cytokines, including TNF-α, on exposure to Stx1 (31). Xaus et al. (48) reported that lipopolysaccharide (LPS)-induced apoptosis in macrophages was mediated by TNF-α acting through TNFR1 in an autocrine manner. TNFR1 comprises part of a death receptor, and when TNFR1 is bound to TNF-α, apoptosis may be triggered through a caspase-8-dependent mechanism (24). Based on these data, we hypothesized that Stx1 may trigger macrophage apoptosis, in part, through the production or release of soluble TNF-α. However, compared to the results obtained with monocytic cells, the induction of apoptosis in Stx1-treated macrophages was delayed and diminished so that only 10.7% of the cells displayed an apoptotic phenotype 12 h after toxin exposure (7). We demonstrate here that THP-1 cell differentiation is associated with increased TNFR and mTNF-α expression. However, pretreatment of the cells with neutralizing anti-TNF-α or anti-TNFR1 antibodies failed to alter apoptosis mediated by Stx1. Thus, macrophage-like THP-1 cells express both the ligand and receptor capable of inducing apoptosis, yet apoptosis occurs in only a fraction of cells treated with Stx1.

Addition of exogenous TNF-α failed to mediate apoptosis in either monocytic (17) or macrophage-like cells. Macrophages appear to utilize protective mechanisms mediated, in part, through NF-κB activation to disrupt TNF-α proapoptotic signaling (1). We have previously shown that Stx1 rapidly triggers the nuclear translocation of NF-κB (p50/p65 heterodimer) in macrophage-like THP-1 cells (34). Human blood monocyte-derived macrophages treated with PDTC are highly sensitive to TNF-α-mediated apoptosis (21), as are cells derived from mice deficient in upstream NF-κB activators (19). Expression of a number of apoptosis inhibitors, including members of the Bcl-2 family and IAPs, are regulated by NF-κB (14). We previously noted, however, that simultaneous treatment of macrophage-like THP-1 cells with Stx1 and LPS resulted in the rapid onset of apoptosis, with approximately 75% of the apoptosis occurring within 12 h (7). Here we found that simultaneous treatment of cells with purified Stx1 and rhTNF-α also slightly increased apoptosis. Collectively, our findings suggest

TABLE 2. Summary of monocytic and macrophage-like THP-1 cell responses to Stx1 (400 ng/ml)

Cells	% Apoptosis[a]	Expression (mean fluorescence intensity) of[b]:				Soluble TNF-α expression (pg/ml)[c]	Caspases involved in apoptosis[d]	Time (h) to[e]:					Ribotoxic stress response[f]
		Gb3	Membrane TNF-α	TNFR1	TNFR2			BID activation	Cytochrome *c* release	Degradation of:			
										IAP-1	IAP-2	XIAP	
Monocytic THP-1 cells	83.7	140.7 ± 12.7	1.6 ± 0.1	2.3 ± 0.8	5.7 ± 1.7	ND	8, 3	4	6	12	24	6	−
Macrophage-like THP-1 cells	10.7	74.5 ± 17.6	29.7 ± 2.2	66.3 ± 8.0	93.2 ± 0.8	271 ± 25	8, 9, 3, 6	12	>12	>24	>24	12	+

[a] Percentage of apoptosis 12 h after treatment with Stx1 (7).
[b] Membrane expression was determined by FACS analysis (17, 31).
[c] Soluble TNF-α expression was determined 18 h after treatment with Stx1 (31). ND, not detected.
[d] Main caspases involved in apoptosis induced by Stx1 (17).
[e] Time to first detection of BID cleavage, cytochrome *c* release into the cytoplasm, or IAP degradation after treatment with Stx1 (17).
[f] +, prolonged activation of JNK and p38 MAPKs.

that TNF-α alone does not induce apoptosis in differentiated THP-1 cells. However, even though Stx1 rapidly activates NF-κB signaling cascades, in the face of protein synthesis inhibition, macrophages may not be capable of mounting the compensatory antiapoptotic response triggered by exogenous TNF-α and/or bacterial products that elicit TNF-α production.

In monocytic cells, Stx1 appeared to activate caspase-3, caspase-6, caspase-8, and caspase-9, and inhibitor studies suggested that caspase-3 and caspase-8 are the major caspases involved in apoptosis (17). Stx1 activated the intrinsic pathway of apoptosis induction, triggering Bid cleavage, mitochondrial membrane disruption, and cytochrome *c* release 4 h after toxin exposure We show here that this broad array of caspases and the intrinsic pathway of apoptotic signaling were also activated by Stx1 in macrophage-like THP-1 cells, although the kinetics of Bid activation and cytochrome *c* release were delayed compared to the results obtained for monocytic cells (Table 2). We reasoned that if the intrinsic pathway was important in Stx1-induced macrophage apoptosis, then nonadherent cells should have an increased loss of mitochondrial membrane potential compared to viable cells remaining plastic adherent, and we found that this indeed was the case. These data suggest that macrophage-like THP-1 cells possess functional apoptotic signaling mechanisms leading to caspase activation and mitochondrial membrane perturbation, yet only a fraction of cells undergo apoptosis after exposure to Stx1.

We and other workers have shown that Stx1 activates the stress-activated JNK and p38 protein kinase cascades (3, 12, 37). While NF-κB has been reported to be involved in cell survival signaling, JNK and p38 MAPK cascades may be associated with cell survival or apoptosis pathways depending on the cell type (47). We used inhibitors of these signaling molecules to examine their role in cell death and survival. Inhibition of NF-κB and JNK signaling resulted in increased macrophage apoptosis following exposure to Stx1, while pretreatment with the p38 inhibitor reduced cell killing. To more directly link NF-κB, JNK, and p38 signaling pathways with caspase activation, we examined Stx1-mediated caspase-3, -8, and -9 activation in cells treated with NF-κB, JNK, and p38 inhibitors. Inhibition of NF-κB and JNK significantly increased caspase-3 and -8 activity, while p38 inhibition caused a significant decline in caspase-9 activity. Recent studies using primary human monocytes showed that LPS signaling through Toll-like receptor 4 leads to NF-κB activation and inhibition of caspase-8 activity. LPS induced apoptosis through caspase-8 activation only when NF-κB signaling was blocked (23). In contrast to LPS, Stx1 activates both NF-κB and caspase 8 in differentiated THP-1 cells, yet the majority of cells survive intoxication. We pretreated cells with caspase-3, -8, and -9 inhibitors prior to Stx1 exposure to show that the JNK and p38 MAPK pathways are activated upstream of caspase activation. However, the general caspase inhibitor ZVAD-fmk blocked Stx1-induced JNK activation through a mechanism that remains to be explored. Finally, we found that the anti- and proapoptotic effects of NF-κB and p38, respectively, probably require rapid activation of these signaling molecules, as delaying inhibitor treatment for as short a time as 4 h after toxin treatment failed to alter the percentage of apoptosis. In contrast, the antiapoptotic effects of JNK involve slower or prolonged activation, as

apoptosis was significantly increased even when the JNK inhibitor was added 4 h after Stx1 exposure.

Antiapoptotic factors that may be regulated by NF-κB and the MAPK pathways remain to be fully explored, but the murine genes encoding IAP-1 and IAP-2 are known to be differentially regulated by NF-κB and p38 (29). The IAPs are a family of antiapoptotic proteins characterized by the presence of zinc-binding baculovirus IAP repeat elements (BIR). BIR are essential for direct IAP binding to caspases (35). In addition to possessing three tandem BIR, IAP-1 and IAP-2 also act as E3 ubiquitin ligases, transferring the 76-amino-acid ubiquitin moiety from ubiquitin-conjugating enzymes to target proteins. Ubiquitinated proteins are subjected to proteasome-mediated degradation. The target proteins of IAP-1 and IAP-2 ubiquitination include caspase-3, and caspase-7 (10), the apoptosis regulatory protein SMAC (9, 36), and the NF-κB signaling molecules IKKγ and RIP (30, 40). Unique among the IAP family proteins, IAP-1 and IAP-2 associate with the cytosolic TNFR2 adaptor proteins TRAF-1 and TRAF-2 (33). We show here that IAP-1 and IAP-2 are degraded in Stx1-treated monocytic THP-1 cells, which rapidly undergo apoptosis (Table 2). In macrophage-like cells, IAP-1 expression is transiently upregulated in response to Stx1, while IAP-2 appears to be constitutively expressed. The maintenance of IAP-1 and -2 expression in Stx1-treated macrophages may result in caspase inactivation, in disruption of apoptotic signaling, and in increased TRAF-1 and -2 ubiquitination leading to disruption of TNFR-mediated signaling. XIAP, known to be an inhibitor of caspase-3, -7, and -9 activity (20), was rapidly degraded in Stx1-treated monocytes. The levels of XIAP gradually declined over a 24-h period after Stx1 treatment of macrophage-like cells and did not return to basal levels, as was the case with IAP-1 and 2.

We showed that the protein synthesis inhibitors Stx1, anisomycin, and CHX all reduced total protein synthesis in differentiated THP-1 cells, although the kinetics of inhibition differed. Stx1 and anisomycin triggered a transient increase in protein synthesis lasting 4 to 6 h after intoxication, followed by a decline in protein synthesis (3). We failed to detect a transient increase in protein synthetic activity in CHX-treated cells. Although the three reagents blocked protein synthesis, CHX did not effectively induce cell death, so that protein synthesis inhibition per se was not required for apoptosis in the time frame examined in this study. However, the capacity to induce the ribotoxic stress response, as defined by prolonged activation of the JNK and p38 MAPK cascades, did correlate with the ability of Stx1 and anisomycin to simultaneously trigger cell survival and apoptotic signaling pathways.

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