

Tripeptidyl Peptidase II Promotes Maturation of Caspase-1 in *Shigella flexneri*-Induced Macrophage Apoptosis

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The invasive enteropathogenic bacterium *Shigella flexneri* activates apoptosis in macrophages. *Shigella*-induced apoptosis requires caspase-1. We demonstrate here that tripeptidyl peptidase II (TPPII), a cytoplasmic, high-molecular-weight protease, participates in the apoptotic pathway triggered by *Shigella*. The TPPII inhibitor Ala-Ala-Phe-chloromethylketone (AAF-cmk) and clasto-lactacystin β -lactone (lactacystin), an inhibitor of both TPPII and the proteasome, protected macrophages from *Shigella*-induced apoptosis. AAF-cmk was more potent than lactacystin and irreversibly blocked *Shigella*-induced apoptosis by 95% at a concentration of 1 μ M. Conversely, peptide aldehyde and peptide vinylsulfone proteasome inhibitors had little effect on *Shigella*-mediated cytotoxicity. Both AAF-cmk and lactacystin prevented the maturation of pro-caspase-1 and its substrate pro-interleukin 1 β in *Shigella*-infected macrophages, indicating that TPPII is upstream of caspase-1. Neither of these compounds directly inhibited caspase-1. AAF-cmk and lactacystin did not impair macrophage phagocytosis or the ability of *Shigella* to escape the macrophage phagosome. TPPII was also found to be involved in apoptosis induced by ATP and the protein kinase inhibitor staurosporine. We propose that TPPII participates in apoptotic pathways.

Gram-negative bacteria of the genus *Shigella* cause bacillary dysentery, a potentially fatal diarrheal disease. In the lower gastrointestinal tract, *Shigella* provokes an acute inflammation that leads to tissue destruction and promotes bacterial invasion (15). Early during infection, *Shigella* is phagocytosed by resident tissue macrophages. *Shigella* escapes the phagolysosome and kills macrophages in vitro (18, 43) and in vivo (45) by inducing apoptosis. Apoptotic macrophages release mature interleukin-1 β (IL-1 β) and IL-18, cytokines which trigger the acute inflammation characteristic of dysentery (36, 44). Bacterial virulence factors are encoded on a 220-kb plasmid and include a type III secretion apparatus and the invasion plasmid antigens (Ipa) A to D (17, 25).

The invasin IpaB is secreted by *Shigella* into the macrophage cytoplasm (37). IpaB is necessary and sufficient to induce apoptosis (6, 42) and binds specifically to caspase-1 (IL-1 β converting enzyme; ICE) (19). Caspases are cysteine proteases that cleave after aspartic acid residues. These enzymes are synthesized as zymogens and are activated by proapoptotic stimuli (39). In macrophages infected with *Shigella*, pro-caspase-1 is proteolytically processed to its mature form which cleaves pro-IL-1 β and activates apoptosis (19).

In addition to caspases, other endogenous proteases have been implicated in apoptosis. The eukaryotic 26S proteasome is an N-terminal threonine protease complex with multiple endoproteolytic activities (trypsin-, chymotrypsin-, and caspase-like). The proteasome is required for the cell's general ATP- and ubiquitin-dependent protein turnover, cell cycle progression, and immunosurveillance (3, 31). Moreover, the proteasome participates in apoptotic pathways triggered by growth factor deprivation, radiation, and chemicals (9, 13, 27).

Another high-molecular-weight protease is the cytoplasmic serine protease tripeptidyl peptidase II (TPPII) (40). TPPII is

an amino-endopeptidase and exists as a homo-oligomer in the cytoplasm (1, 2, 11) or as a membrane-bound complex (33). Tripeptidyl peptidases are involved in general protein degradation (40), but TPPII also cleaves specific substrates such as neuropeptides (33). In lymphoma cells adapted to proteasome inhibitors, a proteolytic system with TPPII-like activity compensates for the loss of proteasome activity (12), and TPPII substitutes some proteasomal functions (11). Both the proteasome and TPPII share a similar overall structure, as determined by electron microscopy (3, 11, 24).

TPPII and the proteasome differ in their substrate spectra and inhibition patterns. The *Streptomyces* metabolite lactacystin and its active, more-cell-permeable form, clasto-lactacystin β -lactone (lactacystin), inhibit the proteasome (8, 10) and, less efficiently, TPPII (11). Ala-Ala-Phe-chloromethylketone (AAF-cmk) irreversibly blocks purified TPPII but not the proteasome. Conversely, the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinal (LLnL) does not inhibit TPPII (11). LLnL blocks the proteasome more potently than *N*-acetyl-Leu-Leu-methioninal (LLM) (32). Finally, 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone (LLL-vs) inhibits the trypsin-, chymotrypsin-, and caspase-like proteasomal activities irreversibly (5). Using these inhibitors, we demonstrate here that TPPII participates in *Shigella*-induced apoptosis upstream of caspase-1 and in the apoptotic pathways triggered by staurosporine and ATP.

MATERIALS AND METHODS

Cells, bacteria, and reagents. Mouse J774 macrophage-like cells, mouse peritoneal macrophages (from B57BL/6 or Swiss Webster mice), and human THP.1 monocytic leukemia cells (American Type Culture Collection [ATCC]) were used in this study. Macrophages were routinely cultured in a humidified atmosphere of 5% CO₂ at 37°C in RPMI 1640 glutamine medium (Mediatech; Fisher Scientific, Pittsburgh, Pa.) supplemented with 10% fetal calf serum (Gibco-BRL, Gaithersburg, Md.), L-glutamine (2 mM), and penicillin and streptomycin (50 μ g/ml each). For infections, J774 cells and peritoneal macrophages were cultured overnight. THP.1 cells were differentiated for 3 days in the presence of 160 nM phorbol 12-myristate 13-acetate (PMA). To induce pro-IL-1 β , the macrophages were treated overnight with 1 μ g of lipopolysaccharide (LPS; *Shigella* serotype 1A; Sigma, St. Louis, Mo.) per ml.

The wild-type *Shigella flexneri* strain M90T, a serotype 5A clinical isolate (35),

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and the virulence plasmid-cured, nonvirulent derivative BS176 were grown aerobically in tryptic soy broth medium (Gibco-BRL) at 37°C. The bacteria were centrifuged and resuspended in serum-free RPMI 1640 medium (SFM) prior to infecting the macrophages.

All chemicals were obtained from Sigma unless otherwise stated. The caspase inhibitors and substrates acetyl-Tyr-Val-Ala-Asp-aldehyde (YVAD), acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk), acetyl-Tyr-Val-Ala-Asp-7-amido-4-methyl-coumarin (YVAD-amc), and carboxybenzyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) were purchased from Bachem (King of Prussia, Pa.). Lactacystin was from Boston Biochem, Inc. (Cambridge, Mass.), LLL-vs was from Calbiochem (La Jolla, Calif.), and carboxybenzyl-Phe-Ala-fluoromethylketone (zFA-fmk) from Enzyme Systems Products (Livermore, Calif.). The inhibitors and substrates were prepared as 5 or 10 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at -20°C. Purified caspase-1 was kindly provided by N. Thornberry (Merck, Rahway, N.J.).

Cytotoxicity assays. Cytotoxicity induced by *Shigella* (multiplicity of infection [MOI] of 10 to 50; 2 to 3 h) (7), neutralized ATP (5 mM, 30 min) (21), or staurosporine (1 μ M, 12 h) was routinely analyzed with the CytoTox96 Kit (Promega, Madison, Wis.) as described earlier (46). The percentage of cytotoxicity was calculated by quantifying the amount of lactate dehydrogenase (LDH) released from dying cells according to the following formula: [(experimental release - spontaneous release)/(total release - spontaneous release)] \times 100 (19). LDH release is not specific for apoptosis; however, *Shigella* (43), ATP (21), and staurosporine (30) represent well-established apoptotic systems. *Shigella*-induced apoptosis of differentiated THP.1 cells was assayed by using the Cell Death Detection ELISA^{PLUS} Kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Prior to the addition of the apoptotic agent, the macrophages were incubated with proteasome inhibitors (2 to 3 h) or caspase inhibitors (1 h) when indicated. The viability of macrophages treated with the inhibitors alone was assayed by LDH release, enzyme-linked immunosorbent assay (ELISA), and oxidation of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (16). Within the time frame of the *Shigella* infections and ATP-induced apoptosis, the inhibitors alone were not cytotoxic for the macrophages, and the toxicity of 5% DMSO was less than 4% (data not shown).

Maturation of caspase-1 and IL-1 β in macrophages infected with *Shigella*. Peritoneal macrophages from B57BL/6 mice were infected with *Shigella* (MOI of 5) and lysed in situ at the indicated time points, and the maturation of caspase-1 was analyzed by Western blotting using the rabbit anti-mouse caspase-1 antibody R10311 (kindly provided by D. Miller, Merck) as described previously (18, 19). When indicated, the macrophages were preincubated with AAF-cmk (10 μ M, 2 h), lactacystin (30 μ M, 4 h), or YVAD-cmk (100 μ M, 1 h).

THP.1 cells were infected with *Shigella* at an MOI of 50. When indicated, the cells were treated with AAF-cmk (10 μ M, 2 h), lactacystin (135 μ M, 3 h), or YVAD-cmk (0.5 mM, 1 h) prior to the infection. Maturation of IL-1 β was analyzed by Western blotting as described above, using a goat anti-human IL-1 β antibody (R&D Systems, Minneapolis, Minn.).

Gentamicin protection assay. To discriminate between phagocytosed *Shigella* and extracellular *Shigella*, a modification of the gentamicin protection assay was used (26). At 20 min postinfection, J774 macrophages infected with *Shigella* (MOI of 50) were washed twice with 0.2 ml of phosphate-buffered saline (PBS)-gentamicin (0.1 mg/ml), incubated for another hour in SFM-gentamicin (0.1 mg/ml), and lysed with PBS-Triton X-100 (0.1%). The lysate was diluted and plated on nutrient broth agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% sodium chloride, and the CFU were counted. When indicated, the macrophages were treated with either AAF-cmk (1 μ M, 1 h), lactacystin (5 μ M, 2 h), YVAD-cmk (100 μ M, 1 h), zVAD-fmk (100 μ M, 1 h), or cytochalasin B (2.5 μ g/ml, 1 h) prior to infection.

Detection of actin tails in *Shigella*-infected macrophages. *Shigella*-infected J774 macrophages were labeled with fluorescein isothiocyanate (FITC)-conjugated phalloidin and propidium iodide as described for epithelial cells (4). Briefly, at 30 min postinfection (MOI of 20), the macrophages were fixed, treated with RNase (1 mg/ml, 1 h), and stained with phalloidin-FITC (50 μ g/ml) and propidium iodide (5 μ g/ml) for 45 min. Prior to the infection, some macrophages were incubated with either AAF-cmk (1 μ M) or lactacystin (5 μ M) for 2 h. The samples were analyzed with a Leica DMRBE confocal microscope equipped with an argon-krypton laser.

Fluorometric assays of TPPII and caspase-1. The hydrolytic activity of TPPII during *Shigella* infection was measured with the fluorogenic peptide substrate Ala-Ala-Phe-7-amido-4-methyl-coumarin (AAF-amc). J774 cells were cultured overnight and infected with *Shigella* (MOI of 10) in PBS as described above. At different time points, the cells were lysed with digitonin (final concentration, 20 μ g/ml), AAF-amc (100 μ M) was added, and the reaction was stopped after 30 min with trifluoroacetic acid (final concentration, 0.2%). Prior to analyzing the hydrolysis of AAF-amc, some macrophages were treated for 2 h with different concentrations of AAF-cmk or lactacystin in PBS. The fluorescence of the leaving group 7-amino-4-methyl-coumarin (amc) was measured at 380-nm excitation and 460-nm emission wavelengths with a MicroMax-2 spectrofluorometer (Instruments SA, Edison, N.J.).

The activity of purified caspase-1 was assayed as described above, using YVAD-amc as a fluorogenic peptide substrate (38). When indicated, aliquots of

purified caspase-1 were treated with different concentrations of either AAF-cmk, lactacystin, or YVAD-cmk (2 h, 4°C) prior to assaying the hydrolysis of YVAD-amc.

RESULTS

Effect of TPPII and proteasome inhibitors on *Shigella*-induced macrophage apoptosis. To identify novel components of the apoptotic pathway triggered by *S. flexneri*, we treated J774 macrophages with various inhibitors of TPPII and the proteasome. The TPPII inhibitor AAF-cmk protected macrophages from the *Shigella* wild-type strain M90T in a dose-dependent manner by more than 95% at concentrations of as low as 1 μ M (Fig. 1A). clasto-Lactacystin β -lactone (referred to as "lactacystin") blocked *Shigella*-induced macrophage cell death by approximately 90% at concentrations of between 1 and 10 μ M. In contrast different proteasome inhibitors at concentrations as high as 100 μ M had little (LLM and LLL-vs) or no (LLnL) effect on *Shigella*-induced apoptosis. The peptide inhibitor zFA-fmk, used as a negative control, did not affect *Shigella*-induced cell death at 100 μ M. As expected, the nonvirulent *S. flexneri* strain BS176 was not cytotoxic. These data indicate that TPPII rather than the proteasome is necessary for *Shigella*-induced macrophage apoptosis.

AAF-cmk, a peptide chloromethylketone, irreversibly blocks the serine protease TPPII (11, 12) and reversibly inhibits TPPI (41). To distinguish between TPPII and TPPI, we washed J774 macrophages treated with AAF-cmk prior to infection with M90T. AAF-cmk (5 μ M) irreversibly protected the macrophages from *Shigella*-induced apoptosis (Fig. 1B), ruling out a role for TPPI. As expected, the peptide aldehydes LLM (100 μ M) and YVAD (50 μ M), a competitive inhibitor of caspase-1 (6), reversibly protected the macrophages from *Shigella*-mediated apoptosis.

Primary macrophages treated with inhibitors of high-molecular-weight proteases prior to an infection with M90T showed a protection profile very similar to J774 cells. The TPPII inhibitor AAF-cmk decreased *Shigella*-mediated apoptosis in mouse peritoneal macrophages by 75% at a concentration of 10 μ M (Fig. 1C). At the same concentration, lactacystin decreased *Shigella* cytotoxicity by 64%, LLnL had almost no effect, and LLM or LLL-vs decreased cytotoxicity by approximately 20%. The caspase-1 inhibitor YVAD-cmk (100 μ M) decreased *Shigella*-induced apoptosis by about 80%.

Human monocytic THP.1 cells differentiated with PMA were also protected from *Shigella*-mediated cytotoxicity by AAF-cmk and lactacystin. However, whereas 10 μ M AAF-cmk completely blocked *Shigella*-induced apoptosis, lactacystin and YVAD-cmk were effective only at 10-fold-higher concentrations (data not shown).

To analyze whether the TPPII inhibitors have an effect on *Shigella* rather than on macrophages, we cultured M90T for 2 h in presence of 1 μ M AAF-cmk or 5 μ M lactacystin. Within this time period, treated bacteria reached the same optical density as untreated cultures, demonstrating that the TPPII inhibitors did not affect the ability of *Shigella* to grow (data not shown). M90T, thus treated and washed with serum-free medium, caused only slightly less cell death in infected macrophages compared to mock-treated M90T (Fig. 1D). In contrast, macrophages preincubated with AAF-cmk (1 μ M) or lactacystin (5 μ M) were protected from *Shigella*-induced apoptosis by 82 or 64%, respectively. These data demonstrate that the TPPII inhibitors block *Shigella*-mediated cytotoxicity by affecting only macrophages and not *Shigella*. As expected, preincubation of macrophages with the TPPII inhibitors had no effect on an infection with the nonvirulent strain BS176.

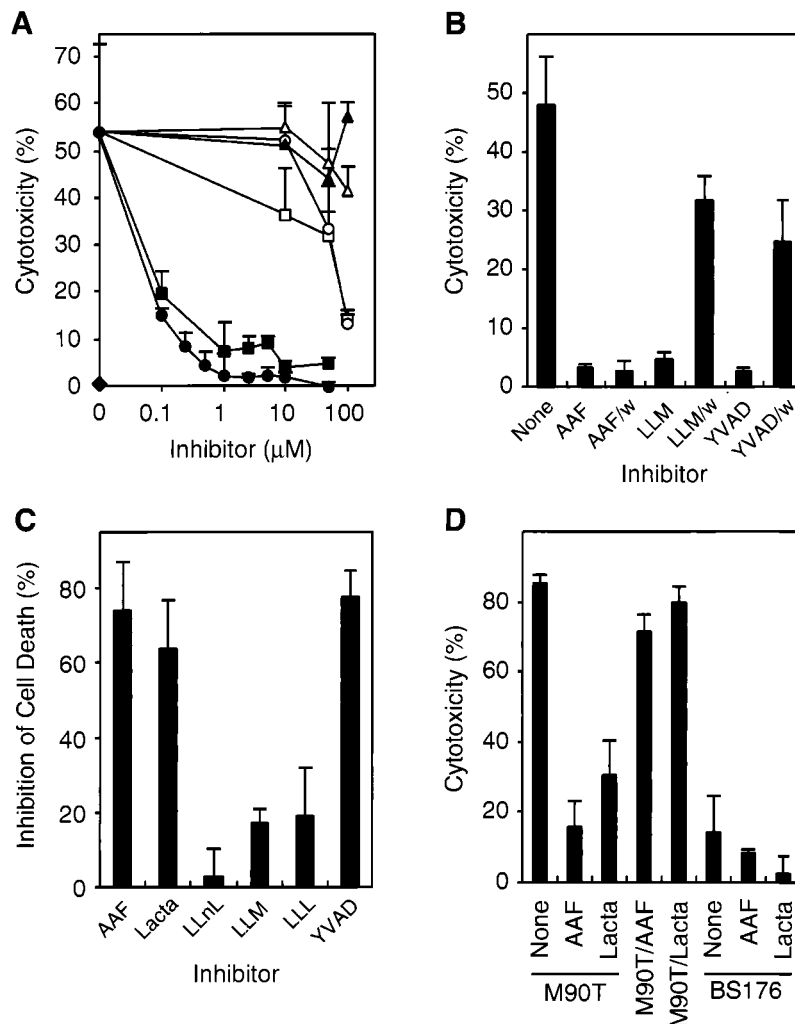


FIG. 1. TPPII is involved in *Shigella*-induced macrophage apoptosis. (A) Mouse J774 macrophages were treated with the TPPII inhibitor AAF-cmk (●), the proteasome-TPPII inhibitor clasto-Lactacystin β -lactone (lactacystin) (■), or the proteasome inhibitors LLnL (▲), LLM (□), LLL-vs (○), or with zFA-fmk (△) and then infected with *S. flexneri* wild-type strain M90T or the nonvirulent strain BS176 (◆) at an MOI of 10. The cytotoxicity was assayed by measuring the LDH release after 2 h. (B) J774 macrophages were treated with AAF-cmk (5 μ M), LLM (100 μ M), or the caspase-1 inhibitor YVAD (50 μ M) and washed (AAF/w, LLM/w, and YVAD/w) prior to M90T infection. (C) Peritoneal macrophages were preincubated with a 10 μ M concentration of the proteasome-TPPII inhibitors indicated or with YVAD-cmk (100 μ M) and infected with M90T. (D) J774 macrophages were infected with M90T grown in the presence of 1 μ M AAF-cmk (M90T/AAF) or 5 μ M lactacystin (M90T/Lacta). In parallel, untreated macrophages (none) or macrophages treated with 1 μ M AAF-cmk (AAF) or 5 μ M lactacystin (Lacta) were infected with mock-treated M90T or BS176. Means and standard deviations of experiments done in triplicate are shown. Similar results were obtained in at least three (A and D) or two (B and C) independent experiments.

Maturation of caspase-1 and IL-1 β in *Shigella*-infected macrophages is inhibited by AAF-cmk and lactacystin. During a *Shigella* infection, caspase-1 matures by limited proteolysis from a 45-kDa precursor to the active enzyme composed of 20- and 10-kDa polypeptides (6, 19). To test the effect of the TPPII inhibitors AAF-cmk and lactacystin on the maturation of pro-caspase-1, we preincubated mouse peritoneal macrophages with either compound and infected the cells with *Shigella*. Lysates of the infected macrophages were prepared at the indicated time points and analyzed by Western blotting with an antibody recognizing the 45-kDa precursor and the 20-kDa processed form of caspase-1. In untreated macrophages, mature caspase-1 was detected as soon as 30 min after infection with the wild-type *Shigella* strain M90T (Fig. 2A). Macrophages infected with the avirulent strain BS176 did not process pro-caspase-1 even by 4 h postinfection. AAF-cmk (10 μ M) and, less potently, lactacystin (30 μ M) blocked the processing

of pro-caspase-1, indicating that TPPII participates upstream of caspase-1 in the apoptotic pathway. Whereas AAF-cmk completely abolished the maturation of caspase-1 throughout the 4-h infection, lactacystin was less efficient, and at 2 h postinfection the 20-kDa caspase-1 fragment began to appear. As previously shown (19), the caspase-1 inhibitor YVAD-cmk (100 μ M) abrogated the processing of pro-caspase-1 during the *Shigella* infection.

M90T but not BS176 triggered the maturation of IL-1 β in differentiated THP.1 cells treated with LPS, indicating that caspase-1 was activated during *Shigella*-induced apoptosis (Fig. 2B). AAF-cmk (10 μ M) and lactacystin (135 μ M) prevented the maturation of IL-1 β in M90T-infected THP.1 cells. As expected, the caspase-1 inhibitor YVAD-cmk (0.5 mM) also abolished the processing of IL-1 β . THP.1 cells not treated with LPS (-LPS) expressed only little pro-IL-1 β .

To test whether AAF-cmk or lactacystin directly inhibit

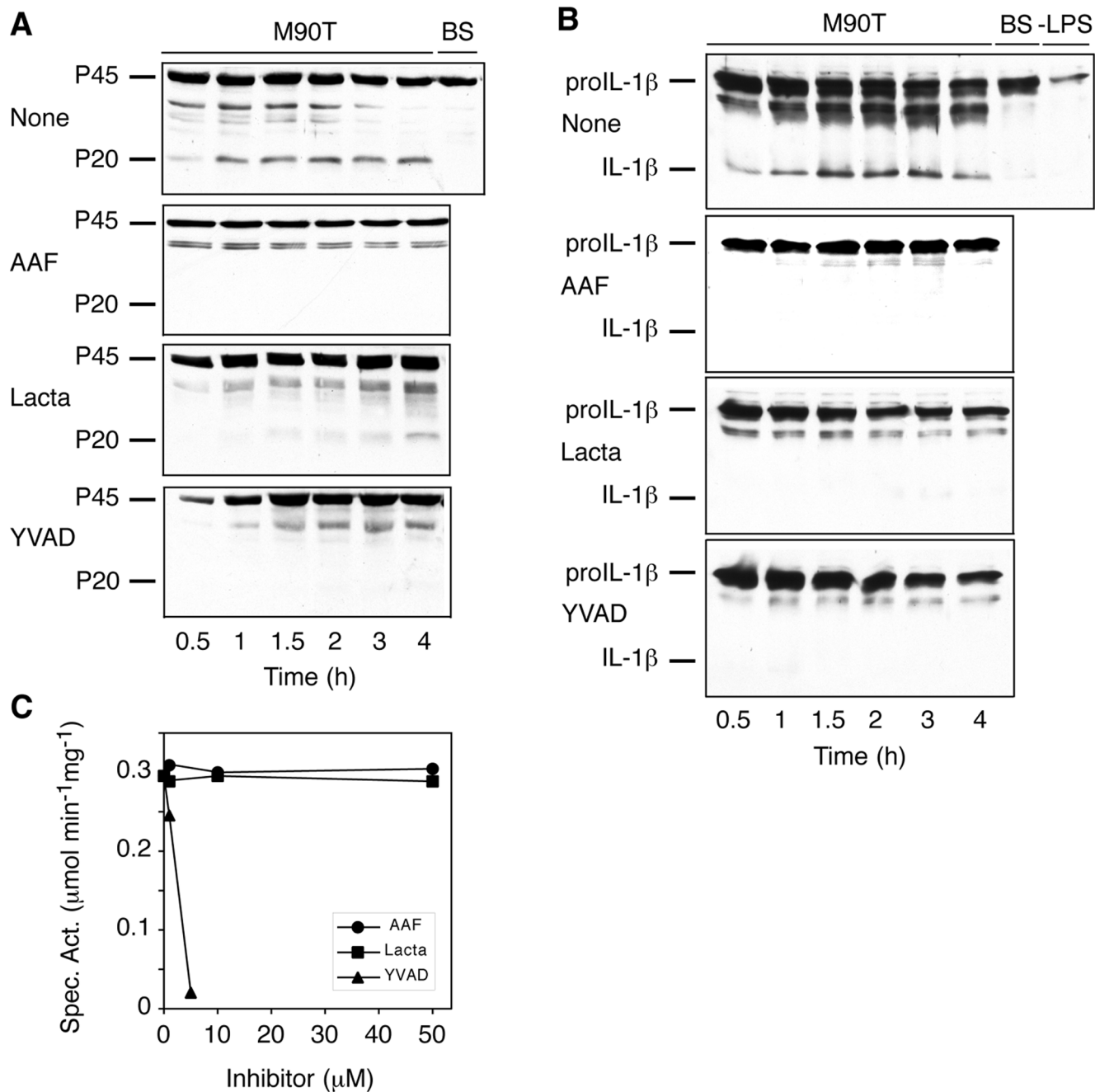


FIG. 2. TPPII is required for the maturation of caspase-1 in *Shigella*-infected macrophages. (A) Peritoneal macrophages (None) and macrophages treated with AAF-cmk (AAF, 10 μM), lactacystin (Lacta, 30 μM), or YVAD-cmk (YVAD, 100 μM) were infected with virulent (M90T, 0.5 to 4 h) or nonvirulent (BS, 4 h) *Shigella* strains. Maturation of caspase-1 was analyzed by Western blotting in lysates of infected macrophages. (B) Differentiated, LPS-stimulated THP.1 cells (None) and cells treated with AAF-cmk (AAF, 10 μM), lactacystin (Lacta, 135 μM), or YVAD-cmk (YVAD, 0.5 mM) were infected with virulent (M90T, 0.5 to 4 h) or nonvirulent (BS, 4 h) *Shigella* strains. Maturation of IL-1β was analyzed by Western blotting in lysates of infected and naive (-LPS) macrophages. (C) The specific activity of purified caspase-1 against the fluorogenic peptide substrate YVAD-amc was determined in the absence or in presence of AAF-cmk, lactacystin, or YVAD-cmk at the concentrations indicated. The specific activity is defined as the number of micromoles of amc released per minute per milligram of protein. The experiments were done at least twice, and results similar to those shown were obtained each time.

caspase-1, we incubated purified caspase-1 with these compounds and assayed the enzymatic activity with the fluorogenic peptide substrate YVAD-amc. Neither AAF-cmk nor lactacystin at concentrations up to 50 μM had any effect on caspase-1 activity (Fig. 2C). Therefore, AAF-cmk and, as shown previously (34), lactacystin do not inhibit caspase-1 directly. In con-

trast, the caspase-1 inhibitor YVAD-cmk (5 μM) almost completely abolished the activity of purified caspase-1.

***Shigella* is protected within macrophages from gentamicin by AAF-cmk and lactacystin.** Intracellular bacteria are protected from the antibiotic gentamicin, which is used to discriminate between intra- and extracellular bacteria (26). As ex-

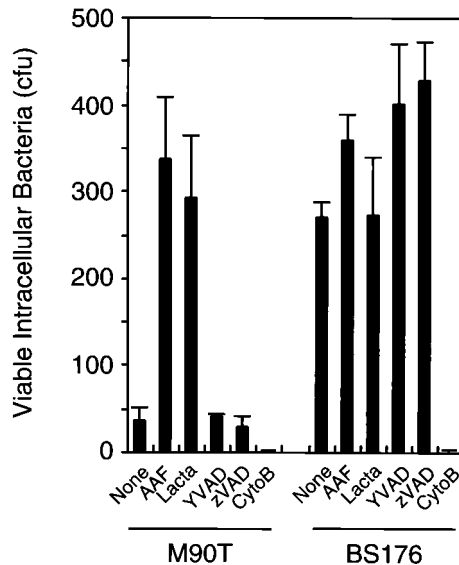


FIG. 3. *Shigella* is protected within macrophages from gentamicin by AAF-cmk or lactacystin. J774 macrophages were treated with the TPPII inhibitors AAF-cmk (AAF, 1 μ M) or lactacystin (Lacta, 5 μ M), with the caspase inhibitors YVAD-cmk or zVAD-fmk (YVAD or zVAD, 100 μ M), or with cytochalasin B (CytoB, 2.5 μ g/ml), an inhibitor of actin polymerization. After infection with either wild-type (M90T) or avirulent (BS176) *S. flexneri* and gentamicin treatment, the CFU were determined. Means and standard deviations of experiments done in quadruplicate are shown. Similar results were obtained in three independent experiments.

pected, the avirulent *S. flexneri* strain BS176 was protected within macrophages from gentamicin (Fig. 3). In contrast, gentamicin killed the wild-type strain M90T and, therefore, apoptotic macrophages appear to become permeable to the antibiotic. To test whether blocking apoptosis would result in protection of M90T from gentamicin, we incubated macrophages with TPPII and caspase inhibitors. Interestingly, treatment of macrophages with either AAF-cmk or lactacystin completely protected intracellular wild-type *Shigella* from gentamicin. The caspase-1 inhibitor YVAD-cmk or the broad-spectrum caspase inhibitor zVAD-fmk, on the other hand, did not protect M90T from gentamicin. However, both caspase inhibitors abolished *Shigella*-mediated cytotoxicity as determined by LDH release (data not shown).

To analyze whether TPPII or caspase inhibitors block bacterial uptake by macrophages, we treated the macrophages with the inhibitors prior to an infection with strain BS176. The inhibitors had no effect on the number of viable avirulent *Shigella* within macrophages, indicating that these inhibitors did not impair the phagocytic activity of the macrophages (Fig. 3). As another control, we preincubated macrophages with cytochalasin B, a potent inhibitor of actin polymerization. No bacteria were recovered from cytochalasin B-treated macrophages, demonstrating that phagocytosis was completely abrogated and that gentamicin quantitatively eradicated extracellular *Shigella*.

Bacterial escape from the phagosome is not impaired by AAF-cmk or lactacystin. Since the TPPII inhibitors AAF-cmk and lactacystin did not affect the phagocytic activity of macrophages, we investigated whether these inhibitors impair the escape of *Shigella* from the macrophage phagosome. Shortly after entry into a host cell, wild-type *Shigella* escapes the phagosome and moves intracellularly by polymerizing actin, resulting in "actin tails" at a single pole of the bacterium (4,

29). To assay the formation of cytoplasmic actin tails, we labeled infected macrophages with phalloidin-FITC and stained both macrophage nuclei and bacteria with propidium iodide. The wild-type *Shigella* strain M90T formed bacterium-associated actin tails in untreated macrophages, as well as in macrophages preincubated for 2 h with 1 μ M AAF-cmk or 5 μ M lactacystin (data not shown). Therefore, AAF-cmk and lactacystin did not prevent the escape of *Shigella* from the phagosome.

TPPII activity is not altered during *Shigella* infection of macrophages. To test whether the activity of TPPII is regulated during an infection of macrophages with *Shigella*, we assayed the hydrolysis of the fluorogenic peptide substrate AAF-amc. TPPII hydrolyzes only peptides with a free amino terminus, and AAF-amc is hydrolyzed by TPPII several orders of magnitude more efficiently than by the proteasome (11). To confirm that the AAF-amc hydrolysis observed was actually due to TPPII activity, we preincubated macrophages with the TPPII inhibitors AAF-cmk and lactacystin and determined the extent of AAF-amc hydrolysis in lysates of macrophages permeabilized with digitonin. AAF-cmk and lactacystin (10 μ M each) blocked the hydrolysis of AAF-amc in macrophage lysates to 94 and 57%, respectively (data not shown), suggesting that the activity measured was due to TPPII.

Up to 1 h postinfection, the specific activity of AAF-amc hydrolysis was similar in lysates of mock-infected macrophages and macrophages infected with either the wild-type strain M90T or nonvirulent BS176 (approximately 40 pmol of amc released min^{-1} mg of protein $^{-1}$; data not shown). Since the hydrolytic activity against a prototypic TPPII peptide substrate was not altered in apoptotic macrophages infected with *Shigella*, TPPII activity is presumably not regulated during the bacterial infection.

TPPII is involved in apoptosis induced by ATP and staurosporine. To test if TPPII is specifically involved in *Shigella*-induced apoptosis or whether it also plays a role in other apoptotic pathways, we analyzed cell death induced by the protein kinase inhibitor staurosporine (30) and ATP (21). At a concentration of 5 μ M, the TPPII inhibitors AAF-cmk and lactacystin decreased staurosporine-induced apoptosis in J774 macrophages by 83 and 75%, respectively (Fig. 4A). Similarly, AAF-cmk and lactacystin (10 μ M each) inhibited ATP-induced apoptosis in mouse peritoneal macrophages by 75 and 54%, respectively (Fig. 4B). In contrast, the proteasome inhibitors LLnL and LLM (data not shown) did not affect apoptosis induced by either staurosporine or ATP. The induction of apoptosis by staurosporine requires a 12-h incubation period. Within this period, AAF-cmk and LLnL were not cytotoxic to the macrophages. However, lactacystin alone caused 40% cell death (data not shown). In summary, the inhibition pattern obtained for staurosporine- and ATP-induced apoptosis indicates that TPPII is also involved in these apoptotic pathways.

DISCUSSION

TPPII participates in *Shigella*-induced macrophage apoptosis. In this report we describe the high-molecular-weight protease TPPII as a previously unidentified component of the apoptotic pathway triggered by *S. flexneri* in macrophages (Fig. 1). The TPPII inhibitors AAF-cmk and lactacystin, but not the proteasome inhibitor LLnL, potentially blocked *Shigella*-induced apoptosis. This inhibition profile is characteristic for purified TPPII and not the proteasome (11). The proteasome inhibitor LLM but not LLnL protected macrophages to some extent from *Shigella*-induced apoptosis, which is inversely correlated to the potential of these compounds to block the proteasome

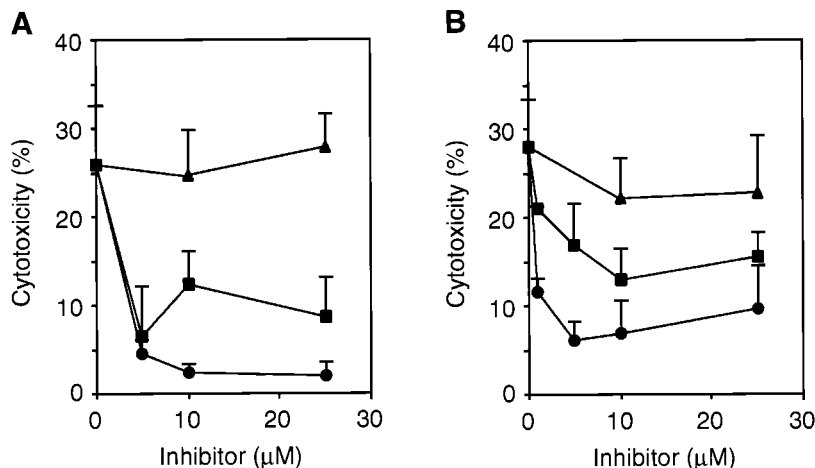


FIG. 4. TPPII is involved in apoptosis induced by staurosporine and ATP. J774 macrophages were incubated with staurosporine (1 μ M, 12 h) (A) or LPS-activated mouse peritoneal macrophages were treated with ATP (5 mM, 30 min) (B) in the absence of in the presence of AAF-cmk (●), lactacystin (■), or LLnL (▲) at the concentrations indicated. The cytotoxicity was determined by measuring the LDH release and is corrected for cell death caused by lactacystin alone. Means and standard deviations of experiments done in triplicate are shown. Similar results were obtained in three (A) or two (B) independent experiments.

(32). In contrast, LLnL inhibited apoptosis more potently than LLM in apoptotic pathways involving the proteasome (14, 34).

AAF-cmk blocks TPPII irreversibly and the lysosomal protease TPPI reversibly (28, 41). AAF-cmk irreversibly blocked *Shigella*-induced apoptosis (Fig. 1), suggesting that cytoplasmic TPPII is involved. Consistent with the idea that AAF-cmk and lactacystin act on a cytoplasmic macrophage protease, we found that the inhibitors (i) block *Shigella*-induced apoptosis by acting on macrophages but not on *Shigella* (Fig. 1), (ii) do not impair phagocytosis of bacteria (Fig. 3), and (iii) do not affect bacterial escape from the phagosome (data not shown). *Shigella* needs to escape the macrophage phagosome in order to induce apoptosis (42) and, therefore, resides in the same subcellular compartment (i.e., the cytoplasm) as TPPII. At present we cannot exclude the possibility that other proteases with an inhibition profile similar to TPPII are also or alternatively involved in *Shigella*-induced apoptosis.

Role of TPPII in *Shigella*-induced apoptosis. TPPII appeared to be upstream of caspase-1 in *Shigella*-induced apoptosis. The TPPII inhibitors AAF-cmk and lactacystin prevented the maturation of caspase-1 and IL-1 β in macrophages infected with *Shigella*, without inhibiting caspase-1 directly (Fig. 2). By activating caspase-1 and consequently IL-1 β , TPPII participates in a proinflammatory apoptotic pathway and contributes to an innate immune response. Similarly, lactacystin has been shown to block the activation of caspase-1 and IL-1 β in macrophages treated with ATP (34). In this and other studies, however, the authors concluded that the proteasome participates upstream of caspases and mitochondrial events in thymocyte apoptosis (14, 20) and neuronal programmed cell death (34).

Macrophages undergoing *Shigella*-triggered apoptosis did not protect the intracellular bacteria from gentamicin (Fig. 3). Wild-type *Shigella* was protected from the antibiotic in macrophages treated with the TPPII inhibitors AAF-cmk and lactacystin, suggesting that TPPII is involved in the process. TPPII might be involved in the exclusion of the antibiotic from the cytoplasm or, alternatively, by preventing its action within the cytoplasm. The caspase inhibitors YVAD-cmk and zVAD-fmk, on the other hand, abolished *Shigella*-induced apoptosis (Fig. 1) (6, 18) but did not protect intracellular *Shigella* from gentamicin. Therefore, caspase-independent processes seem to

occur during *Shigella*-induced apoptosis that are controlled by TPPII.

It is unclear how TPPII participates in *Shigella*-induced apoptosis. The activity of TPPII seems not to be regulated during *Shigella*-mediated apoptosis (data not shown), and TPPII probably operates upstream of caspase-1 (Fig. 2). The *Shigella* invasin IpaB is sufficient to induce macrophage apoptosis and directly binds to and presumably activates caspase-1 (6, 19). Possible roles for TPPII might be to (i) form a complex with IpaB and caspase-1, directly promoting the activation of the caspase, (ii) degrade an inhibitor allowing cell death to be initiated by the IpaB-caspase-1 complex, or (iii) cleave caspase-1 substrates that are part of the apoptotic cascade. A more precise definition of the role of TPPII in apoptosis awaits the identification of its proapoptotic substrate(s).

TPPII is involved in apoptosis triggered by staurosporine and ATP. TPPII might also participate in the apoptotic pathways triggered by staurosporine and ATP. AAF-cmk and lactacystin, but not proteasome peptide inhibitors, protected macrophages from apoptosis triggered by staurosporine and ATP (Fig. 4). The apoptotic pathways triggered by both ATP (23) and staurosporine (22) do not require caspase-1, suggesting that TPPII might also promote the activation of caspases other than caspase-1.

The participation of TPPII in apoptotic pathways is a previously unidentified function for this protease. In addition to its putative role in general protein turnover (40), TPPII also operates as a neuro-endopeptidase (33). Moreover, in EL-4 lymphoma cells adapted to proteasome inhibitors, TPPII or an as-yet-unidentified AAF-amc hydrolyzing activity seems to compensate for the functional loss of the proteasome (11, 12). Cell cycle progression, turnover of ubiquitinated proteins, and generation of some major histocompatibility complex class I-presented peptides was not impaired in these cells. However, it is presently unclear to what extent TPPII has the potential to substitute proteasomal functions. Like TPPII, the proteasome not only functions in immunoregulation but also in apoptosis (9, 13, 27). It currently appears that, depending on the proapoptotic stimulus, either TPPII, the proteasome, or both might participate in a particular apoptotic pathway.

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