Down-Regulation of Bcl-xL and Up-Regulation of Endogenous Bcl-xS and Pseudomonas aeruginosa-Induced Human Mast Cell Apoptosis Is Associated with Down-Regulation of Bcl-xL

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Mast cells are abundant in the tissues adjacent to external surfaces such as lung, intestine, or skin. Mast cells have been repeatedly demonstrated to be critical in the host defense against bacterial infection (1–3). Direct evidence of a role for mast cells in host defense against bacterial infection comes from the study using mast cell-deficient W/Wv mice. In a model of cecal ligation and puncture-induced peritonitis and a model of Klebsiella pneumonia-induced peritonitis or lung infection, animals with a normal number of mast cells survived bacterial challenge, whereas W/Wv mice did not (1, 2). To date, a role for mast cells in the host defense against bacterial infection has been attributed to their released products such as IL-1 and TNF, which in turn recruit other immune cells for the clearance of the pathogen (1, 2, 4). However, little is known about the fate of these mast cells after encountering live bacterial pathogens.

Apoptosis plays a central role in the balance between host defense and the invading pathogen (5). Depending upon the nature of the bacterial pathogen and the population of host cells, apoptosis of the host cells may be detrimental or beneficial to the survival of the host organism. Pseudomonas aeruginosa pneumonia-induced bronchial cell apoptosis is essential for survival, likely through shedding of infected apoptotic bronchial cells (6). In contrast, lymphocyte apoptosis during infection is detrimental, and prevention of lymphocyte apoptosis improves the chances of survival (7). Thus, it is important to differentiate and characterize the apoptotic response in a specific cell population during P. aeruginosa infection. Some cell types such as airway epithelium or endothelial cells are highly resistant to apoptosis in P. aeruginosa pneumonia (5, 8), whereas other cell types such as lymphocytes are highly susceptible to apoptosis during P. aeruginosa infection (5). Mast cells in the lung directly protrude into the airway space that allows the direct interaction of mast cells with bacterial pathogens (9). Although several purified bacterial products such as toxin A from P. aeruginosa (10) or Clostridium difficile (11), or LPS (12) modulate mast cell apoptosis, it is not known whether live P. aeruginosa infection induces mast cell apoptosis.

Caspase-3 activation plays a central role in the execution of apoptosis. Depending upon the specific cell type, two pathways have been reported to be involved in the activation of caspase-3. The death receptor-caspase-8 pathway is essential for apoptosis in type I cells such as lymphocytes (13). Fas-associated death domain-like IL-1-converting enzyme-inhibitory proteins (FLIPs)1 are endogenous inhibitors that counteract caspase-8 pathway activation (14). Although a mitochondria-caspase-9 pathway is required for robust apoptosis in the type II cells such as hepatocytes (15, 16), the balance between the antiapoptotic Bcl family members such as Bcl-xL and Bcl-2 and proapoptotic Bcl family members such as Bcl-xS plays an essential role in maintaining the mitochondrial membrane integrity and regulates mitochondrial pathway-dependent apoptosis (17). Mast cells appear to have mechanisms involving both the FLIPs-associated receptor-caspase-8 pathway and the death receptor-caspase-8 pathway.
Bcl-xS and down-regulation of Bcl-xL, a shift from Bcl-xL to Bcl-xS is proapoptotic through antagonizing survival proteins such as Bcl-2 (20). Due to the antagonistic functions of these proteins, Bcl-xS expression in mast cells. In addition, Bcl-xS expression is the first to show that a shift from Bcl-xL to Bcl-xS is associated with Bcl family protein mitochondrial-dependent and FLIP-associated death receptor pathways. In this study, we demonstrate that human mast cells underwent apoptosis as determined by ssDNA formation in response to live, but not killed P. aeruginosa infection. Similarly, live but not killed P. aeruginosa induced caspase-3 activation in human mast cells. Interestingly, P. aeruginosa infection induced up-regulation of Bcl-xS and down-regulation of Bcl-xL, a shift from Bcl-xL to Bcl-xS expression in mast cells. In addition, P. aeruginosa reduced FLIPs levels in mast cells. To our knowledge, this demonstration is the first to show that a shift from Bcl-xL to Bcl-xS is associated with bacteria-induced apoptosis in mammalian cells. Our results suggest that P. aeruginosa-induced human mast cell apoptosis likely involves Bcl family protein mitochondrial-dependent and FLIP-associated death receptor pathways.

Materials and Methods

Reagents

Mouse anti-sDNA mAb (IgM), biotinylated mouse anti-Bcl-xS/Bcl-xL, rabbit anti-FLIPshort, and rabbit anti-FLIPPlong Abs were purchased from Chemicon International. Mouse anti-rat neutrophil mAb (RP-3, IgM) isotype control was a gift from F. Sendo (Yamagata University, Yamagata, Japan). Rabbit anti-active caspase-3 IgG was purchased from BD Biosciences. Mouse anti-D4-GDI (specific for the 23-kDa form) mAb was purchased from Imgenex. Mouse anti-human Bcl-2 (IgG1) was purchased from Upstate Biotechnology. Goat anti-actin IgG, donkey anti-goat IgG HRP, donkey anti-rabbit IgG HRP, and donkey anti-mouse IgG HRP Ab conjugates were purchased from Santa Cruz Biotechnology. Goat PE-conjugated IgG to mouse IgM was purchased from Caltag Laboratories. 3,3′-Dihexyloxacarbocyanine iodide (DioC6) was from Molecular Probes. Purified P. aeruginosa exotoxin A was purchased from List Biological Laboratories. Camptothecin was obtained from Sigma-Aldrich. FBS, penicillin/streptomycin, IMDM, and RPMI 1640 medium were purchased from Biological Laboratories. Highly purified cord blood-derived mast cells (CBMC) (>95% purity) were obtained by long term culture of cord blood progenitor cells as previously described (23). The percentage of mast cells in the cultures was determined by toluidine blue staining (pH 1.0) of cytocentrifuged samples. Mature mast cells after more than 8 wk in culture were identified by their morphological features and the presence of metachromatic granules, at which time they were used for this study.

FIGURE 1. Induction of human mast cell apoptosis by P. aeruginosa. a and b, HMC-1 cells were treated with increasing concentrations of P. aeruginosa strain 8821 for 24 h before flow cytometric analysis for ssDNA. For the live P. aeruginosa experiment, mast cells were treated with live P. aeruginosa for 3 h at various MOI = 25, 50, or 100, and then a mixture of antibiotics was added to kill P. aeruginosa (200 µg/ml gentamicin, 1% penicillin/ streptomycin (v/v) each 50 U/ml, 100 µg/ml ceftazidime, and 100 µg/ml piperacillin). Live P. aeruginosa induced concentration-dependent generation of ssDNA in HMC-1 5C6 cells. Results are expressed as mean percentage of ssDNA positive cells ± SEM from five independent experiments. *, p < 0.01 compared with HMC-1 cells treated with medium alone. Cells without P. aeruginosa treatment served as controls (NT). Mast cells treated with killed P. aeruginosa (50 killed, MOI = 50; 100 killed, MOI = 100) did not undergo apoptosis. c and d, Human CBMC were treated with medium, live or killed P. aeruginosa 8821 (MOI = 100), for 24 h, then fixed and stained for ssDNA. Similar to HMC-1 cells, CBMC undergo apoptosis after P. aeruginosa treatment. Results are expressed as mean ± SEM. *, p < 0.01 compared with cells treated with medium alone (n = 3). 

Alternative splicing of the Bcl-x pre-mRNA gives rise to two transcripts, coding for either a long-form (Bcl-xL) or a short-form (Bcl-xS) of the protein (19). Bcl-xL inhibits apoptosis through heterodimerization with proapoptotic proteins (20). In contrast, Bcl-xS is proapoptotic through antagonizing survival proteins such as Bcl-xL or Bcl-2 (20). Due to the antagonistic functions of these two splice variants, a shift of the balance between Bcl-xL and Bcl-xS in control of apoptosis has been well recognized, it is not known whether this mechanism of alternative splicing is involved in pathogen-host interaction.

In this study, we demonstrated that human mast cells underwent apoptosis as determined by ssDNA formation in response to live, but not killed P. aeruginosa infection. Similarly, live but not killed P. aeruginosa induced caspase-3 activation in human mast cells. Interestingly, P. aeruginosa infection induced up-regulation of Bcl-xL and down-regulation of Bcl-xS, a shift from Bcl-xL to Bcl-xS expression in mammalian cells. Our results suggest that P. aeruginosa-induced human mast cell apoptosis likely involves Bcl family protein mitochondrial-dependent and FLIP-associated death receptor pathways.

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Murine primary cultured bone marrow-derived mast cells (BMMC) were harvested from the femurs and tibias of C57-black mice from Charles River Breeding Laboratories and maintained as previously described (24). Following 5 wk of culture, mast cell purity of ~98% was achieved as assessed by toluidine blue staining. Mature mast cells were identified by their morphological features and granule prevalence.

**Bacterial preparation and treatment with mast cells**

*P. aeruginosa* strain 8821, a gift from Dr. A. Chakrabarty, University of Illinois (Chicago, IL), is a mucoid strain isolated from a cystic fibrosis patient (25). *P. aeruginosa* was cultured in Luria-Bertani broth and harvested when the culture reached an OD at 640 nm of 2 OD units (early stationary phase). Bacteria were washed in PBS and density adjusted to 1 OD unit before use. For killed *P. aeruginosa* experiments, bacteria were treated with gentamicin (100 μg/ml) for 2 h and exposed directly to UV light illumination for 20 min before experimental use. For live *P. aeruginosa* experiment, mast cells were treated with live *P. aeruginosa* for 3 h at various multiplicity of infection (MOI) values, and then a mixture of antibiotics was added to kill *P. aeruginosa* (200 μg/ml gentamicin, 1% penicillin/streptomycin (v/v) each 50 U/ml, 100 μg/ml ceftazidime, and 100 μg/ml piperacillin). Death of bacteria was confirmed by plating mast cell culture on Luria-Bertani agar plates.

**Detection of ssDNA by flow cytometry**

*P. aeruginosa*-treated or sham-treated mast cells were fixed, permeabilized, and stained with a mAb specific for segments of ssDNA as previously described (26). Briefly, mast cells were fixed for 1–3 days in methanol at −20°C and subsequently heated in formamide at 70°C for 10 min. Non-specific binding was blocked with 1% nonfat dry milk (w/v) in PBS. Cells were stained with anti-ssDNA or IgM isotype control, followed by washing and incubation with a PE-conjugated anti-mouse IgM Ab. After washing, cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

**Preparation of total cell lysate**

Treated cells (0.25 x 10^6–2.5 x 10^6) were homogenized in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaHPO₄, 0.25% sodium deoxycholate, 1% Nonidet P-40, 0.5 mM EDTA, 100 mM sodium pyrophosphate, 100 μg/ml leupeptin, 100 μg/ml aprotinin, 100 μg/ml pepstatin, 1 mM sodium orthovanadate). Cell lysates were subjected to SDS-PAGE and analyzed by Western blotting with mAbs specific for active caspase-3 or actin.

**FIGURE 2.** *P. aeruginosa* induces activation of caspase-3 in mast cells. a, HMC-1 cells were treated with medium (NT) or increasing concentrations of live or killed *P. aeruginosa* (MOI = 25, 50 or 100) for 24 h, then lysed in radioimmunoprecipitation assay buffer. When live *P. aeruginosa* bacteria were used, antibiotics (see Fig. 1) were added at the 3 h and throughout the rest of the incubation period. Sample lysates were subjected to SDS-PAGE and analyzed by Western blotting with mAbs specific for active caspase-3 or actin. b and c, HMC-1 cells were treated with medium or *P. aeruginosa* for 24 h (MOI = 25, 50 or 100), then fixed and permeabilized for staining with FITC-conjugated anti-active caspase-3 Ab for flow cytometric analysis. Representative histograms show that live *P. aeruginosa*-treated HMC-1 5C6, but not medium treated (NT) or killed *P. aeruginosa* (killed 50, MOI = 50; killed 100, MOI = 100) treated cells were stained positive for active caspase-3 (b). Results are expressed as mean percentage of positive staining cells ± SEM of five independent experiments. *, p < 0.05 in c. d and e, Similar to HMC-1 cells, human CBMC after treatment with live, but not killed *P. aeruginosa* 8821 (MOI = 100) for 24 h were stained positive for active caspase-3. Results are expressed as mean ± SEM. *, p < 0.05 (n = 3).

**FIGURE 3.** *P. aeruginosa*-induced D4-GDI cleavage. HMC-1 cells were treated with live *P. aeruginosa* (strain 8821, MOI = 25, 50, or 100) for 3 h. Subsequently, antibiotics were added to kill the bacteria and further incubated for 21 h (total 24 h incubation). Cells without *P. aeruginosa* treatment served as controls (NT). Cell lysates were subjected to SDS-PAGE and Western blotting for the analysis of a 23-kDa cleavage fragment (specifically generated by active caspase-3) of the endogenous caspase-3 substrate D4-GDI. Blots that were probed for actin served as loading control.

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deoxycholate (w/v), 0.1% Nonidet P-40 (v/v), 1 mM Na3VO4, and 1 mM NaF) containing freshly added protease and phosphatase inhibitors (2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM EDTA, 5 mM EGTA, and 2 mM iodoacetamide). Lysates were typically incubated on ice for at least 20 min before centrifugation at 15,000 × g to remove cellular debris. Protein was quantified using a protein quantification reagent according to the manufacturer (Bio-Rad).

Western blotting for active caspase-3, D4-GDI, Bcl-xL/Bcl-xS, and FLIPs
Sample lysates containing 75 μg of protein (for caspase-3), 15 μg (for D4-GDI), 5 μg (for FLIPshort), and 30 μg (for FLIPlong, Bcl-xL/Bcl-xS, Bcl-2, or cytochrome c) were boiled for 5 min and subjected to SDS-10% PAGE. Gels were transferred to polyvinylidene difluoride membrane, and nonspecific binding was blocked using 10% nonfat dry milk. Membranes were then incubated overnight at 4°C with Abs to active caspase-3, D4-GDI, FLIPshort, FLIPlong, Bcl-xL/Bcl-xS, Bcl-2, or cytochrome c and detected by ECL detection reagent (Amersham Biosciences). Membranes were subsequently stripped (62.5 mM Tris-HCl (pH 6.8), 20% SDS (w/v), 100 mM 2-ME) and reprobed for actin.

Detection of active caspase-3 by flow cytometry
Treated mast cells (0.5–1 × 10⁶) were fixed in 4% paraformaldehyde and subsequently stored in 10% DMSO in PBS at −80°C until staining. Cells were thawed and permeabilized with 0.1% saponin in PBS for 1 h followed by incubation in 3% BSA/PBS for 1 h to block nonspecific binding. Cells were then stained with FITC-conjugated rabbit mAb to active caspase-3, washed, and analyzed by flow cytometry.

Detection of mitochondrial membrane potential using DioC₆
Changes of mitochondrial membrane potential were measured using DioC₆ as described elsewhere with minor modifications (27). Mast cells were suspended in 40 nM DioC₆ in medium for 30 min at 37°C. Cells were then transferred to flow cytometry polystyrene tubes and kept on ice. Cells were then analyzed by flow cytometry for green fluorescence.

Statistical analysis
Data were analyzed by one way ANOVA followed by Tukey’s postest, using Instat GraphPad software (version 3.0) to determine the statistical difference between individual treatments. Statistical significance was defined as p < 0.05.

Results
P. aeruginosa induces mast cell apoptosis
To determine whether P. aeruginosa induces mast cell apoptosis, an Ab specific for ssDNA was used because the generation of ssDNA is a specific indicator of apoptosis (26). HMC-1 cells were treated with various concentrations of live P. aeruginosa (strain 8821, MOI = 25, 50, or 100) for 24 h (Antibiotics were added at 3 h and throughout the rest of the incubation period as in Fig. 1). Cells without P. aeruginosa treatment served as controls (NT). Cell lysates were subjected to SDS-PAGE and Western blotting with an Ab that recognizes both Bcl-xL and Bcl-xS. Blots were subsequently stripped and reprobed for Bcl-2 or actin (a). Densitometry analysis of Bcl-xL (b) or Bcl-xS (c) was performed based on three separate experiments. The increase of Bcl-xL to Bcl-xS ratio induced by P. aeruginosa treatment was shown (d).
in mast cells following *P. aeruginosa* infection. To directly examine caspase-3 activation in human mast cells, an Ab that specifically recognizes the activated form of caspase-3 was used. HMC-1 cells were treated with live or killed *P. aeruginosa* with various MOI for 24 h. Cell lysates were used to determine caspase-3 activation by Western blotting. Treatment of mast cells with live *P. aeruginosa* induced an increase of active caspase-3 (Fig. 2a). Similar to ssDNA formation and D4-GDI cleavage, killed *P. aeruginosa* has little effect on caspase-3 activation (Fig. 2a).

To further determine the percentage of mast cell population positive for activated caspase-3, flow cytometry analysis was conducted using intracellular staining with a FITC-labeled Ab specific for the activated caspase-3. The population of mast cells positive for activated caspase-3 after *P. aeruginosa* treatment is dependent on the MOI (Fig. 2, b and c). Approximately 56% of HMC-1 cells were stained positive for activated caspase-3 when mast cells were infected with *P. aeruginosa* at the MOI of 1:100. Consistent with previous results, killed *P. aeruginosa* had little effect on caspase-3 activation (Fig. 2, b and c).

To confirm *P. aeruginosa*-induced caspase-3 activation in primary cultured human mast cells, CBMC were treated with live *P. aeruginosa* at the MOI of 1:100 for 24 h. CBMC were permeabilized and stained with anti-active caspase-3. Similar to HMC-1 cells, CBMC were stained positive for active caspase-3 after treatment with live, but not killed *P. aeruginosa* (Fig. 2, d and e).

*P. aeruginosa* induces D4-GDI cleavage in mast cells

We further confirmed *P. aeruginosa*-induced caspase-3 activation in mast cells by measuring D4-GDI cleavage. D4-GDI is one of the endogenous substrates for caspase-3 (28). Accordingly, cleavage of D4-GDI has been used as an indicator of caspase-3 activity (28). HMC-1 cells were treated with various concentrations of live or killed *P. aeruginosa* (strain 8821) for 24 h, and D4-GDI cleavage was determined by Western blotting. Treatment of *P. aeruginosa* induced significant D4-GDI cleavage leading to the generation of a 23-kDa product, which is specific to caspase-3 activity (Fig. 3). It is noteworthy that only live *P. aeruginosa*, but not killed *P. aeruginosa*, induced enhanced D4-GDI cleavage in mast cells, a pattern consistent with that of ssDNA formation.

*P. aeruginosa* increases Bcl-x<sub>S</sub> and decreases Bcl-x<sub>L</sub> levels in mast cells and decreases mitochondrial membrane potential

Mitochondria play an essential role in the initiation of the apoptotic process by release of proapoptotic substances into the cytosol to activate caspase-3. The balance between the antiapoptotic Bcl family members such as Bcl-x<sub>L</sub> and Bcl-2 and the proapoptotic Bcl family members such as Bcl-x<sub>S</sub> controls apoptosis through several mechanisms including maintaining the integrity of the mitochondrial membrane by preventing the release of proapoptotic substances from the mitochondria (29). To examine whether *P. aeruginosa* regulates the levels of Bcl family members in mast cells, HMC-1 cells were treated with live *P. aeruginosa* with various MOI for 24 h. Cell lysates were used to examine the levels of Bcl-x<sub>S</sub>, Bcl-x<sub>L</sub>, and Bcl-2. Treatment of mast cells with *P. aeruginosa* induced decrease of the antiapoptotic Bcl family member Bcl-x<sub>L</sub> and increased the proapoptotic member Bcl-x<sub>S</sub> (Fig. 4). Interestingly, the level of Bcl-2 was unaffected by *P. aeruginosa* treatment (Fig. 4). Because Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub> are splice variants, we determined the ratio of Bcl-x<sub>S</sub> to Bcl-x<sub>L</sub>. Treatment of mast cells with *P. aeruginosa* induced a consistent increase in the Bcl-x<sub>S</sub> to Bcl-x<sub>L</sub> ratio.

To examine whether Bcl-x<sub>S</sub> expression is a specific effect induced by *P. aeruginosa* treatment, we examined the effect of two apoptosis-inducing agents (camptothecin and exotoxin A) on Bcl-x<sub>S</sub> expression in HMC-1 cells. Camptothecin is an anticancer chemical that induces cell apoptosis by inhibiting topoisomerase I (30). Treatment of HMC-1 with camptothecin (1 μM) induced

FIGURE 5. *P. aeruginosa*, but not exotoxin A or camptothecin induce Bcl-x<sub>S</sub> expression in human mast cells or mouse BMMC. HMC-1 cells were treated for 24 h with camptothecin (Camp) 1 μM (a), 0.1, 1, and 5 μM (b), exotoxin A (ETa) 300 ng/ml live *P. aeruginosa* (Psa live, MOI = 100), killed *P. aeruginosa* (Psa killed, MOI = 100), or without treatment (NT) (c). Cell pellets were used for the examination of D4-GDI, active caspase-3 and Bcl-x<sub>S</sub> to Bcl-x<sub>L</sub> levels by Western blotting and ssDNA formation by flow cytometry. d, Human umbilical CBMC were treated with camptothecin (1 μM), live or killed *P. aeruginosa* (Psa, MOI = 100) for 24 h. Cell pellets were used to examine Bcl-x<sub>S</sub> to Bcl-x<sub>L</sub> expression by Western blotting. e and f, Mouse BMMC were treated with camptothecin (Camp, 1 μM), live or killed *P. aeruginosa* (MOI = 100) for 24 h. Cell lysates were examined by Western blotting with Abs to active caspase-3, D4-GDI, Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub>, or actin. Cells without treatment (NT) were used as controls.
**FIGURE 6.** *P. aeruginosa* treatment induces loss of mitochondrial potential and release of cytochrome c from mitochondria to cytosol. (a and b) HMC-1 cells were treated with various concentrations of live *P. aeruginosa* (MOI = 25, 50, or 100). Then antibiotics (see Fig. 1) were added to kill bacteria and were present throughout the rest of 21 h incubation time (total 24 h incubation). Cells treated with killed *P. aeruginosa* (MOI = 100) or camptothecin (1 μM) served as controls. Cells were then used to stain with DiOC6 and analyzed by flow cytometry. Representative flow cytometry histogram showed that *P. aeruginosa* treatment induces loss of mitochondrial potential in mast cells (a). Results are expressed as mean percentage of positive staining cells ± SEM of three independent experiments. *p < 0.01 compared with no treatment group (b). c) HMC-1 cells were treated with live *P. aeruginosa* (Psa Live), killed *P. aeruginosa* (Psa Killed) (MOI = 100), or camptothecin (Camp, 1 μM) for 24 h. Cells were then subjected to mitochondria/cytosol fractionation to obtain mitochondria-free cytosol fraction. Cytosol proteins were probed for cytochrome c, D4-GDI, or actin by Western blotting. Cells without *P. aeruginosa* treatment (NT) were used as a control.

D4-GDI cleavage, active caspase-3 production, and ssDNA formation (Fig. 5a) and reduced FLIP<sub>short</sub> level (see Fig. 7a below). However, camptothecin at all concentrations tested (0.1, 1, and 5 μM) did not induce Bcl-x<sub>S</sub> expression, although at higher concentrations (1 and 5 μM) it reduced Bcl-x<sub>L</sub> level (Fig. 5b). This result is in contrast to live *P. aeruginosa* infection, which consistently induces Bcl-x<sub>L</sub> expression. Killed *P. aeruginosa* appears to have no effect on Bcl-x<sub>L</sub> expression (Fig. 5c).

Effects of *P. aeruginosa* exotoxin A on Bcl-x<sub>S</sub> formation were examined because we showed previously that exotoxin A induced HMC-1 cell apoptosis (10). HMC-1 cells were treated with exotoxin A (300 ng/ml) for 24 h. Interestingly, no effect of exotoxin A on Bcl-x<sub>S</sub> was observed (Fig. 5c).

To examine whether *P. aeruginosa* induces Bcl-x<sub>S</sub> formation in primary cultured human CBMC, mature CBMC were treated with live or killed *P. aeruginosa* for 24 h. Cell lysates were examined for Bcl-x<sub>S</sub>. As seen in HMC-1, Bcl-x<sub>S</sub> expression was induced by treatment with live but not killed *P. aeruginosa* (Fig. 5d). Camptothecin was used as a control and showed no effect on Bcl-x<sub>S</sub> expression.

To examine whether primary cultured mouse BMMC respond to *P. aeruginosa*, mature mouse BMMC were treated with live *P. aeruginosa*, killed *P. aeruginosa*, or camptothecin. Live *P. aeruginosa* induced increase of Bcl-x<sub>S</sub>, caspase-3 activation, and D4-GDI cleavage in mouse BMMC (Fig. 5, e and f). In contrast, killed *P. aeruginosa* had minor or no effects on Bcl-x<sub>S</sub>, active caspase-3, or D4-GDI levels, a pattern similar to that seen in HMC-1 cells. Similarly, although camptothecin induced caspase-3 activation and D4-GDI cleavage, it did not induce Bcl-x<sub>S</sub> formation (Fig. 5, e and f). Thus, mouse mast cells and human mast cells respond to *P. aeruginosa* and undergo apoptosis likely through similar mechanisms.

To determine whether *P. aeruginosa* treatment affects mast cell mitochondrial membrane permeability, DioC<sub>6</sub> was used. HMC-1 cells were treated with live *P. aeruginosa* with various MOI for 24 h or treated with killed *P. aeruginosa* (MOI = 100) for 24 h. HMC-1 cells were also treated with camptothecin (1 μM) for 24 h as a control. After treatment, cells were stained with DioC<sub>6</sub>. Treatment with camptothecin or live, but not killed *P. aeruginosa* induced loss of mast cell mitochondrial membrane potential (Fig. 6, a and b), suggesting an increase of mitochondrial membrane permeability.

To further determine a role of mitochondrial pathway in *P. aeruginosa*-induced mast cell apoptosis, we examined whether *P. aeruginosa* treatment induces cytochrome c release into cytosol compartment. HMC-1 cells were treated with camptothecin (1 μM), live or killed *P. aeruginosa* (MOI = 100) for 24 h. Cells were then subjected to mitochondria-cytosol separation using a mitochondria-cytosol fractionation kit (BioVision). The release of cytochrome c in the cytosol fraction was examined by Western blotting. Treatment of mast cells with live *P. aeruginosa* induced release of cytochrome c into cytosol (Fig. 6c). In contrast, killed *P. aeruginosa* only induced a low level of cytochrome c release. Treatment with camptothecin also induced a significant release of cytochrome c. Similarly, camptothecin or live but not killed *P. aeruginosa* induced D4-GDI cleavage when cytosol fractions were probed for cleaved D4-GDI product by Western blotting (Fig. 6c).

*P. aeruginosa* down-regulates FLIP<sub>short</sub> and FLIP<sub>long</sub> in mast cells. FLIPs are endogenous proteins that regulate caspase-3 activation through interaction with caspase-8 (14, 31). To determine whether FLIPs are involved in live *P. aeruginosa*-induced mast cell apoptosis, HMC-1 cells were treated with live or killed *P. aeruginosa*.
We determined whether P. aeruginosa cytometry (data not shown). P. aeruginosa (mast cell to bacteria ratio of 1:50) for various times of the incubation period. Cell lysates were examined by Western blotting (Antibiotics as described in Fig. 1 were added at 3 h and throughout the rest of the incubation period). Cell lysates were examined by Western blotting with Abs to FLIPshort (a), FLIPlong (b), or actin. Cells without P. aeruginosa treatment (NT) were used as controls.

strain 8821 for 24 h at the MOI of 1:50. Cell lysates were analyzed for both FLIPshort and FLIPlong by Western blotting. Treatment of mast cells with live P. aeruginosa induced decrease of both FLIPshort and FLIPlong, an effect similar to that of exotoxin A. Killed P. aeruginosa appears to have little effect on FLIP levels (Fig. 7). Treatment of HMC-1 cells with camptothecin induced reduction of FLIPs (Fig. 7). This result is in contrast to its lack of effect on Bcl-xL expression. Surface Fas (CD95) is associated with FLIPs-regulated receptor-caspase-8 pathway and was reported to be up-regulated in epithelial cells by a laboratory strain of P. aeruginosa (1–3). Recently, we demonstrated an active mast cell-bacterial pathogen-induced apoptosis in mammalian cells. One major advantage of using the mechanism of splicing shift producing variants with opposing effect is that one molecule of antiapoptotic Bcl-xL is replaced with one molecule of proapoptotic Bcl-xS, leading to an increased net effect in the control of apoptotic process.

The production of specific Bcl isoforms with opposite effects on the apoptotic response is likely controlled by the promoter usage (33). Several bcl-x gene promoters have been identified (33, 34). The usage of different promoters leads to the generation of different bcl gene products, with promoter 1 primarily producing Bcl-xL and promoter 2 producing Bcl-xS (33). Accordingly, it is possible that upon P. aeruginosa infection human mast cells change the usage of bcl gene promoter leading to the decrease of Bcl-xL and increase of Bcl-xS. However, the promoter usage appears to be tissue specific (33). Thus, it remains to be determined whether P. aeruginosa-induced Bcl-x isoform shift also occurs in other cell types or other pathogen-induced apoptosis because different cell types possess distinct mechanisms in response to different bacterial pathogen. In addition, because mechanisms involved in live P. aeruginosa-induced mast cell apoptosis are likely multifactorial, the causative relationship between Bcl-xL/Bcl-xS levels and mast cell apoptosis requires further study.

It has been suggested that members of the Bcl-xL together with other Bcl-2 gene family members control mitochondrial membrane permeability during apoptosis by regulating the electrical and osmotic homeostasis of mitochondria (29, 35). Using DioC6 as a probe, we observed a loss of mitochondrial potential in mast cells treated with P. aeruginosa. In addition, P. aeruginosa treatment induced release of cytochrome c from mitochondria. These results support a role of mitochondrial pathway in P. aeruginosa-induced human mast cell apoptosis.

An additional mechanism in the initiation of caspase-3 activation is the FLIPs-associated death receptor-caspase-8 pathway. Cellular FLIPs structurally resemble caspase-8 except that they lack proteolytic activity (14). Thus, FLIPs function as intrinsic inhibitors of caspase-8 pathway activation. Treatment of mast cells with live P. aeruginosa reduced the protein levels of both FLIPlong and FLIPshort, suggesting a potential role of death receptor-caspase-8 pathway in P. aeruginosa-induced mast cell apoptosis. We also attempted to determine whether levels of cell surface Fas (CD95) on mast cells were altered by P. aeruginosa treatment because Fas was up-regulated by P. aeruginosa on epithelial cells (32). Little changes of surface Fas were observed when human mast cells were treated with P. aeruginosa for various times.
(3–48 h) (data not shown), suggesting that mast cells and epithelial cells likely respond differently to *P. aeruginosa*.

Interestingly, both ssDNA formation and activation of caspase-3 were induced by live, but not killed *P. aeruginosa*. Further studies are needed to determine the specific bacterial components that are responsible for inducing mast cell apoptosis. It is possible that live *P. aeruginosa* releases bacterial toxins that induce mast cell apoptosis. Our recent study showed that *P. aeruginosa* exotoxin A induces human mast cell apoptosis that is associated with reduced levels of FLIPs, a feature similar to that induced by live *P. aeruginosa*. However, we noticed that unlike live *P. aeruginosa*, exotoxin A did not induce increased expression of Bcl-xS. In addition, we have demonstrated that mitochondrial pathway is activated by live *P. aeruginosa*. This result is in contrast to exotoxin A, which induces mast cell apoptosis through a mitochondrial-independent pathway (10). Thus, exotoxin A may not be a major factor responsible for *P. aeruginosa*-induced mitochondrial pathway-mediated mast cell apoptosis. *P. aeruginosa*-derived azurin and cytchrome c have been implicated in macrophage and mast cell apoptosis (36). Alternatively, *P. aeruginosa* invasion into mast cells may initiate an intracellular event that directly targets the upstream of initiator caspasas.

In summary, we reported for the first time that human mast cells undergo apoptosis after encountering live bacteria, *P. aeruginosa*, through a mechanism that is associated with a shift of alternative splicing from Bcl-xL to Bcl-xS. *P. aeruginosa*-induced caspase-3 activation in mast cell is accompanied by the loss of mitochondrial potential and reduced FLIPs levels, suggesting a potential role for the Bcl mitochondrial pathway and FLIPs-caspase-8 pathway in *P. aeruginosa*-induced mast cell apoptosis.

**Disclosures**

The authors have no financial conflict of interest.

**References**