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**Pseudomonas aeruginosa-Induced Human Mast Cell Apoptosis Is Associated with Up-Regulation of Endogenous Bcl-x<sub>S</sub> and Down-Regulation of Bcl-x<sub>L</sub>**

Christopher E. Jenkins, Ania Swiatoniowski, Melanie R. Power, and Tong-Jun Lin

Mast cells play a critical role in the host defense against bacterial infection. Recently, apoptosis has been demonstrated to be essential in the regulation of host response to *Pseudomonas aeruginosa*. In this study we show that human mast cell line HMC-1 and human cord blood-derived mast cells undergo apoptosis as determined by the ssDNA formation after infection with *P. aeruginosa*. *P. aeruginosa* induced activation of caspase-3 in mast cells as evidenced by the cleavage of D4-GDI, an endogenous caspase-3 substrate and the generation of an active form of caspase-3. Interestingly, *P. aeruginosa* treatment induced up-regulation of Bcl-x<sub>S</sub> and down-regulation of Bcl-x<sub>L</sub>. Bcl-x<sub>S</sub> and Bcl-x<sub>L</sub> are alternative variants produced from the same Bcl-x pre-mRNA. The former is proapoptotic and the latter is antiapoptotic likely through regulating mitochondrial membrane integrity. Treatment of mast cells with *P. aeruginosa* induced release of cytochrome c from mitochondria and loss of mitochondrial membrane potentials. Moreover, *P. aeruginosa* treatment reduced levels of Fas-associated death domain protein-like IL-1β-converting enzyme-inhibitory proteins (FLIPs) that are endogenous apoptosis inhibitors through interaction with caspase-8. Thus, human mast cells undergo apoptosis after encountering *P. aeruginosa* through a mechanism that likely involves both the Bcl family protein mitochondrial-dependent and the FLIP-associated caspase-8 pathways. *The Journal of Immunology*, 2006, 177: 8000–8007.

Phagocyte 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 airspace 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) 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-x<sub>L</sub> and Bcl-2 and proapoptotic Bcl family members such as Bcl-x<sub>S</sub> 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.

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3 Abbreviations used in this paper used: FLIP, Fas-associated death domain protein-like IL-1β-converting enzyme-inhibitory protein; DioC<sub>6</sub>, 3,3′-dihexyloxacarbocyanine iodide; CBMC, cord blood-derived mast cell; HMC, human mast cell; BMMC, bone marrow-derived mast cell; MOI, multiplicity of infection.
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, the balance between Bcl-xL and Bcl-xS in control of apoptosis has been achieved by artificial antisense oligonucleotides and rendered cells to apoptosis (21, 22). Although the importance 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 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-ssDNA mAb (IgM), biotinylated mouse anti-Bcl-xS/Bcl-xL, rabbit anti-FLIPshort, and rabbit anti-FLIPlong 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 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 Invitrogen.

Mast cells and culture conditions

Human mast cells HMC-1 SC6 were maintained in IMDM in a 5% CO2-humidified atmosphere at 37°C. Culture medium was supplemented with 10% FBS and 50 U/ml each of penicillin and streptomycin. 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 cyt centrifuged 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.
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 $\geq 98\%$ 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*, mast cells were treated with live *P. aeruginosa* for 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 ($0.25 \times 10^6$–$2.5 \times 10^6$) were homogenized in ice-cold radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaHPO$_4$, 0.25% sodium deoxycholate) and further incubated for 2 h in a 37°C water bath. Cells without *P. aeruginosa* treatment served as controls (NT). 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 radioimmune precipitation 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.
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 FLIP_shore), and 30 μg (for FLIP_罔ong, Bcl-x_L/Bcl-x_S, 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, FLIP_short, FLIP_罔ong, Bcl-x_L/Bcl-x_S, 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 posttest, 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 or killed P. aeruginosa strain 8821. Treatment of HMC-1 cells with live P. aeruginosa induced ssDNA formation in a concentration-dependent manner (Fig. 1, a and b). Interestingly, no ssDNA formation was detectable when HMC-1 cells were treated with killed P. aeruginosa (Fig. 1, a and b).

P. aeruginosa induces caspase-3 activation in mast cells

Because caspase-3 plays a central role in the execution of apoptosis, several approaches were taken to examine the activation of caspase-3
FIGURE 5. *P. aeruginosa*, but not exotoxin A or camptothecin induce Bcl-xL expression in human mast cells or mouse BMCC. 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 BMCC 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>/Bcl-x<sub>S</sub>, or actin. Cells without treatment (NT) were used as controls.

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 mAb 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 substrate 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>L</sub> and decreases Bcl-x<sub>S</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 antimicrobial chemical that induces cell apoptosis by inhibiting topoisomerase I (30). Treatment of HMC-1 with camptothecin (1 μM) induced
D4-GDI cleavage, active caspase-3 production, and ssDNA formation (Fig. 5a) and reduced FLIP_short level (see Fig. 7a below). However, camptothecin at all concentrations tested (0.1, 1, and 5 μM) did not induce Bcl-x<sub>L</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 <i>P. aeruginosa</i> infection, which consistently induces Bcl-x<sub>L</sub> expression. Killed <i>P. aeruginosa</i> appears to have no effect on Bcl-x<sub>L</sub> expression (Fig. 5c).

Effects of <i>P. aeruginosa</i> exotoxin A on Bcl-x<sub>L</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>L</sub> was observed (Fig. 5c).

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

To examine whether primary cultured mouse BMMC respond to <i>P. aeruginosa</i>, mature mouse BMMC were treated with live <i>P. aeruginosa</i>, killed <i>P. aeruginosa</i>, or camptothecin. Live <i>P. aeruginosa</i> induced increase of Bcl-x<sub>L</sub>, caspase-3 activation, and D4-GDI cleavage in mouse BMMC (Fig. 5, e and f). In contrast, killed <i>P. aeruginosa</i> had minor or no effects on Bcl-x<sub>L</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>L</sub> formation (Fig. 5, e and f). Thus, mouse mast cells and human mast cells respond to <i>P. aeruginosa</i> and undergo apoptosis likely through similar mechanisms.

To determine whether <i>P. aeruginosa</i> treatment affects mast cell mitochondrial membrane permeability, DioC<sub>6</sub> was used. HMC-1 cells were treated with live <i>P. aeruginosa</i> with various MOI for 24 h or treated with killed <i>P. aeruginosa</i> (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 <i>P. aeruginosa</i> 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 <i>P. aeruginosa</i>-induced mast cell apoptosis, we examined whether <i>P. aeruginosa</i> treatment induces cytochrome c release into cytosol compartment. HMC-1 cells were treated with camptothecin (1 μM), live or killed <i>P. aeruginosa</i> (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 <i>P. aeruginosa</i> induced release of cytochrome c into cytosol (Fig. 6c). In contrast, killed <i>P. aeruginosa</i> 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 <i>P. aeruginosa</i> induced D4-GDI cleavage when cytosol fractions were probed for cleaved D4-GDI product by Western blotting (Fig. 6c).

<i>P. aeruginosa</i> down-regulates FLIP_short and FLIP_long 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 <i>P. aeruginosa</i>-induced mast cell apoptosis, HMC-1 cells were treated with live or killed <i>P. aeruginosa
We determined whether *P. aeruginosa* cytometry (data not shown) did not affect the surface Fas levels as determined by flow cytometry (mast cell to bacteria ratio of 1:50) for various times of the incubation period). Cell lysates were examined by Western blotting for caspase-8 pathway and was reported to be up-regulated in epithelial cells that induce *P. aeruginosa*-induced mast cell apoptosis. We used a recently developed technique based on formamide-induced DNA denaturation in order to assess whether *P. aeruginosa* strain 8821 (Psa live, MOI 100) for 24 h (Antibiotics as described in Fig. 1) was 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.

![FIGURE 7. *P. aeruginosa* down-regulates FLIPshort and FLIPlong in mast cells. HMC-1 cells were treated with camptothecin (Camp, 1 μM), etoxoxin A (ETA, 300 ng/ml), killed *P. aeruginosa* (Psa killed, MOI = 100), or live *P. aeruginosa* strain 8821 (Psa live, MOI = 100) for 24 h (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 for caspase-8 pathway and was reported to be up-regulated in epithelial cells that induce *P. aeruginosa*-induced mast cell apoptosis. We used a recently developed technique based on formamide-induced DNA denaturation in order to assess whether *P. aeruginosa* strain 8821 (Psa live, MOI 100) for 24 h (Antibiotics as described in Fig. 1) was 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.](http://www.jimmunol.org/)

**Discussion**

Mast cells play a critical role in the host defense against bacterial infection (1–3). To date, this important role of mast cells has been attributed to the release of mast cell mediators that in turn recruit other immune effector cells such as neutrophil to clear bacterial pathogen (1–3). Recently, we demonstrated an active mast cell-*P. aeruginosa* interaction, which leads to secretion of biologically active mast cell mediators such as IL-1α and IL-β that induce human neutrophil transendothelial migration (4). However, little is known about the fate of mast cells after encountering bacterial pathogen. Because apoptosis has been shown to be one of the critical mechanisms in the host defense against *P. aeruginosa* infection, we investigated whether mast cells undergo apoptosis after encountering *P. aeruginosa* and the mechanisms involved in *P. aeruginosa*-induced mast cell apoptosis. We used a recently developed technique based on formamide-induced DNA denaturation combined with detection of denatured DNA with a mAb against ssDNA that allows specific detection of apoptotic cells (26). We provide compelling evidence that human mast cells undergo apoptosis after incubation with live *P. aeruginosa*.

*P. aeruginosa*-induced apoptosis was confirmed by the detection of caspase-3 activation. An active form of caspase-3 in *P. aeruginosa*-treated mast cells was detected by Western blotting and flow cytometry. *P. aeruginosa*-induced activation of caspase-3 was further verified by the cleavage of D4-GDI, an endogenous caspase-3 substrate. A central role for caspase-3 in the process of apoptosis has been well recognized. Depending on a specific cell type, two major pathways have been well described in the initiation of caspase-3 activation, the FLIPs-associated death receptor-caspase-8 pathway and the Bcl family-regulated mitochondrial pathway. Both pathways exist in mast cells (12, 18). Bcl family members consist of proapoptotic proteins (Bcl-x<sub>S</sub>, Bax, Bad, and others) and the antiapoptotic proteins (Bcl-2, Bcl-2, and Bcl-w). The balance between proapoptotic and antiapoptotic members determines the fate of many types of cells. Bcl-x<sub>S</sub> and Bcl-x<sub>L</sub> are splice variants produced by alternative splicing of Bcl-x pre-mRNA. The antagonistic functions of Bcl-x<sub>S</sub> (antiapoptotic) and Bcl-x<sub>L</sub> (proapoptotic) have prompted several studies in an attempt to shift the alternative splicing in an effort to control the apoptotic process (21, 22). We found that a shift from Bcl-x<sub>S</sub> to Bcl-x<sub>L</sub> in human mast cells was induced by treatment with *P. aeruginosa*. To our knowledge, this demonstration represents the first that shows that alternative splicing of Bcl-x<sub>S</sub> and Bcl-x<sub>L</sub> is involved in 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-x<sub>S</sub> is replaced with one molecule of proapoptotic Bcl-x<sub>L</sub>, 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-x<sub>S</sub>, and promoter 2 producing Bcl-x<sub>L</sub>, Bcl-x<sub>Q</sub>, and Bcl-x<sub>L</sub> (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-x<sub>S</sub> and increase of Bcl-x<sub>L</sub>. However, the promoter usage appears to be species 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-x<sub>S</sub>/Bcl-x<sub>L</sub> levels and mast cell apoptosis requires further study.

It has been suggested that members of the Bcl-x<sub>L</sub> family controls mitochondrial membrane permeability during apoptosis by regulating the electrical and osmotic homeostasis of mitochondria (29, 35). Using Dio5C<sub>a</sub> 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 FLIP<sub>long</sub> and FLIP<sub>short</sub>, 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 \( \textit{P. aeruginosa} \).

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

In summary, we reported for the first time that human mast cells undergo apoptosis after encountering live bacteria, \( \textit{P. aeruginosa} \), through a mechanism that is associated with a shift of alternative splicing from Bcl-xL to Bcl-xS. \( \textit{P. aeruginosa} \)-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 Bclmitochondrial pathway and FLIPs-caspase-8 pathway in \( \textit{P. aeruginosa} \)-induced mast cell apoptosis.

**Disclosures**

The authors have no financial conflict of interest.

**References**