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Acinetobacter baumannii Outer Membrane Protein A Induces Dendritic Cell Death Through Mitochondrial Targeting

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Acinetobacter baumannii **outer membrane protein A (AbOmpA) is a potential virulence factor that induces epithelial cell death, but its pathologic effects on the immune system have yet to be determined. The present study investigated the pathologic events occurring in dendritic cells (DCs) exposed to a cytotoxic concentration of AbOmpA. AbOmpA induced early-onset apoptosis and delayed-onset necrosis in DCs. AbOmpA targeted the mitochondria and induced the production of reactive oxygen species (ROS). ROS were directly responsible for both apoptosis and necrosis of AbOmpA-treated DCs. These results demonstrate that the AbOmpA secreted from *A. baumannii* induces DC death, which may impair T cell biology to induce adaptive immune responses against *A. baumannii*.**

Keywords**:** *A. baumannii*, dendritic cell, immunopathology, ROS

Acinetobacter baumannii is one of the leading causes of nosocomial infections. This opportunistic pathogen causes a variety of human infections, including pneumonia, wound infections, urinary tract infections, septicemia, and meningitis, particularly among critically ill patients (Dijkshoorn *et al*., 2007). Although *A. baumannii* is regarded as a low virulent pathogen, some virulence determinants, including biofilm formation (Lee *et al*., 2008), adherence to and invasion of bacteria in epithelial cells (Choi *et al*., 2008a), and host cell death (Choi *et al*., 2005), have been characterized. However, the immunopathology of *A. baumannii* in association with disease development has yet to be clearly determined.

Non-specific innate host defenses are involved in the clearance of bacterial pathogens and subsequently a robust adaptive immune response is required to overcome bacterial infection. When pathogens are introduced into susceptible hosts, antigen-presenting cells (APCs) capture microbes or microbial products and display microbial antigens to lymphocytes to initiate adaptive immune responses. Dendritic cells (DCs) are professional APCs that possess immune sentinel properties. DC responses against pathogens constitute key elements in the initiation and regulation of adaptive immune responses (Banchereau *et al*., 2000). Therefore, the dysfunction or death of DCs induced by invading pathogens can limit T cell response. Pathogenic bacteria, including *Shigella flexneri* (Kim *et al*., 2008), *Escherichia coli* (De Trez *et al*., 2005), and *Pseudomonas aeruginosa* (Worgall *et al*., 2002) have been demonstrated to inhibit DC maturation or to induce DC death.

Outer membrane protein A of *A. baumannii* (AbOmpA) is a major envelope protein which performs a function in the permeability of small solutes (Jyothisri *et al*., 1999). Additionally, AbOmpA is a potential virulence factor: a bacteria-bound AbOmpA is responsible for adherence to and invasion of bacteria in epithelial cells (Choi *et al*., 2008a) and inhibits complement-mediated bacterial lysis (Kim *et al*., 2009), whereas secreted AbOmpA induces the dysfunction and death of host cells (Choi *et al*., 2005; Kwon *et al*., 2009). *A. baumannii* secretes AbOmpA via outer membrane vesicles (OMVs), and AbOmpA is one of the most abundant proteins associated with OMVs, (Kwon *et al*., 2009), thereby suggesting that AbOmpA secreted from bacteria via OMVs may impair host cell function or induce host cell death. We demonstrated previously that ≥80 nM (3 μg/ml) of AbOmpA induced DC death (Lee *et al*., 2007). However, the molecular mechanisms by which AbOmpA induces DC death remain to be clearly determined. In this study, we employed mouse bone marrow-derived DCs and assessed the pathologic events occurring in DCs exposed to cytotoxic concentrations of AbOmpA.

Materials and Methods

Animals

C57BL/6 (H-2K^b and I-A^b) male mice aged 6-8 weeks were purchased from the Korean Institute of Chemistry Technology (Korea) and housed in an animal facility for at least one week prior to use. All procedures involving animals were approved by the Animal Care Committee of the Kyungpook National University School of Medicine.

Preparation of AbOmpA

The recombinant AbOmpA was prepared as previously described (Lee *et al*., 2007). In brief, the *ompA* gene amplified from *A. baumannii* ATCC 19606^T was ligated into the pET28a vector and transformed into *E. coli* BL21 (DE3). The transformants induced the expression of recombinant proteins with 1 mM isopropyl-β-D-1-thiogalactopyranoside at 25°C for 4 h. After the sonication of bacterial cells, the recombinant proteins were eluted with a HisTrap™ FF column (GE healthcare, USA). The possible contamination of lipopolysaccharide (LPS) was removed using endotoxin removal resin (Sigma-Aldrich, USA). A limulus amebocytes lysate pyrogen kit

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(Sigma-Aldrich) was employed to evaluate LPS contamination, and the quantity of LPS in the final samples was ≤0.01 ng/mg.

Murine bone marrow-derived immature DCs

DCs were prepared from bone marrow-derived cells. Total bone marrow was recovered from the tibia and femurs of C57BL/6 mice. The suspended bone marrow cells were cultured in OptiMEM medium (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 ng/ml of recombinant mouse granulocyte-monocyte colony-stimulating factor (GM-CSF) (R&D Systems, USA), and recombinant mouse interleukin (IL)-4 (R&D Systems). On day 3, the supernatant was replaced with fresh medium containing GM-CSF and IL-4. The non-adherent and loosely adherent DC population was harvested on day 6 or 7, and was analyzed for CD11c expression via flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, USA). The cultured immature DCs (1×10^6 cells/ml) were treated with 80 nM of AbOmpA for the indicated times.

Measurement of cell death

DCs treated with AbOmpA were stained with FITC-conjugated Annexin V and propidium iodide (PI) (BD Pharmingen, USA). The samples were then analyzed via flow cytometry and CellQuest Pro software (BD Biosciences). For each sample, 10^4 cells were acquired for data analysis.

Measurement of intracellular ROS and glutathione levels

Intracellular reactive oxygen species (ROS) were fluorometrically estimated using oxidation-sensitive fluorescent probes, 5,6-carboxy-2',7'-dichlorofluorescein-diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR) (Molecular Probes, USA). DCs treated with AbOmpA were washed three times in 5 mM HEPES-buffered saline and incubated for 15 min in HEPES-buffered saline with 1 μM DCFH-DA and for 2 min with 0.1 μM DHR. The samples were analyzed via flow cytometry. Total intracellular glutathione contents were estimated via an enzymatic recycling assay. The DCs were washed twice in phosphate-buffered saline (PBS) and lysed with lysis buffer. The lysates were incubated for 10 min with 5-sulfosalicyclic acid. The acid-precipitated proteins were then centrifuged for 10 min at 15,000×g and the protein concentrations were determined via a Bradford assay. Total glutathione contents were determined using a standard curve with known quantities of glutathiones.

Western blotting

DCs treated with AbOmpA were lysed with modified RIPA buffer (1.0% NP-40, 1.0% sodium deoxycholate, 150 nM NaCl, 10 mM Tris-HCl; pH 7.5, 5.0 mM sodium pyrophosphate, 1.0 mM $NaVO_4$, 5.0 mM NaF, 10 mM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride) at 4°C. The cell lysates were centrifuged and the protein concentration of each sample was determined via Bradford assays. Thirty micrograms of protein extracts were resolved via 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, USA). The blots were probed with primary antibodies against poly (ADP-ribose) polymerases (PAPR, Calbiochem, USA), caspase-3 (Calbiochem), caspase-8 (Calbiochem), caspase-9 (Calbiochem), and β-actin (Santa Cruz Biotechnology, USA). The membranes were incubated with an appropriate secondary antibody conjugated with horseradish peroxidase. An enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used for detection in accordance with the manufacturer's instructions.

Confocal laser scanning microscopy

DCs were seeded in glass coverslips and treated with AbOmpA. The culture medium was removed and prewarmed medium containing 100 nM of MitoTracker™ (Molecular Probes) was added. The samples were then immersed for 10 min in a solution of 3.5% paraformaldehyde and 0.2% Triton X-100. The nuclei were then stained with dihydrochloride (DAPI) (Molecular Probes). AbOmpA was stained with polyclonal anti-rabbit OmpA antibody and Alexa Fluor® 568-conjugated goat anti-rabbit IgG antibody (Molecular Probes). The subcellular localization of AbOmpA was analyzed with a Nikon C1Si confocal laser scanning microscope system (Japan).

Transmission electron microscopy

DCs were treated with AbOmpA for the indicated times and washed with PBS. After centrifugation, the DCs were fixed with 2.5% glutaraldehyde. The cells were washed three times with 0.2 M Na cacodylate-HCl (pH 7.4) for 10 min each, and post-fixed for 30 min with 1% OsO_4 and 0.15 M Na cacodylate-HCl. The cells were dehydrated in a series of ethanol concentrations, and embedded in Epon. Thin sections of 60-80 nm thickness were obtained using an ultramicrotome (Elexience), mounted on copper grids, and contrasted with uranyl acetate and lead citrate. The sections were analyzed with a Hitachi 7000 transmission electron microscope (TEM, Hitachi Science Systems Ltd, USA).

Statistical analysis

Data are expressed as the Means±SD of the indicated number of experiments. Statistical significance was assessed via Student's *t* tests. *P* values of <0.05 were considered statistically significant.

Results

AbOmpA induces DC death through the mitochondrial targeting

In order to determine the cytotoxic potential of AbOmpA, the DCs were treated for 24 h with 80 nM of AbOmpA. The apoptotic and necrotic cell populations were analyzed via flow cytometry by Annexin V/PI staining. The apoptotic cell population (Annexin V^+/PI^- and Annexin V^+/PI^+ cells) steadily increased up to 12 h and then reduced slightly at 24 h, whereas the necrotic cell population (Annexin V^-/PI^+ cells) was abruptly increased at 24 h (Fig. 1A). Morphological changes of DCs were evaluated by TEM. At 6 h and 12 h of culture, the AbOmpA-treated DCs exhibited typical characteristics of apoptotic cells, including mitochondrial swelling, chromatin condensation, and nuclear fragmentation (Fig. 1B). Both apoptotic and necrotic cells were noted at 24 h of culture. Under these conditions, necrotic cells evidenced nuclear swelling, losses of cell membrane integrity, and the release of cytosolic contents. In order to evaluate the subcellular localization of AbOmpA, the DCs were treated for 12 h with AbOmpA and the cellular distribution of AbOmpA was determined via confocal microscopy. The colocalization of mitochondria (green) and AbOmpA (red) was noted in the merged images (Fig. 1C). Collectively, these results demonstrate that AbOmpA induces early-onset apoptosis and delayed-onset necrosis in DCs via mitochondrial targeting.

AbOmpA induces ROS production originating from the mitochondria

As mitochondria are the major source of ROS, and ROS

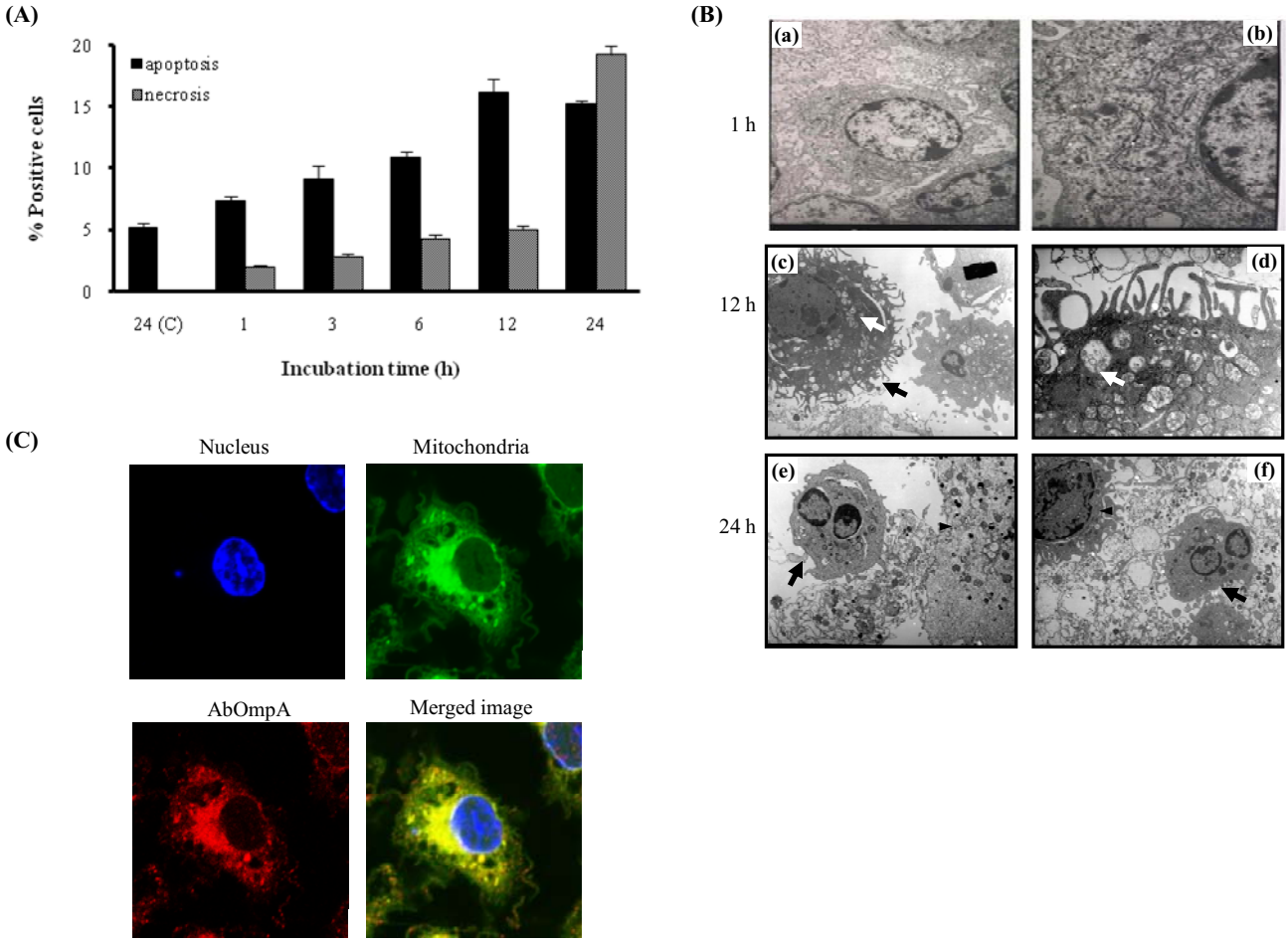

Fig. 1. AbOmpA induces DC death via mitochondrial targeting. DCs were treated with 80 nM of AbOmpA for the indicated times. (A) The cells were gated on $CD11c^+$ cells and analyzed by two-color flow cytometry using an Annexin V/PI staining kit. The Annexin V^+/PI^- and Annexin V^+/PI^+ cells for apoptosis (black bar) and Annexin V^-/PI^+ cells for necrosis (dotted bar) were counted. C, untreated control cells for 24 h. Data are expressed as the Means±SD of three independent experiments. (B) Transmission electron micrographs of the DCs undergoing cell death. Immature DCs were treated with 80 nM of AbOmpA for the indicated times. White and black arrows indicate the mitochondrial swelling and apoptotic cells, respectively. Arrowheads indicate the necrotic cells. Magnification: a, c, e, and f, ×3,000; b, ×8,000; d, ×10,000. (C) Immature DCs were treated for 12 h with 80 nM of AbOmpA. The nuclei and mitochondria were stained with DAPI (blue) and MitoTracker™ (green), respectively. AbOmpA was labeled with rabbit AbOmpA immune serum, followed by Alexa Fluor® 568 secondary antibody (red). The colocalization of AbOmpA and mitochondria evidenced a yellow color in the merged image.

contribute to both apoptotic and necrotic cell death induced by external stimuli (Ricci *et al*., 2003; Skulachev, 2006), we evaluated ROS generation in the AbOmpA-treated cells. Intracellular ROS levels were estimated via flow cytometry using the cell permeable DCFH-DA and DHR. DCF, the fluorescent derivative of DCFH-DA, remains within the cytosol, whereas the fluorescent R123, the product of DHR oxidation, binds selectively to the inner mitochondrial membrane. Upon treatment with AbOmpA, ROS levels in the mitochondria increased as early as 1 h of culture, and reached peak levels at 12 h, as evidenced by R123 fluorescence (Fig. 2A), whereas an elevation of ROS levels in the cytosol was noted as early as 3 h and reached a peak level at 6 h, as demonstrated by DCF fluorescence (Fig. 2B). These findings demonstrate that the intracellular ROS originated from the mitochondria. ROS production is controlled by increased synthesis of reducing equivalents, and the reduction in intracellular glutathione levels has been associated with the hyperproduction of ROS (Richter *et al*., 1995). AbOmpA significantly reduced the levels of intracellular glutathione at as early as 3 h of culture, whereas pretreatment with the anti-oxidant, N-acetyl cysteine, normalized the levels of intracellular glutathione (Fig. 2C).

ROS are responsible for both apoptosis and necrosis of AbOmpA-treated DCs

As AbOmpA induced the mitochondrial swelling and rupture of the outer membrane (Fig. 1B), proapototic molecules in the intermembrane space, such as cytochrome *C*, could be released into the cytosol. Caspases perform a crucial function in apoptotic cell death, and caspase-9 activation is principally dependent on cytochrome *C* in the cytosol. The results of

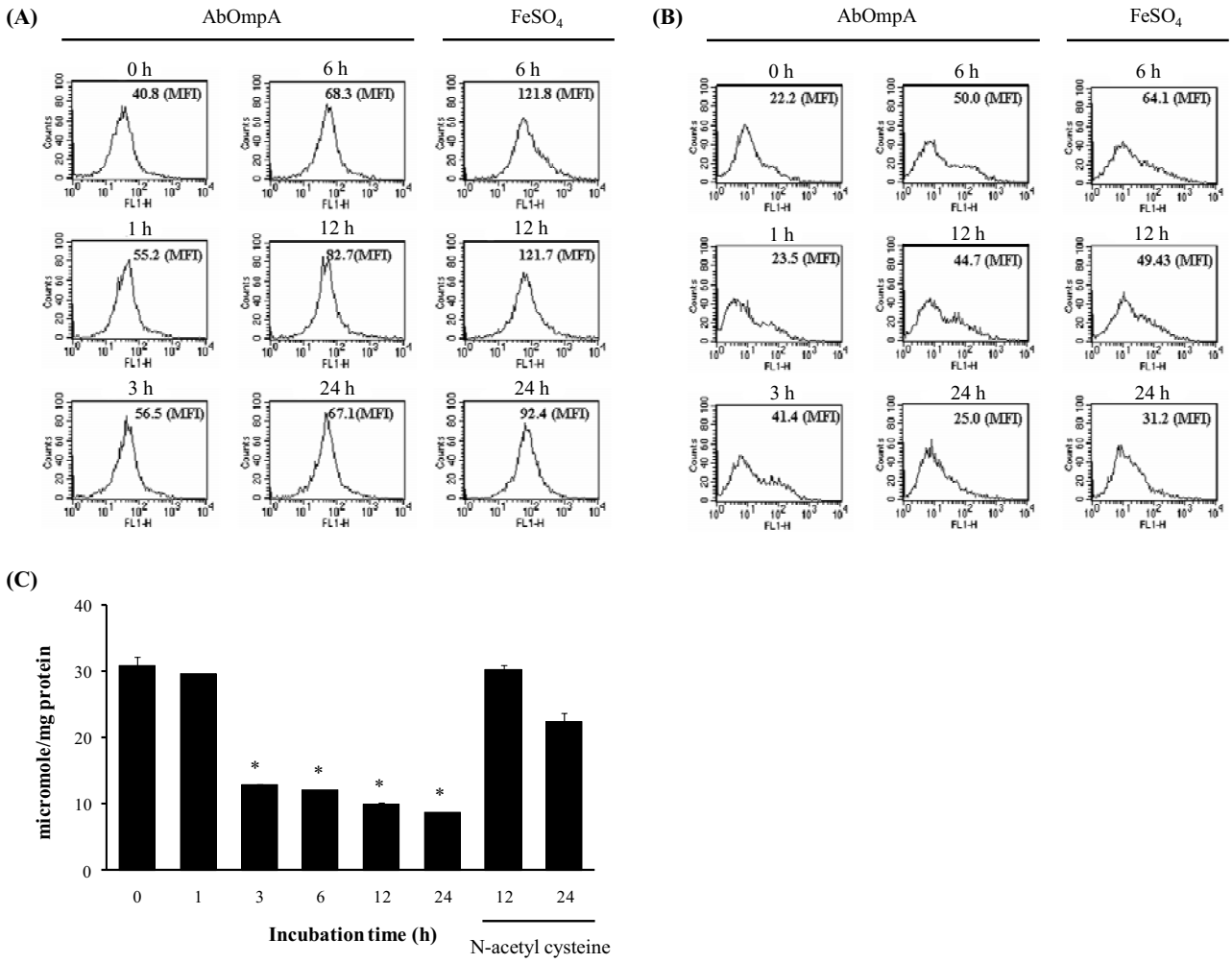

Fig. 2. AbOmpA induces ROS production in DCs. (A and B) DCs were incubated with either 80 nM of AbOmpA or 20 μM of ferrous sulfate for the indicated times. Ferrous sulfate was employed as a positive control. R123 fluorescence (A) and DCF fluorescence (B) were detected by flow cytometry. The mean fluorescent intensity was indicated in the inlet of the box. The results are from one representative experiment of three independent experiments. (C) Intracellular glutathiones were estimated via an enzymatic recycling assay. Glutathione levels were significantly reduced in the AbOmpA-treated DCs (**P*<0.05).

Western blot analysis demonstrated that pro-caspase-9 levels were reduced as early as 1 h, and then pro-caspase-3 and PARP were cleaved 3 h after the culture; however, pro-caspase-8 was not cleaved in the AbOmpA-treated DCs (Fig. 3A), which had been detected previously in AbOmpA-treated epithelial cells (Choi *et al*., 2005). In addition to early-onset apoptosis, delayed-onset of necrosis was observed in the AbOmpA-treated DCs (Fig. 1A). In order to determine whether delayed-onset of necrosis was resulted from AbOmpA-mediated necrotic pathways or secondary effects of apoptosis, DCs were pretreated with pancaspase inhibitor z-VAD-fmk to block caspase-mediated apoptosis completely and then cell death induced by AbOmpA was assessed via flow cytometry. Pancaspase inhibitor did not significantly induce cell death as compared with untreated control cells. Pretreatment of pancaspase inhibitor in the AbOmpA-treated cells significantly inhibited apoptotic cell death (Annexin V^+ cells) (Fig. 3B), whereas the population of necrotic cells (Annexin V^-/PI^+ cells) increased from 13.0% in the AbOmpA-treated cells to 21.9% in the DCs pretreated with z-VAD-fmk at 24 h (Fig. 3C). However, pretreatment with pancaspase inhibitor did not alter the total population of dead cells (sum of apoptotic and necrotic population) relative to the population of dead cells in the AbOmpA-treated DCs. These results indicate that the delayed-onset necrosis induced by AbOmpA is not a secondary effect of the early-onset apoptosis. To determine whether ROS were directly responsible for both the apoptosis and necrosis of AbOmpA-treated DCs, the DCs were pretreated with N-acetyl cysteine. The results demonstrated that N-acetyl cysteine significantly inhibited both apoptosis and necrosis (Fig. 3D), which suggests that ROS originating from the mitochondria are directly responsible for the early-onset apoptosis and the delayed-onset necrosis observed in AbOmpA-treated DCs.

Discussion

The subcellular targeting of bacterial effector molecules to the

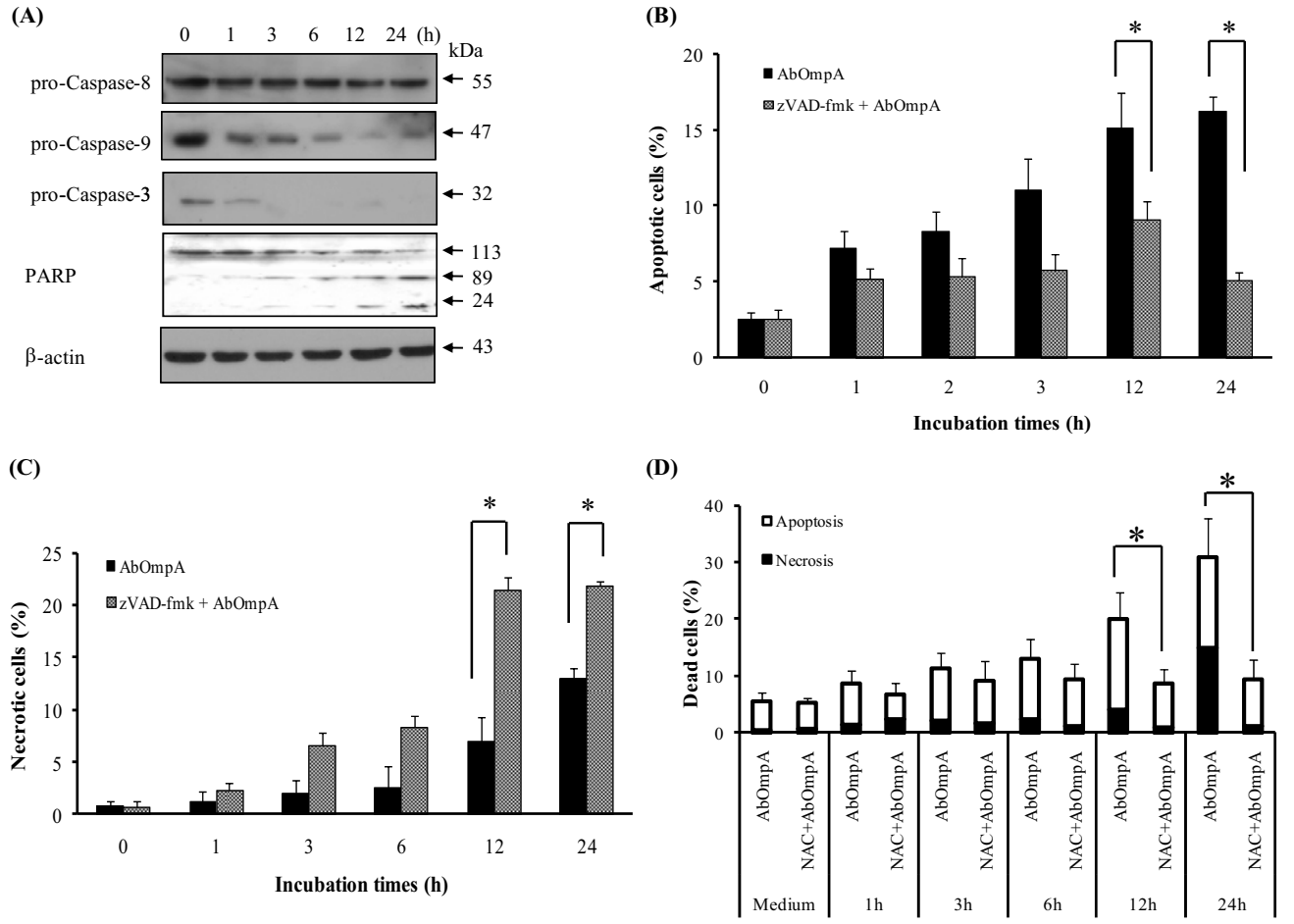

Fig. 3. Caspase activation in AbOmpA-treated DCs and the inhibition of cell death by antioxidant. (A) DCs were treated for the indicated times with 80 nM of AbOmpA. Cell lysates were immunoblotted with anti-pro-caspase-3/-8/-9, and PARP. (B and C) DCs were preincubated for 30 min with z-VAD-fmk and treated with 80 nM of AbOmpA for the indicated times. DCs were stained with Annexin V and PI. The Annexin V^+/PI^- and Annexin V^+/PI^+ cells for apoptosis (B) and Annexin V^-/PI^+ cells for necrosis (C) were counted via flow cytometry. Data are expressed as the Means±SD for three dishes. (D) DCs were pretreated with 5 mM of N-acetyl cysteine (NAC) and incubated with AbOmpA for the indicated times. DCs were stained using an Annexin V/PI staining kit and 10^4 cells were counted via flow cytometry. The data are expressed as the mean values of two independent experiments. Pretreatment with NAC significantly inhibited both the apoptosis and necrosis of DCs treated with AbOmpA (*$P<0.05$).

mitochondria impairs the physiological function of target cells and evenly controls cell viability, which may be linked to local and systemic pathologies in infected hosts. To extend these observations to the immune system, a murine DC model was introduced to understand the immunopathology of AbOmpA in *A. baumannii* infection. The experimental proofs developed here showed that AbOmpA targeted the mitochondria and induced ROS production, which in turn induced DC death.

The secretion of bacterial effectors is a prerequisite for the subcellular targeting in the host cells. We demonstrated recently that *A. baumannii* secreted AbOmpA via OMVs (Kwon *et al*., 2009). AbOmpA packaged in the OMVs interacted with and were internalized by the host cells. Considering the finding that AbOmpA induced epithelial cell death via both mitochondrial and nuclear targeting (Choi *et al*., 2005, 2008b), the subcellular targeting of AbOmpA was determined in DCs exposed to cytotoxic concentrations of AbOmpA. The mitochondrial targeting of AbOmpA was prominently noted in DCs, but nuclear targeting was only rarely observed. These findings indicate that mitochondrial targeting is a mainstream source of DC death. Several bacterial proteins regulate cell death at the mitochondrial level. Bacterial effector proteins, including the EspF of enteropathogenic *E. coli* (Nougayrède and Donnenberg, 2004), PorB of *Neisseria gonorrheae* (Müller *et al*., 2000), VacA of *Helicobacter pylori* (Galmiche *et al*., 2000), and ActA of *Listeria monocytogenes* (Pistor *et al*., 1994), are associated with the mitochondria and induce cell death. In this study, the AbOmpA of *A. baumannii* also targeted the mitochondria and induced both early-onset apoptosis and delayed-onset necrosis in DCs.

An increase in the permeability of the outer mitochondrial membrane results in a swelling of the matrix, the disruption of the outer mitochondrial membrane, and the release of mitochondrial pro-apoptotic molecules into the cytosol, which are typical cascades of apoptotic cell death occurring via the mitochondrial death pathway (Skulachev, 2006). By way of contrast, an increase in the permeability of both the outer and inner mitochondrial membranes induces necrotic cell death.

Mitochondrial changes such as ROS production and the activation of caspases are early events, and are responsible for early-onset apoptosis in AbOmpA-treated DCs. On the other hand, delayed-onset necrosis was noted in the AbOmpA-treated DCs. ROS generated by death stimuli can induce lysosomal membrane permeabilization and subsequently result in the release of lysosomal enzymes, which can cause lysosomal degradation-mediated cell death (Crompton, 2003). N-acetyl cysteine pretreatment inhibited both apoptosis and necrosis in DCs, thereby suggesting that ROS are directly responsible for both the apoptosis and necrosis of AbOmpA-treated DCs.

Antigen-loaded DCs express costimulatory molecules on their surface, which in turn interact with T cells to deliver their antigens. When the DCs were treated with 5 nM of AbOmpA, the expression levels of CD40, CD80, CD86, and MHC class I and class II molecules were higher than those detected in the DCs treated with identical concentrations of LPS (Lee *et al*., 2007), thereby suggesting that AbOmpA is a potential immunoadjuvant. However, a relatively high concentration (80 nM) of AbOmpA induced DC death and limited the expression of costimulatory molecules in DCs (data not shown). This result indicates that DCs undergoing cell death limit the expression of costimulatory molecules, which may result in defective T-cell responses. However, this study has some limitations to understand immunopathology of *A. baumannii* regarding the interaction of DCs with AbOmpA during *in vivo* infection, because murine bone marrow-derived DCs stimulated with IL-4 and GM-CSF were used. It is necessary to observe the effects of AbOmpA on the steady-state DCs from the tissues during *A. baumannii* infection.

In conclusion, the results of this study provide us with new insights into *A. baumannii* pathogenesis via the immunopathology of AbOmpA. Upon the secretion of AbOmpA from a high bacterial burden via OMVs and subsequent delivery into the cytosol of host cells, DCs in a local environment may be expected to evidence high concentrations of AbOmpA. Because DCs are a key element in the initiation and regulation of adaptive immune responses, DC death and dysfunction may result in defective T cell responses against *A. baumannii*.

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