

Apoptosis Induced by *Pseudomonas aeruginosa* in Antigen Presenting Cells Is Diminished by Genetic Modification with CD40 Ligand

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ABSTRACT

Persistent colonization with *Pseudomonas aeruginosa* (PA) is a hallmark of the lung disease associated with cystic fibrosis (CF). Based on the concept that PA is not cleared from the lung by the host response in individuals with CF, we analyzed the capacity of PA to induce cell death in human alveolar macrophages (AM) and murine dendritic cells (DC), antigen presenting cells that play a central role in the initiation of pulmonary host defenses against pathogens, and evaluated if genetic modification can lead to protection against PA induced cell death. AM and DC were susceptible to cell death induced by the laboratory PA isolates PAO1, PAK and PA103, as well as a mucoid derivative of PAO1 and PA isolates derived from sputum of individuals with CF. Apoptosis, analyzed by TUNEL assay, was detectable in AM and DC as early as 3 h after infection with PA. In contrast, the same strains and doses of PA had little effect on the lung epithelial cell line A549 and primary cultures of human bronchial epithelial cells *in vitro*. Pretreatment of DC with the caspase inhibitors VAD-fmk and YVAD-cmk reduced PA induced cell death ($p < 0.05$). Finally, genetic modification of DC to express

CD40L using an adenovirus vector decreased the susceptibility of DC to cell death induced by PAO1 compared with DC infected with a control Ad vector ($p < 0.01$). The data demonstrate that DC and AM are susceptible to apoptosis induced by PA and that this response can be partially reversed by genetic modification with CD40L, a CD4+ T cell molecule that plays a central role in activating antigen presenting cells. These observations suggest a potential mechanism contributing to the persistence of PA in CF and suggest that genetic manipulation of antigen presenting cells with anti-apoptotic genes may be able to strengthen host defenses in CF. (*Pediatr Res* 52: 636–644, 2002)

Abbreviations

Ad, adenovirus
AM, alveolar macrophages
CF, cystic fibrosis
DC, dendritic cells
PA, *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (PA) can cause infections of the respiratory tract, particularly in immunocompromised patients or individuals with cystic fibrosis (1–3). Of particular interest is the interaction of PA with cells of the respiratory system, a major site for infections with PA. The mechanism by which PA can establish infection in the lung is undoubtedly complex and linked in part to the ability of the PA population to develop biofilms, and in part by the capacity of PA to interfere with the

host defense mechanisms arrayed against pathogens (1–5). Critical to the pulmonary defense against extracellular bacteria is the sampling and processing of the bacteria by antigen presenting cells, including alveolar macrophages (AM) and dendritic cells (DC). AM play a central role in clearing extracellular bacteria from the lung and DC interspersed in the airway epithelial cells and in the lung parenchyma are the most potent antigen presenting cells in the lung (6–9).

The focus of the present study is to analyze whether antigen-presenting cells can be protected from PA induced apoptosis by prior genetic modification. Several recent studies have shown that PA can be cytotoxic, and can induce apoptosis in a variety of cell types such as epithelial cells, macrophages and endothelial cells (10–16). Multiple virulence factors of PA, in particular the type III secretion system, have been proposed to be important for this process (11, 13, 14, 16). Induction of apoptosis in antigen presenting cells is a major virulence factor

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for enteral pathogens such as *Shigella*, *Salmonella* and *E. coli* (17–21), leading to impairment of the intestinal host defense system and thus contributing to the spread of the infection. The induction of apoptosis with release of cytokines in intestinal macrophages by *Salmonella* or *Shigella* is an important characteristic of the host-pathogen interaction for these bacteria (17, 19, 21, 22).

In the context that induction of apoptotic cell death by PA in antigen presenting cells might be one mechanism for this virulence in the lung environment and thus contribute to the progression of the infection, the present study extends the concept of PA induced apoptosis to evaluate AM and DC, two cell types critical to the clearance of extracellular bacteria from the lung. The data demonstrate that a variety of established laboratory as well as clinical isolates of PA are capable of inducing apoptosis in AM and DC. Importantly, we also show that at least partial protection against PA induced apoptosis of antigen presenting cells can be achieved by prior genetic modification of the antigen presenting cell with CD40L, a member of the TNF superfamily normally expressed on activated CD4+ lymphocytes. CD40L acts *via* ligation to its receptor CD40, expressed on antigen presenting cells (23). In addition to being a critical mediator of T cell dependent B cell activation (24), CD40 ligation on DC has been shown to lead to enhanced activation and differentiation as well as to decreased tumor induced apoptosis (25). Since DC genetically modified to express CD40L and pulsed with heat inactivated PA provide enhanced protection and induction of a strong anti-PA antibody response against chronic pulmonary PA infection (26), genetic modification of antigen presenting cells to express CD40L may prove useful for novel strategies of protecting the susceptible host against PA.

METHODS

Bacteria strains. The PA strains used included the well characterized laboratory strains PAOI, PAO/NP, PAK and PA103 (provided by A. Prince, Columbia University, NY, U.S.A.), a strain isolated from the sputum of an individual with CF (AD2A provided by J. Burns, University of Washington, WA, U.S.A.), and the mucoid laboratory strain PDO300, a mucA22 derivative of PAOI constructed by crossing a mucA22 fragment from FRD1 into the PAOI genome by gene replacement (provided by D. Ohman, Commonwealth University of Virginia, VA, U.S.A.). PA LPS and *E. coli* LPS was obtained from Calbiochem, San Diego, CA, U.S.A. Bacteria were grown from frozen stocks in tryptic soy broth (Difco, Detroit, MI, U.S.A.) at 37°C to midlog phase, washed three times with PBS, pH 7.4 (PBS) and resuspended in infection media at the desired concentration as determined by spectrophotometry. For different inoculum doses within an individual experiment serial dilutions were done and the inoculum volume kept constant. Numbers of bacteria were confirmed by determining the colony forming units (cfu) of diluted aliquots on MacConkey agar plates (Difco). PDO300 colonies always demonstrated a mucoid phenotype. Bacteria were heat inactivated at 56°C for 1 h.

Cells. Murine bone marrow-derived DC were generated from 6 to 8 wk old C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) and grown in complete RPMI 1640 medium (10% fetal bovine serum, 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 units/mL penicillin) supplemented with 10 ng/mL recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF, R&D systems, Minneapolis, MN, U.S.A.) and 2 ng/mL recombinant mouse IL-4 (IL-4, R&D systems) as previously described (26, 27).

Human alveolar macrophages were obtained by bronchoalveolar lavage of healthy volunteers (28). The lavage fluid was filtered through one layer of gauze, centrifuged (400 g, 10 min) and washed three times in PBS, pH 7.4. Cells were resuspended in complete RPMI 1640 medium and plated. Macrophage content (always >90%) was determined by Giemsa stain on cytospin preparations. Cell viability (always >90%) was determined by trypan blue exclusion. After 3 h, the macrophages were washed to remove nonadherent cells.

The human respiratory epithelial cell line A549 was maintained in Dulbecco's Modified Eagle Medium (10% fetal bovine serum, 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 units/mL penicillin). Primary human bronchial epithelial cells were obtained from Clonetics (Walkersville, MD, U.S.A.) and maintained in Bronchial Epithelial Basal Medium with growth factors and supplements as supplied by Clonetics.

Adenovirus vectors. AdmCD40L is a replication-deficient Ad5-based recombinant adenovirus with E1 and E3 deletions in which the mouse CD40L cDNA is under control of the cytomegalovirus immediate-early promoter and enhancer (29). The control AdNull vector is structurally similar but contains no transgene (30). Preparation and titration of the adenovirus vectors were as previously described (31, 32). All vectors used in this study were free of replication competent adenovirus (33).

Cytotoxicity of PA. To evaluate the cytotoxicity of PA strains on antigen presenting cells, DC and AM were plated in 96 well plates at 5×10^4 /well and infected with PA at doses ranging from 0.1–100 bacteria/cell in RPMI, 25 mM HEPES, pH 7.4. Immediately after addition of the bacteria, the plates were centrifuged at $500 \times g$ for 10 min to synchronize the initiation of the infection. Cytotoxicity was assessed by measuring LDH release into the supernatant after 3 h using a colorimetric assay (Roche, Indianapolis, IN, U.S.A.) and measuring the OD at 450 nm. Maximum LDH release was determined by addition of 1% Triton X to uninfected cells at the initiation of the infection. A549 and HBEC were infected in a similar fashion in their media supplemented with 25 mM HEPES, pH 7.4.

PA-induced apoptosis in antigen presenting cells. To assess whether PA induces apoptosis in antigen presenting cells, DC and A549 cells were infected with PA as described above in 96 well plates for 3 h and apoptosis was assessed by a colorimetric assay for DNA fragmentation (R&D Systems) as described by the manufacturer. Cells spiked with PA for 0 h served as a negative control, cells treated with DNase served as a positive control. To evaluate the induction of apoptosis using a different method, DC and A549 cells were plated in coverslip dishes and infected with PA for 3 h. The cells were then washed twice and

continued in culture in the presence of gentamicin (200 $\mu\text{g}/\text{mL}$; Sigma Chemical Co., St. Louis, MO, U.S.A.) for 0 or 3 h. Cells were then fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min and DNA strand breaks were detected by *in situ* terminal deoxynucleotidyltransferase (TUNEL) assay (Promega, Madison, WI, U.S.A.) using fluorescein thiocyanate conjugated dUTP. Positive controls were generated by treating uninfected cells with DNase. Nuclei were counterstained with propidium iodide. The number of apoptotic cells was determined by fluorescent microscopy. Apoptosis was also evaluated using binding of annexin V. DC were infected with PAO1 at 1 cfu/cell for 4 h, washed and stained with annexin V-FITC (PharMingen), counterstained with 2 μM TO-PRO3-iodide (Molecular Probes, Eugene, OR, U.S.A.) to detect dead cells and then analyzed by flow cytometry.

Effect of caspase inhibition on PA induced cellular cytotoxicity. To analyze whether PA induced cytotoxicity can be influenced by caspase inhibition, murine DC were incubated with the irreversible caspase inhibitors VAD-fmk (40 μM , nonspecific inhibitor of caspase-3 and 8) or YVAD-cmk (40 μM , nonspecific inhibitor of caspase-1; both from Clontech, Palo Alto, CA, U.S.A.) in RPMI for 1 h before addition of PAO1 (10 cfu/cell) or PA103 (1 cfu/cell) for 1 h. PA induced cell death was then assessed by measuring LDH release in the supernatant as described above.

Effect of genetic modification with CD40L

To evaluate the effect of genetic modification with CD40L on PA induced cell death in antigen presenting cells, DC were infected with AdCD40L or Ad Null at 100 moi. After 24 h the cells were infected with PAO1 or PDO300 for 3 h and cell death was evaluated by TUNEL assay and LDH release as described above. Expression of CD40L, MHC class II and ICAM-1 (CD54) was evaluated by flow cytometry (Coulter, Miami, FL, U.S.A.) using phycoerythrin (PE)-labeled antibodies (all from PharMingen, San Diego, CA, U.S.A.). An isotype-matched mouse mAb was used as negative control (PharMingen).

Statistical Analysis

All data are reported as mean \pm SE. Statistical evaluation was performed using the one-tailed *t* test, and a value of $p < 0.05$ was accepted as a significant difference.

RESULTS

PA Induced cell death of antigen presenting cells. To analyze the effect of PA on antigen presenting cells, human AM and murine DC were infected with the laboratory PA strains PAO1, PAO/NP, PAK, and PA103, and cell death evaluated by measuring LDH release after 3 h. All PA strains caused cytotoxicity of both AM and DC, in contrast to the lung epithelial cell line A549, where only PA103 caused cell death at this time point (Fig. 1). The effect was dose dependent and was dependent on the PA strain (PAO1 and PA103 > PAO/NP > PAK).

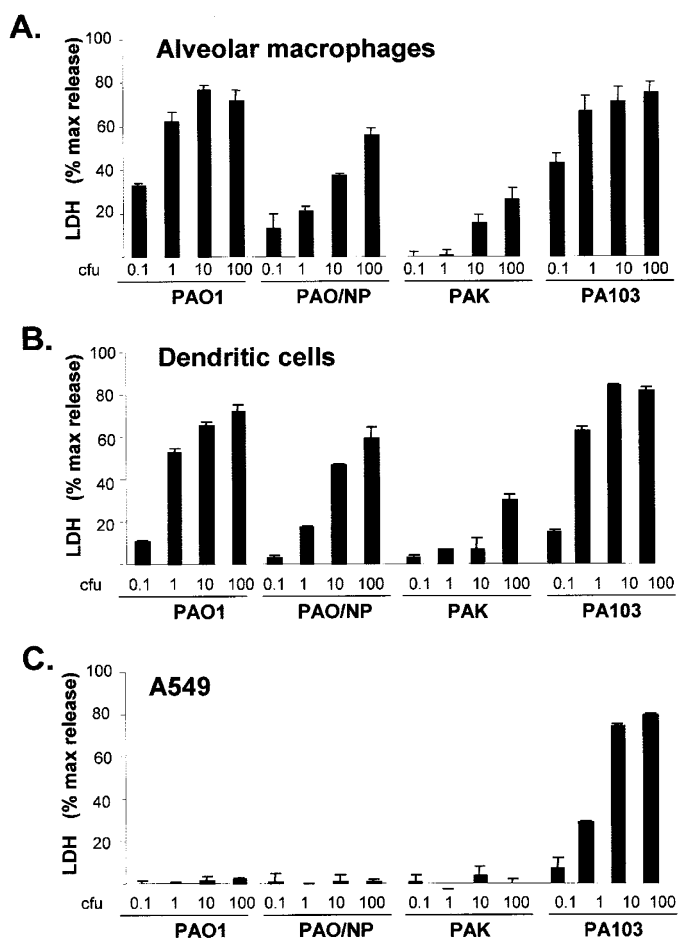


Figure 1. Cytotoxicity of antigen presenting cells induced by different laboratory and clinical strains of *Pseudomonas aeruginosa*. Human AM obtained by bronchoalveolar lavage from healthy volunteers. DC were derived from bone marrow of C57Bl/6 mice. The AM, DC, and the human lung epithelial cell line A549 were infected with various laboratory strains of PA at doses from 0.1–100 cfu/cell. Following addition of the bacteria, the plates were centrifuged to synchronize the initiation of the infection. Cytotoxicity was assessed by measuring LDH release into the supernatant using a colorimetric assay following 3 h infection. (A) AM; (B) dendritic cells, and (C) A549 cells. Data are presented as LDH in supernatant/maximum cellular release of LDH by lysis with triton-X above the baseline LDH release for uninfected cells. Shown is a representative of three experiments. Each datapoint represents mean of triplicate wells.

To analyze whether clinical PA isolates derived from individuals with cystic fibrosis or a mucoid laboratory isolate are equally able to induce cell death in DC as laboratory strains of PA, a clinical isolate derived from an individual with CF, AD2A, and a mucoid PA mutant PD0300 were used to infect DC. The clinical PA isolate (Fig. 2A) as well as the mucoid mutant PDO300 (Fig. 2B) induced dose-dependent cell death in DC comparable to the parental strain PAO1, suggesting that cell death in DC can be induced by various phenotypes. The clinical and mucoid strains seemed to be less cytotoxic compared with PAO1 or PA103. Similar results were obtained when other clinical isolates, derived at different stages of disease from the CF lung were evaluated (not shown). To evaluate the role of bacterial membrane components, in particular LPS, DC were exposed to 100 cfu/mL of heat inacti-

vated PAO1 or PA103 and LPS, derived from *E. coli* or PA. No significant cell death was observed after 3 h (Fig. 2C), suggesting that the induction of cell death is an active process by live PA.

To evaluate whether the observed cell death was due to apoptosis, DC and A549 cells were infected with the same PA strains; apoptosis was evaluated using the TUNEL assay. Apoptotic AM and DC were detectable by TUNEL assay following infection with PAO1 after 3 h, whereas no significant apoptosis was observed in the A549 cell infected with the same dose of PAO1 (Fig. 3). At 6 h following infection, apoptotic A549 cells were seen in the cells infected with the strain PA103, whereas exposure of A549 cells to PAK, PAO1 or PAO/NP did not result in significant apoptosis (Fig. 4). In contrast, apoptosis in DC was detectable at 3 h with not only PA103, but was also observed with PAO1, PAO/NP and PAK (Fig. 4). Similar results were obtained using a colorimetric assay measuring DNA breaks (not shown). Using annexin V binding as another assay for the detection of apoptosis showed increased dead and apoptotic cells in DC infected with PAO1 (Fig. 5). Quantification showed $42.7 \pm 7.4\%$ annexin V positive cells infected with PAO1 compared with $10.0 \pm 2.8\%$ in uninfected controls, the number of To-Pro-3-iodide positive DC was $60.7 + 5.0$ for infected compared with $17.8 + 2.2$ for uninfected cells. These results suggest that AM and DC are more susceptible to PA induced cell death compared with epithelial cells and that at least part of the observed cell death is due to apoptosis.

To evaluate whether the observed results for A549 cells also hold true for other primary cultures of human lung epithelial cells, human bronchial epithelial cells and A549 cells were infected with PAO1. At 10 and 100 cfu/cell, doses that cause more than 70% cell death in AM or DC, there was less than 10% cell death in the A549 cells and less than 18% in the HBEC (not shown).

Suppression of PA induced DC cell death by caspase inhibition. To evaluate whether caspase activation is part of

the PA induced apoptotic response of DC to PA exposure, PA induced cell death was evaluated following caspase inhibition in DC. Cell death was significantly reduced following incubation with the caspase inhibitor VAD-fmk and 2VAD-cmk for exposure of DC to PAO1 ($p < 0.05$ compared with controls, Fig. 6A) and PA103 ($p < 0.001$ compared with controls, Fig. 6B), suggesting that caspase activation is involved in the cellular apoptotic response in the DC.

Decreased apoptosis following genetic modification of DC with CD40L. DC modified with AdCD40L demonstrated expression of CD40L on the surface compared with AdNull infected or uninfected DC (Fig. 7A). The expression of MHC class II (IA-b) and CD54 (ICAM-1) did not change in AdCD40L infected cells compared with AdNull infected cells (Fig. 7B,C). To analyze whether PA induced cell death in DC was influenced by expression of CD40L, DC infected with AdCD40L were exposed to PA. DC modified with CD40L and infected with PAO1 showed 32% and 30% less cell death compared with naive DC or DC modified with AdNull respectively ($p < 0.001$, all comparisons, Fig. 8A). Also, DC modified with CD40L and exposed to PDO300 showed 45% and 55% less cell death compared with naive DC or DC modified with AdNull respectively ($p < 0.001$, all comparisons, Fig. 8B). Modification with AdNull or AdCD40L alone without PA exposure had no effect (not shown). The number of apoptotic DC, determined by the photometric apoptosis assay, following PAO1 infection was decreased in the cells modified with AdCD40L by 36% and 49% compared with controls or AdNull modified cells following infection with PAO1 at 1 cfu/cell ($p < 0.01$ all comparisons, Fig. 9A) and was decreased by 59% and 48% compared with controls or AdNull modified cells following infection with PAO1 at 10 cfu/cell ($p < 0.001$ all comparisons, Fig. 9B). The degree of cytotoxicity (Fig. 8) was higher than the amount of apoptosis observed (Fig. 9). Although these experiments were done independently, this may be a reflection of apoptosis and necrosis occurring simultaneously. Evaluating the number of apoptotic cells by TUNEL assay showed, compared with uninfected cells (Fig. 10A–C), an increased number of apoptotic cells in the AdNull or non-Ad modified controls following infection with PAO1 (Fig. 10D,E) or PDO300 (Fig. 10G,H) compared with AdCD40L-modified cell (Fig. 10F, I). Quantification of the cells showing apoptotic nuclei demonstrated a decrease of 57% and 55% compared with controls or AdNull modified cells for PAO1 ($p < 0.001$ compared with both controls, Fig. 11A) and a decrease of 72% and 68% compared with controls or AdNull modified cells for PDO300 ($p < 0.001$ compared with both controls, Fig. 11B). The overall number of apoptotic cells was lower than in the experiment described in Fig. 4. This, in addition to the 50% lower dose, may be a reflection of the biologic variability of bacteria grown for different experiments. This data demonstrates that PA induced cell death in DC can be at least partially decreased using prior genetic modification to express CD40L.

DISCUSSION

The present study demonstrates that primary cultures of antigen presenting cells are more sensitive to PA induced

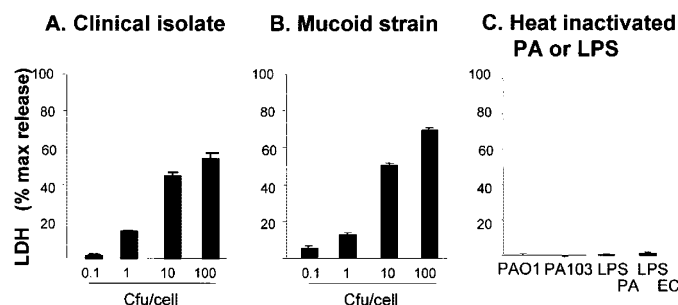


Figure 2. Dendritic cell cytotoxicity mediated by a clinical isolate, mucooid strain and membrane components of *Pseudomonas aeruginosa*. Murine dendritic cells, derived from bone marrow from C57Bl/6 mice were infected with: (A) A clinical isolate (AD2A) derived from sputum from an individual with CF at doses from 0.1–100 cfu/cell; (B) Mucooid laboratory PA isolate PDO300; and (C) Heat inactivated PA and LPS derived from PA or *E. coli*. Following addition of the bacteria to the DC, the plates were centrifuged to synchronize the initiation of the infection. Cytotoxicity was assessed by measuring LDH release into the supernatant using a colorimetric assay following 3 h infection. Data are presented as LDH in supernatant/maximum cellular release of LDH by lysis with triton-X above baseline signal for uninfected cells. Shown is a representative of three experiments. Each datapoint represents mean of triplicate wells.

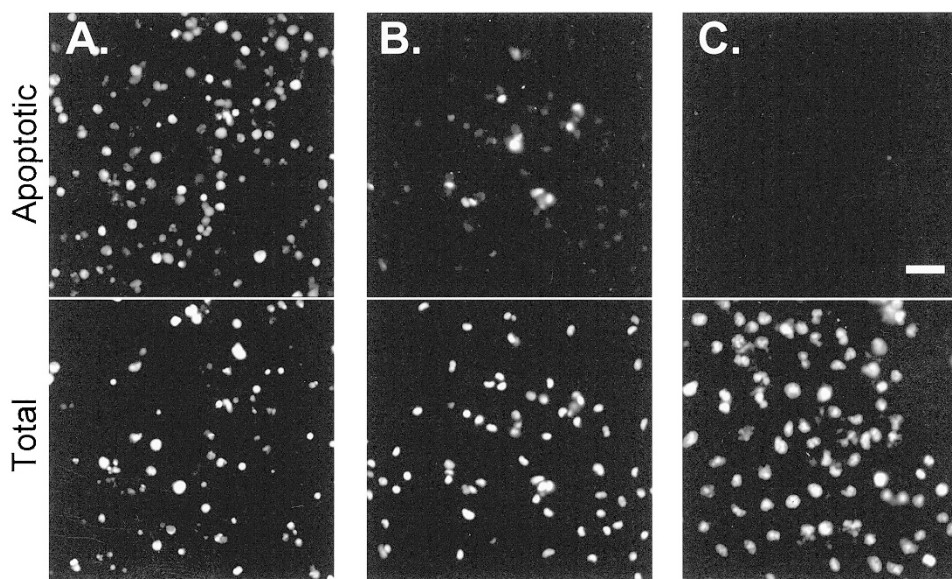


Figure 3. *Pseudomonas aeruginosa* induction of apoptosis in murine antigen presenting cells. Murine DC, AM and A549 cells were infected with the laboratory strain PAO1 at a dose of 10 cfu/cell for 3 h in coverslip dishes. (A) DC; (B) AM, and (C) A549 cells. Apoptosis was assessed by fluorescent terminal nucleotide end labeling (TUNEL) and analyzed by fluorescent microscopy. Positive controls were generated by DNase treatment of uninfected cells. Bar = 50 μ m, all panels.

apoptosis than primary cultures of lung epithelial cells. The apoptotic response of the antigen presenting cells to PA was dependent on live bacteria as no cell death was observed at identical doses with heat inactivated bacteria nor with LPS at a dose approximately 10 \times higher than the LPS present on the bacteria, and was equally observed with a clinical isolate and a mucoid derivative. Inhibition of caspases reduced the degree of cell death. Importantly, genetic modification of DC to express CD40L diminished the cell death induced by PA. Together, these observations support the concept that PA may suppress host defenses, in part, by inducing cytotoxicity of the cells that encounter the pathogen and present it to the host mechanisms that help suppress the infection. The role of APC in the pathogenesis of CF is not clear, however, the immune system is not able to clear PA from the lung efficiently. Opsonic phagocytosis may be defective in the lungs of CF patients chronically colonized with PA, as host and bacterial proteases cleave complement and phagocytosis receptors (34). This could lead to deficient antigen presentation. In this context, the elimination of antigen presenting cells *via* apoptosis could lead to impairment of innate immune functions and could thus be a factor in the establishment of chronic colonization with PA. Importantly, genetic modification of the antigen presenting cells may function to prevent this action of the PA.

Induction of apoptosis by PA. Various bacterial pathogens, especially Gram-negative organisms such as *Salmonella*, *Shigella* and *E.coli*, have the ability to induce apoptosis in host cells *via* a variety of bacterial factors (17–22). Recently it has become evident that PA can also induce apoptosis in host cells, so that this mechanism may be an important factor for the pathogenicity of this organism (10–16). As the lung is one of the primary sites for infection with PA, especially for patients with cystic fibrosis, most studies have focused on cells related to this organ. Studies analyzing bacterial factors responsible

for the induction of apoptosis have focused on the role of the type III secretion system, although other bacterial factors have also been shown to play a role (10, 11, 13, 16). Generally, type III dependent factors can induce apoptosis or cytoskeletal changes in macrophages. For example, type III mediated induction of apoptosis in intestinal macrophages by *Shigella* or *Yersinia* leading to the release of proinflammatory cytokines is an important characteristic of the host pathogen interaction for these bacteria (17–19, 21, 22). The type III secretion system is highly active in PA strains such as PA103 and PAO1, the strains that induced cell death most potently in the antigen presenting cells in the present study. PA103 induced apoptosis has been reported for the murine macrophage cell line J774, bone marrow derived murine macrophages and human peripheral monocytes (13). Immunization against PcrV, a component of the type III secretion system, prevents PA103 mediated cytotoxicity for AM in mice *in vivo* leading to increased survival and decreased pulmonary toxicity (35). Another study reported type III secretion system dependent swelling of cells and nuclei with disintegration of the plasma membrane in human leukocytes and J774 cells in response to PA isolates derived from individuals with CF (36). Nucleoside diphosphate kinase secreted by a mucoid strain of PA has been reported as another virulence factor in macrophages, inducing ATP-dependent cytotoxic activity mediated through P2Z receptors (16, 37).

The exact trigger for the cell death of the antigen presenting cells in our study is not known. Apoptosis and necrosis are visible following exposure to PA. This may be due to secondary necrosis following apoptosis, but may also reflect a direct cytotoxic effect in addition to apoptosis. Based on the variety of factors produced by the bacteria under our growth condition the latter is more likely. Comparing cytotoxicity as determined by LDH release with the number of apoptotic cells could

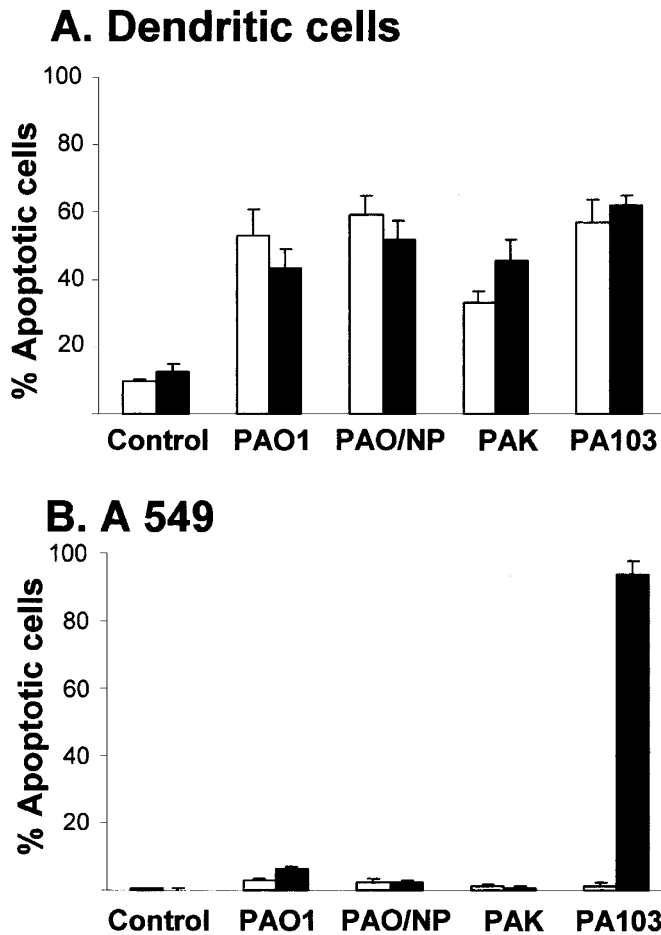


Figure 4. Quantification of apoptotic DC following exposure to *Pseudomonas aeruginosa* as assessed by TUNEL assay. Murine DC and A549 epithelial cells were infected with various laboratory strains of PA at a dose of 10 cfu/cell for 3 h (□) or 6 h (■) in coverslip dishes. Apoptosis was assessed by fluorescent terminal nucleotide end labeling (TUNEL) and analyzed by fluorescent microscopy (see Fig. 3) by counting the percentage of apoptotic cells in 5 high power fields.

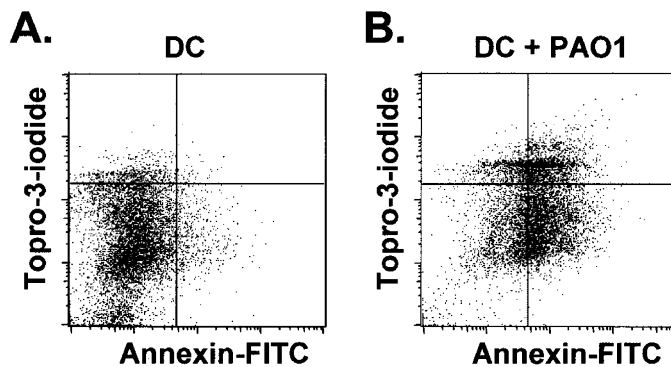


Figure 5. Evaluation of apoptosis in DC following exposure to PA using binding of annexin V. DC were infected with PAO1 (10 cfu) for 3 h. (A) uninfected DC; (B) DC + PAO1. The cells were stained with annexin V-FITC and topro-3 iodide and analyzed by flow cytometry. There is increased annexin staining in the DC exposed to PAO1 indicating apoptosis. Shown is a representative of four experiments.

suggest that if the apoptosis percentages are low, the mechanism of action of *P. aeruginosa* is probably a direct effect on cell permeability and if the apoptosis and cytotoxicity percent-

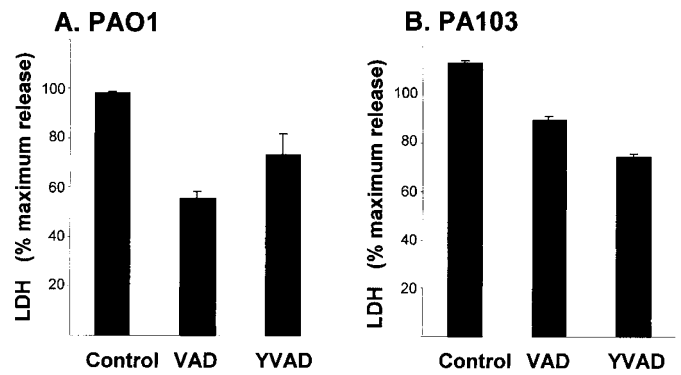


Figure 6. Induction of cell death by *Pseudomonas aeruginosa* in murine DC is partially suppressed by caspase inhibition. DC were infected with PAO1 (10 cfu) or PA103 (1 cfu) in the presence of the caspase inhibitors VAD-fmk or YVAD-cmk. Cytotoxicity was assessed by measuring LDH release into the supernatant using a colorimetric assay following 3 h infection. Data are presented as LDH in supernatant/maximum cellular release of LDH by lysis with triton-X above baseline LDH release for uninfected cells. Shown is a representative of three experiments. Each datapoint represents mean of triplicate wells.

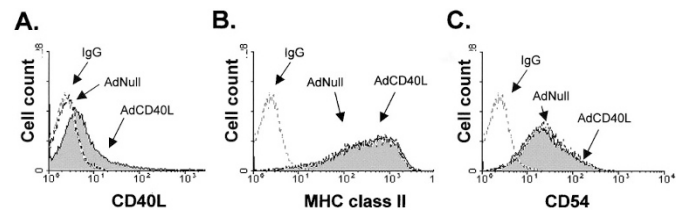


Figure 7. Expression of CD40L on DC genetically modified to express CD40L. DC were infected with AdCD40L for 24 h and analyzed for the expression of (A) CD40L, (B) MHC class II, and (C) ICAM (CD54) using PE-conjugated antibodies by flow cytometry. Shown in each panel is the result for the AdCD40L and AdNull infected cells as well as Ig-control on AdCD40L infected cells.

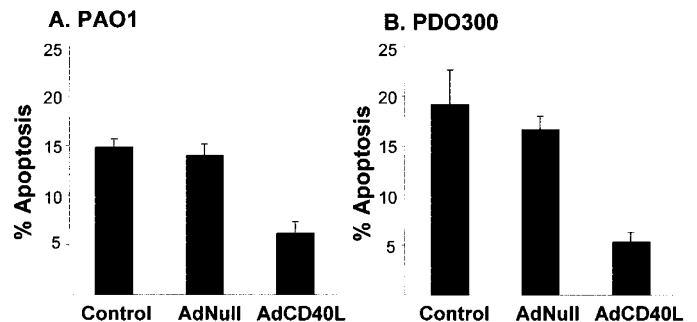


Figure 8. DC modified to express CD40L are less susceptible to cytotoxicity induced by *Pseudomonas aeruginosa*. DC infected with AdCD40L (100 moi) and 24 h later infected with PAO1 or PDO300 at 1 cfu/cell. AdNull infected or uninfected DC served as controls. Cytotoxicity was assessed by measuring LDH release into the supernatant using a colorimetric assay following 3 h infection. Data are presented as LDH in supernatant/maximum cellular release of LDH by lysis with triton-X above baseline LDH release for uninfected cells. (A) DC infected with PAO1, and (B) DC infected with PDO300. Each datapoint represents mean of triplicate measurements.

ages are high and comparable, the main target of the bacteria could be the apoptotic pathway followed by secondary necrosis. The most potent strains in inducing cell death were PAO1 and PA103, both of which express the type III secretion system as a pathogenetic factor with only PA103 expressing the type

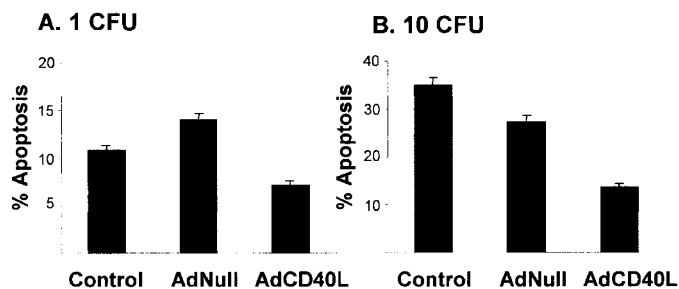


Figure 9. Inhibition of *Pseudomonas aeruginosa*-induced apoptosis in DC modified to express CD40L. DC genetically modified with AdCD40L (100 moi) were infected 24 h later with PAO1. AdNull infected DC served as control. Apoptosis was assessed by colorimetric detection of DNA fragmentation. (A) DC infected with 1 cfu/cell; (B) DC infected with 10 cfu/cell. Data are presented as % apoptosis above baseline (cells cultured with PA for 0 h) of DNase treated cells. Each datapoint represents the mean \pm SEM of triplicate wells.

III cytotoxin *exoU*. The apoptotic responses did not differ significantly. Adhesion of the bacteria *via* pili may be a factor, as PAO/NP, a strain that lacks pilin, was less potent at similar doses, but was still able to induce cell death of the alveolar macrophages and DC. The complex rich medium used for all

of our experiments may not permit the identification of PA-specific factors for the induction of apoptosis, but on the other hand enabled the comparisons of various strains under optimal growth conditions. Using more defined (*e.g.* iron-depleted) media instead of complex medium, may more closely reflect the growth condition in the CF lung (38) and will be analyzed in future experiments, together with the effects of epithelial lining fluid derived from patients with CF. Differences were also seen in the response to isolates generated from sputum of individuals with CF, although no clear pattern was detectable for early or late isolates. One characteristic feature of the PA strains found in the CF lung is their mucoid character. Mucoidity of isolates propagated *in vitro* is often lost when grown under standard conditions. The laboratory mucoid mutant used in our study, PDO300 was equally able to induce cell death in DC compared with its nonmucoid parental strain PAO1. The laboratory mucoid phenotype may not however reflect the complex forms of mucoidity and biofilms in the CF airways.

Host cell specific factors related to the induction of PA induced cell death have also been studied. For example, respiratory epithelial cells have been reported to be less susceptible than other mucosal epithelium to PA cytotoxicity and apoptosis

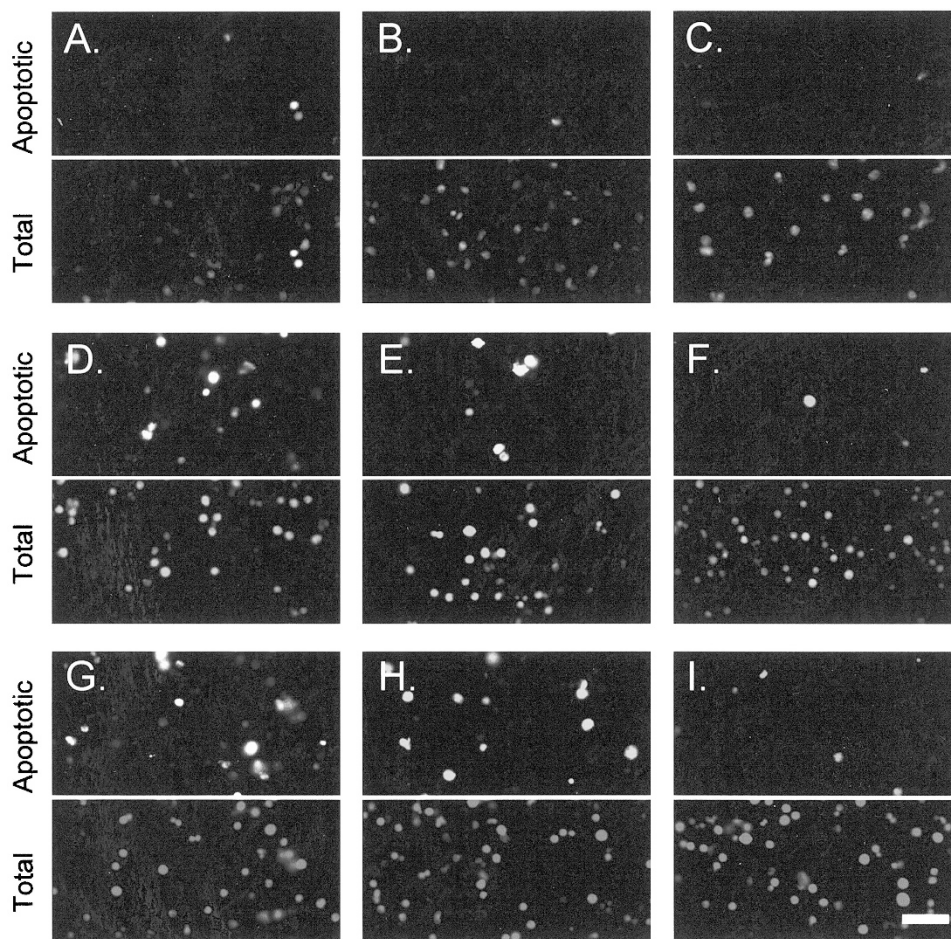


Figure 10. Decreased *Pseudomonas aeruginosa*-induced apoptosis in DC modified to express CD40L. Murine DC genetically modified with AdCD40L (100 moi) were infected 24 h later with PAO1 or PDO300 in coverslip dishes. AdNull infected DC or uninfected DC served as control. (A) Control cells uninfected, (B) AdNull uninfected, (C) AdCD40L uninfected, (D) control cells + PAO1, (E) AdNull + PAO1, (F) AdCD40L + PAO1, (G) control cells + PDO300, (H) AdNull + PDO300, and (I) AdCD40L + PDO300. Apoptosis was assessed by fluorescent terminal nucleotide end labeling (TUNEL) and analyzed by fluorescent microscopy. Bar = 50 μ m, all panels.

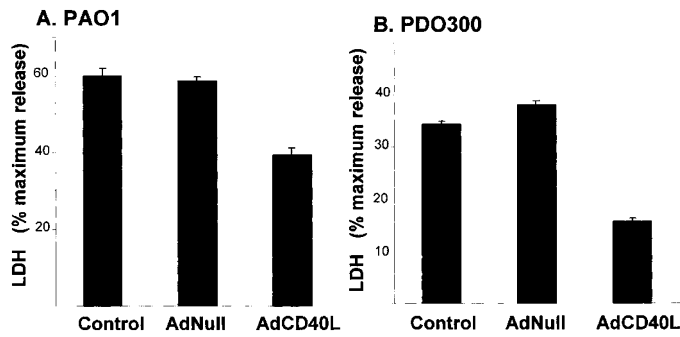


Figure 11. Quantification of apoptotic DC genetically modified to express CD40L following exposure to *Pseudomonas aeruginosa* as assessed by TUNEL assay. Murine DC were infected with the laboratory strains PAO1 and PDO300 at a dose of 5 cfu/cell for 3 h in coverslip dishes. Apoptosis was assessed by fluorescent terminal nucleotide end labeling (TUNEL) and analyzed by fluorescent microscopy by counting the percentage of apoptotic cells in 5 high power fields.

in vitro and *in vivo* (13, 14), possibly due to inaccessibility of the PA to the basolateral surface of the epithelia. The lung epithelial cells tested in the present study were less susceptible to PA induced cell death compared with antigen presenting cells under conditions without tight junctions. As the lung is an important site of infection with PA, especially in individuals with CF, recent studies have examined induction of apoptosis in cells of the respiratory tract *in vitro* and *in vivo*. Induction of apoptosis in lung epithelial cells by various PA strains was shown in CF and non-CF cell lines and was independent of the expression of the cystic fibrosis transmembrane conductance regulator gene, the gene responsible for CF (14).

Caspase activation, a central pathway in the cellular responses to apoptotic stimuli (39), was likely involved in the cell death induced by PA. Prior treatment with the caspase inhibitors VAD-fmk and YVAD-cmk lead to decreased cell death induced by PA. These inhibitors, are rather nonspecific and do not allow identification of the individual caspases involved, however, they demonstrate that apoptosis *via* the caspase pathway is occurring. Fas/FasL interaction has recently been shown to be important for apoptosis induced by PA in the respiratory tract of mice *in vivo* (12). This pathway may play a role in the cell death induced in the antigen presenting cells, although murine DC have been shown to be resistant to apoptosis mediated *via* this pathway (40). Previous studies from our laboratory have demonstrated that DC pulsed with live or heat inactivated PA can induce protective immunity against pulmonary PA infection in a mouse model. The protective effect seemed equal for the heat inactivated as for the live bacteria, although we observed more cell death and less *in vitro* IL-12 production following preparation of the DC incubated with live bacteria (41). Apoptosis of the DC could have had an effect and influenced the immune response.

CD40L and the prevention of apoptosis. As CD40L has been shown to regulate apoptosis in B cells (24), genetic manipulation using CD40L may be a way to circumvent PA induced cell death. CD40L, a member of the tumor necrosis factor gene superfamily, is usually expressed on activated lymphocytes and functions mainly *via* its interaction with

CD40 as a mediator of T cell dependent B cell activation, proliferation and differentiation (23). Retrovirus-mediated expression of CD40L in tumor cells inhibited tumor-induced apoptosis following ligation to CD40 (25). Genetic modification of DC to express CD40L before infection with PA significantly reduced the amount of PA induced cell death for the nonmucoid strain PAO1 as well as the mucoid strain PDO300. AdNull infection in itself seemed to have a small effect on apoptosis. Although the differences were statistically not significant and not consistent, Ad infection may influence cell survival independent of the transgene expression. Concomitant expression of CD40L with antigens in DC has been shown to activate DC and induce antibody production by B cells independent of CD4⁺ cells, although the mechanism of how gene transfer of CD40L to DC activates the DC has not been elucidated (26). Expression of CD40L acting within the cells, or expression of the protein on the surface leading to activation through interaction with CD40 on the same or neighboring cells are potential mechanisms. Surface expression of CD40L in the present study was detectable for about 50% of the cells, although the number of CD40L transduced cells may be higher, as CD40L can be shed from the surface (23). Whether or not the expression of CD40 induced by gene transfer of CD40L is necessary is not known, as all the antigen presenting cells used in the present study expressed CD40 on their surface. Antibodies against CD40 to block this receptor usually lead to activation and thus may not answer the question. Genetic modification of cell to resist PA-induced apoptosis could be used to improve host defenses against PA. The concept that expression of CD40L in antigen presenting cells will prevent cell death induced by bacterial pathogens is a novel concept that warrants further investigation.

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