Actinobacillus pleuropneumoniae exotoxin ApxI induces cell death via attenuation of FAK through LFA-1

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ApxI exotoxin is an important virulence factor derived from *Actinobacillus pleuropneumoniae* that causes pleuropneumonia in swine. Here, we investigate the role of lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), a member of the β2 integrin family, and the involvement of the integrin signaling molecules focal adhesion kinase (FAK) and Akt in ApxI cytotoxicity. Using Western blot analysis, we found that ApxI downregulated the activity of FAK and Akt in porcine alveolar macrophages (AMs). Preincubation of porcine AMs with an antibody specific for porcine CD18 reduced ApxI-induced cytotoxicity as measured by a lactate dehydrogenase release assay and decreased ApxI-induced FAK and Akt attenuation, as shown by Western blot analysis. Pretreatment with the chemical compounds PMA and SC79, which activate FAK and Akt, respectively, failed to overcome the ApxI-induced attenuation of FAK and Akt and death of porcine AMs. Notably, the transfection experiments revealed that ectopic expression of porcine LFA-1 (pLFA-1) conferred susceptibility to ApxI in ApxI-insensitive cell lines, including human embryonic kidney 293T cells and FAK-deficient mouse embryonic fibroblasts (MEFs). Furthermore, ectopic expression of FAK significantly reduced ApxI cytotoxicity in pLFA-1-cotransfected FAK-deficient MEFs. These findings show for the first time that pLFA-1 renders cells susceptible to ApxI and ApxI-mediated attenuation of FAK activity via CD18, thereby contributing to subsequent cell death.

*Actinobacillus pleuropneumoniae* (App) is a Gram-negative bacterium that causes severe hemorrhagic and necrotizing pleuropneumonia in pigs, leading to great economic loss in industry. App-derived Apx exotoxins, i.e., ApxI-IV, belong to the repeats-in-toxin (RTX) family and are the most important virulence factors involved in the pathogenesis of App¹. Apx exotoxins exert cytotoxic effects on porcine alveolar macrophages (AMs), neutrophils, and lymphocytes, impairing the host defense mechanisms²⁻⁴. ApxI has a strong cytotoxic effect and causes cellular damage and apoptosis in porcine AMs, which provide the first line of defense against bacterial infection in the lungs via their phagocytic and lytic abilities⁵⁻⁸.

RTX toxins, including the α-hemolysin (Hly) of *Escherichia coli*, leukotoxin (Lkt) of *Mannheimia haemolytica*, leukotoxin (Ltx) of *Aggregatibacter actinomycetemcomitans*, and adenylate cyclase toxin (CyaA) of *Bordetella pertussis*, interact with β₂ integrin and lead to cell death⁹⁻¹⁰. Human LFA-1 is a receptor for Hly and Ltx, and the binding of either toxin to LFA-1 causes cytolysis¹¹. Bovine LFA-1 is a receptor for Lkt, which binds CD11a and CD18 subunits¹². In addition, β₂ integrin plays an important role in Apx toxin-induced events. The CD18 subunit of lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) results in ApxIII-induced cell death¹³, and CD18 mediates ApxI-induced activation of the p38, c-Jun N-terminal kinase, and nuclear factor-κB pathways for the expression of proinflammatory cytokines¹⁶⁻¹⁷. However, the current knowledge regarding how β₂ integrin interacts with Apx toxins and the signaling mechanisms underlying the cytotoxicity of Apx toxins is limited.

β₂ integrins consist of α (CD11) and β₂ (CD18) subunits and are expressed primarily on leukocytes to mediate cell adhesion, migration, differentiation, survival, and proliferation¹⁶. The members of the β₂ integrin family include LFA-1, macrophage-1 antigen (Mac-1; CD11b/CD18), complement receptor 4 (CD11c/CD18), and αDβ₂ (CD11d/CD18). Upon ligand binding, inactive β₂ integrins switch to an active state, interact with adaptor

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molecules, and initiate a signaling network via α-actinin, 14–3–3 protein, or focal adhesion kinase (FAK), leading to corresponding cellular functions\(^n\). FAK and Akt are important regulators of integrin-derived survival signals. The role of the FAK signaling pathway in β\(_3\) integrin-mediated cell survival is well defined, and Akt modulates diverse prosurvival and antidote functions\(^m\). The activation of prodeath pathways and/or impairment of prosurvival pathways may pave the way for cell death\(^m\).

Although it has been thoroughly demonstrated that activation of the β\(_3\) integrin-FAK pathway supports cell survival, the role of FAK and Akt in β\(_3\) integrin-mediated cell survival has not been extensively investigated. Previous studies reported that CyaA, Hly, and Lkt attenuated Akt activity\(^n\), suggesting that RTX toxins downregulate cell survival pathways. Whether LFA-1 mediates the cytotoxic effects of ApxI via FAK and Akt is unclear. Therefore, the present study examined the role of LFA-1 in ApxI cytotoxicity and delineated the possible involvement of FAK and Akt in this event.

**Materials and methods**

**Chemicals, reagents, and antibodies.** Poly-D-lysine (PDL), polymyxin B, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbo-xanilide inner salt (XTT), Hoechst 33342, phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), 2-amino-6-chloro-a-cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester (SC79), bovine serum albumin (BSA), and an anti-β-actin antibody were purchased from Sigma-Aldrich (Merck, Germany). Phosphate-buffered saline (PBS) was purchased from Thermo Fisher Scientific (USA).

The CD11a-specific monoclonal antibodies MUC76A and CD11a-EXT were purchased from Kingfisher Biotech (USA) and AllBio (Taiwan), respectively. The CD18-specific antibodies PNK-1 and LS-C312785 were purchased from Bio-Rad (USA) and Lifespan Biosciences (USA), respectively. Isotype-matched mouse IgG1 and IgG2a were purchased from Thermo Fisher Scientific (USA) and Kingfisher Biotech (USA), respectively. The antibodies against phospho-FAK Tyr\(_{397}\) and phospho-Akt Ser\(_{473}\) were purchased from Cell Signaling Technology (USA), and an antibody recognizing phospho-FAK Tyr\(_{925}\) was purchased from Bioss (USA).

**Cell culture.** *Porcine AMs.* Porcine AMs were obtained from 6- to 8-week-old specific pathogen-free pigs via lavage and stored in liquid nitrogen using previously described procedures\(^n\). The protocol for the euthanasia of the pigs was approved by the Institutional Animal Care and Use Committee of Agricultural Technology Research Institute (permit number 10781) and was in accordance with the Guide for the Care and Use of Laboratory Animals (Council of Agriculture, Executive Yuan, ROC) and the ARRIVE Guidelines. Euthanasia was performed by intramuscular injection of azaperone (2 mg/kg body weight; Stroless, China Chemical & Pharmaceutical Research Institute, Taiwan) and a combination of equal parts by weight of tiletamine and zolazepam (4 mg/kg body weight; Zoetil**\(_{50}\), Virbac, France) to induce sedation and anesthesia, respectively, followed by electrocution. Prior to use in experiments, porcine AMs were thawed and incubated at 37°C in a humidified CO\(_2\) incubator overnight. To use the culture medium for porcine AMs was RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

*Human embryonic kidney (HEK) cells.* HEK 293T cells (Clontech, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified CO\(_2\) incubator.

*Mouse embryonic fibroblasts (MEFs).* FAK-deficient MEFs (ATCC CRL2644) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified CO\(_2\) incubator.

*Preparation of crude ApxI exotoxin.* An isolate of *A. pleuropneumoniae* serotype 10 (strain 13039) was a gift from the Animal Health Research Institute, Council of Agriculture, ROC. The preparation of the exotoxin and measurement of the cytotoxic activity using an XTT assay were performed according to previously described procedures\(^n\). One cytotoxic unit (CU) of ApxI was defined as the quantity of toxin that caused a 50% reduction in mitochondrial activity in porcine AMs.

*Plasmid preparation.* pCX-MCS1 was a gift from Dr. Chin-Kai Chuang (Agricultural Technology Research Institute, Taiwan, ROC). The pCX-CD11a, pCX-CD18 and pCX-GFP plasmids were constructed by separately cloning the coding sequence of porcine CD11a (GenBank accession DQ013284) with XhoI and Hin\(_{dIII}\) linkers, the coding sequence of porcine CD18 (GenBank accession U13941) with XhoI and KpnI linkers, and the coding sequence of green fluorescent protein (National Center for Biotechnology Information reference sequence WP_153939948) with EcoRI and Hin\(_{dIII}\) linkers into the multiple cloning site in pCX-MCS1. The FAK expression plasmid and corresponding control plasmid were purchased from OriGene (USA). The plasmids were transformed into *E. coli* TOP10 (Invitrogen, USA) and prepared from bacterial cultures using a Hispeed Plasmid Midi Kit (Qiagen, Germany) according to the manufacturer’s instructions.

*Transfection.* HEK 293T cells or MEFs were seeded in PDL-coated 35-mm cell culture plates (1 × 10\(^6\) HEK 293T cells or 2 × 10\(^8\) MEFs/plate) or PDL-coated 12-mm coverslips in a 24-well plate (1 × 10\(^5\) HEK 293T cells/well) and incubated at 37°C in a 5% CO\(_2\) atmosphere overnight. The culture medium was replaced with medium without antibiotics on the day of transfection. Lipofectamine 2000 (Invitrogen, USA) was used for transfection according to the manufacturer’s instructions. For confocal microscopy, HEK 293T cells on coverslips were transfected with 0.5 μg of pCX-CD11a and 0.5 μg of pCX-CD18, 1 μg of pCX-CD11a, 1 μg of pCX-CD18, or
1 μg pCX-MCS1. For transfection of HEK 293T cells for the LDH release assay or Western blot analysis, HEK 293T cells in 35-mm plates were transfected with 2 μg of pCX-CD11a and 2 μg of pCX-CD18 or 4 μg of pCX-GFP. For MEF transfection, the cells were transfected with 1.5 μg of pCX-CD11a and 1.5 μg of pCX-CD18 along with 0.3 μg of the FAK-expressing plasmid or corresponding control plasmid or with 3.3 μg of the corresponding empty vector as the control group. HEK 293T cells or MEFs were transfected for 24 h prior to subsequent experiments.

**Treatment with ApoxI, drugs, and antibodies.** For experiments with ApoxI treatment, cells were washed with low-serum medium (LSM; RPMI-1640 supplemented with 2 mM L-glutamine, 1% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml polymixin B), and incubated with with ApoxI in LSM. Transfected HEK 293T cells and MEFs were incubated with 0–25 CU/ml and 0–8 CU/ml ApoxI, respectively, for 5 h for LDH release assay. Porcine AMs without activator or antibody pretreatment were incubated with 0 or 2.5 CU/ml ApoxI for 0–60 min for Western blot analysis. In experiments with activator treatment, porcine AMs were incubated with LSM containing 200 nM PMA, 4 μg/ml SC79, or 0.1% DMSO for 0, 15, 30, or 60 min prior to Western blot analysis. In experiments with activator pretreatment and ApoxI treatment, porcine AMs were incubated with LSM containing 200 nM PMA, 4 μg/ml SC79, or 0.1% DMSO for 15 min prior to incubation with ApoxI. For Western blot analysis, PMA- and SC79-pretreated porcine AMs were incubated with 0 or 2.5 CU/ml ApoxI for 0–5 min and 0–60 min, respectively. For LDH release assay, PMA-and SC79-pretreated porcine AMs were incubated with 90 min with 0–10 CU/ml and 0–5 CU/ml ApoxI, respectively. In experiments with antibody pretreatment, porcine AMs were incubated with LSM containing 10 μg/ml of a monoclonal antibody (MUC76A, PNK-1, or isotype control) on ice for 60 min prior to incubation with 2 CU/ml ApoxI for 8 h, and 0 or 2.5 CU/ml ApoxI for 10 min for LDH release assay and Western blot analysis, respectively. To minimize LPS contamination in the exotoxin preparation, polymixin B was added to a final concentration of 10 μg/ml throughout this study.

**Lactate dehydrogenase (LDH) release assay.** ApoxI-induced cell death was measured as the activity of LDH released from damaged cells using a cytotoxicity detection kit (Roche, Switzerland). Briefly, porcine AMs or transfected cells were detached using Accutase (Gibco), suspended in LSM, and seeded in 96-well culture plates at a density indicated elsewhere. The cells were treated with ApoxI for the indicated periods, and the cell culture supernatant was collected by centrifugation at 200 × g for 10 min. The levels of cytotoxicity were 10–35% at 1.6–25 CU/ml ApoxI in the pCD11a/pCD18-cotransfected cells, and 1–10% in the controls (Fig. 1b). Cotransfection of pCD11a/pCD18 led to a 3.5- to 11-fold increase in the expression of CD11a and CD18.

**Confocal microscopy.** Transfected HEK 293T cells on PDL-coated coverslips were washed with PBS, fixed with 1% paraformaldehyde for 10 min, incubated with 2 mg/ml ammonium chloride in PBS containing 0.05% Tween-20 for 10 min, and blocked with 5% BSA in PBS for 30 min. The cells were incubated with monoclonal antibodies MUC76A and PNK-1 (1 μg/ml of each antibody) for 30 min at 4 °C, followed by incubation with Alexa Fluor 488- and PE-conjugated secondary antibodies. The nuclei were stained with 1 μg/ml Hoechst 33342 for 15 min. The cell surface expression of LFA-1 was evaluated under a confocal microscope (FV1000; Olympus, Japan).

**Western blot analysis.** Cell lysates were collected via centrifugation at 14,000 × g, and the protein concentrations were determined according to a previously described procedure. SDS–polyacrylamide gel electrophoresis was used to separate 20–50 μg of cell lysate, and the separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were probed with primary antibodies specific for porcine CD11a, porcine CD18, phospho-FAK735, phospho-FAK763, phospho-AktSer473, or β-actin and were then incubated with the corresponding secondary antibody. The intensities of the immunoreactive bands were quantified using ImageJ software (National Institutes of Health, USA) and normalized to the intensity of the loading control β-actin.

**Statistical analysis.** Data obtained from at least three independent experiments were analyzed using Student's t-test. GraphPad Prism 6 (GraphPad) was used for statistical analysis. The error bars indicate the standard deviations. A difference was considered significant when p < 0.05.

**Results**

**Ectopically expressed porcine LFA-1 confers susceptibility to ApoxI in human embryonic kidney 293T cells.** To examine the role of porcine LFA-1 (pLFA-1, pCD11a/pCD18) in ApoxI cytotoxicity, ApoxI-insensitive HEK 293T cells were used. First, the HEK 293T cells were transfected with a plasmid carrying porcine cd11a, cd18 or cotransfected with both plasmids for 24 h and subjected to confocal microscopy analyses for the expression of pCD11a and pCD18. pCD11a and pCD18 were simultaneously detected primarily at the cell membrane in pCD11a/pCD18 cotransfected cells. In contrast, neither pCD11a nor pCD18 was detected at the cell membrane in HEK 293T cells transfected only with a plasmid encoding pCD11a or pCD18 (Fig. 1a). Subsequently, cytotoxicity of ApoxI was investigated in HEK 293T cells cotransfected with plasmids expressing pCD11a and pCD18, green fluorescent protein (GFP), and non-transfected cells. These cells were treated with 1.6 to 25 CU/ml of ApoxI for 5 h. The level of LDH released to the culture medium was measured as an indicator of cell membrane damage and/or cytolysis. ApoxI-induced cytotoxicity in the pCD11a/pCD18-cotransfected cells was significantly higher than the control groups, including the GFP-transfected cells and the nontransfected cells. The levels of cytotoxicity were 10–35% at 1.6–25 CU/ml ApoxI in the pCD11a/pCD18-cotransfected cells, and 1–10% in the controls (Fig. 1b). Cotransfection of pCD11a/pCD18 led to a 3.5- to 11-fold increase in...
Figure 1. Porcine LFA-1 confers susceptibility to ApxI in HEK 293T cells. (a) HEK 293T cells were transfected with plasmids expressing porcine CD11a (pCD11a) and/or porcine CD18 (pCD18) or the corresponding empty vector. Ectopic expression of pCD11a and pCD18 was examined using confocal microscopy. Confocal images show the subcellular localization of pCD11a (pseudogreen), pCD18 (pseudored), and nuclei (pseudoblue). (b) Cells were transfected with plasmids expressing pCD11a and pCD18, green fluorescent protein (GFP), or non-transfected (None) for 24 h, followed by incubation with 1.6–25 CU/ml of ApxI for 5 h. The culture supernatants were collected and analyzed using the LDH release assay. Data are from three independent experiments of triplicate determinants. Error bars represent the standard deviations. *p < 0.05; **p < 0.01; ***p < 0.001. (c) Cells were transfected with plasmids expressing pCD11a and pCD18 or GFP. Expression of pCD11a and pCD18 was examined using Western blot analysis. Immunoblots were also probed with an anti-β-actin antibody as the loading control. The grouping of blots was cropped from different portions of the same gel and exposed separately. Uncropped blot images for (c) are presented in Supplementary Figure S1.
cytotoxicity levels to ApxI compared to control groups. The expression of pCD11a and pCD18 in cotransfected HEK 293T cells was confirmed using Western blot analysis (Fig. 1c). Taken together, cotransfection of porcine cd11a and cd18 was essential for pLFA-1 expression on the cell surface of HEK 293T cells. pLFA-1 is pivotal to ApxI cytotoxicity, as evidenced by the findings that ectopic expression of pLFA-1 rendered ApxI-insensitive cells susceptible to ApxI.

**CD18 mediates the cytotoxicity of ApxI in porcine AMs.** To evaluate the involvement of the two subunits of LFA-1 in ApxI-induced cell death, porcine AMs were preincubated with specific antibodies against CD11a or CD18 for 1 h and treated with 2 CU/ml of ApxI for 8 h, and the level of LDH release was measured. Preincubation with a CD18 antibody significantly reduced ApxI-induced cytotoxicity by 18% (Fig. 2a). However, ApxI-induced cytotoxicity in cells preincubated with a CD11a antibody was not affected (Fig. 2b). These findings suggest that CD18 serves as an important mediator of ApxI-induced cell death in porcine AMs.

**ApxI attenuates FAK and Akt activity in porcine AMs.** A plethora of studies indicate that the integrin signaling molecules, such as FAK, phosphoinositide 3-kinase (PI3K) and Akt, play important roles in cell survival. To investigate whether FAK was involved in ApxI cytotoxicity, FAK activity in porcine AMs treated with ApxI for 0–60 min was examined using Western blot analysis to assess the levels of phospho-FAK_{397} (p-FAK_{397}) and phospho-FAK_{925} (p-FAK_{925}). Upon exposure to 2.5 CU/ml of ApxI, the level of p-FAK_{397} decreased significantly during the first 20 min, but it was unaffected at the same time points in cells treated with control medium (0 CU/ml of ApxI) (Fig. 3a,b). The level of p-FAK_{397} declined significantly to 29% at 5 min of exposure to ApxI, then recovered to the basal level at 30 min. The level of p-FAK_{925} decreased to 77% during 1 h-exposure to ApxI, which did not differ significantly from the time-matched control (Fig. 3a,d). Akt activity in ApxI-treated porcine AMs was also examined using detection of the phospho-Akt_{473} (p-Akt_{473}) level. The analytical results showed that the level of p-Akt_{473} increased after 5 min of incubation regardless of the presence or absence of ApxI treatment (Fig. 3a,c). The level of p-Akt_{473} in ApxI-treated cells increased 3-fold after a 10-min ApxI incubation and remained constant for 60 min, and these levels were significantly lower than the...
control medium-treated group. Taken together, the time course studies reveal that ApxI significantly attenuated the activity of FAK and Akt in porcine AMs.

**ApxI-mediated attenuation of FAK/Akt activity requires CD18 in porcine AMs.** To examine whether CD18 was involved in ApxI-mediated attenuation of FAK/Akt activity, porcine AMs were preincubated with a CD18-specific antibody for 1 h and treated with 2.5 CU/ml ApxI for 10 min. The levels of p-FAK\text{Ty}397 and
p-AktSer473 were examined using Western blot analysis. The analytical results showed that the levels of p-FAK Tyr397 were 44% and 60% in the ApxI-treated cells with prior incubation with an isotype-matched antibody and CD18 antibody, respectively (Fig. 4a, b). Similarly, the levels of p-Akt Ser473 in ApxI-treated cells were 41% with CD18 antibody preincubation compared to the isotype control, which was 23% (Fig. 4a, c). Collectively, a CD18-specific antibody reversed the ApxI-induced reduction in FAK and Akt activity, which suggests a role of CD18 in mediating ApxI-induced FAK/Akt attenuation in porcine AMs.

Figure 4. Pretreatment with a CD18-specific antibody reduces ApxI-induced decreases in the levels of p-FAK Tyr397 and p-Akt Ser473 in porcine AMs. Porcine AMs were preincubated with an anti-CD18 antibody (α-CD18) or isotype control (Isotype) on ice for 1 h. ApxI was added to a final concentration of 2.5 CU/ml for an additional 10 min, and cell lysates were subjected to (a) Western blot analysis for p-FAK Tyr397, p-Akt Ser473, and β-actin. The grouping of blots was cropped from different portions of the same gel and exposed separately. Uncropped blot images for (a) are presented in Supplementary Figure S3. The average intensities of (b) p-FAK Tyr397 and (c) p-Akt Ser473 are from three independent experiments and normalized to the intensity of β-actin. The intensity of the ApxI-treated group was further normalized to the intensity of the non-ApxI-treated group. Asterisks indicate significant differences compared to the isotype control. **p < 0.01.
PMA and SC79 fail to restore the activity of FAK and Akt, respectively, and rescue Apxl-induced cell death in porcine AMs. We hypothesized that if the reductions of FAK and Akt activity were keys to Apxl-induced cell death, then enhancement of FAK or Akt activity would protect cells from Apxl-induced cell death. Therefore, we examined whether Apxl-induced FAK and Akt attenuation and cell death were modulated via chemical activators of FAK or Akt, i.e., PMA and SC79, respectively.

To test the effect of PMA on FAK activation, porcine AMs were incubated with PMA for 0–60 min, and the activity of FAK and Akt was determined using Western blot analysis. The levels of p-FAK(Tyr397) increased significantly 2.6-fold after a 15-min PMA treatment and remained constant for up to 60 min of PMA treatment (Fig. 5a,b). However, the level of p-Akt(Ser473) decreased after PMA treatment (Fig. 5a,c). These results indicated that PMA activated FAK, but not Akt, activity in porcine AMs. To investigate the effects of PMA on Apxl-mediated attenuation of FAK and Akt activity, porcine AMs were incubated with PMA prior to Apxl stimulation, and the activity of FAK and Akt was determined. A steady increase in p-FAK(Tyr397) was detected in Apxl–untreated cells pretreated with PMA (Fig. 5d,e). However, the level of p-FAK(Tyr397) in Apxl-treated cells was reduced 56% after 5 min of exposure to Apxl. The levels of p-Akt(Ser473) in Apxl–treated groups were 7–56% lower than the time matched, Apxl–untreated groups, but the differences between the Apxl–treated and –untreated groups were statistically insignificant. (Fig. 5d,f). To further examine the effect of PMA on Apxl cytotoxicity, porcine AMs preincubated with PMA or DMSO (vehicle) were stimulated with Apxl, and cell death was evaluated. After exposure to 1–10 CU/ml Apxl, the cytotoxicity levels were 2–32% and 5–59% in vehicle- and PMA-treated groups, respectively (Fig. 5g). PMA pretreatment produced a 1.5- to 2.3-fold increase in Apxl cytotoxicity compared to the vehicle pretreatment group. Taken together, PMA did not reverse Apxl-induced attenuation of the levels of p-FAK(Tyr397) and p-Akt(Ser473) and failed to rescue cells from Apxl cytotoxicity.

To test the effect of SC79 on Akt activity, porcine AMs were incubated with SC79 for 0–60 min, and the activity of Akt was determined using Western blot analysis. The levels of p-Akt(Ser473) increased 10- to 12-fold after SC79 treatment and increased 6–8-fold in the vehicle control group (Fig. 6a,b). SC79 treatment increased Akt activity 1.5-fold compared to vehicle control. To further investigate the effect of SC79 on Apxl-induced attenuation of Akt activity, porcine AMs pretreated with SC79 were stimulated with Apxl, and the level of p-Akt(Ser473) was examined. The results showed a significant decrease in the level of p-Akt(Ser473) within 1 h of exposure to Apxl (Fig. 6c,d). These levels fell to 18.1% at 15 min and declined to 6.5% at 1 h. The level of p-Akt(Ser473) was approximately 73% in the time-matched controls. SC79 did not overcome the Apxl-induced decrease in the level of p-Akt(Ser473). To further examine the effect of SC79 on Apxl-induced cell death, porcine AMs pretreated with SC79 were stimulated with 0–5 CU/ml Apxl for 90 min, followed by the LDH release assay. The levels of cytotoxicity increased significantly 20–40% in porcine AMs pretreated with SC79 compared to porcine AMs pretreated with vehicle (Fig. 6e). SC79 also failed to reduce Apxl-induced cytotoxicity in porcine AMs.

Collectively, PMA and SC79 failed to restore the FAK and Akt activity affected by Apxl, respectively, and rescue cell death from Apxl treatment.

Ectopic expression of FAK decreases Apxl-induced effects. To test whether enhancement of FAK activity via ectopic expression protected cells from Apxl cytotoxicity, FAK-deficient MEFs were transfected with multiple plasmids expressing FAK, pCD11a, and pCD18, or the corresponding empty vectors. The transfected cells were stimulated with 0–8 CU/ml of Apxl for 5 h, and the levels of LDH release were measured. The analytical results showed that the levels of cytotoxicity were 11–28% at 0.5–8 CU/ml Apxl in the pLFA-1-transfected cells and 2–13% in the vector controls. A 2- to 7-fold increase in cytotoxicity levels was observed in the pLFA-1-transfected cells compared to vector controls (Fig. 7a). The levels of cytotoxicity in the cells cotransfected with pLFA-1 and FAK were 7–18% at 0.5–8 CU/ml Apxl, which was significantly lower than the pLFA-1-transfected cells (Fig. 7a). Ectopic expression of pLFA-1 and FAK were confirmed in FAK-deficient MEFs transfected with pCD11a and pCD18 with or without FAK (Fig. 7b). Taken together, the results indicated that pLFA-1 conferred sensitivity to Apxl, and ectopic FAK ameliorated Apxl cytotoxicity in FAK-deficient MEFs.

Discussion

Integrin signaling plays a critical role in cell survival, cell proliferation, cell motility, cytoskeletal organization, cell differentiation and gene expression. FAK is a central coordinator of integrin signaling and interacts with various signaling molecules to regulate multiple biological processes. The prosurvival role of β1 integrin/FAK signaling has been extensively investigated and found to occur via the activation of downstream Src, PI3K and Akt to sup- press apoptosis in anchorage-dependent cells. β2 integrin Mac-1–derived signals suppress neutrophil apoptosis via the Akt and ERK survival pathway and regulate the survival and proliferation of human acute myeloid leukemia cells via Syk/STAT activation. To the best of our knowledge, the present study is the first to show that Apxl induces cytotoxicity via the β1 integrin signaling and delay of FAK and Akt function, which is in accordance with a previous study showing that A. actinomycetemcomitans Ltx, and M. haemolysitca Lkt induced cell death in HeLa cells. Further investigation is required to examine the interaction between Apxl and LFA-1 in order to demonstrate the ligand/receptor interaction. Our current and past studies consistently showed that the CD18 subunit mediated the Apxl-induced effects, including cell death and the secretion of proinflammatory cytokines, in porcine AMs. Vanden Bergh et al. also showed that the CD18 subunit of LFA-1 played a pivotal role in Apxl-induced cytotoxicity.
Figure 5. PMA fails to overcome ApxI-induced reduction in FAK and Akt activity and cytotoxicity. (a–c) Porcine AMs were treated with 200 nM PMA or 0.1% DMSO (Vehicle) for 0–60 min. Cell lysates were subjected to (a) Western blot analyses for p-FAK Tyr397 and p-Akt Ser473. The grouping of blots was cropped from different portions of two gels and exposed separately. Black lines delineate the boundary between not contiguous lanes of the same gel. Uncropped blot images for (a) are presented in Supplementary Figure S4. The average intensities of (b) p-FAK Tyr397 and (c) p-Akt Ser473 are from three independent experiments and normalized to the intensity of β-actin. Asterisks indicate significant differences compared to the 0-min treatment group. * p < 0.05. (d–f) Porcine AMs pretreated with 200 nM PMA for 15 min were stimulated with 0 or 2.5 CU/ml of ApxI for 0–5 min. Cell lysates were collected and subjected to (d) Western blot analyses for p-FAK Tyr397, p-Akt Ser473, and β-actin. The grouping of blots was cropped from different portions of two gels and exposed separately. Uncropped blot images for (d) are presented in Supplementary Figure S5. The average intensities of (e) p-FAK Tyr397 and (f) p-Akt Ser473 are from three independent experiments and normalized to the intensity of β-actin. Asterisks indicate significant differences compared to the non-ApxI treatment group at the identical time point. * p < 0.05; ** p < 0.01. (g) Porcine AMs were incubated with 200 nM PMA or 0.1% DMSO (Vehicle) for 15 min, then incubated with 0–10 CU/ml of ApxI for 90 min. The percent cytotoxicity was quantified using the LDH release assay. Data are from three independent experiments of triplicate determinants. * p < 0.05.
role in ApxIII-induced leukocytolysis. Because CD18 is a common subunit among β2 integrin family members, the possible involvement of other β2 integrin members in the effects of Apx cannot be excluded. The present study identified the ApxI-induced attenuation of FAK and Akt in porcine AMs. The levels of p-FAK\(^{397}\) and p-Akt\(^{473}\) were significantly reduced shortly after exposure to ApxI. Phosphorylation at Tyr397 is an important step in FAK activation and its subsequent interaction with Src and other downstream signaling molecules to transduce the integrin signal supporting cell survival. Decreases in FAK and Akt activity likely indicate the first wave of ApxI-induced signaling events. Comparison of the kinetics of FAK and Akt attenuation upon ApxI exposure suggested that FAK was downstream of β2 integrin and upstream of Akt in ApxI-modulated signaling. However, ApxI may attenuate the activity of FAK and Akt via independent pathways, a possibility that warrants further study.

Based on our finding that ApxI attenuated FAK/Akt activity, we hypothesized that downregulation of FAK/Akt signaling could lead to cell death and that an increase in FAK/Akt activity could rescue cells from ApxI cytotoxicity. We used the chemical activators PMA and SC79 to increase FAK and Akt activity, respectively. However, these activators failed to overcome the ApxI-induced attenuation of FAK and Akt activity and enhanced...
the consequent cell death. PMA significantly increased FAK activation in our study but had an inhibitory effect on the basal activity of Akt. The effect of PMA on Akt activity was inconsistent in various cell types and involved different regulatory mechanisms. For example, PMA increases the level of p-AktSer473 in breast cancer cells and mast cells via PKCδ activation and interaction with PKCβ, respectively30,31, but PMA attenuates Akt activity in endometrial cancer cells and mouse keratinocytes via PKCα, and PKCδ and PKCε32,33. The present study suggests that the inhibitory effect of PMA on the basal level of Akt activity may explain the potentiation of cell death after ApxI stimulation.

Downregulation of Akt by RTX toxins has been reported previously. M. haemolytica Lkt toxin attenuated the level of p-AktSer473, and B. pertussis CyaA and E. coli Hly reduced the level of p-AktThr308 and p-AktSer47322–24. Consistent with these studies, we also identified ApxI-induced attenuation of Akt. We further evaluated the ability of the Akt activator SC79 to reverse ApxI cytotoxicity. In contrast to our prediction, SC79 failed to increase Akt activity in response to ApxI and enhanced ApxI-induced cell death. The enhancement of ApxI-induced cell death by SC79 may correspond to the extremely low level of Akt activity. Wiles et al. also reported that the inhibitory effect of E. coli Hly on Akt was not overcome by administration of tumor necrosis factor-α and epidermal growth factor or by overexpression of constitutively active Akt mutants, which enabled PtdIns(3,4,5)P3-independent translocation or activation of Akt23. However, inhibitors of protein phosphatases PP1 and PP2A reduced the attenuation of p-AktSer473 by Hly. Their study revealed that Hly attenuated Akt activity via the participation of PP1 and PP2A. Whether similar mechanisms are active during ApxI-induced Akt attenuation is unclear; however, our data consistently suggest that ApxI downregulates the activity of FAK and Akt prior to cell death regardless of the presence or absence of PMA or SC79, respectively.

Figure 7. Ectopic expression of FAK attenuates ApxI-induced cytotoxicity in pLFA-1-transfected FAK-deficient MEFs. (a) FAK-deficient MEFs were transfected with plasmids expressing porcine LFA-1 (pLFA-1), in combination with a plasmid encoding FAK (pLFA-1 + FAK) or the corresponding empty vectors (Vector) for 24 h. The transfected cells were incubated with ApxI for 5 h. The culture supernatants were collected and analyzed using the LDH release assay. Data are from three independent experiments of triplicate determinants. *p < 0.05. (b) FAK-deficient MEFs were transfected with plasmids expressing porcine LFA-1 (pLFA-1) alone, in combination with a plasmid encoding FAK (pLFA-1 + FAK), or the corresponding empty vectors (Vector). Levels of pCD11a, pCD18, and p-FAKγ397 were examined using Western blot analyses. Immunoblots were also probed with an anti-β-actin antibody as the loading control. The grouping of blots was cropped from different portions of the same gel and exposed separately. Uncropped blot images for (b) are presented in Supplementary Figure S8.
ApxI impairs the host defense system via downregulation of β2 integrin-FAK survival signaling in porcine AMs. ApxI-induced cell death, and FAK attenuation imparts this event. This study provides valuable insight into how ApxI impairs the host defense system via downregulation of β2 integrin-FAK survival signaling in porcine AMs.

The present study used an alternative approach to determine the role of FAK in ApxI cytotoxicity and evaluate the effect of ectopically expressed FAK in FAK-deficient MEFs. We found that FAK acted as a prosurvival signaling molecule, because the expression of ectopic FAK decreased ApxI-induced cell death in FAK-deficient MEFs transfected with pLFA-1. Notably, pLFA-1-transfected FAK-deficient MEFs remained susceptible to ApxI, suggesting that other unidentified mechanisms may underlie the cytotoxic effect of ApxI.

In conclusion, the evidence presented in this study suggests that a signaling pathway consisting of the β2 integrin LFA-1, FAK, and Akt is essential for cell survival and attenuated by ApxI (Fig. 8). LFA-1 plays a pivotal role in ApxI-induced cell death, and FAK attenuation imparts this event. This study provides valuable insight into how ApxI impairs the host defense system via downregulation of β2 integrin-FAK survival signaling in porcine AMs.

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Figure 8. A schematic model illustrates the interaction of ApxI with LFA-1 and the signaling events affected by ApxI. Porcine LFA-1 (pCD11a/pCD18) serves as a potential receptor for ApxI. The CD18 subunit mediates the decreases in the levels of p-FAK Tyr397 and p-Akt Ser73 in response to ApxI. Attenuation of FAK and Akt activity contributes to ApxI-induced cell death. Whether ApxI attenuates Akt activity in a FAK-dependent manner (dotted line) must be further investigated. The diagram was drawn using Microsoft PowerPoint 2013 (https://www.microsoft.com/).
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Author contributions

S.C.L. and S.L.H. conceived the original idea, analyzed and interpreted the data, and wrote the manuscript. S.C.L., Y.T.C., C.Y.W., and J.Y.W. designed and performed the experiments. Z.W.C. and J.P.W. constructed plasmids and collected porcine AMs. Z.W.C., J.P.W., and J.H.L. analyzed and interpreted the data. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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