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Philippe G.A.C. Vanden Bergh, Laurent L.M. Zecchinon, Thomas Fett, Daniel Desmecht. Porcine CD18 mediates *Actinobacillus pleuropneumoniae* ApxIII species-specific toxicity. *Veterinary Research*, 2009, 40 (4), 10.1051/vetres/2009016 . hal-00903108

HAL Id: hal-00903108

<https://hal.science/hal-00903108>

Submitted on 11 May 2020

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Porcine CD18 mediates *Actinobacillus pleuropneumoniae* ApxIII species-specific toxicity

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(Received 19 November 2008; accepted 8 April 2009)

Abstract – *Actinobacillus pleuropneumoniae*, the causative agent of porcine pleuropneumonia, produces Apx toxins that are recognized as major virulence factors. Recently, we showed that ApxIIIa-cytotoxic activity specifically targets *Sus scrofa* leukocytes. Since both LtxA from *Aggregatibacter actinomycetemcomitans* (aggressive periodontitis in humans) and LktA from *Mannheimia haemolytica* (pneumonia in ruminants) share this characteristic, respectively towards human and ruminant leukocytes, and because both use the CD18 subunit to interact with their respective LFA-1, we hypothesized that ApxIIIa was likely to bind porcine CD18 to exercise its deleterious effects on pig leukocytes. A β_2 -integrin-deficient ApxIIIa-resistant human erythroleukemic cell line was transfected either with homologous or heterologous CD11a/CD18 heterodimers using a set of plasmids coding for human (ApxIIIa-resistant), bovine (-resistant) and porcine (-susceptible) CD11a and CD18 subunits. Cell preparations that switched from ApxIIIa-resistance to -susceptibility were then sought to identify the LFA-1 subunit involved. The results showed that the ApxIIIa-resistant recipient cell line was rendered susceptible only if the CD18 partner within the LFA-1 heterodimer was that of the pig. It is concluded that porcine CD18 is necessary to mediate *A. pleuropneumoniae* ApxIIIa toxin-induced leukolysis.

Actinobacillus pleuropneumoniae / ApxIIIa / porcine / LFA-1 / CD18

1. INTRODUCTION

Actinobacillus pleuropneumoniae is the bacterial causative agent of porcine pleuropneumonia, a frequent and highly infectious disease generating significant economic losses related to deficits in zootechnical profits and intensive use of antibiotics [17, 21]. Virulence factors of *A. pleuropneumoniae* include Apx exotoxins (ApxIA, ApxIIA, ApxIIIa and ApxIVA), lipopolysaccharides, polysaccharidic capsule, fimbriae, iron collecting systems, proteases, superoxide dismutase, etc. [3]. The Apx toxins are recognized as major virulence factors and

belong to the Repeats in ToXin (RTX) protein family [13]. They share the same structural characteristics which are a series of glycine- and aspartate-rich nonapeptide repeats which constitute the main calcium-binding sites of the protein [16]. Even though the *apxIVA* gene is not disrupted by an insertion element [25], all serotypes are able to synthesize ApxIVA (only in vivo) whose autocatalytic and cross-linking activities [22] make it different from other Apx toxins that are of the pore-forming toxin (PFT) type. Some of the Apx toxic activities were already detected in previous studies. ApxIA exerts a strong hemolytic activity and a strong cytotoxic activity whereas ApxIIa possesses a weak hemolytic activity and a

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moderate cytotoxic activity. ApxIII_A does not display haemolytic activity but a strong cytotoxic activity on porcine neutrophils and pulmonary alveolar macrophages [3, 4, 12, 13, 23].

Several studies have shown that lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18, $\alpha_L\beta_2$), the most abundant and widespread β_2 -integrin, is centrally involved in the pathogenesis of some diseases caused by RTX PFT-producing bacteria. The virulence of *A. actinomycetemcomitans* (localized aggressive periodontitis in humans), *Mannheimia haemolytica* (pneumonia in ruminants), and pathogenic strains of *Escherichia coli* (extraintestinal infections) closely depends on a ligand/receptor interaction between their respective toxin (LtxA, LktA, and HlyA) and LFA-1 [6, 7, 14], which triggers the synthesis and release of a wide array of cytokines and chemoattractants that exacerbate inflammation and ultimately result in widespread leukolysis [29]. Moreover, LtxA and LktA were shown to use the CD18 subunit of human and bovine LFA-1, respectively, to mediate leukolysis [5, 7]. Since widespread leukolysis is also seen in pig lungs during actinobacillosis and because ApxIII_A (i) resembles HlyA (54% identity), LktA (49%) and LtxA (45%) and (ii) specifically lyses porcine leukocytes [28], this study was designed to examine the hypothesis that ApxIII_A toxicity similarly relies on a specific ligand-receptor interaction with pig CD18.

In this perspective, a β_2 -integrin-deficient ApxIII_A-resistant erythroleukemic cell line was transfected either with homologous or with heterologous CD11a/CD18 heterodimers using a set of plasmids coding for human (ApxIII_A-resistant), bovine (-resistant) and porcine (-susceptible) CD11a and CD18 subunits. Cell preparations that switched from ApxIII_A-resistance to -susceptibility were then sought to identify the subunit involved.

2. MATERIALS AND METHODS

2.1. Reagents, cell line and antibodies

RPMI-1640 with 25 mM Hepes and 0.3 mg/mL L-glutamine, Dulbecco Modified Eagle Medium (DMEM) with 4.5 mg/mL glucose and 0.58 mg/mL

L-glutamine, and Dulbecco phosphate buffered saline (DPBS) with calcium and magnesium were purchased from Lonza BioWhittaker. Both culture media were supplemented with 10% [v/v] heat-inactivated fetal bovine serum along with amphotericin-B 250 µg/mL (Gibco, Merelbeke, Belgium) and penicillin-streptomycin 10 000 U/mL. The K-562 cell line was obtained from American Type Culture Collection (#CCL-243) and was maintained in DMEM medium at 37 °C in a humidified atmosphere of 5% CO₂. MAbs MCA1972 (anti-pig CD18) and MCA2308 (anti-pig CD11a) were purchased from Abd Serotec (Düsseldorf, Germany), mAb BAQ30A (anti-bovine CD18) from VMRD (Pullman, USA), mAb 555382 (anti-human/bovine CD11a) from BD Biosciences (Erembodegem, Belgium) and AlexaFluor® 488-conjugated goat anti-mouse IgG from Invitrogen (Carlsbad, CA, USA).

2.2. Preparation of ApxIII_A toxin

Plasmid pJFF1003, containing the *apxIII_A* gene, was kindly provided by Dr P. Kuhnert and Prof. J. Frey (Institute of Veterinary Bacteriology, University of Bern, Switzerland) and recombinant ApxIII_A toxin was produced following Maier et al. protocol [18]. This plasmid contains, inserted in the pET14b vector, the *apxIII_{CABD}* operon controlled by a strong constitutive endogenous promoter. Transformed *E. coli* Rosetta™ (Novagen, Leuven, Belgium) were seeded on Luria-Bertani (LB) agar plates with ampicillin (50 µg/mL) and incubated overnight at 37 °C. Several clones were then cultivated, each in 200 mL LB broth with ampicillin (50 µg/mL) and the Complete® protease inhibitor cocktail (Roche, Brussels, Belgium), one tablet for 50 mL culture with shaking (200 rpm) at 37 °C until an optical density of 1.2 at 600 nm was reached. No isopropyl- β -D-thiogalactopyranoside (IPTG) was added since *apxIII_{CABD}* genes are efficiently expressed from their own promoter [18]. Next, toxin-containing supernatant was recovered by centrifugation for 20 min at 10 000 g and filtrated on a membrane with 0.2 µm sizing pores (Nalgen, Leuven, Belgium). Thereafter, the toxin precipitation from the cell-free supernatant was achieved by adding 22 g of solid polyethylene glycol 4 000 (Sigma, Bornem, Belgium) per 100 mL of supernatant and stirring for 45 min at 4 °C. Finally, ApxIII_A was pelleted by centrifugation for 1 h at 10 000 g and dissolved in sterile DPBS (1 mL for 200 mL of starting culture). The ImageJ 1.37c software¹ gave an estimation of

¹ <http://rsb.info.nih.gov/ij/>

~100 µg/mL (~0.83 µM, ~5.10¹⁴ toxins/mL) Ap-xIIIa (120 kDa) after electrophoresis on Coomassie blue-stained sodium dodecyl sulfate gels (Invitrogen) after using 1 µg of BSA (Sigma) as the standard.

2.3. Ectopic expression of diverse LFA-1 on β_2 -integrin-deficient human cells

Engineering of plasmid driving expression of bovine [9, 10] and human [10] LFA-1 subunits, and porcine CD11a [26, 27] was previously described. To retrieve full-length porcine PoCD18 cDNA, total RNA from the spleen of freshly slaughtered pigs (*Sus scrofa domestica*) was first extracted with TRIzol (Invitrogen) as described by the manufacturer, and reverse transcribed using Improm II (Promega, Leiden, Netherlands). PoCD18 cDNA was then generated by long distance PCR using High Fidelity PCR Enzyme Mix (Fermentas, St. Leon-Rot, Germany) with primers designed from the proximal and distal UTR of the published PoCD18 cDNA (GenBank U13941): 5'-gcaggacatgctgtgccg-3' (forward) and 5'-ttcaccagcacccttag-3' (reverse). The following cycling parameters were applied: 5 min at 94 °C, then 35 cycles including: (i) 30 s at 94 °C, (ii) 30 s at 60 °C, and (iii) 2 min 30 s at 72 °C, followed by a final extension at 72 °C for 10 min. Resulting PCR products were then gel-purified using the Qiaquick Gel Extraction Kit (Qiagen, Venlo, Netherlands), TA-cloned into pCRII-TOPO vector (Invitrogen), and seeded on ampicillin plates (50 µg/mL). Minipreps were obtained from colonies grown in 5 mL LB-ampicillin (50 µg/mL) broth and clones were sequenced on an ABI-3100 Genetic Analyzer using Big Dye terminator chemistry (Applied Biosystems, Foster, USA). PoCD18 cDNA was deduced from sequences obtained from nine independent clones and then subcloned into pcDNA5 expression vector (Invitrogen). Next, the vector was amplified and purified with Nucleo Bond[®] Xtra Midi Plus EF (Macherey-Nagel, Belgium) and its concentration was measured by a NanoDrop[®] spectrophotometer ND-1000 (Thermo Fischer Scientific, Zellik, Belgium).

The K-562 cell line was electroporated with 7 plasmid pairs that were presumed to drive cell-surface expression of 3 homologous LFA-1 heterodimers, PoCD11a/PoCD18, BoCD11a/BoCD18 and HuCD11a/HuCD18 and 4 heterologous LFA-1, BoCD11a/PoCD18, HuCD11a/PoCD18, PoCD11a/BoCD18 and PoCD11a/HuCD18. For electroporation, the Nucleofector[™] technology (Amaxa Biosystems,

Koeln, Germany) was used for LFA-1 transfection in the K-562 cell line, using the T-16 optimized protocol recommended by the manufacturer. Briefly, cells were passaged two days before nucleofection in order to reach a cell density of 5 to 6 × 10⁵ cells/mL. Prior to nucleofection, 10⁶ K-562 cells were centrifuged and the supernatant was completely discarded so that no visible residual medium covered the pellet. Then, 2.5 µg of each plasmid in 1 to 5 µL H₂O were added to the pellet which was resuspended in Nucleofector[™] Solution V (at room temperature) to a final concentration of 10⁶ cells/100 µL. Thereafter, the solution was transferred into an Amaxa-certified cuvette to run the T-16 program. Next, 500 µL of pre-warmed RPMI-1640 were added to the cuvette and the solution was transferred into a 15 mL tube and incubated 10 min at 37 °C. Nucleofected cells were then placed into the well of a six well plate containing 1.5 mL of prewarmed DMEM and incubated for 24 h at 37 °C. Transfected K-562 were then washed with DPBS and rescued into 500 µL of RPMI-1640.

2.4. Probing of ectopic expression of CD11a/CD18 heterodimers at the cell surface

The success of each of the 7 double electroporation procedures to drive subsequent K-562 membrane expression of the LFA-1 intended was evaluated by flow cytometric detection of CD18 and CD11a-positive cells. Briefly, approximately 10⁵ transfectants were washed three times in 1 mL DPBS/BSA1%, blocked further in ice-cold DPBS/BSA1% for 20 min, and successively exposed to relevant primary (for 20 min on ice) and AlexaFluor[®] 488-conjugated secondary (goat anti-mouse IgG for 20 min on ice) antibodies. Effective transfectants were identified using the BD FACSCanto[™] flow cytometry system and associated BD FACSDiva software for detecting and counting fluorescent cells. For each experiment, the membrane expression rate obtained was defined as the mean between CD11a and CD18 positive cells. Positive and negative control cells for porcine LFA-1 cell-surface expression consisted in similarly processed porcine peripheral blood mononucleated cells (PBMC) extracted from fresh blood by the Accuspin[™] System-Histopaque[®] 1077 (Sigma) [28] and human β_2 -integrin free K-562 cells respectively. The autofluorescence area (negative cells) was determined using K-562 cells similarly processed except that the primary antibody was replaced by an isotype-matched nonpertinent mAb.

2.5. Assaying toxin activity

ApxIII^A-induced cytotoxicity was probed by measuring the relative proportion of the target cell population that underwent cell death within 1 h after toxin addition to the medium. Sensitive cells were defined as those showing propidium iodide (PI) incorporation and their counting was made by flow cytometry. A typical experiment consisted of addition of 50 μ L of the toxin stock solution in 50 μ L of the control (see below) or principal (K-562 transfectants) cell suspensions in RPMI (2.10^6 cells/mL). After 1 h incubation at 37 °C, the ongoing processes were stopped by addition of 1 mL ice-cold DPBS. The cell pellet then retrieved from a 5 min duration centrifugation at 200 g was resuspended into 500 μ L of a DPBS (495 μ L) and PI (5 μ L, 250 μ g/mL, from Invitrogen) mixture and was analyzed within 10 min. Target cells treated with 10% paraformaldehyde were used as positive controls of necrosis. Positive and negative controls for toxin susceptibility consisted of porcine PBMC and human β_2 -integrin-deficient K-562 cells, respectively.

2.6. Statistical analysis

Statistical analysis was carried out using Student paired *t*-test². *P* values were calculated and the term "significant" corresponds to a *P* value less than 0.05.

3. RESULTS

3.1. Ectopic expression of CD11a/CD18 heterodimers by K-562 cells

Flow cytometric profiling of nontransfected K-562 cell populations revealed that the distribution of autofluorescence values emitted in the 530/30 nm window was nearly identical whatever the primary mAb (anti-CD18, anti-CD11a or nonpertinent) used in combination with Alexa 488-conjugated secondary antibodies (Fig. 1). This set of control measurements allowed objective definition of the spectra typical of autofluorescence. By comparison, double electroporation experiments consistently resulted in a dramatic shift to the right of fluorescence values, thus demonstrating the

presence of LFA-1-expressing subpopulations (Fig. 1). Within the series of experiments reported here, the ranges of membrane expression obtained were the following: ~30 to 60% (PoCD11a/PoCD18), ~60 to 80% (BoCD11a/PoCD18), ~60 to 75% (HuCD11a/PoCD18), ~40 to 60% (PoCD11a/BoCD18), ~55 to 75% (PoCD11a/HuCD18), ~60 to 80% (BoCD11a/BoCD18) and ~65 to 80% (HuCD11a/HuCD18). Distributions of PI-specific fluorescence emitted by mock- or ApxIII^A-exposed nontransfected K-562 cell populations were also established (Fig. 1). By doing so, the autofluorescence spectrum corresponding to the highly predominant PI-negative cell population was objectively defined along with the rate of spontaneous cell death (< 5%).

3.2. Only K-562 cells expressing PoCD18-containing LFA-1s become ApxIII^A-susceptible

K-562 cell populations obtained after double electroporation were exposed to the toxin and the rate of cytotoxicity was found to be dramatically increased in cell preparations expressing the PoCD11a/PoCD18, BoCD11a/PoCD18 and HuCD11a/PoCD18 heterodimers (Fig. 1). Conversely, cytotoxicity rate recorded among PoCD11a/BoCD18, PoCD11a/HuCD18, BoCD11a/BoCD18 and HuCD11a/HuCD18 expressing preparations remained within the range displayed by control K-562 cells (Fig. 1). When the PI/forward scatter (FSC) dot plots profile displayed by these latter four cell preparations and by nontransfected K-562 cells upon exposition to ApxIII^A were retrieved, only a single low-PI/high-FSC cell population was detected (data not shown). Inversely, in PoCD11a/PoCD18, BoCD11a/PoCD18 and HuCD11a/PoCD18 expressing cell preparations, PI accumulation consistently correlated with a dramatic decrease in FSC (size reduction), which is compatible with ongoing cell death (Fig. 2). For each double transfection experiment, assessment of LFA-1 expression rate and measurement of PI incorporation upon ApxIII^A exposition was made in three different cell subfractions. Each double transfection experiment was also carried out several times, thus generating a spectrum of

² <http://www.graphpad.com/quickcalcs/ttest1.cfm> (GraphPad Software).

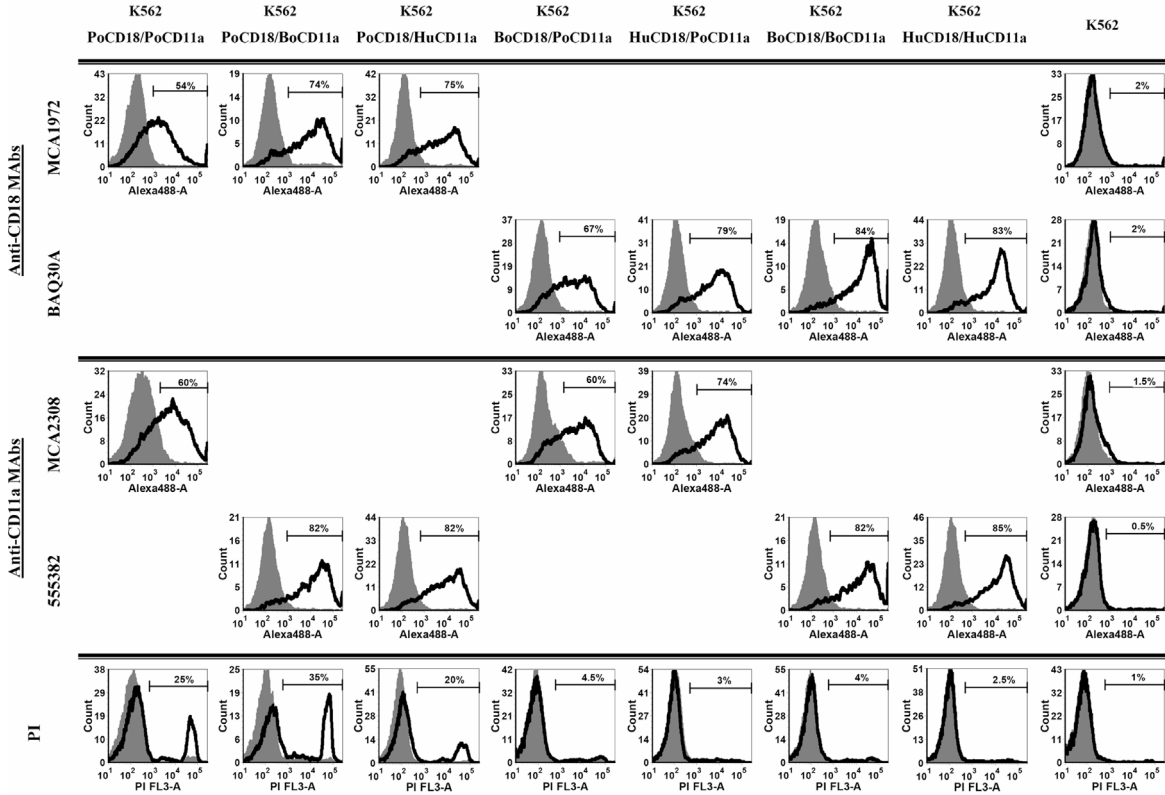


Figure 1. Flow cytometric phenotyping of nontransfected (last column on the right) and double transfected K-562 cells. Ectopic LFA-1 cell-surface expression was probed by detection of cell-surface expression of either the CD18 (rows 1–2) or the CD11a (3–4) subunit. Distributions of control and principal single cell fluorescence intensities were obtained after using an isotype-matched non pertinent (filled, grey) or an anti-CD18 or -CD11a primary antibody (open, white) respectively, before detection with Alexa Fluor 488-conjugated secondary Abs. Cells undergoing cell death were defined as those showing PI incorporation (row 5) and their relative proportion was established in mock- (unimodal grey distribution) and ApxIIIa-exposed (uni- or bimodal white) non- or cotransfected K-562 cells. Range of Alexa 488/PI positivity is given in each panel along with percentage of positive cells. See text for details.

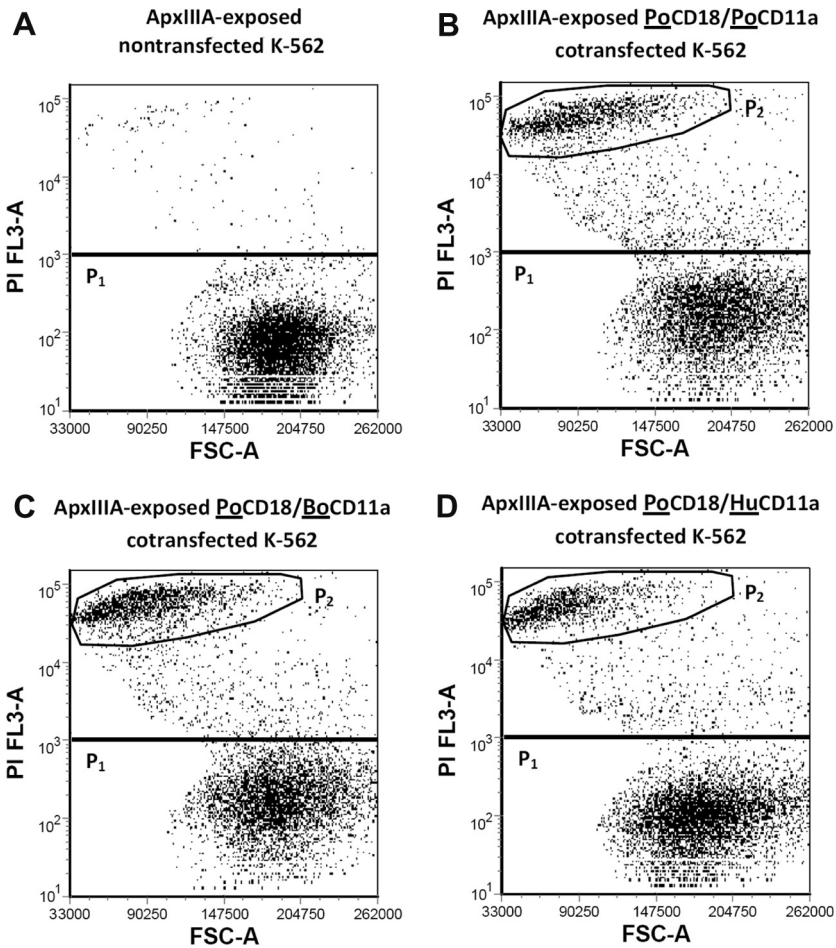


Figure 2. K-562 cells accumulate PI upon ApxIIIa exposition when expressing porcine CD18-containing LFA-1s. PI versus forward scatter (FSC) dot plot from flow cytometric analysis of ApxIIIa-exposed control (top, left) and double transfected K-562 cells. Only the P2 populations expressed the intended LFA-1.

expression rates for each LFA-1 species tested. When all the experiments were gathered, a clear linear relationship was detected between densities of PoCD11a/PoCD18, BoCD11a/PoCD18 and HuCD11a/PoCD18 expressing cells and susceptibility to ApxIIIa-induced cell death (Fig. 3). Such a relationship was totally absent when data from PoCD11a/BoCD18, PoCD11a/HuCD18, BoCD11a/BoCD18 and HuCD11a/HuCD18 expressing cells were gathered (Fig. 3). Finally, ApxIIIa-induced cytotoxicity in BoCD11a/

PoCD18-expressing K-562 cell preparations was toxin concentration-dependent (Fig. 4).

4. DISCUSSION

In order to demonstrate that porcine CD18 mediates ApxIIIa-induced leukolysis, we cotransfected the ApxIIIa-resistant, β_2 -integrin-deficient human erythroleukemic K-562 cell line with pairs of cDNA that were presumed

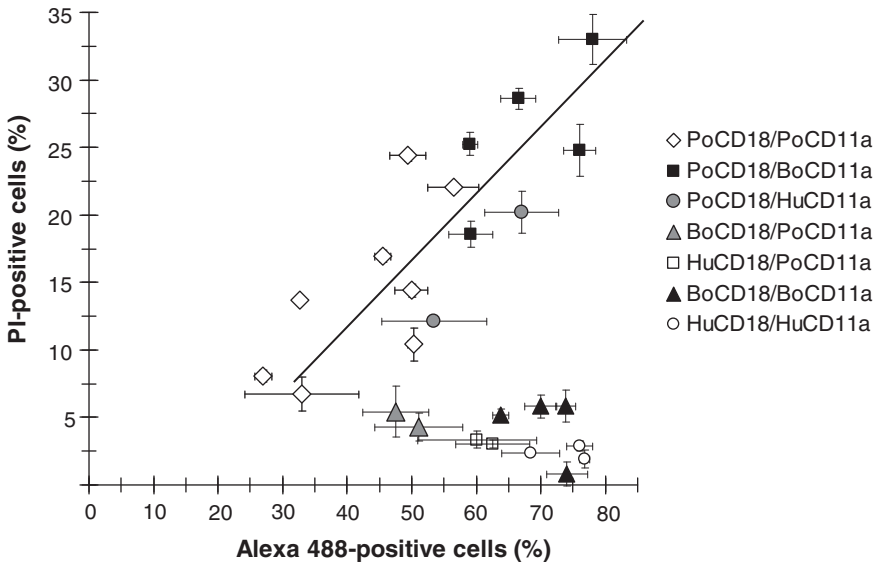


Figure 3. Susceptibility to ApxIIIa-induced cell death closely depends on the rate of LFA-1 ectopic expression in human erythroleukemic K-562 cells provided the contributing CD18 subunit is that of the pig. Values (squares, circles, triangles and lozenges) are means \pm SD from three representative experiments in which mean LFA-1 expression rate (horizontal SD) and mean PI incorporation (vertical SD) were measured by flow cytometry. LFA-1 cell-surface expression rate typical of each experiment was defined as the mean proportion of Alexa 488-positive cells calculated from two independent labelling procedures, one using MCA1972 (anti-CD18) and the other MCA2308 or 555382 (anti-CD11a) as primary antibody.

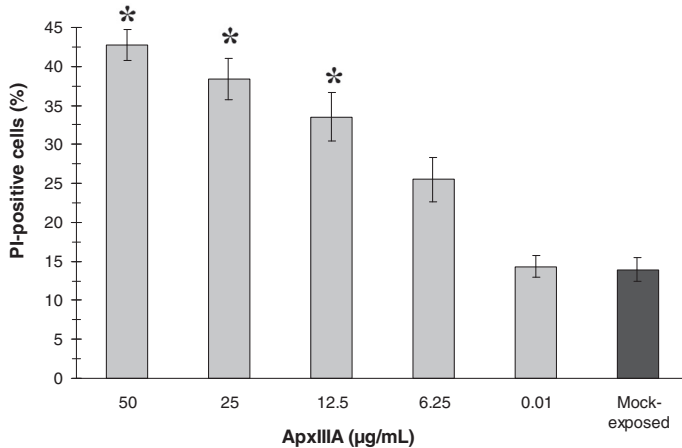


Figure 4. Susceptibility of BoCD11a/PoCD18-expressing human erythroleukemic K-562 cells to ApxIIIa is dose-dependent. Transfected cells with similar LFA-1 expression rates were first incubated for 1 h with decreasing concentrations of ApxIIIa and then analysed for PI incorporation by flow cytometry. ApxIIIa induced concentration-dependent cell death (grey boxes) while culture medium (RPMI-1640) did not (negative control, black). Values are means \pm SD from triplicates. Asterisks point to concentrations causing significantly higher cytotoxicity than that spontaneously recorded from mock-exposed double transfectants ($P < 0.05$).

to drive cell-surface expression of a set of 3 homologous and 4 heterologous LFA-1 heterodimers. After a 1 h incubation at 37 °C with the crude ApxIIIa preparation, nontransfected K-562 cells and cotransfectants expressing homologous bovine and human LFA-1 showed levels of PI incorporation similar to those recorded for mock-exposed K-562 cells, thus proving their ApxIIIa-resistant status. Cotransfectants expressing homologous pig LFA-1 accumulated PI and showed a reduction in size, both characteristics being compatible with the development of cell death (Figs. 1 and 2). This interpretation fits with most current mechanistic assumptions about PFT RTX toxins standing that hydrophilic membrane pores are created following molecule insertion in the lipid bilayer which leads to calcium influx and potassium efflux [1, 2, 18, 19]. The created pore diameter is estimated to be approximately 2 nm which is sufficiently small to prevent leakage of large cytoplasmic molecules such as proteins [18]. Since intracellular osmotic pressure is higher than that of extracellular fluid, there would be a passively entering flow causing an initial fast and irreversible swelling followed by bursting and ultimately necrosis and size reduction [24].

When K-562 cotransfectants expressing heterologous CD11a/CD18 heterodimers were exposed to ApxIIIa, two profiles were readily seen depending on the porcine partner involved. With PoCD11a, distributions of PI-related fluorescence values upon exposition to ApxIIIa mimicked those recorded in mock-exposed K-562 cells, thus suggesting that the pig CD11a subunit is not directly enrolled in the ApxIIIa-PoLFA-1 ligand/receptor interaction. Conversely, ectopic expression of heterologous LFA-1s made of the PoCD18 subunit partnering with either the human or bovine CD11a resulted in the K-562 cell population switching from ApxIIIa-resistance to susceptibility. Moreover, the cytotoxicity rate recorded upon toxin incorporation was directly proportional to the rate of PoCD18-containing homologous and heterologous LFA-1 expression (Fig. 3) and a clear correlation was shown between ApxIIIa concentration and rate of cell death in K-562 cotransfectants expressing heterologous BoCD11a/PoCD18 (Fig. 4). Taken together,

the results unambiguously suggest that PoCD18 is necessary to mediate *A. pleuropneumoniae* ApxIIIa toxin-induced leukolysis.

This is thus the third example (after LtxA and LktA) of a species-specific and leukocyte-specific RTX cytotoxin using the CD18 subunit. Since LktA from *M. haemolytica* was recently shown to bind ruminant β_2 -integrin Mac-1 (CD11b/CD18) too, which, again, results in ruminant leukocyte necrosis [15], it might be that ApxIIIa binds porcine Mac-1 as well, which could also contribute to the pathogenesis of actinobacillosis. Pushing the parallelism further, the CD18 domain critically enrolled in ApxIIIa-CD18 ligand/receptor interaction could be one of its constitutive EGF modules as recently demonstrated for LtxA/HuCD18 [7] and LktA/BoCD18 [8], although this is still disputed [11]. Binding and killing of target cells by RTX toxins were also shown to depend on the recognition of N-linked oligosaccharide chains linked to β_2 -integrin receptors [20], which could also apply to ApxIIIa.

Acknowledgements. Philippe Vanden Bergh is the recipient of a studentship from the "Fonds pour la formation à la Recherche dans l'Industrie et l'Agriculture", rue d'Egmont 5, B-1000 Bruxelles, Belgium.

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