Transfection of non-susceptible cells with *Ovis aries* recombinant lymphocyte function-associated antigen 1 renders susceptibility to *Mannheimia haemolytica* leukotoxin

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Abstract

*Mannheimia haemolytica* is an important etiological agent of pneumonia in domestic sheep (DS, *Ovis aries*). Leukotoxin (Lkt) produced by this organism is the principal virulence factor responsible for the acute inflammation and lung injury characteristic of *M. haemolytica* caused disease. Previously, we have shown that the leukocyte-specific integrins, β\(_2\) integrins, serve as the receptor for Lkt. Although it is certain that CD18, the β subunit of β\(_2\) integrins, mediates Lkt-induced cytolysis of leukocytes, it is not clear whether CD18 of all three β\(_2\) integrins, LFA-1, Mac-1 and CR4, mediates Lkt-induced cytolysis of DS leukocytes. Since polymorphonuclear leukocytes, which express all three β\(_2\) integrins, are the leukocyte subset that is most susceptible to Lkt, we hypothesized that all three β\(_2\) integrins serve as the receptor for Lkt. The objective of this study was to determine whether DS LFA-1 serves as a receptor for *M. haemolytica* Lkt. We cloned the cDNA for DS CD11a, the α subunit of LFA-1, and co-transfected it along with the previously cloned cDNA for DS CD18, into a Lkt-non-susceptible cell line. Transfectants stably expressing DS LFA-1 were bound by Lkt. More importantly, Lkt lysed the DS LFA-1 transfectants in a concentration-dependent manner. Pre-incubation of Lkt with a Lkt-neutralizing monoclonal antibody (MAb), or pre-incubation of transfectants with MAbs specific for DS CD11a or CD18, inhibited Lkt-induced cytolysis of the transfectants. Exposure of LFA-1 transfectants to low concentrations of Lkt resulted in elevation of intracellular [Ca\(^{2+}\)]. Taken together, these results indicate that DS LFA-1 serves as a receptor for *M. haemolytica* Lkt.

Keywords: *Mannheimia haemolytica*; Leukotoxin; Receptor; Domestic sheep; LFA-1

1. Introduction

*Mannheimia haemolytica* is an important bacterial pathogen of pneumonia in domestic sheep (DS, *Ovis aries*) and bighorn sheep (BHS, *Ovis canadensis*)
(Foreyt and Jessup, 1982; Miller et al., 1991; Brogden et al., 1998; Ackermann and Brogden, 2000). In conjunction with active viral infection and stress factors, *M. haemolytica* migrates to the lungs and causes acute pneumonia (Mosier, 1997). Leukotoxin (Lkt) and lipopolysaccharide (LPS) are the primary virulence factors causing lung injury characteristic of *M. haemolytica* (Zecchinon et al., 2005). Lkt, which is specific for ruminant leukocytes, is a 102 kDa glycoprotein belonging to the RTX (repeats in toxin) family of toxins produced by several Gram-negative bacteria (Welch, 1991). At high concentrations the toxin induces trans-membrane pore formation (Clinkenbeard et al., 1989; Jeyaseelan et al., 2001). At sub-lytic concentrations, Lkt activates alveolar macrophages and polymorphonuclear leukocytes (PMNs) resulting in the release of proinflammatory cytokines (Yoo et al., 1995).

We and others have previously shown that β2 integrins serve as the receptor for Lkt on the target cells (Wang et al., 1998; Ambagala et al., 1999; Li et al., 1999; Jeyaseelan et al., 2000). β2 integrins are leukocyte-specific integrins which have a common β subunit, CD18. The β subunit CD18 associates with three distinct α subunits, CD11a, CD11b and CD11c, giving rise to three well-characterized β2 integrins, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and CR4 (CD11c/CD18) (Gahmberg et al., 1997; Hogg et al., 2002). Recent studies in our laboratory involving recombinant expression of CD18 into Lkt-non-susceptible cells have shown unambiguously that CD18 mediates Lkt-induced cytolysis of leukocytes of cattle (Deshpande et al., 2002), BHS (Liu et al., 2007) and DS (Dassanayake et al., 2007). However, it is not clear whether CD18 of all three β2 integrins, LFA-1, Mac-1 and CR4, mediates Lkt-induced cytolysis of DS leukocytes. Since PMNs, which express all three β2 integrins, are the leukocyte subset that is most susceptible to Lkt, it is logical to hypothesize that all three β2 integrins serve as the receptor for Lkt. The objective of this study was to determine whether DS LFA-1 serves as a receptor for *M. haemolytica* Lkt.

2. Materials and methods

2.1. Cell lines and growth conditions

The human embryonic kidney cell line, HEK-293 (ATCC® Number: CRL-1573™) was cultured in complete culture medium (DMEM medium [Invitrogen] supplemented with 10% [v/v] heat-inactivated fetal bovine serum along with l-glutamine 4 mM and gentamicin 50 μg/ml [Sigma]). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2. HEK-293 cells stably transfected with DS LFA-1 (CD11a/CD18) were selected and maintained in complete culture medium containing selection antibiotics; geneticin, 800 μg/ml (G418; Invitrogen) and blasticidin, 20 μg/ml (InvivoGen).

2.2. Monoclonal antibodies and leukotoxin

Anti-human CD11a and CD18 monoclonal antibodies (MAbs) HUH73A (IgG1) and HUH82A (IgG2a), (Saalmuller et al., 2005), which cross react with DS CD11a and CD18 were obtained from Washington State University Monoclonal Antibody Center. MAb MM601 (IgG1) was used in Lkt-neutralization experiments, and the MAb MM605 (IgG2a) conjugated with FITC was used in flow cytometry to demonstrate Lkt-binding (Gentry and Srikumaran, 1991). The MAbs 8G12 (IgG1) (Klucas and Anderson, 1988) and MM113 (IgG2a) (Srikumaran et al., 1990) were obtained from the Department of Veterinary and Biomedical Sciences at the University of Nebraska-Lincoln, and used as isotype-matched controls.

The Lkt from *M. haemolytica* (serotypes A1, A2 and A6) was prepared as described by Gentry and Srikumaran (1991), filter-sterilized and stored at −20 °C in aliquots until needed. All the experiments were performed with the same batch of Lkt.

2.3. Molecular cloning of cDNA encoding CD11a and CD18

Peripheral blood was collected from suffolk breed of DS by venipuncture and subjected to density gradient centrifugation using Ficoll-Paque (Amerham Pharmacia Biotech). PMNs were isolated by hypotonic lysis of the erythrocyte pellet (Deshpande et al., 2002). The isolated PMNs were induced with phorbol myristate acetate (PMA, 25 ng/ml for 15 min, Fett et al., 2005) and total cellular RNA was extracted using TRIzol reagent (Invitrogen). cDNA synthesis was performed using Superscript III™ first-strand
synthesis kit (Invitrogen). Forward and reverse primers (CD11a RACE1: 5'- GTCGCCAGTAAATCCCAAGA 3'; RACE2: 5'- GCACCTCAATCTCAACCCT 3') used to amplify the cDNA for DS CD11a were designed based on previous RACE experiments (Fett et al., 2005; GenBank Accession nos. AY 731092 and AY731091). The entire gene encompassing the 5' and 3' UTR regions was amplified using PfuUltra™ II Fusion HS (Stratagene) as described the manufacturer. The PCR fragments were gel-purified using QIAquick® Gel Extraction Kit (Qiagen), and cloned into pCR®4-TOPO vector (Invitrogen). Ten nanograms of plasmid DNA isolated from one of the positive clones was used as template to amplify the ORF along with 5' UTR region using forward (5'- CACCGTCGCCAGTAAATCCCAAGAGC 3') and reverse (5' TCATCTCCACCTTCACTCCATCC 3') primers by PfuUltra™ II Fusion HS. The amplified CD11a, cDNA was cloned into the mammalian expression vector, pcDNA6.2/GW/D-TOPO (Invitrogen) and sequenced. The cDNA sequence encoding DS CD11a was submitted to GenBank (DQ975205). The 2313 bp cDNA for DS CD18 (DQ470837) was cloned into pcDNA3.2/GW/D-TOPO in a similar fashion (pRD/CD18, Dassanayake et al., 2007). The resulting constructs, pKL/DS CD11a (Fig. 1) and pRD/CD18 were used to co-transfect HEK-293 cells. All sequencing reactions were performed by Big Dye™ and analyzed using BioEdit v7.0.5 (Ibis therapeutics) software.

2.4. Co-transfection of cDNA encoding DS CD11a/CD18 into HEK-293 cells

Purified plasmid DNA of pKL/DS CD11a and pRD/CD18 were used to co-transfect HEK-293 cells using Transfast™ as per manufacturer’s protocol (Promega) with minor modifications. Forty-eight hours post-transfection, cells were transferred into 75 cm² flasks containing selection medium (complete growth medium containing geneticin, 800 μg/ml and blasticidin, 20 μg/ml). Transfected cell populations which continued to grow in the selection medium were stained with anti-CD18 MAb (HUH82A) and anti-CD11a MAb (HUH73A) followed by FITC-conjugated goat anti-murine Ig antibodies. The stained double transfectants sorted by a fluorescence-activated cell sorter (FACSVantage SE, Becton-Dickinson) were plated into 96-well tissue culture plates at one cell per well. The single clones expressing DS LFA-1 after 3 weeks of maintenance on selection medium were amplified in 75 cm² tissue culture flasks and used for further analysis.

2.5. Flow cytometric analysis of cell surface expression of DS CD11a and CD18

Cell surface expression of DS CD11a and CD18 by the transfectants was determined by flow cytometric analysis according to previously published procedures (Deshpande et al., 2002) with minor modifications. Briefly, 2.5 × 10⁵ cells were resuspended in 50 μl of FACS buffer (3% horse serum and 0.01% sodium azide in 1× PBS) and incubated with 50 μl of anti-CD11a MAb HUH73A, anti-CD18 MAb HUH82A, or isotype-matched control MAb (15 μg/ml) at 4°C for 20 min. Following three washes in FACS buffer, the cells were incubated with 50 μl of FITC-conjugated goat anti-murine Ig antibodies (Caltech Laboratories; 1:200 dilution) at 4°C for 20 min. Similar treatment was given to the parent cells (HEK-293), which were used as negative control. The cells were washed with FACS buffer, resuspended in 200 μl, PBS containing 2% formaldehyde and analyzed by a flow cytometer (FACSort, Becton-Dickinson).

![Diagram](Fig. 1. Schematic representation of mammalian expression vector pKL/DS CD11a containing cDNA for DS CD11a. The cDNA for DS CD11a was amplified from total RNA of DS PMNs, and cloned into a mammalian expression vector pcDNA6.2/GW/D-TOPO to yield pKL/DS CD11a.)
2.6. Flow cytometric analysis of Lkt-binding to DS LFA-1

The transfectants were tested for Lkt-binding by flow cytometry according to previously published procedures (Gopinath et al., 2005). FITC-conjugated MM605 (1:75 dilution) was used to detect the Lkt bound to DS LFA-1. The parent cells (HEK-293) were used as negative control.

2.7. MTT dye reduction cytotoxicity assay for detection of Lkt-induced cytotoxicity

The susceptibility of the transfectants to M. haemolytica Lkt-induced cytolysis was determined by a previously described MTT dye reduction cytotoxicity assay (Gentry and Srikumaran, 1991). The parent cells (HEK-293) were used as the negative control. The percent cytotoxicity was calculated as follows:

\[
\text{%Cytotoxicity} = \left[ 1 - \frac{\text{OD of toxin-treated cells}}{\text{OD of toxin-untreated cells}} \right] \times 100
\]

2.8. Inhibition of Lkt-induced cytolysis of transfectants expressing DS LFA-1 by anti LFA-1 and anti-Lkt MAbs

In order to demonstrate that the recombinantly expressed LFA-1 mediates Lkt-induced cytolysis of the transfectants, the cytotoxicity assay was performed as described in Section 2.7, with the exception that the cells were pre-incubated with either anti-CD11a MAb (HUH73A) or anti-CD18 MAb (HUH82A) at 100 μg/ml, for 90 min at 4 °C before the addition of Lkt. The transfectants were pre-incubated with isotype-matched control MAbs (8G12 or MM113) to serve as the negative controls.

In order to show that the cytolysis of the transfectants was specifically induced by Lkt, the Lkt was incubated with the Lkt-neutralizing MAb (MM601) at 1 μg/ml for 40 min at 4 °C, before incubation with the transfectants. The isotype-matched MAb 8G12 served as the negative control. Lkt preparations from M. haemolytica (serotype A1) were used for these assays. The percent inhibition of cytotoxicity was calculated as follows:

\[
\text{%Inhibition of cytotoxicity} = \left[ \frac{1 - \text{%cytotoxicity in the presence of antibody}}{\text{%cytotoxicity in the absence of antibody}} \right] \times 100
\]

2.9. Detection of Lkt-induced intracellular [Ca²⁺]i elevation in transfectants expressing DS LFA-1

Intracellular [Ca²⁺]i elevation in transfectants expressing DS LFA-1 exposed to Lkt was measured by fluorescent calcium indicator (Molecular Probes). Cells were incubated with Lkt (40 U) for 45 s and intracellular [Ca²⁺]i elevation was measured by flow cytometry. At least 5000 cells were counted to evaluate the intracellular [Ca²⁺]i elevation. Culture supernatant from a M. haemolytica Lkt deletion mutant (Murphy et al., 1995) was used as the negative control.

2.10. Statistical analysis

Statistical analysis was carried out using student t-test and P-values were determined using http://www.graphpad.com/quickcalcs/ttest1.cfm (GraphPad Software, Inc.). The term significant indicates a P-value less than 0.05.

3. Results

3.1. Cloning of the cDNA encoding DS CD11a and CD18

The cDNA encoding DS CD18 was cloned earlier in our laboratory (Dassanayake et al., 2007). In order to develop transfectants expressing DS LFA-1 (CD11a/CD18) we cloned the cDNA for DS CD11a in this study (Fig. 1). Five independent clones were sequenced. The overall gene structure and organization of the DS CD11a is similar to that of the DS CD11a in the previous report (AY731091, Fett et al., 2005). The DS cDNA for CD11a consists of 3555 nucleotides encoding an ORF of 3495 bp. The deduced protein sequence consists of 1164 amino acids (aa). This differs...
from the previously reported sequence for DS CD11a by 2 aa: D544E and Y1107H. The first substitution falls between the 2 and 3 cation binding motifs, and the second is the last aa in the cytoplasmic tail. These substitutions do not significantly alter the molecular weight, isoelectric point, antigenic index or the overall charge in comparison with the protein encoded by AY731091 gene. From the above comparison we concluded that the CD11a cloned in our laboratory is an allelic variant of the previously reported gene. The cDNA for DS CD11a exhibits 99% and 98% identity at nucleotide and aa level with that of O. canadensis (DQ 459072) and Capra hircus (AY773019), respectively. It also exhibits 96% identity with that of Bos taurus (AY267467) and 85% with Sus scrofa (DQ613285) at nucleotide level but only 94% and 77% at aa level, respectively.

3.2. Transfection of HEK-293 cells with cDNA encoding DS CD11a and CD18 results in cell surface expression of DS LFA-1

Two weeks following transfection of HEK-293 cells with the cDNA for DS CD11a and CD18, cell populations resistant to blasticidin (selects for DS CD11a expression) and geneticin (selects for DS CD18 expression) in the selection medium began to appear. Flow cytometric analysis with the anti-DS CD11a (HUH73A) and anti-DS CD18 (HUH82A) MAbs revealed surface expression of DS CD11a and CD18 (LFA-1) by these cells. These cells were pooled, stained with MAbs specific for DS CD11a and CD18, and subjected to fluorescence-activated cell sorting to isolate transfectant clones. Several clones expressing DS CD11a and CD18 were obtained. One of these clones, SCS 5, stably expressing DS CD11a and CD18 (Fig. 2A) and DS CD18 (Fig. 2B) was selected for further analysis.

3.3. Leukotoxin binds to the transfectants expressing DS LFA-1

In order to determine whether the recombinant expression of DS LFA-1 on HEK-293 cells enables Lkt to bind to them, the transfectants (SCS 5) were incubated with Lkt followed by FITC-conjugated anti-Lkt MAb MM605. This analysis revealed specific binding of Lkt to the LFA-1 transfectants (SCS 5), but not the parent cells (HEK-293, Fig. 3), indicating that recombinantly expressed LFA-1 on HEK-293 cells rendered them permissive for Lkt binding.

3.4. DS LFA-1 expression in HEK-293 cells renders them susceptible to Lkt-induced cytolysis

Lkt binding to the transfectants does not necessarily mean that they are susceptible to Lkt-induced cytolysis. Hence the transfectants expressing DS LFA-1 (SCS 5)
the parent cells (HEK-293) were tested for susceptibility to Lkt-induced cytolysis by MTT dye reduction cytotoxicity assay. We used Lkt from *M. haemolytica* serotypes A1, A2 and A6. The transfected cells were effectively lysed by Lkt from all the three serotypes in a concentration-dependent manner, whereas the parent cells were not (Fig. 4).

### 3.5. Anti-LFA-1 and anti-Lkt MAbs inhibit Lkt-induced cytolysis of transfectants expressing DS LFA-1

In order to demonstrate that Lkt-induced cytolysis of the transfectants was in fact mediated by the recombinantly expressed DS LFA-1, we performed the cytotoxicity assay after pre-incubating the transfectants with MAbs specific for DS CD11a or CD18. Both MAbs inhibited Lkt-induced cytolysis (>75%) confirming the fact that the recombinantly expressed LFA-1 mediated the Lkt-induced cytolysis of the transfectants.

Lkt from *M. haemolytica* used in this study was in the form of culture supernatant which is likely to contain very small amounts of LPS and possibly other secretory and excretory products. Hence we repeated the cytotoxicity assay after incubating the Lkt preparation with the Lkt-neutralizing MAb MM601. Pre-incubation of Lkt with MM601 inhibited the Lkt-mediated cytolysis of transfectants (>50%) whereas the isotype-matched control MAb 8G12 had no effect, clearly indicating that cytolysis of the transfectants was specifically induced by Lkt and not by any other components present in the Lkt preparation.

### 3.6. Lkt induces intracellular \([\text{Ca}^{2+}]_i\) elevation in transfectants expressing DS LFA-1

Intracellular \([\text{Ca}^{2+}]_i\) elevation in target cells following exposure to low concentrations of Lkt has been accepted as an indication of Lkt-receptor interaction (Dileepan et al., 2005; Thumbikat et al., 2005). Hence we exposed the LFA-1 transfectants to Lkt and measured intracellular \([\text{Ca}^{2+}]_i\) elevation. Forty five seconds exposure to 40 U of Lkt was sufficient to elevate \([\text{Ca}^{2+}]_i\) levels in the LFA-1 transfectants while Lkt had no effect on the parent cells (Fig. 5A). Culture supernatant from a Lkt deletion mutant of *M. haemolytica* also had no effect (Fig. 5B), confirming that the intracellular \([\text{Ca}^{2+}]_i\) elevation is specifically induced by Lkt.
4. Discussion

In this study, experiments were designed to determine whether LFA-1 (CD11a/CD18) serves as a receptor for Lkt. Previously our laboratory had cloned and sequenced DS CD18 (Dassanayake et al., 2007). In this study, DS CD11a was cloned in order to develop transfectants co-expressing CD18 and CD11a. The predicted aa sequence of DS CD11a cloned for this work differs from the previously reported sequence for DS CD11a by 2 aa: D544E and Y1107H (AY731091, Fett et al., 2005). The CD11a we cloned is an allelic variant of the previously reported one. Plasmids containing DS CD11a and CD18 were co-transfected into HEK 293 cells. These cells were chosen for transfection because they do not express any known \( \beta_2 \) integrins. As a result, these cells are not bound or lysed by Lkt, and are ideal for studies aimed at dissecting the role played by an individual \( \beta_2 \) integrin in mediating Lkt-induced cytolysis without the compounding effects of the other known \( \beta_2 \) integrins. Double staining (for CD11a and CD18) was followed by fluorescence-activated cell sorting to ensure that single clones were obtained expressing both CD11a and CD18. One such clone, SCS 5, was used in subsequent analysis. It has been established that the \( \alpha \) and \( \beta \) subunits, CD11 and CD18, have to associate with each other in order to be transported to, and expressed on, the cell surface (Gahmberg et al., 1998). In accordance with this observation, in a previous study, CD11a and CD18 molecules were co-precipitated from transfectant cell lysate with either the anti-CD11a or anti-CD18 MAbs (Deshpande et al., 2002). These data support the conclusion that the DS CD11a and CD18 are expressed as a dimer on the cell surface of the transfectant clone SCS 5.

Flow cytometric analysis revealed that Lkt bound to the DS LFA-1 transfectants, but not the parent cells (Fig. 3) suggesting that LFA-1 serves as a receptor for Lkt. Lkt has been shown to bind to non-ruminant leukocytes without inducing cytolysis (Jeyaseelan et al., 2000). However, concentration-dependent cytolysis of the transfectants by Lkt in this study clearly indicates that DS LFA-1 serves as a receptor for Lkt.

* M. haemolytica* serotype 2 is the serotype that is commonly isolated from the pneumonic lungs of DS while serotype 1 is commonly isolated from the pneumonic lungs of cattle (Angen et al., 1999;
In a study by Thumbikat et al. (2005), anti-CD11a induced cytolysis of bovine LFA-1 transductants. In a study by Dileepan et al. (2005), anti-CD11a MAb did not inhibit Lkt-induced cytolysis. In a study by Dassanayake et al., 2007). In a study by Dileepan et al. (2002; Liu et al., 2007; Deshpande et al., 2002). In our previous studies, transfection of cDNA for CD18 from cattle, BHS or DS alone into Lkt-non-susceptible mouse cell line P815 rendered it susceptible to Lkt-induced cytolysis of DS LFA-1 transfectants by anti-CD18 MAb and anti-CD11a MAb confirmed that cytolysis of the transfectants was mediated by the recombinantly expressed LFA-1. The inhibition of Lkt-induced cytolysis by anti-CD18 MAb is very likely due to the direct inhibition of interaction of CD18 with Lkt. However, inhibition of cytolysis by anti-CD11a MAb is likely due to this MAAb binding to CD11a and sterically hindering the interaction of CD18 with Lkt. This conclusion is based on the following observations: (1) In our previous studies, transfection of cDNA for CD18 from cattle, BHS or DS alone into Lkt-non-susceptible mouse cell line P815 rendered it susceptible to Lkt-induced cytolysis (Deshpande et al., 2002; Liu et al., 2007; Dassanayake et al., 2007). (2) In a study by Dileepan et al. (2005), anti-CD11a MAb did not inhibit Lkt-induced cytolysis of bovine LFA-1 transductants. (3) In a study by Thumbikat et al. (2005), anti-CD11a MAb did not inhibit Lkt binding to bovine alveolar macrophages.

Lkt induces intracellular \([\text{Ca}^{2+}]\), elevation in target cells including PMNs and alveolar macrophages, and this elevation of intracellular \([\text{Ca}^{2+}]\) is primarily due to the influx of extracellular \([\text{Ca}^{2+}]\), through voltage-gated channels (Ortiz-Carranza and Czuprynski, 1992; Hsuan et al., 1998; Dileepan et al., 2005). Hence intracellular \([\text{Ca}^{2+}]\), elevation is considered as an indicator of Lkt binding to its receptor (Dileepan et al., 2005). Hence we measured intracellular \([\text{Ca}^{2+}]\), elevation in the transfectants and parent cells. Lkt induced intacellular \([\text{Ca}^{2+}]\), elevation in the transfectants, but not in the parent cells, confirming that LFA-1 serves as a receptor for Lkt.

In conclusion, recombinant expression of DS LFA-1 in *M. haemolytica* Lkt-non-susceptible cells rendered them susceptible to binding and lysis by Lkt, indicating that DS LFA-1 serves as a receptor for Lkt. Inhibition of Lkt-induced cytolysis of DS LFA-1 transfectants by anti-LFA-1 antibodies, and elevation of intracellular \([\text{Ca}^{2+}]\), in response to Lkt binding confirmed the use of DS LFA-1 as a receptor by *M. haemolytica* Lkt.

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**References**


