# Integrin-EGF-3 domain of bovine CD18 is critical for *Mannheimia haemolytica* leukotoxin species-specific susceptibility

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## Abstract

*Mannheimia haemolytica* leukotoxin (LktA) is the primary virulence factor contributing to the pathogenesis of lung injury in bovine pneumonic pasteurellosis. Results from the authors' previous studies demonstrated that the site required for LktA binding leading to susceptibility to its biological effects resides within amino acid residues 500–600 of the extracellular region of bovine CD18. Experiments were designed to identify a much smaller functional domain within this 100 amino acid region of bovine CD18 that is critically required for species-specific susceptibility to LktA effects. Chimeric bovine X human CD18 with different integrin epidermal growth factor(I-EGF) like domains switched between bovine and human CD18 were generated and coexpressed with bovine CD11a in the human K562 cell line. The resulting chimeric transductants were tested for susceptibility to LktA-induced effects. The results demonstrate unequivocally that the I-EGF-3 domain of bovine CD18 (amino acid residues 541–581) is critical for conferring species-specific susceptibility to *M. haemolytica* leukotoxin.

## Introduction

Bovine pneumonic pasteurellosis continues to be one of the most important disease problems facing the American beef and dairy industries (Mosier, 1997). *Mannheimia haemolytica* (ST1 or ST6) is the primary etiologic agent of the disease (Ackermann & Brogden, 2000). The bacterium possesses an array of potential virulence factors; of these, a large body of evidence suggests leukotoxin (LktA) as the most important factor contributing to lung injury (Jeyaseelan *et al.*, 2002).

Previous studies in the authors' laboratory and in those of others demonstrated that LktA binds to the CD18 subunit of bovine β2 integrins (Ambagala *et al.*, 1999; Li *et al.*, 1999; Jeyaseelan *et al.*, 2000; Deshpande *et al.*, 2002). β2 Integrins are heterodimeric cell surface glycoproteins composed of a common β chain, CD18, that associates with four distinct α chains to give rise to four different β2 integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (p150/95) and CD11d/CD18. The bovine CD18 has been characterized (Shuster *et al.*, 1992; Huang *et al.*, 1997; Zecchinon *et al.*, 2004a). Data indicate that the amino acid residues between 123 and 363 located in the N-terminal region of CD18 are highly conserved among different species (Shuster *et al.*, 1992; Zecchinon *et al.*, 2004a, b). The C-terminus of the extracellular region of CD18 has four cysteine-rich tandem repeats (amino acid residues 449–617), which are comparable in structure to epidermal growth factors (EGF). These are known as integrin epidermal growth factor-like (I-EGF-like) domains, namely, I-EGF-1 (spans amino acid residues 449–496), I-EGF-2 (497–540), I-EGF-3 (541–581) and I-EGF-4 (582–617) (Zecchinon *et al.*, 2004a, b). The I-EGF-like domains contain eight cysteine residues, which have been shown to be involved in disulfide bonds (Lu *et al.*, 2001; Beglova *et al.*, 2002).

Previous studies showed that the site required for LktA binding and susceptibility to its biological effects reside within amino acid residues 500–600 of the extracellular region of CD18 (Dileepan *et al.*, 2005a). Experiments were designed with the purpose of identifying a smaller functional domain within this 100 amino acid region of bovine

CD18 (i.e. from amino acid 500 to 600) responsible for conferring susceptibility to LktA effects. Here, the identification of a region of 40 amino acid residues within the bovine CD18 that are critical for imparting species-specific susceptibility to the biological effects of LktA is reported.

## Materials and methods

### Preparation of purified *M. haemolytica* leukotoxin

Production and purification of LktA from *M. haemolytica* strain D153 have been described by the authors previously (Yoo *et al.*, 1995) and all studies were carried out with the same batch of purified LktA.

### Generation of bovine X human chimeric I-EGF-switched CD18 constructs

Six bovine X human chimeric CD18 constructs were generated by exchanging the nucleotide sequences in the cDNA encoding amino acids in the I-EGF-2 domain (497–540), I-EGF-3 domain (541–581), and in the I-EGF-4 domain (582–617) in the bovine CD18 with the corresponding sequences from human CD18, and vice versa. The chimeric construct generated by domain swapping is shown in Fig. 1. Human CD18 (Invitrogen Corp, Carlsbad, CA) and bovine CD18 (provided by Marcus Kehrli, NADC, Ames, IA) cDNA plasmids were subcloned into the pMigR1 retroviral vector (Dileepan *et al.*, 2005a). Bovine CD11a cDNA in pGEM-T vector generated in the authors' laboratory (Dileepan *et al.*, 2005b) was subcloned into the pMSCV-puro retroviral vector (Dileepan *et al.*, 2005a).

Each construct was generated in two steps using the 'MEGA-WHOP' technique (Miyazaki & Takenouchi, 2002; Miyazaki, 2003) as described previously (Dileepan *et al.*, 2005a), using primers as indicated in Table 1. Clones generated by domain swapping were sequence verified (Advanced Genetic Analysis Center, University of Minnesota, Minneapolis, MN) and the sequences of each construct were aligned with both human CD18 and bovine CD18 using MEGALIGN (DNAStar, Madison, WI). The results indicated that all six constructs contained the appropriate swapped regions, with no nonspecific mutations.

### Recombinant expression of chimeric, I-EGF-switched CD18 together with bovine CD11a on K562 cells

The six different bovine X human chimeric, I-EGF-switched CD18 cDNA constructs were recombinantly coexpressed

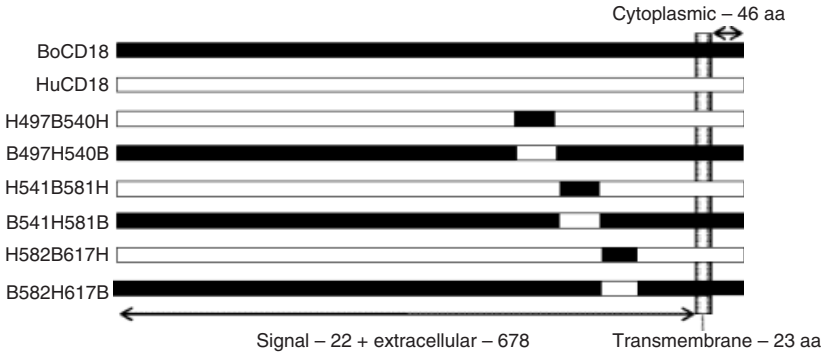


**Fig. 1.** Schematic representation of the six bovine X human chimeric CD18 by exchanging the nucleotide sequences in the cDNA encoding EGF-2 domain (497–540); EGF-3 domain (541–581); and EGF-4 domain (582–617) in the bovine CD18 with corresponding sequences from human CD18 and vice versa. The figure also shows schematic depiction of the bovine CD18 (BoCD18) and human CD18 (HuCD18) amino acids.

**Table 1.** Primers used for domain swapping (with the human sequences underlined)

| Construct | Forward primer | Reverse primer |
|---|---|---|
| B497H540B | 5′-CAGGAGCTGGAGGGCAGCTGCCGGAAGGACAACAAC-3′ | 5′-GCCGTCGTAGCGTTCGCAGTTGATGGTGTCACACTC-3′ |
| H497B540H | 5′-CAGGAGCTGGAAGGAAGCTGCCGCAAGGACAACAGC-3′ | 5′-GCCGTTGTAGCGCTCACAGTTGACGTTGTCGCACTC-3′ |
| B541H581B | 5′-AAGAAGATCTACGGCCAGTTCTGCGAGTGCGACAACGTCAA CTGTGA GCGCTACAAC-3 | 5′-GGCCGCGGCCGCTGCACTCGACGCCGTCCAAGTTGA GGC AGCCCTCAGTGGTCC-3′ |
| H541B581H | 5′-GCAGTACTGCGAGTGTGACACCATCAACTGCGAACGCTAC GAC-3 | 5′-ACTACACTCAACACGCCGCGGGTTCAGGCAGCCCTGA GTGGA-3′ |
| B582H617B | 5′-GCATGCCAGTGCCTCAAGTCCACTCAGGGCTGCCTGAAC CCGC-3 | 5′-GCAGGGGGCGAAGCCCGCACAGGGCACGGGGCAGCCG GGGCA-3′ |
| H582B617H | 5′-GCGTGCCAGTGCGAGAGGACCACTGAGGGCTGCCTC AACTTGGAC-3′ | 5′-GCAGGAGATGTACTTGCCACAGGGTGAGGGGCAGCCCGGG CACTC-3′ |

with bovine CD11a cDNA in the human K562 cell line (Hickstein *et al.*, 1993) by transduction followed by selection with puromycin as the authors have described previously (Dileepan *et al.*, 2005a, b). Transductants expressing chimeric CD18/BoCD11a were purified using the Magnetic Cell Sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) column and expanded as described previously (Dileepan *et al.*, 2005a, b). The resulting transductants were designated H497B540H, B497H540B, H541B581H, B541H581B, H582B617H and B582H617B (Fig. 1) following the nomenclature adopted by Zang & Springer (2001). For example, the designation H497B540H indicates that residues 497–540 are from the bovine CD18 and residues 1–496 and 541 to the C-terminus are from the human CD18.

### Demonstration of the surface expression of the chimeric CD18 antigen

Surface expression of chimeric CD18 in the six transductants was confirmed by fluorescence-activated cell sorting (FACS) analysis using CD18-specific monoclonal antibody (mAb) BAQ30A (Fig. 2). BAQ30A was used because it cross reacts with both bovine and human CD18 antigens. FACS was performed as described previously (Dileepan *et al.*, 2005a, b). Fluorescence was analyzed by a FACSCalibur flow cytometry system using CELLQUEST software (Becton Dickinson Immunocytometry Systems, San Jose, CA), and expressed as mean fluorescence intensity.

### Determination of LktA-induced intracellular calcium elevation

Elevation of intracellular calcium $[Ca^{2+}]_i$ in chimeric transductant cells exposed to LktA was measured by video fluorescence imaging as previously described (Dileepan *et al.*, 2005a, b). Fluorescence signals were determined from regions of interest. $[Ca^{2+}]_i$ was calculated by the ratio method described by Grynkiewicz *et al.* (1985). The BoLFA-1 (BoCD11a/BoCD18) cell line served as positive control and the parent K562 cell line served as negative control.

### Determination of LktA-induced cytotoxicity

LktA-induced cytolysis was determined by a previously described XTT dye (Sigma) reduction cytotoxicity assay (Jeyaseelan *et al.*, 2000) using 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT sodium salt, Sigma). The percent cytotoxicity was calculated using the formula: Percentage cytotoxicity = [1 − (OD of LktA-treated cells/OD of LktA-untreated cells)] × 100. Whereas the BoLFA-1 (BoCD11a/BoCD18) cell line served as positive control, the parent K562 cell line served as negative control.

### Reagents

RPMI 1640 medium with L-glutamine and Hank's balanced salt solution (HBSS) were purchased from Celox Laboratories Inc., (St Paul, MN). All other reagents were obtained from Sigma Chemical Company (St Louis, MO). The transductant cell line BoLFA-1 was generated in the authors' laboratory using previously described procedures (Dileepan *et al.*, 2005b). mAb BAQ30A was purchased from VMRD Inc. (Pullman, WA).

### Statistical analysis

Data were analyzed statistically by the paired *t*-test and *P* values were determined using GRAPHPAD PRISM statistical analysis software (version 3.02, San Diego, CA). The term significant indicates a *P* value of $< 0.05$.

## Results

Surface expression of chimeric CD18 antigens in the various transductants was confirmed by FACS using anti-CD18-specific mAb BAQ30A (Fig. 2). The parent cell line K562 cells, which do not express CD18 antigen, was used as a negative control. Results indicate high levels of expression of CD18 antigen in all chimeric transductants, and expression levels were at comparable levels between different chimeric transductants.

The transductant cells were subjected to two different functional assays to determine their susceptibility to LktA, namely LktA-induced intracellular calcium $[Ca^{2+}]_i$ elevation and cytotoxicity. LktA ($50\,LU\,mL^{-1}$) induced significant $[Ca^{2+}]_i$ elevation in B497H540B, H541B581H and B582H617B, but not in the H497B540H, B541H581B and H582B617H transductants. No $[Ca^{2+}]_i$ elevation was observed in the B541H581B transductant, which had the entire bovine backbone with only the I-EGF-3 (541–581) replaced with the human counterpart. By contrast, $[Ca^{2+}]_i$ elevation in the transductant H541B581H was observed, which had the entire human backbone with only the I-EGF-3 replaced with a bovine counterpart. However, the magnitude of $[Ca^{2+}]_i$ elevation in this transductant was much lower compared with the response seen with the BoLFA-1 cells (Fig. 3).

A pattern of susceptibility to LktA similar to that seen for $[Ca^{2+}]_i$ responses was seen in the cytotoxicity assays as well (Fig. 4). Exposure to $100\,LU\,mL^{-1}$ LktA resulted in significant cytotoxicity in the chimeric transductants B497H540B, H541B581H and B582H617B comparable to BoLFA-1 cells. By contrast, transductants H497B540H, B541H581B and H582B617H were nonsusceptible to LktA-induced cytotoxicity. Furthermore, while the B541H581B transductant was nonsusceptible, the transductant H541B581H showed considerable amount of cytotoxicity on exposure to LktA. Taken

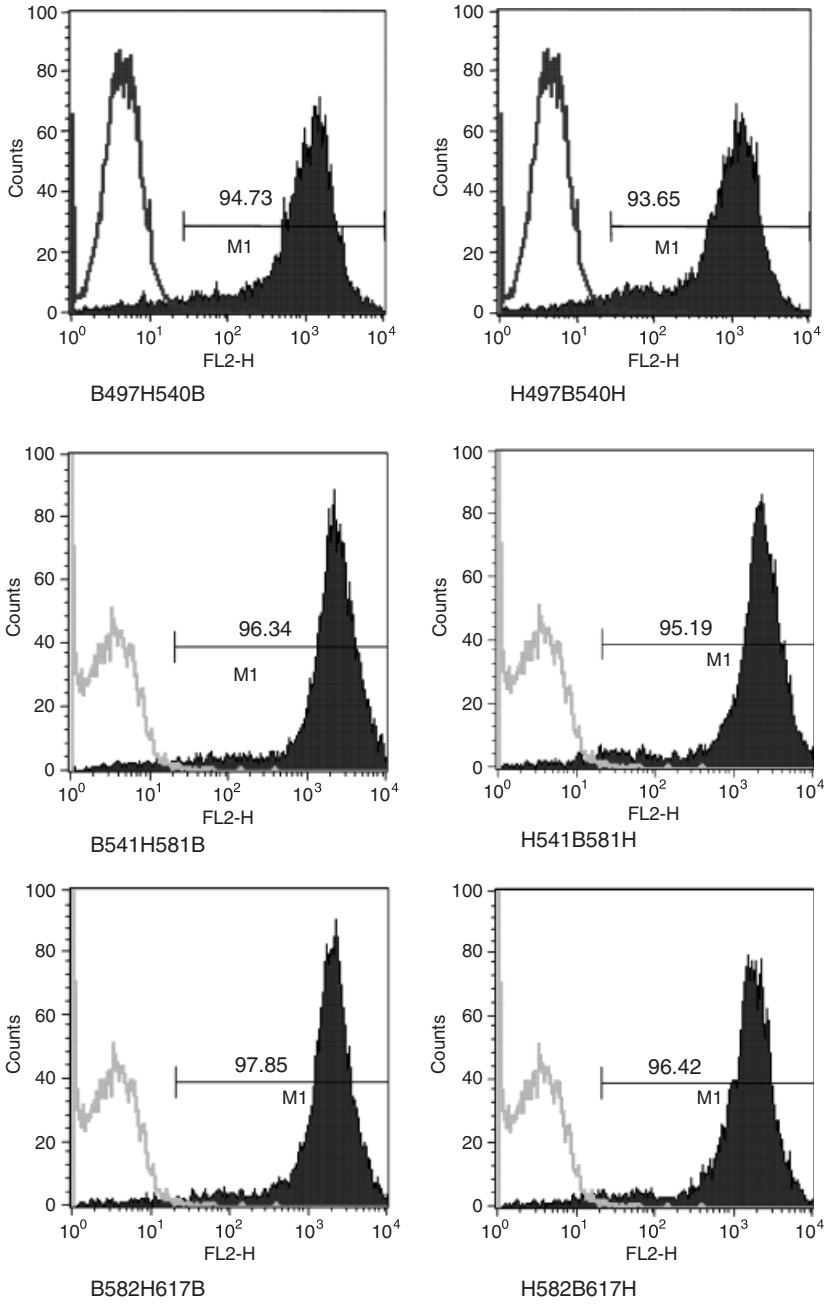


**Fig. 2.** Surface expression of chimeric CD18 antigens in the various transductants was confirmed by FACS using anti-CD18-specific mAb BAQ30A. The parent cell line K562 cells (open trace) which do not express CD18 antigen were used as a negative control. *X*-axis shows fluorescence intensity (FL2-H) and the *Y*-axis shows cell number (Counts) and the numbers shown within the panels are the percentages of positive CD18 cells. Results show high levels of expression of CD18 antigen in all chimeric transductants (solid traces). Data presented are representative of one of three experiments performed.

together, these results indicate that the EGF-2 and the EGF-4 domains, which encompassed amino acids 497–540 and 582–617, respectively, were not involved in imparting susceptibility to LktA. Of interest was the observation that the B541H581B transductant, in which the EGF-3 domain of bovine CD18 was replaced with the corresponding human CD18 counterpart, lost susceptibilty to LktA-induced effects. In addition, the H541B581H transductant, in which the EGF-3 domain of human CD18 was replaced with the corresponding bovine CD18 counterpart, restored susceptibility to LktA-induced effects (intracellular $Ca^{2+}$ elevation and cytotoxicity). This susceptibility however was of a lower

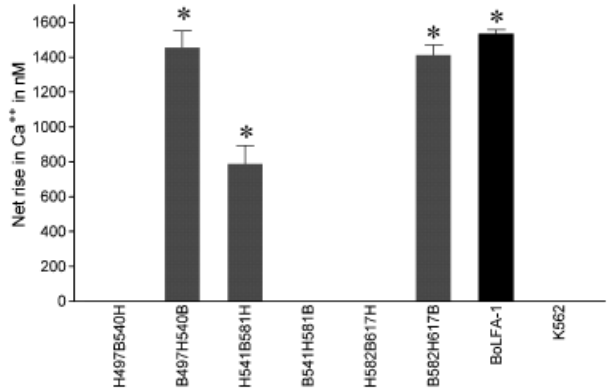


**Fig. 3.** LktA-induced intracellular calcium $[Ca^{2+}]_i$ elevation in the B497H540B, H541B581H and B582H617B, but not in the H497B540H, B541H581B and H582B617H transductants. Results are expressed as mean ± SEM of three separate experiments. The BoLFA-1 (BoCD11a/BoCD18) and parent K562 cell lines served as controls. Values that are significantly different from the negative control value ($P < 0.05$) are indicated by asterisks.

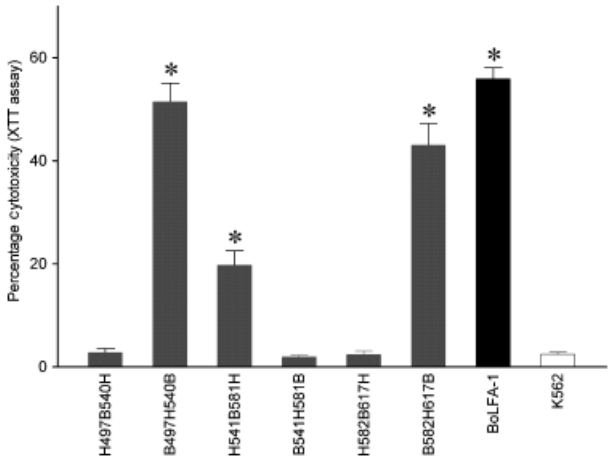


**Fig. 4.** Significant LktA-induced cytolysis was seen in the chimeric transductants B497H540B, H541B581H and B582H617B compared with the parent cell line. By contrast, transductants H497B540H, B541H581B and H582B617H showed lower cytotoxicity upon exposure to LktA comparable to that obtained with parent cell line. The BoLFA-1 (BoCD11a/BoCD18) and parent K562 cell lines served as controls. Results are expressed as mean ± SEM of three separate experiments. Values that are significantly different from the negative control value ($P < 0.05$) are indicated by asterisks.

magnitude compared with that observed with the positive control BoLFA-1 cells. These results suggest that the EGF-3 domain of bovine CD18, which encompasses amino acids 541–581, is critically required for species-specific susceptibility to *M. haemolytica* LktA.

## Discussion

Chimeric CD18 constructs were generated, which contained bovine sequences corresponding to I-EGF-2, -3 or -4 within the human CD18 backbone. Gain-of-function is demonstrated, i.e., LktA-induced intracellular $Ca^{2+}$ elevation and cytotoxicity, in the H541B581H transductant containing the bovine I-EGF-3 domain but not in others. The functional role of this region is also confirmed by demonstrating loss-of-function in the transductant B541H581B that contained the human I-EGF-3 domain in the bovine CD18 backbone. This provides strong evidence that the I-EGF-3 domain of bovine CD18 encompassing amino acid residues 541–581 are critical for imparting species-specific susceptibility to LktA in bovine leukocytes. This susceptibility of the transductant H541B581H to LktA was of a lower magnitude than that observed in the positive control BoLFA-1 transductant. However, surface expression levels in the H541B581H and BoLFA-1 transductants were comparable. It is quite possible that the region(s) adjoining the I-EGF-3 domain may be critical for providing the appropriate conformation for optimal susceptibility to LktA effects.

In this study, evidence has not been provided for LktA binding to any of the transductants that were generated. Previous investigations from the authors' laboratory and of others have clearly shown that LktA binding to leukocytes does not exhibit species specificity (Sun *et al.*, 1999; Jeyaseelan *et al.*, 2000, 2001). For example, LktA binds to porcine leukocytes without eliciting any biological effects (Sun *et al.*, 1999; Jeyaseelan *et al.*, 2000, 2001). Furthermore, LktA also binds to the CD11a subunit of LFA-1 (Dileepan *et al.*, 2005b; Thumbikat *et al.*, 2005). Thus, to evaluate species-specific susceptibility, functional assays such as LktA-induced signaling and cytotoxicity rather than binding were relied on.

This report shows that the I-EGF-3 domain of bovine CD18 imparts species-specific susceptibility to *M. haemolytica* leukotoxin. Whether specific amino acid residues within this domain are involved in conferring this functional role remains to be determined. On the basis of the results, it can be speculated that peptide sequences identical to the I-EGF-3 domain or antibodies to this region of bovine CD18 may be useful in preventing LktA-induced lung injury in bovine pneumonic pasteurellosis.

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