EXPRESSION OF β2 INTEGRINS (CD11a/CD18)

IN K562 CELLS

By

AMIT DEWAN

Bachelor of Veterinary Sciences & Animal Husbandry

G.B. Pant University of Agriculture & Technology

Pantnagar, India

2001

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2005

EXPRESSION OF β2 INTEGRINS (CD11a/CD18)

IN K562 CELLS

Thesis Approved:

Dr. Jerry Ritchey

Thesis Adviser

Dr. Anthony Confer

Dr. Jeremiah Saliki

Dr. John Wyckoff III

A. Gordon Emslie

Dean of the Graduate College

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my mentor and major advisor, Dr. Jerry Ritchey for his constructive guidance, supervision, encouragement, inspiration, moral and financial support during my entire graduate program. I would also like to extend my appreciation to my graduate committee members, Drs. Anthony Confer, John Wyckoff III, and Jeremiah Saliki for their support, guidance, valuable insights and willingness to serve. I would also like to thank the Departments of Physiological Sciences and Veterinary Pathobiology for providing me the teaching assistantship during the first as well as second & third year of my graduate program, respectively. And Drs. Larry Stein, James Lish, and Rebecca Morton for supporting my appointment as a teaching assistant.

Finally, I would like to express my deep gratitude to the College of Veterinary Medicine, now Center for Veterinary Health Sciences for providing me this opportunity to undertake an advanced education.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION……..1

Background……..2
Host Respiratory Defense Mechanisms…….....2
Factors/Stressors that Impair Respiratory Defenses…….....5
Viral Infections and Mycoplasma…….....6
Development of Bronchopneumonia……..11
Mannheimia hemolytica…….... 15
Bacterial Factors Associated with Virulence……..17
Leukotoxin: Mode of Action and Effects…….....26
Summary and Statement of Research Problem…… ...30

II. MATERIALS AND METHODS……..34

Blood Collection……34
Collection of PBMCs from blood……34
Stimulation of PBMCs with Concanavalin A……..34
RNA isolation……35
Reverse Transcription (RT) of mRNA into cDNA……..35
Primer Design……36
Generation of Inserts by Polymerase Chain Reaction (PCR)…….37
Selection of Cloning and Expression Vectors……...38
Digestion & Ligation of Inserts with Cloning & Expression Vectors…… 40
Transformation of Constructs into E.coli strain DH5α……..41
Sequencing of Inserts in Recombinant Vectors/Constructs……42
Cultivation/Propagation of Tissue Culture Cells……42
Transfection of Constructs into K562, Vero and HL-60 cells……..43
Immunostaining for Detection of Cell Surface Expression of Integrins by Confocal Microscopy……..47
Membrane Isolation from Vero Cells Transfected with

bovine CD18 Recombinant Vector……48
Western Blotting……49
Trypan Blue Dye Exclusion Test……50
Leukotoxin Activity and LDH Release Assay……50
Flow Cytometric Analysis……51

III. RESULTS……53

Generation of Inserts……55
Cloning & Ligation of Inserts in pUC19 & Expression Vectors….56
Sequencing of Inserts……58
Cultivation/Propagation of Cell Lines……58
Strategy & Transfection of Recombinant Vectors into Cell Lines……59
Immunostaining & Confocal Microscopy Results of Transfected Vero Cells along with Positive & Negative Control……60
Western Blotting Results……62
Assessment of LKT Sensitivity……63
Trypan Blue Dye Exclusion Test……63
LDH Release Assay……63
Flow Cytometric Analysis of Transfected K562 Cells……64
Immunostaining & Confocal Microscopy Results of Transfected K562 Cells……66

IV. GENERAL DISCUSSION AND CONCLUSIONS……83

REFERENCES……96

LIST OF TABLES

Table Page

1. Description of the placement of coding sequences Into the expression vectors……40

2. Transfection of Vero cells with bovine CD18 recombinant vector alone in a 6-well plate format……44

3. Transfection of Vero cell clones with human CD11a recombinant vector in a 6-well plate format……44

4. Co-transfection of K562 cells with bovine CD18 and human CD11a recombinant vectors in a 6-well plate format……...45

5. Transfection of HL-60 cells with bovine CD18 recombinant vector in a 6-well plate format……46

6. Description of the monoclonal antibodies used during this study……....48

7. List of the transfection strategies, expected results and their appropriate interpretations following transfection of K562 cells……...54

8. Trypan blue dye exclusion test data……76

LIST OF FIGURES

Figure Page

1. 0.6% agarose gels stained with ethidium bromide with amplification products for bovine and human CD11a and CD18……...68

2. Cloning of inserts into pUC19 cloning vector……...69

3. Subcloning of inserts into pcDNA3.1 expression vectors……...70

4. Morphology of the cell lines used in this study.……71

5. Confocal microscopy images of positive and negative control cells exhibiting surface expression of β_2 integrins……72

6. Confocal microscopy images of Vero cells exhibiting surface expression of bovine CD18……...73

7. Confocal microscopy images of Vero cells exhibiting surface expression of chimeric human CD11a/bovine CD18……74

8. Western blots showing the surface expression of bovine CD18 on transfected Vero cells……75

9. LDH release assay data……...77

10. Flow cytograhs exhibiting the cell surface expression of bovine & human β_2 integrins in transfected K562 cells 48 hrs posttransfection……...79

11. Confocal microscopy images of K562 cells exhibiting rescued surface expression of bovine and human CD11a/bovine CD18 48 hrs posttransfection……80

12. Confocal microscopy images of K562 cells exhibiting co-expression of bovine CD11a/CD18……..81

13. Confocal microscopy images of K562 cells exhibiting co-expression of human CD11a/CD18……..82

LIST OF PICTURES

Picture Page

1. Macro and microscopic view of lung lesions in bovine pneumonic pasteurellosis……14

ABBREVIATIONS

ADCC	Antibody-dependent Cellular Cytotoxicity'
AMs	Alveolar Macrophages
ARS	Agricultural Research Service
BALT	Bronchial Associated Lymphoid Tissue
BAMs	Bovine Alveolar Macrophages
BGH	Bovine Growth Hormone
BL3	Bovine Lymphoma 3
BLAD	Bovine Leukocyte Adhesion Deficiency
b.p.	Base Pairs
BPI3V	Bovine Parainfluenza 3 Viruses
BRD	Bovine Respiratory Disease Complex
BRSV	Bovine Respiratory Syncytial Virus
BVDV	Bovine Viral Diarrhea Virus
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
CR4	Complement Receptor 4
DAB	Diaminobenzidine
ddH_2O	Double Distilled Water
DEPC	Diethyl Pyrocarbonate

DNA	Deoxy Ribonucleic Acid
dNTPs	Deoxynucleoside Triphosphate
FBS	Fetal Bovine Serum
HCL	Hydrochloric Acid
HlyA	*E.Coli* hemolysin
hrs	Hours
IBRV	Infectious Bovine Rhinotracheitis Virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
ICAM-1	Inter Cellular Adhesion Molecule-1
IDT	Integrated DNA Technologies
IL-1	Interleukin-1
IL-8	Interleukin-8
IROMPs	Iron-Regulated Outer Membrane Proteins
KCl	Potassium Chloride
kDa	kilo Dalton
LAD	Leukocyte Adhesion Deficiency
LBP	Lipopolysaccharide Binding Protein
LDH	Lactate Dehydrogenase
LFA-1	Lymphocyte Function associated Antigen-1
LKT	*Mannheimia hemolytica* Leukotoxin
LPS	Lipopolysaccharide
LTB4	Leukotriene B4

LTC4	Leukotriene C4
LTD4	Leukotriene D4
MAbs	Monoclonal Antibodies
MAC	Membrane Attack Complex
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium Chloride
mg	Milligram
ml	Milliliter
µg	Microgram
µl	Microliter
MHC	Major Histocompatibility Complex
min	Minutes
MOPS	3-(N-morpholino)-Propanesulphonate
MPS	Mononuclear Phagocyte System
mRNA	messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
NK	Natural Killer Cells
nm	Nano Molar
OMPs	Outer Membrane Proteins
PAF	Platelet Activating Factor
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline containing 0.05%

	Tween-20
PCR	Polymerase Chain Reaction
PLA2	Phospholipase A2
PIMs	Pulmonary Intravascular Macrophages
PMN	Polymorphonuclear
RBCs	Red Blood Cells
rpm	Revolutions Per Minute
rRNA	ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RTX	Repeats in Toxins
s	Seconds
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
URT	Upper Respiratory Tract
USDA	United States Department of Agriculture
TEM	Transmission Electron Microscopy
T_m	Melting Temperature
TNFα	Tumor Necrosis Factor α
TUs	Toxic Units

Chapter 1

Introduction

Bovine Pneumonic pasteurellosis, commonly called shipping fever, is a severe, acute respiratory disease of cattle. The disease results in death losses, decreased animal performance and productivity, as well as other medical and labor costs that cause a tremendous economic impact on the stocker and feeder cattle industry. In fact, it has been estimated that shipping fever results in an estimated one billion dollars in losses to the North American beef cattle industry annually (Whitely et al., 1992).

The pathogenesis of shipping fever is relatively complex and is often referred to as the bovine respiratory disease complex. Initially, there is **impairment of the host's innate respiratory defense mechanisms** by one or a combination of factors or stressors. The **stressors** include weaning, changes in feed, transportation over long distances, overcrowding, dehydration, fatigue, abrupt changes in weather/temperature, and perhaps most-importantly, **respiratory viral infections**. Following impairment of the respiratory defense mechanisms, secondary bacterial infections result in a severe **bronchopneumonia** that produce most of the morbidity and mortality associated with the disease. The organism most frequently and consistently isolated from the lungs of cattle that have died with shipping fever has been ***Mannheimia hemolytica*** **biotype A, serotype 1(A1)**. *M. hemolytica* produces numerous **virulence factors** to aid

establishment of disease, and arguably, the most important of these virulence factors is **leukotoxin**.

Leukotoxin is a heat-labile exotoxin that has been shown to be specific for ruminant leukocytes. Leukotoxin acts in a dose dependent manner to cause death of the leukocytes by either apoptosis (low dose) or cytolysis (high dose). Therefore, leukotoxin severely impairs the ability of the pulmonary host defenses as it results in destruction of the immune cells recruited to remove the proliferating *Mannheimia*. Recently, it has been shown that leukotoxin acts by specific binding to the cell surface receptor **LFA-1 (CD11a/CD18)**. LFA-1 is important in the process of extravasation of leukocytes and is comprised of an **alpha (CD11) and beta (CD18) subunit** that form a heterodimer. What is not certain is which specific subunit is critical for leukotoxin binding, CD11a alone, CD18 alone, or a combination of both? A better understanding of the relationship between LKT and LFA-1 would prove beneficial to the treatment and management of bovine pneumonic pasteurellosis.

Background

I. Host Respiratory Defense Mechanisms:

An understanding of pulmonary defense mechanisms is critical before discussing how they are impaired in the establishment of disease. The normal pulmonary

defense mechanisms can be divided based upon their anatomic location of either air-conducting regions or gas exchange regions of the respiratory tract. A comprehensive review of the pulmonary defense mechanisms is beyond the scope of this thesis. The following discussion summarizes the major components and has been summarized from Immunobiology, 5th Edition by Charles A. Janeway, Jr., Paul Travers, Mark Walport, and Mark J. Shlomchik; and How the Immune System Works, 2nd Edition by Lauren Sompayrac.

A) Airconducting regions:

1) Nasal turbinates: The nasal turbinates play an important role by creating an enormous turbulence of airflow that results in the forcing of particles larger than 10 µm onto the surface of the nasal mucosa. Here the particles become entrapped and are removed by mucociliary escalator.
2) Bifurcation of major airways: The particles that are between 2-10 µm are trapped by this bifurcation whereas particles less than 2 µm may to settle in the bronchioles and the gaseous exchange regions.
3) Mucociliary clearance: The mucociliary blanket is the most important defense mechanism of the conductive system. This blanket is a complex, multi-laminar mucus layer that moves along rhythmically beating cilia. The mucus plays an important role in trapping the inhaled particles. Toxic soluble gases can also mix with the mucus blanket thereby lowering the concentration of the toxic gases that reach the lower lung.

4) Bronchial Associated Lymphoid Tissue (BALT): BALT is located at the bifurcation of the bronchi and bronchioles. BALT plays an important role in on-site immunologic processing of the inhaled antigens. BALT traps the pathogens and presents the antigens derived from them to the circulating lymphocytes, thus inducing adaptive immune responses. In the absence of pathogens/antigens, BALT provides sustaining signals to the circulating lymphocytes, so that they continue to survive and recirculate until they encounter their specific antigen.
5) Humoral Immunity: Humoral Immunity includes IgA antibodies that guard the respiratory mucosal surfaces as they are very effective against mucosal invaders including bacteria. The dimeric structure of IgA enables them to cross-link or agglutinate pathogens together into clumps that are large enough to be swept out of the body with the mucus.

B) Gas Exchange Regions:

1) Alveolar Macrophages (AMs): Alveolar Macrophages derived from the blood monocytes play an active role in phagocytosis and killing of bacteria without inciting an inflammatory response. AMs leave the alveoli and migrate to the transitional region where they are exported by the mucociliary elevator and phagocytose entrapped particles (larger than 10 µm).
2) IgG antibodies play an important role in complement cascade activation and binding the surface of pathogens for opsonization by alveolar

macrophages. IgG plays a role in helping natural killer (NK) cells to destroy the pathogens by 'antibody-dependent cellular cytotoxicity' (ADCC) mechanisms. In ADCC, the antibody specifically binds both to the pathogen through its antigen binding regions and to the Fc receptors on the surface of NK cells. Thus, the IgG antibody actually forms a bridge between the pathogen and the NK cell facilitating the destruction of the pathogen by the NK cell.

3) Pulmonary Intravascular Macrophages (PIMs): PIMs are a resident cell population, which in structure and function resemble mature macrophages of the mononuclear phagocyte system (MPS) in various domestic species, particularly the ruminants. PIMs are involved in lipid metabolism and are the major source of vasoactive substances, which significantly influence both the dynamics of pulmonary circulation and the surfactant turnover of the ruminant lung (Atwal et al., 1989). PIMs are also invoved in efficiently clearing blood-borne particles.

II. Factors/Stressors that Impair Respiratory Defenses:

Stressors that lead to the impairment of pulmonary defense mechanisms (Frank G.H. et al., 1986) include:

A) Environmental Stressors like heat, cold, wind, rain, dust, toxic fumes, etc.

B) Nutritional stressors like ration changes, irregular feeding schedules, inadequate access to clean water, etc.

C) Management stressors like weaning, transportation, comingling, overcrowding, etc.

Stress in general causes immunosuppression that promotes colonization by bacteria like *Mannheimia hemolytica* (Filion et al., 1984). Some physical stressors that have been associated with pneumonic pasteurellosis cause increased plasma cortisol concentration, increased glucocorticoids, reduced phagocytic and bactericidal capabilities of macrophages, inhibition of antibody responses and lymphocyte and neutrophil functions (Roth et al., 1984). These provide an opportunity for respiratory viruses to establish themselves in the respiratory tract. The damage caused to the respiratory tract by the respiratory viruses further impairs pulmonary defense mechanisms. Because of the important role played by the respiratory viruses in the full-fledged establishment of bacterial pneumonia, viral infections deserve special consideration.

III. Viral Infections and Mycoplasma:

Although bacterial pneumonia secondary to *Mannheimia hemolytica* appears to be the cause of death in most acute cases of BRD, the role of viruses in this disease complex cannot be underestimated. Viral infections alone usually cause self-limiting disease; however, respiratory viruses modulate the host-defense

mechanisms and alter the upper respiratory tract (URT) and lung environment, such that bacterial infection can occur. Viral infection has been shown to influence adherence and colonization of bacteria by altering the host cell surface membrane receptors and the microenvironment where bacterial attachment occurs (Babiuk et al., 1995). This is followed by damage and necrosis of the URT epithelium, causing loss of cilia or alteration of mucus and ciliated cell function, compromising the mucociliary clearance of bacteria. This in turn, leads to increased colonization and bacterial growth. There are important viruses that directly affect the bovine respiratory tract and have long been established as important components in the BRD complex.

1) Bovine herpes virus 1 or infectious bovine rhinotracheitis virus (IBRV) is a ubiquitous respiratory virus that usually affects cattle older than 6 months. Respiratory infection varies in severity depending upon the strain involved, and the symptoms can range from inapparent or mild clinical signs to severe disease (usually as a result of secondary bacterial pneumonia) with about 10% mortality (Kapil and Basaraba, 1997). Infection with IBRV causes necrosis of the URT epithelium that in turn compromises mucociliary clearance of bacteria, leading to increased colonization, multiplication, and ultimately bronchopneumonia.

2) Bovine parainfluenza 3 virus (PI3V) is the most consistently isolated virus in cases of BRD (Toth et al., 1988). This ubiquitous paramyxovirus replicates within ciliated epithelium of the URT as well as alveolar epithelium and macrophages,

and alone, causes only mild clinical signs in young calves (2-8 months) and occasionally yearling cattle (Bryson et al., 1979). Experimental calves exposed to an aerosol of PI3V demonstrated reduced pulmonary bacterial clearance of *Mannheimia hemolytica* particularly at day 7 and day 11 post-exposure (Lopez et al., 1976). This inhibition of pulmonary clearance caused by the virus may be a key factor in the pathogenesis of pneumonic pasteurellosis. Furthermore, as with IBRV, PI3V appears to cause a pre-disposition to bacterial pneumonia secondary to virus mediated alterations in bactericidal capacity of macrophages, decreased phagocytosis of bacteria, and immunosuppression (Martin et al., 1989).

3) Bovine respiratory syncytial virus (BRSV) commonly infects young healthy cattle causing an asymptomatic or mild acute respiratory disease. In newly weaned calves, BRSV causes a severe necrotizing bronchiolitis and bronchointerstitial pneumonia that can result in death (Scott et al., 1994). BRSV infections mainly occur as annual winter outbreaks affecting young calves during the first 6–9 months of life. The virus infects respiratory epithelium in both the upper and lower respiratory tract (Morrison et al., 1999; Viuff et al., 1996; and Viuff et al., 2002). The clinical response to BRSV varies from sub-clinical to severe, acute pneumonia (Kimman et al., 1988). The virus first replicates in the epithelial cells in the upper part of the respiratory tract, and later, around day 6 post infection (p.i.), in the lower respiratory tract (Viuff et al., 2002). Enhanced respiratory rate and coughing can persist for a longer time, probably due to airway damage or secondary bacterial infections (Tjornehoj et al., 2003). The

cytotoxic T-lymphocyte response seems to be important in immune protection of calves as it is found that the depletion of cytotoxic T-cells (CD8+) before experimental BRSV infection results in prolonged nasal shedding of virus and more extensive replication of virus in the lungs (Gaddum et al., 1996; Taylor et al., 1995). BRSV is also known to impair certain functions of alveolar macrophages. Specifically, non-opsonized phagocytosis was reported to decrease during the early post-inoculation period in a calf model (Olchowy et al., 1994). Fc-receptor mediated phagocytosis was either unchanged or slightly increased following BRSV inoculation. A variable effect was noticed on AM phagosome lysosome fusion with increased fusion activity on days 2 through 5, 7 and 12, but reduced activity on days 6 and 10 post-inoculation (Olchowy et al., 1994). Alterations in AM function are the most critical impairment of the host defense mechanisms within the gas exchange regions of the lung.

4) Bovine Viral Diarrhea Virus (BVDV): Respiratory tract infections from opportunistic bacteria may occur in unthrifty cattle persistently infected with BVD (Peterhans et al., 2003). BVDV can be recovered with high frequency from pneumonic lungs of cattle. Initial replication of this virus occurs in the respiratory tract and tonsils (Bolin et al., 1992) and it is believed that certain strains of this virus have the capacity of producing a mild respiratory tract disease. However, there is little direct experimental evidence that establishes BVDV as a primary pathogen causing pneumonia. Experimental evidence also supports the conclusion that BVDV enhances the incidence and severity of respiratory tract

disease caused by *Mannheimia hemolytica* (Potgieter et al., 1997). AMs recovered from experimentally infected calves showed significant reduction in Fc-receptor (FcR) and complement receptor (C3R) expression, phagocytosis and microbicidal activity, and the production of neutrophil chemotactic factors (Welsh et al., 1995). However, macrophages exposed to the virus *in vitro*, only showed significantly reduced FcR and C3R expression and phagocytic activity (Welsh et al., 1995). These results demonstrate that BVDV can reduce local immune defense mechanisms in the lung following infection by the respiratory route, and in conjunction with the other immunosuppressive agents, BVDV would favor a pre-disposing role for the virus in the pathogenesis of respiratory disease in cattle.

5) Mycoplasmas are another group of organisms that may contribute to pneumonic pasteurellosis. Although mycoplasmas are commonly isolated from the upper and lower respiratory tracts of cattle with or without clinical pneumonia, their role in BRD is difficult to evaluate. *Mycoplasma bovis* and *Mycoplasma dispar*, the two most common isolates, potentially may be important underlying factors playing a role in BRD because of their immunosuppressive and subclinical pneumonia causing capacity as illustrated by feedlot calves with clinical signs of respiratory disease (Allen et al., 1992). The lung lesion caused by *M. bovis* is partly due to the host's immune response, presumably contributing to the control of the infection, and that the B-lymphocytes infiltrating the lung are

a major source of the local and systemic IgG antibody that is detected after infection (Howard et al., 1986).

IV. Development of Bronchopneumonia:

The impairment of pulmonary defenses as a result of complex interactions between the stressors and viral agents can lead to the establishment of bacterial pneumonia. The most distinctive feature of this pneumonia is the development of inflammation at the bronchiolar-alveolar junctions. As a result, it is usually known as bronchopneumonia. The following summary of the pertinent features of the development and characteristics of bronchopneumonia has been obtained from Pathology of Domestic Animals, Volume 2, 3rd Edition by K.V.F. Jubb, Peter C. Kennedy, and Nigel Palmer. The vulnerability of bronchiolar-alveolar junctions to the damage caused by *M. hemolytica* is mainly due to three factors. First, these junctions serve as a major site for the deposition of particles (0.5-3.0 µm in diameter) that are capable of reaching deep lung. As a result, most bacteria settle at this site. Secondly, the bronchiolar epithelium is devoid of a mucus blanket and a resident macrophage population. Lastly, the cellular (macrophages) and the non-cellular materials cleared from large volumes of alveolar parenchyma clog the narrow lumen of the bronchioles. The clearance of the clogged bronchiolar lumen is further hampered by the lack of collateral ventilation in this region, a unique feature of ruminant lungs. Therefore, the absence of as well the failure of these defense mechanisms to halt the

unchecked colonization and growth of bacterial populations such as *M. hemolytica, Pasteurella multocida, Hemophilus somnus,* and *Corynebacterium pyogenes* that normally colonize the URT, into the lower airways subsequently results in variable degrees of inflammation of the lung tissue. This ultimately leads to the precipitation of bronchopneumonia.

The bronchopneumonia is characteristically cranioventral in distribution (Picture 1A). The cranioventral nature of the bronchopneumonia is mainly attributed to the increased deposition of particles in the cranial regions due to shorter and more abruptly branching airways, or defenses that are more easily compromised, or both. Other factors that might contribute to this cranioventral pattern include the gravitational influences that impede the clearance of the cranioventral regions, and the smaller size of ventral airspaces and their greater vulnerability to collapse.

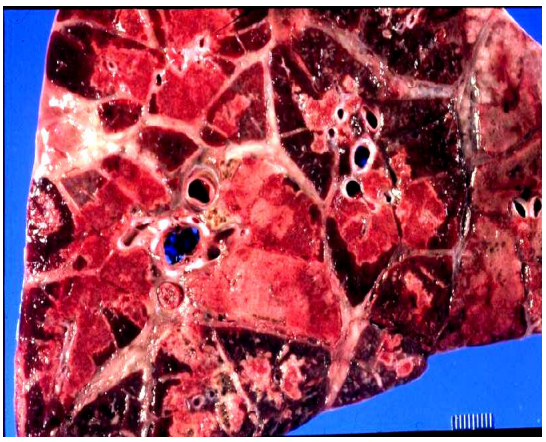
The bronchopneumonia involves a patchy distribution of inflammation that generally involves more than one lobe. This pattern results from an initial infection of bronchi and bronchioles that extends into the adjacent alveoli. In addition to the extensive reddish-black to grayish-brown cranioventral regions of consolidation with prominent gelatinous thickening of interlobular septa (Picture 1B) and fibrinous pleuritis, areas of coagulation necrosis are a characteristic feature. They usually contain large numbers of bacteria, particularly at the

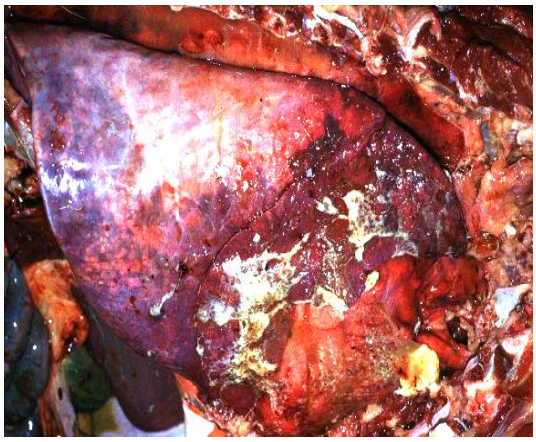
periphery adjacent to the compacted debris of inflammatory cells that forms the white boundary seen grossly.

Microscopically, the bronchioles and the adjacent alveoli are filled with neutrophils (Picture 1C & D), and an admixture of variable amounts of cell debris, mucus, fibrin, and macrophages. The bronchiolar epithelium appears to be hyperplastic or necrotic depending upon the severity of the lesion. Also, few foci of inflammation can also be seen in the peribronchiolar connective tissue. Alveoli peripheral to the severely inflamed bronchiolar regions are partially atelectatic and contain variable amounts of edematous or serofibrinous exudate, erythrocytes, and macrophages.

The bronchopneumonia that becomes confluent, fulminating or highly aggressive, and tends to involve major portion or the entire lobe is termed as lobar pneumonia. Lobar pneumonia is the result of overwhelming spread of inflammation throughout the lung without reference to bronchiolar regions. Grossly, the interlobular septa appear prominently distended with serofibrinous exudate along with the development of irregular, discrete zones of necrosis with swollen pale borders. Microscopically, the vessels passing through the severely inflamed regions tend to develop vasculitis with occasional formation of thrombi. A common feature to most lobar pneumonias is massive proliferation of bacteria within the developing necrotic foci.

Picture 1

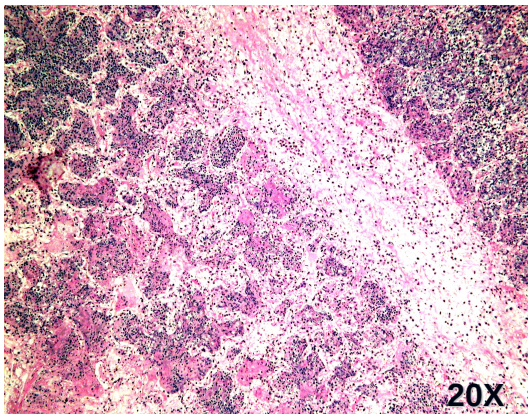


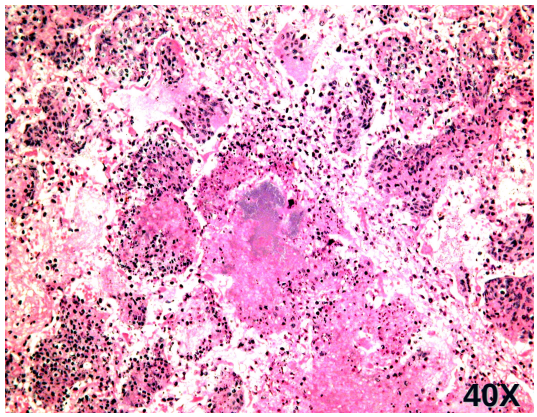


A. Lobar Pneumonia (in situ) **B.**Lobar Pneumonia (Cross Section)

Macroscopic view of lung lesions in Bovine Pneumonic Pasteurellosis

(Photographs provided by Dr. Anthony Confer)





C **D**

Microscopic view of lung lesions in Bovine Pneumonic Pasteurellosis

(Photographs provided by Dr. Jerry Ritchey)

V. *Mannheimia hemolytica:*

The etiology that is usually associated with bovine pneumonic pasteurellosis is *Mannheimia* (*Pasteurella*) *hemolytica* biotype A, serotype 1 (A1) (Confer et al., 1988; Frank et al., 1988). Genetic analysis (16S rRNA sequencing and DNA-DNA hybridizations) of *Pasteurella* organisms has resulted in a new classification for several strains of the organism commonly involved in respiratory tract disease in cattle. *Mannheimia hemolytica* is the new taxonomic classification for *Pasteurella hemolytica*, as suggested on the basis of results from a recently published study (Angen et al., 1999).

According to Kim Alan Brogden (June, 2000), of the USDA-ARS National Animal Disease Center, historically, two biotypes have been recognized for the taxon *Pasteurella hemolytica*: biotype A, and biotype T. Biotype A isolates ferment arabinose whereas biotype T isolates ferment trehalose. The trehalose-positive isolates were found to represent a distinct species (*Pasteurella trehalosi* that currently contains serovars 3, 4, 10, and 15). The trehalose-negative organisms were found to represent the distinct genus (*Mannheimia*) with five species (*Mannheimia glucosida*, *Mannheimia granulomatis*, *Mannheimia hemolytica*, *Mannheimia ruminalis*, and *Mannheimia varigena*). The organisms now classified as *Mannheimia hemolytica* contain serovars 1, 2, 5-9, 12-14, 16, and 17.

Adlam and Rutter (1989) describe *M. hemolytica* is a gram-negative, non-motile, non-spore forming, small coccobacillus or rod (0.2 µm to 2.0 µm) that is a member of the family *Pasteurellaceae.* The family *Pasteurellaceae* includes many pathogenic bacteria of the genera *Pasteurella, Hemophillus* and *Actinobacillus*: These organisms have worldwide distribution and are capable of affecting a broad range of species, including humans, domestic animals, lower mammals and birds. Most *Pasteurellae* are commensals on the mucous membranes of the URT and intestinal tract of wild and domestic animals. *M. hemolytica* is a facultative anaerobe and its culture characteristics include oxidase and catalase positivity, with varying ability to cause hemolysis. The organism can grow in unenriched media; however, the growth is enhanced with supplements of fetal bovine serum or blood. Bacterial colonies are usually obvious by 24 hrs, and are characterized by moderately-sized, round grayish, hemolytic colonies with a sweet odor.

Mannheimia hemolytica biotype A, serotype 1 and 2 (A1 and A2) are normally present in low numbers in the nasal passage of clinically healthy cattle, and the organisms that are predominantly isolated are biotype A, serotype 2 (A2). These organisms are rarely associated with shipping fever (Frank, 1979; 1988).

However, when healthy cattle are stressed secondary to viral infections and/or changes in feed, nutritional deficiencies, weaning, transportation over long distances, overcrowding, dampness, dehydration, injury, fatigue, or inclement

weather temperature drop leads to the precipitation of pneumonic pasteurellosis. The main event between immunosuppression of the animal and the precipitation of pneumonia involves the journey of commensal *M. hemolytica* biotype A, serotype 1 (A1) from the upper (nasal passage) to the lower respiratory tract, ultimately leading to the colonization of lungs. As a result, the organism most frequently and consistently isolated from the lungs of cattle that have died with shipping fever has been *M. hemolytica* biotype A, serotype 1 (A1).

VI. Bacterial Factors Associated with Virulence:

During the last decade, advances in molecular biological techniques have helped unravel and identify many virulence factors of bacterial pathogens including those of *M. hemolytica*. Even so, there are still many gaps in the understanding of the pathogenic mechanisms of this organism. So far, the most important virulence factors identified for *M. hemolytica* include fimbriae/pili, capsule, LPS, outer membrane proteins, heat-shock proteins, o-sialoglycoprotease, neuraminidase, and arguably the most important of all – leukotoxin.

Colonization of the URT mucous membrane seems to be the first and foremost step towards the establishment of *M. hemolytica*. However, not much information is available on the mechanisms of adhesion and colonization of the bovine URT by *M. hemolytica*. It is not clear whether *M. hemolytica* adheres to the URT mucosa or whether it remains in the mucus layer without adhering (Confer et al.,

1995). Two major factors that lead to the colonization of the URT by gram-negative bacteria are alterations in the mucociliary apparatus and the loss of fibronectin, an adhesive glycoprotein from epithelial cell surfaces (Pavia et al., 1987). Loss of fibronectin, which exposes receptors on cells and permits binding of gram-negative bacteria, appears to be a key event favoring colonization of the human URT (Proctor et al., 1987; Woods et al., 1987). In cases of human URT infections, adherence could potentially take place through pili. The presence of pili in *M. hemolytica* has only been demonstrated by one group of researchers, and has not been verified by other independent groups (Gonzalez and Maheshwaran, 1993; Confer et al., 1995). Hence, the expression of pili and their role in adherence of *M. hemolytica* remains unclear. Outer membrane proteins (OMPs) and capsular polysaccharide preparations of *M. hemolytica* have been shown to adhere to bovine tracheal mucus (Botcher et al., 1993; Confer et al., 1995). In addition, neuraminidase produced by *M. hemolytica* can cleave bovine mucin and could be a potential factor in promoting adherence of the organism to mucous membranes (Botcher et al., 1993) (Confer, 1995).

Fimbriae/Pili:

Bacteria employ a number of mechanisms through which they attach to the host tissues. One of the better-studied mechanisms involves the use of rod-shaped protein structures called fimbriae. The existence of fimbrial structures on *M. hemolytica in vivo* and *in vitro* has been reported. Two types of fimbriae have been demonstrated on *M. hemolytica* A1 grown *in vitro* (Morck et al., 1987). A

larger, 12 nm diameter, rigid structure, and smaller, 5 nm diameter flexible structure were seen using transmission electron microscopy (TEM). Pulmonary lavage fluid recovered from an experimentally infected calf also revealed fimbriae of about 10 nm diameter on the bacterial cells (Morck et al., 1988). Also, linear structures suggestive of fimbriae were seen adherent to the tracheal epithelium in a naturally infected calf (Morck et al., 1989). Potter and coworkers (1988) purified the larger, rigid fimbriae by mechanical shearing and centrifugation from *M. hemolytica* A1, and characterized their subunit molecular weight as 35-kDa by SDS-PAGE and immunoblotting with monoclonal antibodies raised against native fimbriae. All further electron microscopic studies using similar procedures have failed to demonstrate the presence of fimbriae on the surface of *Mannheimia hemolytica* (Richards A, unpublished data, 1989; Murphy G, unpublished data, 1992; Gonzalez and Maheswaran, 1993).

Capsule:

The capsule serves a number of protective functions, including a possible role in adherence to mucus or epithelial cell surfaces. The main function of the capsule is to protect the bacteria from the host's inflammatory response, including complement activation and phagocyte-mediated killing.

Capsules are known to be very poor immunogens, especially those with sialic acid or hyaluronic acid residues (Lifely et al., 1985). Many members of the pathogenic family Pasteurellaceae, including *M. hemolytica*, are encapsulated.

The capsule is produced during the logarithmic phase of growth (Odendaal et al., 1999). Those purified *M. hemolytica* capsule has been analyzed and found to be acidic, negatively charged, with high molecular weight polysaccharides, composed of repeating units of two or three sugars or amino sugars (Wilson et al., 1992). Although, the capsule of *M. hemolytica* is not a good immunogen, a high serum antibody response to purified *M. hemolytica* A1 capsular polysaccharide (Brogden et al., 1989) correlates with resistance to transthoracic challenge with virulent *M. hemolytica* A1 in cattle (Confer et al., 1989). Whether or not the capsule of *M. hemolytica* plays any role in URT adherence has yet to be determined.

Endotoxin/Lipopolysaccharide (LPS):

By weight, 12-25% of the bacterium is composed of LPS (Rimsay et al., 1981; Keiss et al., 1964) and the majority of the LPS is of the smooth type (Emau et al., 1987). LPS interacts with leukocytes, macrophages/monocytes, endothelium, hepatocytes, adreno-cortical cells, RBCs and platelets and localizes on the cell membrane, and/or in the cytosol, mitochondria, lysosomes, endoplasmic reticulum and the nucleus (Spitzer, 1986). LPS enters the cells by first binding with LPS binding protein (LBP) to form LPS-LBP complex. This complex subsequently interacts with LPS's specific receptor, CD14, via the core polysaccharide or by insertion into the cell membranes via the lipid A portion of the molecule (Morrison et al., 1981; Haeffner-Cavaillon et al., 1985). LPS also binds to low and high-density lipoproteins in plasma and a variety of cell types

may ingest LPS via receptors for these proteins (Tobias et al., 1985). LPS may directly interact with the mitochondrial outer membrane and destroy the membrane's proton gradient leading to the loss of oxidative glycolysis. This leads to anaerobic glycolysis, production of lactic acid and oxygen radicals, breakdown of the lysosomal membrane and ultimately leading to cell death (Guo et al., 1994).

Bovine pulmonary artery endothelium is sensitive to the stimulatory and toxic effects of endotoxin (Gartner et al., 1988). It has been documented that the exposure of bovine endothelial cultures to LPS causes dose dependent shape changes, cell retraction, increased cell membrane permeability and pyknosis (Meyrick et al., 1986). These changes are accompanied by decreased synthesis of DNA, RNA and protein (Gartner et al., 1988).

Endotoxin entering the lung via the airway or blood can directly interact with alveolar and pulmonary intravascular macrophages leading to their activation, both directly and indirectly, via complement activation (Morrison et al., 1978, 1981). In response, these macrophages produce a variety of proinflammatory (Issekutz et al., 1987; Roubin et al., 1985), procoagulant (Tipping et al., 1988; Nakstad et al., 1987), and reactive oxygen intermediates (ROIs) and proteases (Werb et al., 1983; Dyer et al., 1985). The cytokines (IL-1, TNFα) and lipid mediators (PAF, LTB4, LTC4 and LTD4) are major factors in the inflammatory response. IL-1 and TNFα are chemotactic for neutrophils, monocytes and

lymphocytes and they activate neutrophils to degranulate and produce ROIs (Billingham et al., 1987). Highly purified IL-1 is not directly chemotactic, but does stimulate degranulation and the respiratory burst in neutrophils. These cytokines also increase the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) on endothelial cell surfaces and the family of CD18 adhesion molecules on neutrophil cell surfaces (Billingham et al., 1987). These adhesion molecules are essential for neutrophil's endothelial adhesion and emigration into the lung parenchyma (alveoli). IL-1 and TNFα can also act in an autocrine manner to activate macrophages (Billingham et al., 1987).

Outer Membrane Proteins:

Outer membrane proteins have been shown to be involved in *in vivo* growth of *M. hemolytica*. Of these, iron-regulated outer membrane proteins (IROMPs) appear to be the most important. For *in vivo* growth in an iron-restricted environment such as lung tissue, bacteria produce IROMPs that function to sequester and transport iron into the bacterial cell. Three IROMPs have been discovered in *M. hemolytica* grown under iron-restricted conditions. Antibodies to these IROMPs occur in convalescent serum from calves recovering from *M. hemolytica* infection. Also, iron is required in the media for optimal growth and production of leukotoxin (Gentry et al., 1986). These studies suggest a role for IROMPs for *in vivo* growth of *M. hemolytica*, and may be important in protective immunity against shipping fever.

In the last five years, outer membrane proteins (OMPs) of *M. haemolytica* have always been, but recognized in the last 10 years as potential antigens for acquired immunity. Antibodies to *M. hemolytica* OMPs are associated with resistance to *M. hemolytica* challenge in cattle. Many of the OMPs are exposed on the surface of *M. haemolytica* S1 strains and, henceforth, are readily recognized by leukocytes and circulating antibodies (Pandher et al., 1999). In a recent study, Confer et al. (2003) demonstrated that the addition of a recombinant OMP, rPlpE to commercial *M. hemolytica* vaccines could greatly enhance vaccine efficacy. They also reported a significant reduction in complement-mediated killing of *M. hemolytica* in bovine immune serum depleted of anti-PlpE antibodies through adsorption with PlpE. This suggests that antibodies against PlpE may contribute to host defense against the bacterium.

Heat Shock Proteins:

Heat shock proteins (HSPs) are also known as stress proteins. As the name suggests, heat shock proteins provide protection to the bacterium from stressful conditions encountered within the host. These proteins are induced when the bacteria are subjected to different types of environmental stresses like heat, cold or oxygen deprivation. One major function of HSPs is that they act as chaperones to make sure that newly synthesized cell proteins are folded into the right shape and in the right place under these conditions. Enhanced production of an approximately 54-kDa protein was detected in heat-shocked cultures of *M. hemolytica*. The heat-shock-inducible protein of *M. hemolytica* cross-reacted with

antibodies to 60-kDa heat-shock proteins of *Mycobacterium tuberculosis*, *Chlamydia*, and *Escherichia coli* GroEL (Mosier et al., 1998). Results of this study suggest that the 54-kDa heat-shock protein may be a growth-condition-dependent immunogen that is one component of resistance to pneumonic pasteurellosis.

O-sialoglycoprotease:

O-sialoglycoprotease facilitates the adherence of *M. hemolytica* to the cell surface. O-sialoglycoprotease also has the ability to cleave fibronectin from the cell surface *in vitro* (Woods et al., 1987), thus exposing the surface receptors. It has been observed that brief trypsinization of bovine nasal epithelium leads to increased adherence of *M. hemolytica* A1. O-sialoglycoprotease may also specifically cleave and inactivate secretory IgA (Kooney et al., 1984).

Neuraminidase:

M. hemolytica produces neuraminidase and neutral proteases that may alter the microenvironment and enhance its ability to colonize the URT (Frank et al., 1981; Tabatabai et al., 1981; Otulakowski et al., 1983). Neuraminidase is a virulence factor associated with several gram-negative and gram-positive bacteria (Milligan et al., 1978; Wadstrom et al., 1978). In an *in vitro* study, *M. hemolytica* A1 produces larger amounts of this enzyme than the less virulent A2 strain (Tabatabai et al., 1981). Neuraminidase has been shown to decrease the gel-forming capacity and viscosity of bovine respiratory mucus (Milligan et al., 1978;

Wadstrom et al., 1978). This may lead to decreased clearance efficiency of the mucociliary apparatus and permit bacteria to penetrate the mucus blanket and approach the epithelium.

Leukotoxin:

Leukotoxin is a heat-labile protein exotoxin that is oxygen stable, non-dialyzable, hemolytic, water-soluble, and is produced by *M. hemolytica* during the logarithmic phase of growth (Baluyut et al., 1981). Leukotoxin has a molecular weight of 101-105 kDa when determined by SDS-PAGE (Lo et al., 1987; Chang et al., 1987). It is highly immunogenic and antiserum raised to the 105-kDa protein neutralizes its leukotoxic activity (Chang et al., 1987). Cattle with high leukotoxin antibody titers have higher survival rates in natural and experimental cases of pneumonic pasteurellosis than those with low antibody titers (Confer et al., 1988).

Leukotoxin has been shown to be specific for ruminant leukocytes: neutrophils, lymphocytes, and monocytes/macrophages (Shewen et al., 1982). Leukotoxin acts by forming pores in the cell membrane that permit the influx of calcium into cells (Gerbig et al., 1989; Clinkenbeard et al., 1989). Neutrophils and macrophages respond to leukotoxin by cell swelling, organelle and cytoplasmic membrane disruption and chromatolysis. The same changes occur in alveolar macrophages when leukotoxin is instilled into the lung (Whiteley et al., 1991). The lytic effect of leukotoxin appears to be dependent on the presence of calcium

in the medium (Gerbig et al., 1989; Clinkenbeard et al., 1989). Calcium influx into the cells may induce the activation of phospholipases that release platelet activating factor and arachidonic acid with subsequent formation of chemotactic and vasoactive lipids. These events further lead to the induction of proinflammatory cytokines IL-1 and TNF-α. Uncontrolled calcium influx causes the cell membranes to loose their integrity as a result of continued phospholipase degradation. This calcium influx leads to the activation followed by cell death.

VII. Leukotoxin: Mode of Action and Effects

As leukotoxin is arguably the most important virulence factor, it deserves special consideration. LKT is a member of RTX (Repeats in Toxins) family. According to their target cell specificity, RTX are divided into two classes, leukotoxins and hemolysins. Leukotoxins act on leukocytes only. Hemolysins lyse erythrocytes as well as various nucleated cell types. Nucleotide sequence alignments indicate that *M. hemolytica* LKT, *E.Coli* hemolysin (HlyA), *Actinobacillus actinomycetemcomitans* leukotoxin, and *Actinobacillus pleuropneumoniae* hemolysin are nearly identical (Lally et al., 1989). LKT plays an important role in the pathogenesis of bovine pneumonic pasteurellosis. It specifically acts on ruminant leukocytes (Clinkenbeard and Upton, 1991) including cattle, sheep, goats, deer, siaga antelope and sable antelope (Confer et al., 1990). Non-ruminants leukocytes are resistant to the action of LKT (Confer et al., 1990).

Molecular separation of LKT and LPS is difficult (Yoo et al., 1995). Czuprynski and Welsh (1995) proposed that LPS might be necessary for maximal production of some RTX family toxins and might act as a cofactor in some biological effects of these toxins. Toxicity is initiated by the binding of LKT to the target cells. It is generally believed that LKT binding specificity determines LKT target cell specificity. Flow cytometric analysis showed that LKT binds to bovine leukocytes but not to pig or human leukocytes (Brown et al., 1997). However, it has been documented that LKT can bind to Raji cells, which are a human lymphocytic cell line (Sun et al., 1999). LKT does not bind to protease K-treated bovine neutrophils, which indicates that some proteins on the cell surface are involved in the binding process (Brown et al., 1997). Lally and coworkers (1997) found that antibodies against β2-integrin inhibited *E.coli* hemolysin (HlyA) activity and nonsusceptible K562 cells transfected with β2 integrin (human CD11a/CD18 or LFA-1) became susceptible to HlyA and *A. actinomycetemcomitans* leukotoxin. Therefore, they proposed that the β2 integrin, LFA-1, acts as a receptor for *A. actinomycetemcomitans* leukotoxin and HlyA.

After binding to the target cells, RTX family toxins change their conformation (Moayeri and Welch, 1997) and insert into target cell membranes to form transmembrane pores (Murphy et al., 1995). Some LKT mutants without the transmembrane pore-forming domain can still bind to BL3 (bovine lymphoma cell line) cells and act as competitors of wild-type LKT (Cruz et al., 1990). The initial pore size produced by LKT is slightly less than 0.9 nm (Clinkenbeard et al.,

1989). This pore formation causes rapid leakage of intracellular potassium and results in subsequent cell swelling (Clinkenbeard et al., 1989). The above events are not dependent on the presence of extracellular calcium (Clinkenbeard et al., 1989); however, LKT can induce an extracellular calcium influx through a voltage-gated channel (Hsuan et al., 1998). As a result, the intracellular calcium concentration increases from a resting concentration of 0.1µM to 1.5 µM. The increased intracellular calcium levels induced by LKT may signal the activation of phospholipase, apoptosis, release of cytokines, degranulation and production of ROIs, and ultimately lysis of the cell (Cudd et al., 1999).

Clarke and co-workers (1994) demonstrated that *M. hemolytica* increases the production of leukotriene B4 (LTB4) in a tissue chamber model. Clinkenbeard and co-workers (1994) demonstrated that LKT induces LTB4 production from bovine neutrophils *in vitro*. Wang and co-workers (1998) found that LKT activates phospholipase A2 (PLA2), which then cleaves eicosanoids from membrane phospholipids in BL3 cells. Eicosanoids may be converted into LTB4 by the action of 5-lipoxygenase.

LKT also induces cytokine synthesis and release. Yoo and co-workers (1995) documented that purified LKT induces expression of IL-1 and TNF-α and this can be detected by northern blot. They also demonstrated that 0.5 U/ml LKT is required to induce bovine alveolar macrophages (BAMs) to transcribe TNF-α and IL-1β mRNA. At higher concentration of LKT, BAMs were lysed. Czuprynski and

co-workers (1995) demonstrated that heat-treated LKT or LKT treated with neutralizing mAb lost the ability to induce cytolysis but continued to induce cytokine release. LKT also induces intracellular calcium elevation in LKT susceptible bovine alveolar macrophages (BAM), and subsequently induces the expression of IL-1β, TNF-α and IL-8. Tyrosine kinase inhibitor blocks LKT induced expression of these cytokines in BAMs (Hsuan et al., 1999).

Following incubation with sub-lytic doses of LKT, bovine leukocytes demonstrate morphologic changes that are consistent with apoptosis such as cytoplasmic membrane blebbing, chromatin condensation and margination (Stevens and Czuprynski, 1996). Wang et al., 1998, and Sun et al., 1999 reported that LKT induced BL3 cells to undergo internucleosomal DNA fragmentation that is regarded as the hallmark of apoptosis. Sub-lytic doses of LKT also induce degranulation and generation of ROIs from neutrophils. Release of enzymes from neutrophil's cytoplasmic granules and ROIs damage the tissues and play an important role in pathogenesis of bovine pneumonic pasteurellosis. High doses of LKT lyse the target cells. Experiments have shown that large plasma membrane defects develop on target cells resulting in the leakage of large intracellular molecules such as lactate dehydrogenase (LDH) (Clinkenbeard et al., 1989). Deletion of LKT gene from *M. hemolytica* results in the deletion of leukolytic ability of bacterial culture supernatant (Murphy et al., 1995).

M. hemolytica LKT exhibits low level of non species-specific hemolytic activity (Murphy et al., 1995). This hemolytic activity of LKT is much less efficient than its leukolytic activity. The ability of LKT to cause hemolysis suggests that it is able to bind to erythrocytes by a mechanism independent of its species-specific leukolytic activity. LKT binding to erythrocytes may not be mediated by a protein receptor. Instead, binding to erythrocytes may involve direct interaction of LKT with membrane phospholipids. The exact mechanism remains to be elucidated.

Li and Clinkenbeard (1999) have documented CD18 is the species-specific receptor of *M. hemolytica* LKT. The LKT binding domains of bovine CD18 are apparently species-specific, because *M. hemolytica* LKT does not recognize human CD18. Pro-LKT could recognize or bind to the bovine CD18 receptor, suggesting that LKT acylation, which is necessary for its activity, is not required for target cell specific binding (Fedorova and Highlander, 1997).

VIII. Summary and Statement of Research Problem:

Identification of the host cell receptor(s) of *M. hemolytica* LKT is a prerequisite for understanding the pathogenesis of bovine pneumonic pasteurellosis. Significant progress has been made towards identifying the receptor for the RTX family toxins. Lally and coworkers (1997) identified the $ß_2$ integrin LFA-1 as the receptor for the leukotoxin of *A. actinomycetemcomitans* and the HylA of *E.coli.*

Subsequently, $ß_2$ integrins have been identified as the receptors for the LKT of *M. hemolytica* (Ambagala et al., 1999; Jeyaseelan et al., 2000; Li et al., 1999; Wang et al., 1998). $ß_2$ integrins are leukocyte-specific integrins, which have a common ß subunit, CD18 that noncovalently associates with three distinct α chains, CD11a, CD11b, and CD11c, to give rise to three different $ß_2$ integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (CR4), respectively.

The most important role of $ß_2$ integrins is to mediate the intercellular adhesion essential for normal immune and inflammatory responses (Springer et al., 1990). The $ß_2$ integrins promote intercellular adhesion by binding with complementary molecules present on other immune cells or endothelium. The best characterized ligand for a $ß_2$ integrin, CD11a/CD18 (LFA-1) is a distinct protein of the immunoglobulin superfamily, intercellular adhesion molecule-1 (ICAM-1) (Marlin et al., 1987; Rothlein et al., 1986). ICAM-1 is expressed by endothelial cells, lymphocytes, and tissue macrophages following their stimulation by certain cytokines, such as TNFα, IL-1, and IL-8 (Dustin et al., 1986). As the ligand for LFA-1, ICAM-1 functions in the trafficking of leukocytes between the peripheral blood and the sites of inflammation in tissues. In addition to cell-cell adhesion, other immune functions such as phagocytosis and chemotaxis are also dependent on $ß_2$ integrins (Arnaout et al., 1990). $ß_2$ integrins have also been found to promote apoptosis in human leukocytes (Walzog et al., 1997), an observation that correlates with the apoptosis induced by low dose of *M.*

hemolytica leukotoxin (Stevens et al., 1996). The disease, leukocyte adhesion deficiency (LAD), resulting from the loss of $ß_2$ integrin surface expression, emphasizes the importance of $ß_2$ integrins in normal immunological function. LAD has been reported in both humans and cattle (Arnaout et al., 1990). LAD patients are highly susceptible to bacterial pathogens and suffer from recurrent infections. In humans and cattle, the disease results from mutations in the gene encoding CD18 (Kishimoto et al., 1989; Tajima et al., 1993). Specifically, the molecular basis of bovine leukocyte adhesion deficiency (BLAD) is a single point mutation (adenine to guanine) at position 383 of the CD18 gene that causes an aspartic acid to glycine substitution at amino acid 128 in the glycoprotein. This mutation lies within a large extracellular region that is highly conserved across the CD18 subunits of cattle, human and mouse (Kishimoto et al., 1989; Shuster et al., 1992). BLAD calves have been used in conjunction with the *M. hemolytica* pneumonia model to study neutrophil infiltration patterns in the lung (Ackermann et al., 1999; Ackermann et al., 1996). These studies concluded that CD18 is important for the emigration of neutrophils through bronchi and bronchioles.

While there is agreement that $ß_2$ integrins are the receptors for *M. hemolytica* leukotoxin, there is no consensus as to which subunit (or both) serves as the specific receptor for the LKT. Previous studies have revealed that LKT binds to all three $ß_2$ integrins, suggesting that the ß subunit CD18, which is common to all three $ß_2$ integrins, is the subunit that mediates LKT-induced cytolysis of bovine leukocytes. However, the possibility of the CD11 subunits contributing to the

binding of the LKT cannot yet be ruled out. Previous studies have revealed that *Candida albicans* binds to the CD11b/CD18 on human leukocytes (Forsyth et al., 1998). Although in the absence of CD18, the CD11b subunit alone binds *C. albicans*, a single point mutation in the CD18 abolishes the binding of the fungus to CD11b/CD18. Therefore, it could be extrapolated that even if *M. hemolytica* LKT binds only to CD18, the CD11 subunit might contribute to the appropriate conformation needed for the binding of the LKT.

On the basis of the previous work by Ambagala et al., 1999; Jeyaseelan et al., 2000, Lally et al., 1997, and Li et al., 1999, **we hypothesized that LKT binding and cytotoxicity is mediated by bovine CD18 subunit alone.** Further, the irrefutable identification of CD18 as the LKT receptor could be made only by recombinant expression of CD18 and CD11 subunits, individually or together on LKT-non-susceptible cells, and thereby rendering them susceptible to LKT binding and subsequent lysis.

Chapter 2

Materials and Methods

Blood Collection: Blood was collected for peripheral blood mononuclear cells (PBMCs) to serve as a source of RNA for the amplification of the integrin coding sequences. 15 ml of whole blood was collected using jugular puncture from a clinically healthy heifer in a sodium citrate tube.

Collection of PBMCs from blood:

Whole bovine blood was centrifuged @ 2000 rpm for 7 min and plasma removed and discarded. The cellular fraction was resuspended 2X the original volume in saline and layered on 12 ml of ficoll (Histopaque^{R}, Sigma), which was subsequently centrifuged at 800g for 40 min without brake. The PBMC layer was removed with a quick draw pipette gun. The collected PBMCs were washed twice with 1X phosphate-buffered saline (PBS) at 2000 rpm for 5 min each at room temperature.

Stimulation of PBMCs with Concanavalin A: The pelleted cells were suspended in 3 ml of RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS, Cat # 35-011-CV, Mediatech, Cellgro, VA). Live cells were counted by trypan blue dye exclusion and the cell count was adjusted to $1X10^{6}$ cells/ml and 5 ml of the

cell suspension (5 X 10^6 cells) were plated (Sigma) in a 6-well plate with 10 µg/ml Concanavalin A for 4 hrs at 37 °C in the incubator.

RNA isolation: Total cellular RNA was isolated from stimulated bovine PBMCs using a guanidine thiocyanate technique. Briefly, 1 ml of RNA Stat-60 (TEL-TEST, INC., Friendswood, TX) was added to the pelleted cells ($5X10^6$) and the cells were lysed by pipetting up and down multiple times. 200 µl of chloroform was added to the cell lysate and the RNA was subsequently isolated according to the kit instructions, quantified with a spectrophotometer, and adjusted to 100 ng/µl with nuclease free water.

Reverse Transcription (RT) of mRNA into cDNA: The bovine RNA sample was reverse transcribed into cDNA in one large RT reaction. Briefly, 6 µg of RNA (60µl of RNA suspension) was combined with 500 pmol of Oligo dT, heated to 72 °C for 7 min, and subsequently chilled on ice. This was further adjusted with additional reagents to the following conditions: 5X RT buffer (50 mM Tris-HCL pH 8.3, 3 mM $MgCl_2$, 75 mM KCl), 20 mM DTT, 0.5 mM dNTP, 1 U/ml RNAsin, 10 U/µl Superscript RT (Invitrogen) in a total volume of 150 µl made up with diethyl pyrocarbonate (DEPC)-treated water. The RT mixture was incubated at 45 °C for 60 min, heat-inactivated at 94 °C for 10 min, and quickly chilled on ice. Commercially available human leukocyte cDNA was procured from Clontech, BD Biosciences.

Primer Design: Bovine CD18 as well as human CD18 and CD11a sense and antisense primers were designed from previously published sequences in Genbank. *HindIII* (**AAGCTT**) and *XbaI* (**TCTAGA**) restriction sites were incorporated in the sense and antisense primers for directional cloning, respectively.

Bovine CD18: (Sequence published by Shuster et al., 1992)

Sense-5'- ATAT**AAGCTT**GGGGACATGCTGCGCCAG 3'

Antisense-5'-CAAT**TCTAGA**GCACCCCTAACTCTCGGCAAACTTAGGG-3'

Human CD18: (Sequence published by Kishimoto et al., 1987)

Sense-5'- ATAT**AAGCTT**CAGGACATGCTGGGCCTCCGCCC 3'

Antisense-5'- CAAT**TCTAGA**GTGCTCCTAACTCTCAGCAAACTTGG-3'

Human CD11a: (Sequence published by Corbi et al., 1988)

Sense-5'- ATAT**AAGCTT**CCTCGAGTGCTGGA-3'

Antisense- 5'- CAAT**TCTAGA**GCACCTCACAGGCCTGG-3'

Due to lack of a published bovine CD11a sequence, 3 sets of consensus primers were designed from alignment of the conserved regions of published human and murine CD11a sequences.

BovineCD11a:

Sense-5'- ATAT**AAGCTT**CCTAAGCGCATGATCAGTTTCC-3'

5'- ATAT**AAGCTT**GGGCCACCTGAC-3'

5'- ATAT**AAGCTT**GCCCCTGGGGCCACAGG-3'

Antisense- 5'- CAAT**TCTAGA**CTCAGTCCTTGCCAC-3'

5'- CAAT**TCTAGA**CTTAGTCCTTGTCAC-3'

The following bovine CD11a primers were designed from the sequence published in Genbank in January 2004 (Fett et al., 2004).

Sense - 5'- ATAT**AAGCTT**CCCAAGAGCCCTCTGAGACT-3'

Antisense - 5'- CAAT**TCTAGA**GCACCTCAATCTCCACCACT-3'

All the primer sets were acquired from Integrated DNA Technologies, Inc. (Coralville, IA) on a fee for service basis. All the sense and antisense primers were diluted to a concentration of 200 µM, which was designated as 'Stock'. For the actual PCR, the stock was further diluted to a working concentration of 25 µM.

Generation of Inserts by Polymerase Chain Reaction (PCR): Bovine CD18 was generated using bovine cDNA in a 50 µl PCR using PCR master mix (Catalog # M7502, Promega, 2800 Woods Hollow Road, Madison, WI). Briefly, the procedures recommended by the manufacturer were

followed, with the following cycling parameters: 1 min at 94 °C, then 35 cycles including: (i) 40 s at 94 °C, (ii) 30 s at 68 °C, and (iii) 2 min 30 s at 72 °C, followed by a final extension at 72 °C for 10 min. Human CD18 was generated with human cDNA and CD18 primers using similar PCR parameters as that of bovine CD18 except that the T_m, for human CD18 was increased to 70 °C.

Human CD11a was generated by using human cDNA with Advantage^R –GC cDNA PCR Kit (Catalog # K1907-1, BD Biosciences Clontech, Palo Alto, CA). This kit contains reagents specifically formulated to amplify cDNAs with high G:C content. Briefly, the procedures recommended by the manufacturer were followed, with the following cycling parameters: 1 min at 94 °C, then 35 cycles including: (i) 40 s at 94 °C, (ii) 30 s at 56 °C, and (iii) 3 min 30 s at 72 °C, followed by a final extension at 72 °C for 10 min.

Bovine CD11a was generated using bovine cDNA in a long distance PCR using Elongase amplification technology (Invitrogen). Briefly, the procedures recommended by the manufacturer were followed, with the following cycling parameters: 2 min 30 s at 94 °C, then 35 cycles including: (i) 30 s at 94 °C, (ii) 30 s at 56 °C, and (iii) 3 min 30 s at 68 °C, followed by a final extension at 68 °C for 10 min.

Selection of Cloning and Expression Vectors: pUC19 was chosen for the purpose of individual/separate cloning of the four desired PCR products namely

bovine and human CD11a and CD18. pUC19 contained a multiple cloning site with *HindIII* (AAGCTT) and *XbaI* (TCTAGA) restriction sites and a gene that conferred resistance to ampicillin. Also, pUC19 enabled the mass production of the inserts before their insertion into the expression vectors. pUC19 was a generous gift from Dr. Alain Stintzi, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK. As per the cloning strategy, the integrin subunits were individually/separately cloned in pUC19 followed by transformation in *E.coli* strain DH5α^{TM}, and mass production by maxiprep (Qiagen) for their ultimate incorporation into the respective expression vectors.

Two types of expression vectors were chosen, pcDNA3.1/Neo (Cat # V790-20, Invitrogen) and pcDNA3.1/Zeo (Cat # V860-20, Invitrogen). These vectors were chosen for their high-level, constitutive/co-expression in a variety of mammalian cell lines. Both the vectors had a cytomegalovirus (CMV) enhancer-promoter for high-level expression, an ampicillin resistance gene, and a pUC origin for selection and maintenance in *E. coli* strains like DH5 α^{TM}. The only difference was that each vector had a different selectable marker *i.e.* one had neomycin (pcDNA3.1/Neo) and other zeocin (pcDNA3.1/Zeo). The different selectable markers allowed for co-selection of the recombinant expression vectors in a cell line. The integrin subunits cloned in pUC19 were finally incorporated in pcDNA3.1 expression vectors as described in the next section.

Digestion and Ligation of Inserts with Cloning and Expression Vectors: The individual PCR products of bovine and human CD11a and CD18 along with pUC19, pcDNA3.1/Neo and pcDNA3.1/Zeo were digested with *HindIII* (Cat # R6041, Promega) and *XbaI* (Cat # R6181, Promega). Briefly, 1µg of the cDNA (bovine CD18 /human CD18 /bovine CD11a/human CD11a) was combined with 0.25 µl each of *HindIII* (10U/µl) and *XbaI* (8-12U/µl) along with 3 µl of 10X of the appropriate buffer in a total volume of 30 µl made up with nuclease-free water. This mixture was then incubated at 37 °C (water bath) for 60 min, and subsequently electrophoresed on a 0.6% agarose gel. The digested product(s) were then ligated with pUC19, pcDNA3.1/Neo and pcDNA3.1/Zeo using T4 DNA Ligase (Cat # M1801, Promega) and 10X ligase buffer (Cat # C1263, Promega) according to the manufacturer's instructions. The following table denotes the placement of the coding sequence into the expression vectors.

Table 1

Expression Vector	**Insert(s)/PCR Products**
pcDNA3.1/Neo	Bovine CD18 Human CD18
pcDNA3.1/Zeo	Bovine CD11a Human CD11a

In order to check the authenticity of the specific ligation reactions, the ligated products (inserts + expression vectors) were digested with *HindIII* and *XbaI* in the same manner as described above.

Transformation of Recombinant Vectors/Constructs into *E.coli* strain DH5α:

The ligated product(s)/construct(s) were transformed into *E.coli* strain DH5α according to the manufacturer's instructions. Briefly, 50 µl of DH5α^{TM} cells (Cat # 18258-012, Invitrogen life technologies, Inc., CA) were mixed with 1.0 µg of the respective/individual constructs and incubated on ice for 20 min. The mixture was further subjected to heat shock at 42 °C (water bath) for 2 min followed by 1.5 min incubation on ice. Subsequent addition of 430 µl of pre-warmed (37 °C) LB broth to the mixture was followed by incubation at 37 °C (water bath) for 45 min. Following incubation, the mixture was plated onto LB agar containing 100 µg/ml ampicillin (Cat # BP1760-25, Fisher Biotech) to screen for the successful transformation of the ligated product(s). The plates were incubated at 37 °C overnight and individual colonies were picked and expanded in LB broth supplemented with 100 µg/ml ampicillin. The transformation was confirmed by harvesting the plasmid from 2 ml broth preparations (miniprep, Qiagen) and digesting them with *HindIII* and *XbaI*. The successfully transformed constructs were mass produced in LB broth and harvested using QIAGEN HiSpeed Plasmid Maxi Kit (Cat # 12663) for sequencing and transfection.

Sequencing of Inserts in Recombinant Vectors/Constructs: The 5'-CGCAAATGGGCGGTAGGCGTG-3' Cytomegalovirus (CMV) and 5'-TAGAAGGCACAGTCGAGG-3' Bovine Growth Hormone (BGH) sequences inherently present in the expression vectors were used as forward and reverse primers, respectively for sequencing the respective inserts of bovine and human CD11a and CD18 at Recombinant DNA/Protein Resource Facility (Core Facility), Oklahoma State University, Stillwater and Oklahoma Medical Research Foundation (OMRF) on a fee-for-service basis. Sequences returned were found to match the bovine and human CD11a and CD18 from BLAST analysis/sequence alignment.

Cultivation/Propagation of Tissue Culture Cells: The cell lines K562 (human erythroleukemia cell line) and HL-60 (human myeloblastic cell line) were obtained from and cultured as described by American Type Culture Collection, Rockville, MD. Briefly, the cells were propagated in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 20 µg of penicillin-streptomycin/ml and 10% heat inactivated fetal bovine serum (FBS). Medium for HL-60 cells was supplemented with 20% FBS instead of 10%. The BL3 (bovine lymphoma cell line) and Vero (African green monkey kidney cell line) cells were obtained from the laboratories of Drs. K.D. Clinkenbeard and R. Eberle, respectively. BL3 cells were propagated in Minimum Essential Medium (MEM) supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10.0 µg of Gentamicin/ml and

10% FBS. The Vero cells were propagated in Dulbecco's MEM supplemented with 2 mM L-glutamine, 10 mM HEPES, 1.5 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, 20 µg of penicillin-streptomycin/ml and 10% FBS. The cell lines were cultured in 75-cm^2 Corning tissue culture flasks maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Transfection of Recombinant Vectors/Constructs into K562, Vero and HL-60 cells: Vero cells were plated at a density of 4-6 X 10^5 cells/well in a 6-well plate 24 hrs before transfection to provide 80-90% confluency at the time of transfection. Calcium phosphate transfection system (Catalog # 18306-019, Invitrogen Life Technologies Inc.) was used to sequentially and/or co-transfect Vero cells with various permutations and combinations of constructs according to the manufacturer's instructions. The following tables in a 6-well plate format depict the type & amount (µg) of recombinant vectors (constructs), and carrier DNA transfected per well.

4-6 X 10^5 **Vero cells/well** were plated in a 6-well plate 24 hrs prior to transfection (Table 2):

10.0µg pcDNA3.1/Neo + Bov CD18 + 10.0µg Carrier DNA	10.0µg pcDNA3.1/Neo + Bov CD18 + 10.0µg Carrier DNA	10.0µg pcDNA3.1/Neo + Bov CD18 + 10.0µg Carrier DNA
10.0µg pcDNA3.1/Neo + Bov CD18 + 10.0µg Carrier DNA	10.0µg Carrier DNA only **(Control)**	Not Transfected **(Control)**

Table 3

6.5µg pcDNA3.1/Neo + Bov CD18 6.5µg pcDNA3.1/Zeo + Hum CD11a + 7.0µg Carrier DNA	6.5µg pcDNA3.1/Neo + Bov CD18 6.5µg pcDNA3.1/Zeo + Hum CD11a + 7.0µg Carrier DNA	6.5µg pcDNA3.1/Neo + Bov CD18 6.5µg pcDNA3.1/Zeo + Hum CD11a + 7.0µg Carrier DNA
6.5µg pcDNA3.1/Neo + Bovine CD18 6.5µg pcDNA3.1/Zeo + Human CD11a + 7.0µg Carrier DNA	10.0µg Carrier DNA only **(Control)**	Not Transfected **(Control)**

Salmon sperm DNA @ 10 mg/ml was used as carrier DNA. The non-adherent/suspension K562 cells were sequentially and/or co-transfected with various combinations of constructs, whereas the non-adherent HL-60 cells were transfected with bovine CD18 construct alone using Calcium Phosphate or Lipofectamine^{TM} 2000 transfection reagent (Cat # 11668-027, Invitrogen Life Technologies Inc.) according to the manufacturer's instructions. Transfection of the K562 and HL-60 cells was similar to that described with the Vero cells. The tables below illustrate the transfection strategy.

6-8 X 10^5 **K562 cells/well** were plated in a 6-well plate on the day of transfection (Table 4):

4.0 µg pcDNA3.1/Neo + Bov CD18 4.0 µg pcDNA3.1/Zeo + Hum CD11a	4.0 µg pcDNA3.1/Neo + Bov CD18 4.0 µg pcDNA3.1/Zeo + Hum CD11a	4.0 µg pcDNA3.1/Neo + Bov CD18 4.0 µg pcDNA3.1/Zeo + Hum CD11a
4.0 µg pcDNA3.1/Neo + Bov CD18 Only **(Control)**	4.0 µg pcDNA3.1/Zeo + Hum CD11a Only **(Control)**	Not Transfected **(Control)**

6-8 X 10^5 **HL-60 cells/well** were plated in a 6-well plate on the day of transfection (Table 5):

4.0 µg pcDNA3.1/Neo + Bov CD18	4.0 µg pcDNA3.1/Neo + Bov CD18	4.0 µg pcDNA3.1/Neo + Bov CD18
4.0 µg pcDNA3.1/Neo + Bov CD18	4.0 µg pcDNA3.1/Neo Only **(Control)**	Not Transfected **(Control)**

As per the published evidence of CD11a and CD18 being expressed on the cell surface as a heterodimer (Lally *et al*., 1997), the Vero and the K562 cells transfected with constructs containing one type of insert (bovine CD11a/human CD11a/bovine CD18/human CD18) were treated as controls for the cells co-transfected with bovine and human CD11a and CD18 constructs.

At 48 hrs post-transfection, the appropriate antibiotic (Geneticin^R or Zeocin^R) was added to the transfected cell cultures. The cells transfected with constructs containing the neomycin resistance cassette received Geneticin^R, whereas the ones containing the Zeocin^R resistance cassette received Zeocin^R. Cells transfected with both pcDNA3.1/Neo and pcDNA3.1/Zeo were cultured in

medium containing both antibiotics. The drugs were used at varying final concentrations of 100-500 µg/ml (Geneticin^{R}) and 100-250 µg/ml (Zeocin^{R}) depending upon the type of cell line and the recombinant vector(s) transfected. Cells transfected for detecting transient surface expression of integrins were not supplemented with antibiotics.

Immunostaining for Detection of Cell Surface Expression of Integrins by Confocal Microscopy: The transfectants were tested for cell surface expression of bovine CD18, CD11a, human CD18, CD11a by immunostaining, using antibodies listed in Table 6. Immunostaining was carried out by plating 1.0 X 10^4 transfected Vero cells, and parent Vero cell controls in chamber slides. The slides were incubated at 37 °C overnight to obtain 70-80% confluency. 1.0 X 10^4 K562 transfectants, and BL3 cells (positive control) were adhered to the glass slide by use of a cytospin instrument at 2000 rpm for 5 min. On the day of immunostaining, the transfected, and parent Vero, K562, and BL3 cells were washed 3X with warm media & immunostaining buffer (5% FBS in PBS), fixed with 4% paraformaldehyde for 30 min and quenched with 50 mM NH_4Cl in PBS for 5 min at room temperature. The cells were subsequently washed three times with immunostaining buffer and blocked with 5% non-fat milk for 1 hr at room temperature. Primary antibodies such as murine anti-bovine CD18 MAb, murine anti-bovine CD11a/CD18 MAb, and non-specific murine (MOPC-21) MAb were applied at 100 µl (1:500 dilution in PBS) for 2 hrs at 4 °C. Following three washes in immunostaining buffer, the cells were incubated with 100 µl (1:1000

dilution in PBS) of Alexa Fluor^{R} 488-labeled goat anti-mouse IgG_1 secondary antibody (Cat # A-21121, Molecular Probes, Eugene, Oregon) for 1 hr at 4 °C. The cells were washed three times with immunostaining buffer, mounted with DAKO fluorescent mounting medium (Cat # S3023, DAKO Corporation, Carpinteria, CA), coversliped and analyzed with a Leica confocal microscope.

Table 6

Anti body Specificity	**Clone**	**Isotype**	**Source**
Bovine CD18	BAQ30A	IgG1	VMRD, Inc., Pullman, WA
Human CD18	H20A	IgG1	VMRD, Inc., Pullman, WA
Bovine CD11a/CD18	BAT75A	IgG1	VMRD, Inc., Pullman, WA
Human CD11a	HI111	IgG1	BD Pharmingen, Inc., San Diego, CA
Non-specific murine antibody	MOPC21	IgG, Whole molecule	Jackson ImmunoResearch Laboratories, Inc., PA

Membrane Isolation from Vero Cells Transfected with Bovine CD18 Recombinant Vector: Membrane extracts were prepared utilizing a technique described by Li et al. (1999) with a few modifications. Briefly, 1.0 X 10^9 Vero cells (Vero cells transfected with bovine CD18 recombinant vector and showing fluorescence – clones 1, 2, and 3) were washed 3 times with PBS and suspended in 10 ml of 50 mM 3-(N-morpholino)-propanesulphonate, pH 7.0

(MOPS buffer) that contained one protease inhibitor cocktail tablet (Boehringer Mannheim, Indianapolis, IN). The suspension was subjected to 10-passes of a Potter-Elvenhjem tissue grinder with a 0.1 mm clearance and subsequently centrifuged at 1400 g for 15 min. The pelleted, unbroken cells and nuclei were removed whereas the supernatant containing the membrane fraction was collected, centrifuged at 2500 g for 15 min and washed thrice with MOPS buffer. The membranes were then extracted with 1% Triton X 100 in MOPS buffer for 30 min at 4 °C.

Western Blotting: The membrane extracts in Triton X-100 were mixed 1:5 (v/v) with 6X non-reducing SDS-PAGE sample buffer and subjected to SDS-PAGE on 8% gels (Cat # E-4325-0008, ISC Bioexpress). Following transfer to nitrocellulose membranes for 1h, the membranes were blocked with 3% non-fat dried milk (diluted in MOPS buffer), and washed three times with PBS containing 0.05% Tween-20 (PBST). The membranes were then sequentially incubated with either 1:500 murine anti-bovine CD18, murine anti-bovine CD11a/CD18 or non-specific murine (MOPC-21) MAbs for 3 hrs, and then 1:1000 biotin conjugated rabbit anti-mouse IgG (Cat # 315-065-003, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hr and 1:2000 freshly prepared peroxidase conjugate (Cat # PK40000, Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature. Following incubations, the membranes were washed thrice with PBST and the bound peroxidase was

developed with 3, 3'-diaminobenzidine (DAB) substrate (Catalog # SK-4100, Vector Laboratories Inc., Burlingame, CA).

Inhibition of Leukotoxin-induced Leukolysis/Trypan Blue Dye Exclusion Test: A trypan blue dye exclusion test was conducted to assess the sensitivity of Vero cells transfected with the bovine CD18 recombinant vector (clones 1, 2, and 3) to LKT. Parent Vero cells and BL3 cells were used as negative and positive controls, respectively. In addition, some cultures were preincubated with antibodies against bovine CD18 to confirm that any cytolysis seen (or abrogated by anti-bovine CD18 antibodies) was due to specific expression of bovine CD18. Briefly, $2X10^5$ BL3 cells and 80-90% confluent transfectant & Vero cells in a 96-well plate were incubated with or without 10 µl (1:100 dilution in RPMI-1640) of the antibodies mentioned in Table 6 at 37 °C for 45 min. Subsequently, 0.1 µg (0.5 TU/ml) of LKT in 10 µl of RPMI-1640 was added to the pre-incubated cells followed by additional 45 min incubation at 37 °C. Exposed cells were cooled on ice and mixed with 100 µl 0.4% trypan blue. The viable cells were enumerated in triplicate using a hemocytometer, and the counts were normalized to the negative control: parent Vero cells, cells treated with MOPC-21 and cells with no LKT treatment.

Leukotoxin Activity and LDH Release Assay: LKT activity was quantified as toxic units (TU) by measurement of intracellular lactate dehydrogenase (LDH) leakage caused by incubation of 50% dilution of the LKT preparation

(Clinkenbeard et al., 1989b) with the transfectants (Vero cells transfected with bovine CD18 recombinant vector alone), parent cells (Vero, negative control) and BL3 cells (positive control). Briefly, 0.5 X 10^6 cells/well in a 96-well plate were incubated with 50% dilution (0.5 TU/ml) of the LKT for 2 hrs at 37 °C. The exposed cells were further collected by centrifugation at 700 g for 10 min and 100 µl of supernatant from each treatment was transferred into a new 96-well flat bottom microtiter plate, which was then warmed to 37 °C. LDH activity was measured by addition of 100 µl of 37 °C assay reagent LDH-L 50 (Sigma, St. Louis, MO) in flat-bottomed 96-well microtiter plates. Substrate conversion was measured in a thermally-controlled kinetic microtiter plate reader (Thermomax, Molecular Devices, Palo Alto, CA) at 340 nm for 2 min at 37 °C.

Flow Cytometric Analysis for Cell Surface Expression of Integrins: The transfectants (K562 cells co-transfected with bovine and human CD11a and CD18 recombinant vectors), parent cells (K562, negative control), and BL3 cells (positive control) were tested for cell surface expression of the integrins, using murine anti-bovine CD18 MAb, anti-human CD18 MAb, anti-bovine CD11a/CD18 MAb, anti-human CD11a MAb or a non-specific murine MAb (Table 6) in a flow cytometric analysis according to the following procedure. Briefly, 5X10^5 K562 cells, transfectants and BL3 cells were incubated with 100 µl (1:100 dilution in PBS) each either of BAQ30A, H20A, BAT75A, HI111, or a non-specific murine MAb (MOPC-21) at 4 °C for 1 hr. Following three washes in FACS buffer (3% fetal bovine serum in PBS), the cells were incubated with 100 µl (1:500 dilution in

PBS) of fluorescein isothiocyanate-labeled goat antibodies specific for mouse IgG (Cat # 115-095-166, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 4 °C for 1 hr. The cells were washed three times with FACS buffer, resuspended in 0.5 ml PBS, and analyzed by FACS caliber machine.

Chapter 3

Results

Identification of host cell receptor(s) of *Mannheimia hemolytica* leukotoxin is critical for understanding the pathogenesis of bovine pneumonic pasteurellosis. The objective of this study was to elucidate the host cell receptor (bovine CD11a or CD18 or the heterodimer bovine CD11a/CD18) for *Mannheimia hemolytica* LKT binding and subsequent cytotoxicity. To achieve this objective, we transfected several LKT-resistant cell lines with different combinations of bovine (LKT-sensitive) and human (LKT-resistant) CD11a and CD18 recombinant vectors. Following transfection, the cells were screened for transient or stable surface expression of the ß2 integrin (CD11a/CD18) by flow cytometry, confocal microscopy (immuno-staining) and western blotting. LKT sensitization was assessed by LDH release assay and trypan blue dye exclusion following *in vitro* LKT challenge.

According to the original plan, LKT-resistant K562 cells (human chronic myelogenous leukemia cells) were supposed to be transfected with different permutations of bovine and human CD11a and CD18 constructs. The following table lists the transfection strategies, expected results and their appropriate interpretations:

Table 7

K562 Transfectant	Expected Expression	Expected Response to LKT challenge	Interpretation
Human or bovine CD11a or CD18 only	Negative	Resistant	Heterodimer formation necessary for surface expression
Bovine CD11a/CD18	Positive	Susceptible	Either bovine CD11a, or CD18, or both necessary for LKT-mediated cytolysis
Human CD11a/CD18	Positive	Resistant	Confirmation of human receptor resistance to LKT binding and toxicity
Hum CD11a/Bov CD18	Positive	Susceptible	LKT sensitivity restricted to bovine CD18
Bov CD11a/Hum CD18	Positive	Resistant	LKT sensitivity restricted to bovine CD18

Previously, Ritchey and coworkers made several attempts to optimize the transfection of K562 cells with bovine CD18 recombinant vector. All such attempts met with failure as the transfected K562 cells did not survive for more than 10-14 days in the medium supplemented with Geneticin^{R} (personal communication with Dr. Jerry Ritchey, August 2001). It was subsequently decided to use some other LKT-resistant cell line that had been widely used for transfections and was known to exhibit high stable recombinant protein expression. For that purpose, we chose Vero cells (African green monkey kidney cells). Another additional cell line HL-60 cells (human promyelocytic leukemia cells) were also transfected with different combinations of bovine and human CD11a and CD18 recombinant vectors. Prior to the actual transfection of recombinant vectors in cell lines, the following results were obtained.

Generation of Inserts: Bovine and human blood was collected for generating cDNAs for obtaining bovine and human CD11a and CD18 coding sequences. Briefly, the total cellular RNA was collected from *in vitro* concanavalin A-stimulated bovine and human peripheral blood mononuclear cells. The RNA was reverse transcribed and the cDNA amplified using primers selected from the published sequences of bovine and human CD11a and CD18. To facilitate directional cloning, the sense primers were incorporated with a *HindIII* and the antisense primers with an *XbaI* restriction site. There were no internal *HindIII* or *XbaI* sites in the coding region of any of the inserts and *XbaI* was readily incorporated into the stop codon region.

The primers amplified a single band (Figure 1) of the predicted size for bovine & human CD18 (~ 2400 b.p.) and human CD11a (~ 3500 b.p.). The generation of bovine and human CD18 and human CD11a was accomplished with only a few rounds of PCR optimization to acquire a clean product. However, we encountered an extreme difficulty in the generation of bovine CD11a coding sequence. First, the mRNA or cDNA sequence of bovine CD11a was not published at the initiation of these studies. We designed 3 sets of consensus primers from alignment of human and murine CD11a sequences, incorporating *HindIII* and *XbaI* restriction sites. Initially, an appropriately-sized PCR product was generated (~ 3400 b.p.). However, upon sequencing, it was determined that this product did not have reasonable homology with any known CD11a sequence from any species. Many additional attempts were made by trying different

combinations of primers out of the 3 designed sets of consensus primers as well as by modifying PCR parameters (primer and cDNA concentrations, $MgCl_2$ concentrations, annealing temperatures). These attempts also did not result in generation of an appropriate sized bovine CD11a product.

Following multiple failures, we consulted with Dr. Udaya deSilva in the Department of Biochemistry and Molecular Biology, Oklahoma State University for trouble-shooting suggestions. Considerations were: first, at 3400 b.p., the amplification may have been precluded by inadequate length of the cDNA from inefficiency at the RT step. Secondly, the amplification may have been hampered by the presence of secondary structures or GC rich regions in the bovine cDNA. To compensate for these possible problems, we changed the parameters for RT-PCR (using a third-generation RT enzyme) and also included the use of an Advantage-GC cDNA PCR kit during amplification to more efficiently melt the G:C rich regions in the cDNA. These efforts were also unsuccessful. In January 2004, new sense and antisense primers were designed from recently published sequence of bovine CD11a (Fett et al., 2004). Using these primers and modifications of the RT and PCR parameters, an appropriate-sized (~ 3500 b.p.) product was generated as shown in Figure 1.

Cloning and Ligation of Inserts in pUC19 and Expression Vectors: All four inserts were first cloned into pUC19 (small, high copy number, *E.coli* plasmids, 2686 b.p. in length) followed by subcloning into expression vectors –

pcDNA3.1/Neo and pcDNA3.1/Zeo. pUC19 contained a multiple cloning site with *HindIII* (AAGCTT) and *XbaI* (TCTAGA) restriction sites and a gene that conferred resistance to ampicillin. Further, it produces an α-peptide of ß-galactosidase, which complements the *lac* deletion mutation in *E.coli* strains such as DH5α^{TM}. This implies that when such strains (like DH5α^{TM}) are transformed by non-recombinant vectors (i.e. pUC19 alone), blue colonies are generated on plates containing X-gal, whereas white or colorless colonies are formed on the same plates by vectors with inserts. The inserts and pUC19 were individually digested with *HindIII* and *XbaI* and further ligated with T4 DNA Ligase as described in the materials and methods section. Figure 2 shows the results of *HindIII* and *XbaI* digestion of pUC19 containing an insert.

Expression vectors pcDNA3.1/Neo (5428 b.p.) and pcDNA3.1/Zeo (5015 b.p.) were selected for their high-level, stable and non-replicative transient expression in a variety of mammalian cell lines. Both the vectors had cytomegalovirus (CMV) enhancer-promoter sequences for high-level constitutive expression, a large multiple cloning site in forward (+) and reverse (-) orientations to facilitate directional cloning, the bovine growth hormone (BGH) polyadenylation signal and transcription termination sequences for enhanced mRNA stability, an ampicillin resistance gene, and pUC origin for selection and maintenance in *E. coli* strains like DH5α^{TM}. The only difference between the two vectors was the resistance cassette, Neomycin^{R} vs Zeocin^{R}. Bovine and human CD11a were individually ligated into pcDNA3.1/Zeo, whereas bovine and human CD18 were ligated into

pcDNA3.1/Neo. Figure 3 shows the results of digestion of ligated expression vectors and inserts with *HindIII* and *XbaI*.

Sequencing of Inserts: The bovine and human CD11a and CD18 inserts were sequenced in pcDNA3.1 using CMV forward and BGH reverse primers. The sequencing results were successfully matched with the respective published sequence data available from National Center for Biotechnology Information (NCBI).

Cultivation/Propagation of Cell Lines: Figure 4 illustrates the morphology of the cell lines at 20X. K562 and Vero cell lines were chosen for LKT resistance secondary to the lack of CD11/CD18 surface expression. Though HL-60 cells naturally express human CD11a/CD18 on their surface, these cells were chosen to be transfected with bovine CD18 recombinant vector alone in an attempt to produce a chimeric heterodimeric surface expression of a chimeric human CD11a/bovine CD18 heterodimer. In the majority of experiments conducted during this study, BL3 cells were used as positive control as they exhibited a natural surface expression of bovine LFA-1 or CD11a/CD18 and were sensitive to LKT. In some experiments, bovine PBMCs were used as positive control. All the above-mentioned cell lines were non-adherent or suspension cells except the Vero cells, which adhered to the surface of the cultivation/propagation flask or plate.

Strategy and Transfection of Recombinant Vectors into Cell Lines: After the initial failure of K562 cells transfected with bovine CD18 subunit to survive in the Geneticin^{R} supplemented medium, Vero cells were chosen for transfection with different combinations of recombinant vectors because these cells are leukotoxin resistant and exhibit a well-known ability and stability for expressing recombinant proteins. Vero cells were transfected with bovine and human CD18 recombinant vectors using calcium phosphate and were selected with Geneticin^{R} for further testing of stable CD18 expression. Although, a single subunit (either CD11or CD18) is not expected to have surface expression by itself (Marlin et al., 1986), the selected Vero cell clones were tested for surface expression of bovine CD18 by immunostaining and western blotting. The Clones were also checked for LKT sensitivity by trypan blue exclusion and LDH release assay.

Due to the low unexpected expression of a single subunit (bovine CD18) exhibited by Vero cells, all further transfection studies were discontinued in these cells. K562 cells were again pulled out of the freezer and chosen for transfection on account of their ability to be efficiently tested by flow cytometry and previously demonstrated stable recombinant human CD11a/CD18 expression (Lally *et al*., 1997). These cells were transfected with bovine CD18 alone as well as different combinations of bovine and human CD11a and CD18 recombinant vectors using the same calcium phosphate transfection system. 48 hrs following transfection, one-half of the transfected cells were subjected to flow cytometric analysis and immunostaining to detect the transient surface expression of the above-

mentioned inserts, while the other half of the transfected cells were supplemented with selection antibiotics for 2-3 weeks post-transfection to select clones that might exhibit stable cell surface expression of the heterodimeric integrin.

HL-60 cells, which naturally express human CD11a/CD18, were transfected with bovine CD18 recombinant vector alone followed by 48 hrs post-transfection selection with geneticin (500 µg/ml) to detect stable surface expression of chimeric human CD11a/bovine CD18 heterodimer.

Immunostaining and Confocal Microscopy Results of Transfected Vero Cells along with Positive and Negative Control: Figure 5 (A), (B), and (C) depict the confocal, transmitted, and the overlay images of the fluorescent bovine PBMCs (positive control) indicating cell surface expression of CD11a/CD18 when stained with bovine anti CD11a/CD18 MAb (BAT75A). The confocal, and transmitted images of the wild type K562 (D, and E) & Vero cells (F, and G) stained with the same BAT75A MAb exhibited a lack of CD11a/CD18 surface expression as expected.

Briefly, following transfection with bovine CD18 recombinant vector alone, fifteen clones continued to grow in the selective (Geneticin^{R}) media. Three out of fifteen clones indicated low level of cell surface expression of bovine CD18 based upon

fluorescent immunostaining. Results of experiment with one representative clone out of three are shown in Figure 6.

The three Vero cell clones identified with weak fluorescence were grown on chamber slides and transfected with human CD11a recombinant vector in an attempt to enhance the low expression of bovine CD18 by eventual expression of a chimeric heterodimer human CD11a/bovine CD18. 48 hrs post-transfection, one half of the transfected Vero cell clones were tested for transient human CD11a/bovine CD18 expression, while the other half was supplemented with Geneticin^{R} and Zeocin^{R} inorder to obtain clones that stably express the chimeric heterodimer.

Figure 7 (A), (B), and (C) exhibit the fluorescent confocal, transmitted, and overlay images of one of the Vero cell clones rescued with human CD11a when detected with BAQ30A MAb and fluorescent secondary antibody reagent. There was low transient surface expression of the supposed chimeric heterodimer human CD11a/bovineCD18 that could not be interpreted different from the level of expression of the clones transfected with bovine CD18 alone. Panels (D), and (E) along with (F), and (G) represent the confocal, and transmitted images of wild type Vero cells transfected with human CD11a, and bovine CD18 when detected with H1III, and BAQ30A MAbs, respectively. The Vero cells transfected with human CD11a, and bovine CD18 recombinant vectors alone as controls did not fluoresce. The Vero clones selected for stable expression of chimeric human

CD11a/bovine CD18 following rescue with human CD11a subunit did not survive long enough in the selective media to be tested.

Western Blotting Results: The membrane extracts from the Vero cell clones expressing low levels of bovine CD18 were subjected to SDS-PAGE on 8% gels. Two out of the three clones in Figure 8 (A), lanes 1, and 3 exhibit a band between 78 and 132 kDa and within the range of 95-100 kDa bovine CD18 when incubated with BAQ30A as the primary antibody. No band was detected on blots incubated with an irrelevant antibody (MOPC-21) or PBS alone (negative controls). A band at 78 kDa was present in all the blots, including the negative controls, indicating non-specific binding of the secondary antibody or peroxidase conjugate to a protein in this region. All subsequent attempts to detect the bovine CD18 expression (band) using membrane extracts prepared from subsequent Vero cell clones met with failure.

Assessment of LKT Sensitivity: Sensitivity of CD18-expressing Vero clones to *M. hemolytica* LKT was tested by *in vitro* LKT challenge and the subsequent cytotoxicity was measured by

a) Trypan blue dye exclusion, and
b) LDH release assay

To confirm that the cytotoxic effect was mediated by bovine CD18, some cultures were pretreated with anti CD18 MAb to interfere with the receptor:leukotoxin binding, and thus protect LKT-sensitive cells from LKT challenge.

Trypan Blue Dye Exclusion Test: Trypan blue dye exclusion was conducted to test the apparent bovine CD18-expressing Vero clones for LKT sensitivity. Attempts to assess the inhibitory effect of BAQ30A, and MOPC21 MAbs on transfectants (Vero cells transfected with bovine CD18 recombinant vector alone - clones 1, 2 and 3), parent cells (Vero, negative control) and BL3 cells (positive control) pre-incubated with *M. hemolytica* LKT were performed. The pre-incubation of BL3 cells with BAQ30A caused inhibition of LKT-induced cytolysis, but the non-specific murine MAb (MOPC21) did not inhibit LKT-induced cytolysis (Table 8). None of the Vero cell clones 1, 2 or 3 that exhibited fluorescence through bovine CD18 immunostaining showed any cytolysis when incubated with LKT. The counts were normalized to the parent Vero cells, non-specific murine MAb (MOPC21) and no LKT + no MAb.

LDH Release Assay: The LDH release assay was used to monitor the LKT-induced target cell lysis. LKT activity was quantified as toxic units (TU) by measurement of intracellular lactate dehydrogenase (LDH) leakage caused by 50% dilution of the LKT preparation. TU was determined by graphing specific LDH leakage versus the culture supernatant dilution factor. One TU was defined as the dilution factor at which the specific LDH leakage was 50% for 4×10^6 BL3

cells ml^{-1} exposed for 2 hrs at 37 °C. BL3 cells (positive control) showed LDH release (~450 U/ml) compared to the negative control (parent Vero cells) (Figure 9). Similar to the findings with trypan blue dye exclusion, the LDH release from the Vero cell clones was similar to that seen with the wild-type Vero cells (Figure 9).

Flow Cytometric Analysis of Transfected K562 Cells: On account of equivocal expression of bovine CD18 on Vero cell clones detected by a number of techniques, limited ability of Vero cells to be tested by flow cytometric analysis, and/or inherent sturdiness of Vero cells to incubation with LKT, further transfection or testing of Vero cell clones was discontinued. K562 cells being non-adherent and previously tested by Lally et al. (1997) for recombinant human CD11a/CD18 expression were chosen for transfection with different combinations of recombinant vectors. Also, by this time, we had generated the bovine CD11a recombinant vector. *As per* the earlier mentioned strategy, K562 cells were transfected with different combinations of bovine and human CD11a and CD18.

$ß_2$ integrins are expressed as heterodimers of α and ß subunits (Gahmberg et al., 1998). It has been documented that the α subunit (CD11) and the ß subunit (CD18) have to associate with each other to be transported to the plasma membrane and expressed on the cell membrane (Marlin et al., 1986). Therefore, it was of interest to determine whether bovine CD18 was expressed as a

heterodimer with bovine and/or human CD11a as well as human CD18 with bovine and/or human CD11a in co-transfected K562 cells. To confirm heterodimeric expression, or to disprove the notion of CD18 expression alone as a single subunit as seen in Vero cell clones, K562 cells were initially transfected with bovine CD18 recombinant vector alone and selected in Geneticin^{R}-supplemented medium to generate clones that stably expressed bovine CD18. The clones obtained by culture in selective media tested negative for cell surface expression of bovine CD18 using flow cytometric analysis.

Figure 10 shows the results of flow cytometric analysis used to detect the transient cell surface expression of bovine and human CD18 heterodimerized with their α subunit counterparts bovine and human CD11a, respectively, in K562 cells transfected with bovine CD11a and CD18, and human CD11a and CD18 recombinant vectors. The results indicated low level of expression for bovine CD11a/CD18, and human CD11a/CD18. According to the findings of Martin *et al*. (1986) and our expectation, none of the control cells transfected singly with bovine or human CD11a or CD18 alone showed any level of surface expression of α (CD11a) or β (CD18) subunit. The co-transfected K562 cells selected with Geneticin^{R} and Zeocin^{R} for stable cell surface expression of bovine and human β_2 integrins could not be tested as they did not survive and perished within 10-14 days post-transfection. The generation of stable expressing clones was attempted multiple times, but every time, the transfected cells did not survive long enough in the selective media to be subsequently tested.

Immunostaining and Confocal Microscopy Results of Transfected K562 Cells: K562 clones transfected with bovine CD18 alone and isolated from the selective medium were co-transfected with bovine and/or human CD11a in an attempt to induce surface expression of the heterodimer. 48hrs following transfection, one half of the transfected K562 cell clones rescued with bovine or human CD11a were tested for transient heterodimer expression, while the other half was supplemented with selection antibiotics, Geneticin^{R} and Zeocin^{R} inorder to obtain clones with stable expression.

Figure 11 (A), (B), and (C) exhibit the fluorescent confocal, transmitted, and the overlay images of bovine CD18-K562 cell clones rescued with bovine CD11a when detected with BAT75A MAb. While (D), (E), and (F) depict the fluorescent confocal, transmitted, and the overlay images of fluorescent K562 cell clones rescued with human CD11a when detected with BAQ30A MAb. Based on the fluorescent intensity, there was weak cell surface expression of both bovine CD11a/CD18 and chimeric human CD11a/bovine CD18. The K562 cells selected with Geneticin^{R} and Zeocin^{R} for the stable expression of the above-mentioned heterodimers did not survive long enough to be tested after multiple attempts.

Figure 12 (A), (B), and (C) exhibit the fluorescing confocal, transmitted, and overlay images of K562 cells co-transfected with bovine CD11a and CD18 when detected with BAQ30A MAb 48 hrs post-transfection. The fluorescence intensity of the images indicated some level of transient surface expression of bovine

CD11a/CD18. Panels (D), and (E) along with (F), and (G) represent the confocal, and transmitted images of wild type K562 cells transfected with bovine CD11a alone, or bovine CD18 alone when detected with H1III, and BAQ30A MAbs, respectively. As expected, the control K562 cells transfected with bovine CD11a or bovine CD18 recombinant vectors alone did not fluoresce. The co-transfected K562 cells selected for stable expression of bovine CD11a/CD18 did not survive long enough in the selective media to be tested.

Figure 13 (A), (B), and (C) exhibit the fluorescent confocal, transmitted, and overlay images of fluorescent K562 cells co-transfected with human CD11a and CD18 when detected with H20A MAb 48 hrs post-transfection. The fluorescence intensity of the images indicated some level of transient surface expression of human CD11a/CD18. Panels (D), and (E) along with (F), and (G) represent the confocal, and transmitted images of K562 cells transfected with either human CD11a or CD18 alone when detected with H1III, and H20A MAbs, respectively. The control K562 cells transfected with human CD11a or human CD18 recombinant vectors alone did not fluoresce as expected. Similar to the other attempts, the co-transfected K562 cells selected for stable expression of human CD11a/CD18 did not survive long enough in the selective media to be tested after multiple attempts.

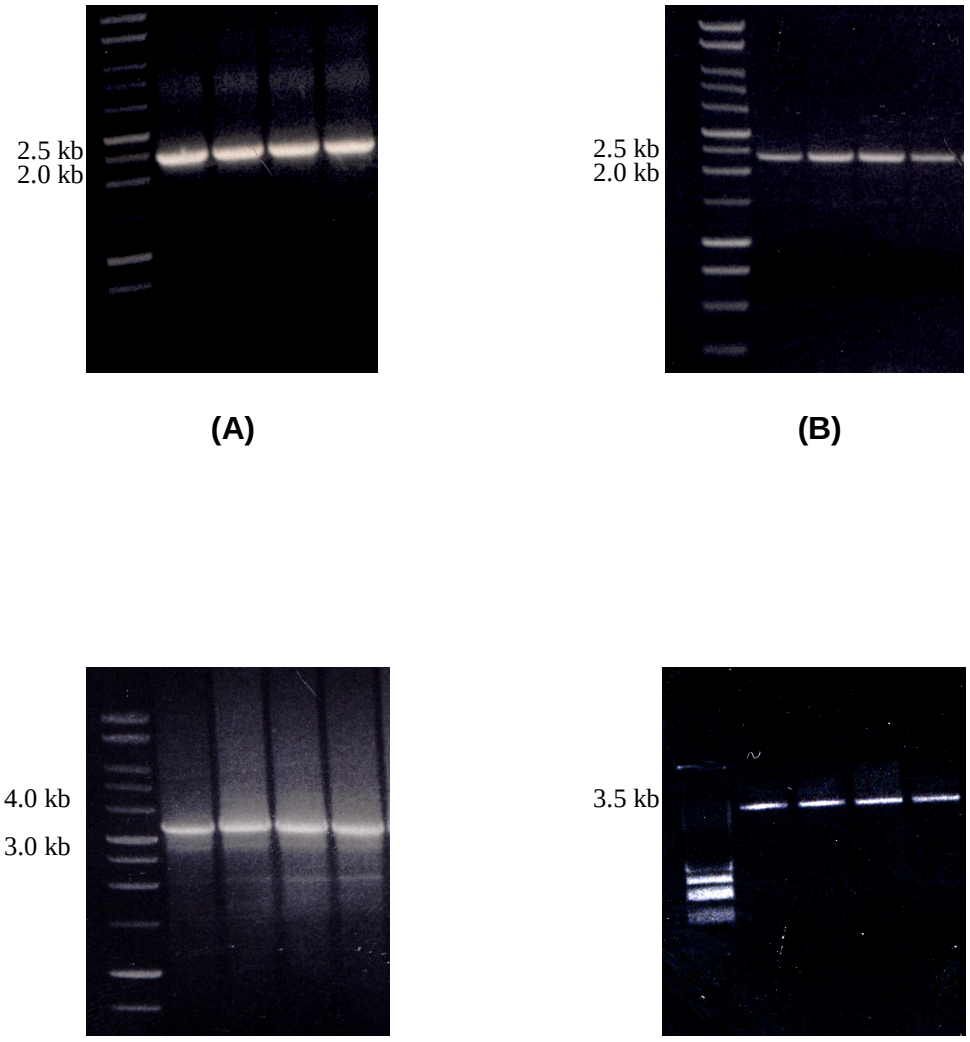


Figure 1. **0.6% agarose gels stained with ethidium bromide with amplification products** for bovine CD18 (panel **A**), human CD18 (panel **B**), bovine CD11a (panel **C**), and human CD11a (panel **D**). All products were of expected size: CD18 (~ 2400 b.p.), CD11a (~ 3500 b.p.). Lane 1 in all the panels is 1.0 kb ladder.

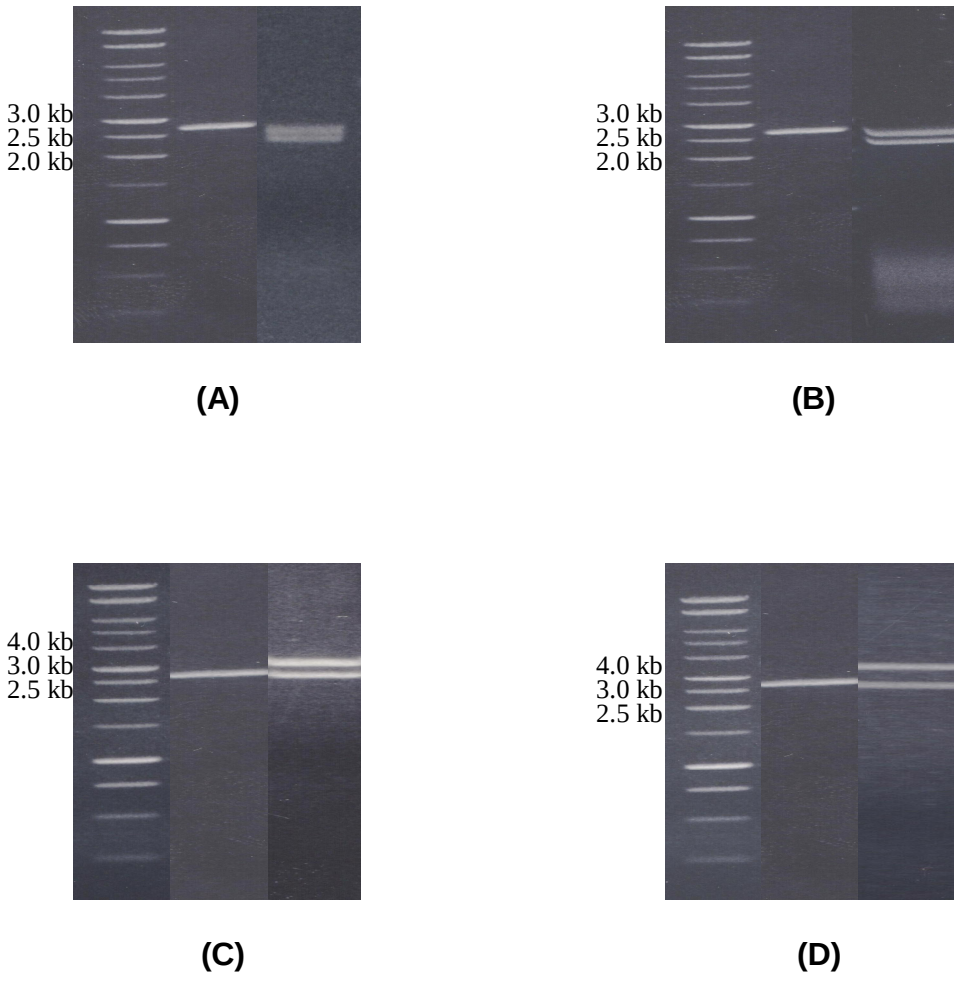


Figure 2. **Cloning of inserts into pUC19 cloning vector.** All panels are 0.6% agarose gels stained with ethidium bromide. Lane 1 & 2 in all the panels is 1.0 kb ladder and linearized pUC19 with *HindIII*and *XbaI,* respectively. Panel **A**, lane 3 is digested pUC19 and bovine CD18; Panel **B**, lane 3 is digested pUC19 and human CD18; Panel **C**, lane 3 is digested pUC19 and bovine CD11a; Panel **D**, lane 3 is digested pUC19 and human CD11a with *HindIII* and *XbaI*.

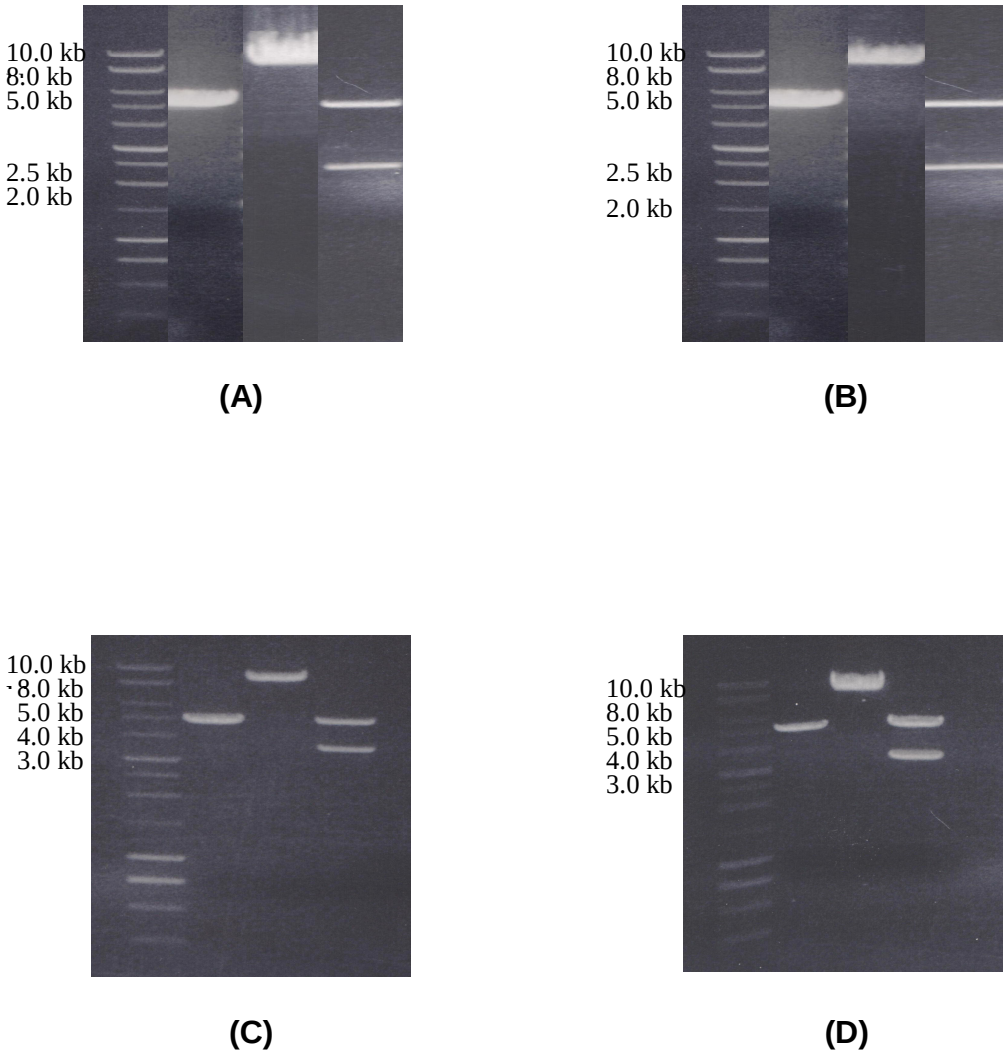
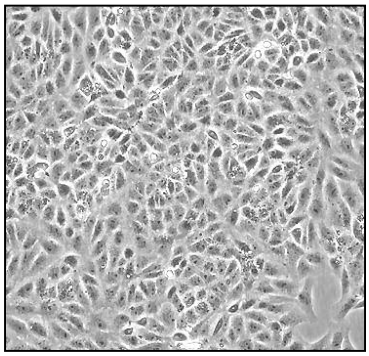
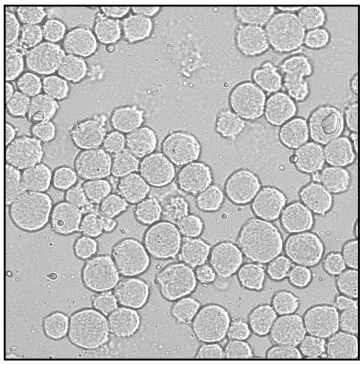


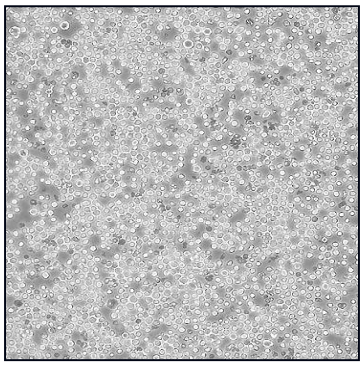
Figure 3. **Subcloning of inserts into pcDNA3.1 expression vectors**. All panels are 0.6% agarose gels stained with ethidium bromide Panel **A**, lane 4 is digested pcDNA3.1/Neo and bovine CD18; Panel **B**, lane 4 is digested pcDNA3.1/Neo and human CD18; lane 1, 2 & 3 in panel **A** & **B** are 1.0 kb ladder, linearized pcDNA3.1/Neo with *HindIII* & *XbaI* and linearized pcDNA3.1/Neo + bovine or human CD18, respectively. Panel **C**, lane 4 is digested pcDNA3.1/Zeo and bovine CD11a; Panel **D**, lane 4 is digested pcDNA3.1/Zeo and human CD11a; lane 1, 2 & 3 in panel **C** & **D** are 1.0 kb ladder, linearized pcDNA3.1/Zeo with *HindIII* & *XbaI* and linearized pcDNA3.1/Zeo + bovine or human CD11a, respectively.



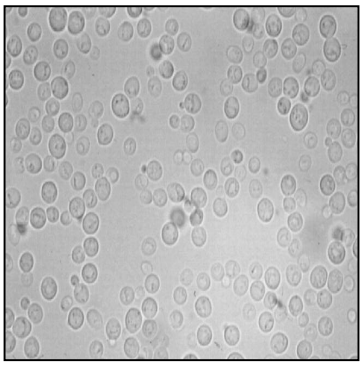
(A)



(B)



(C)



(D)

Figure 4. **Morphology of the cell lines used in this study. (A)** Vero cells, adherent, fibroblast-like; (B) K562 cells, suspension, lymphoblast; (C) HL-60 cells, suspension, myeloblastic; (D) BL3 cells, suspension, lymphoblast. All the cell lines have been photographed at 20X.

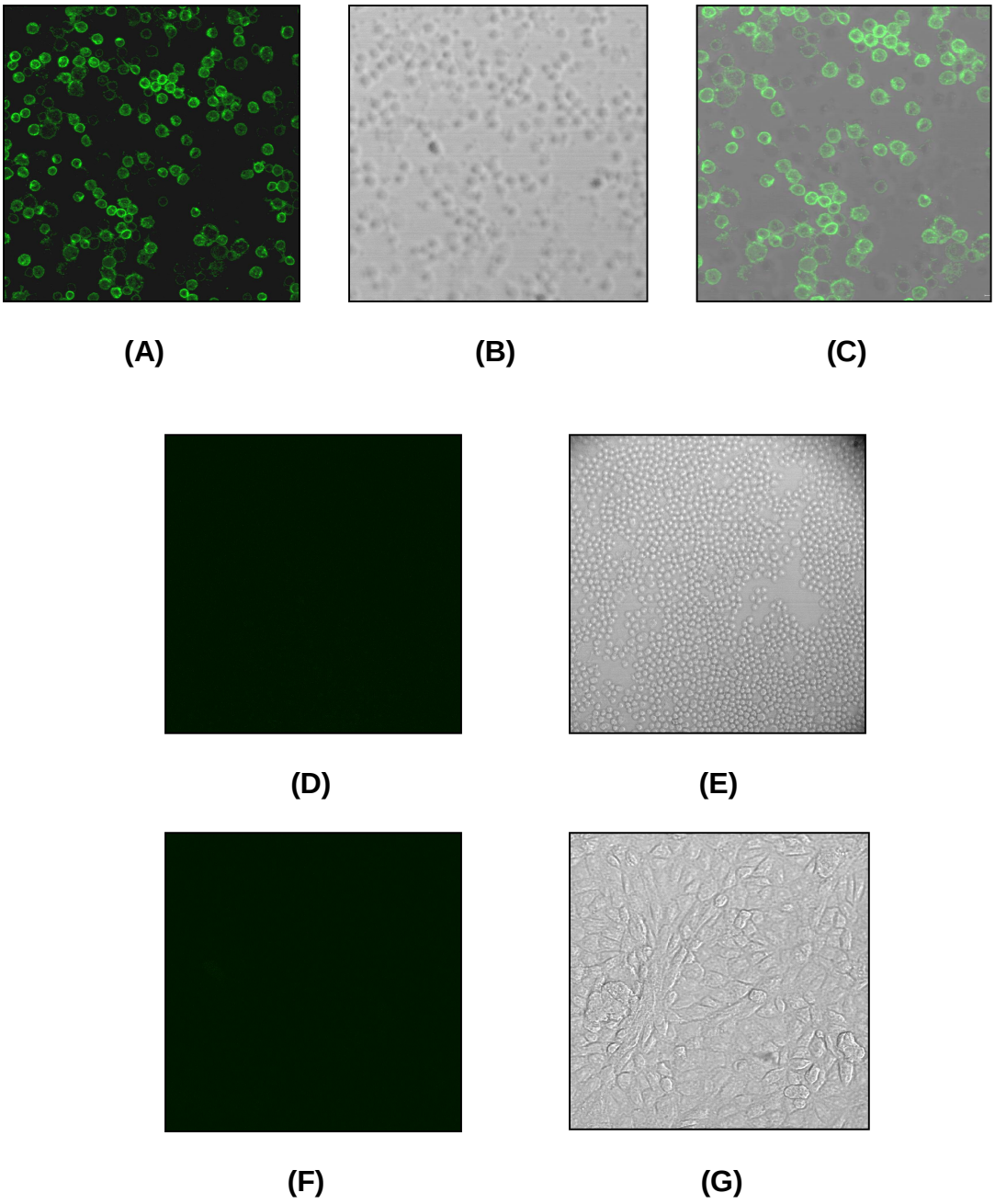


Figure 5. **Confocal microscopy images of positive and negative control cells exhibiting surface expression of β_2 integrins. (A)**, **(B), and (C)** Confocal, transmitted, and overlay images of bovine PBMCs (positive control) exhibiting CD11a/CD18 surface expression. **(D), and (E)** Confocal, and transmitted images of wild type K562 cells exhibiting no CD11a/CD18 expression. **(F), and (G)** Confocal, and transmitted images of wild type Vero cells exhibiting no CD11a/CD18 expression. Anti CD11a/CD18 MAb (BAT75A) was used to detect the cell surface expression.

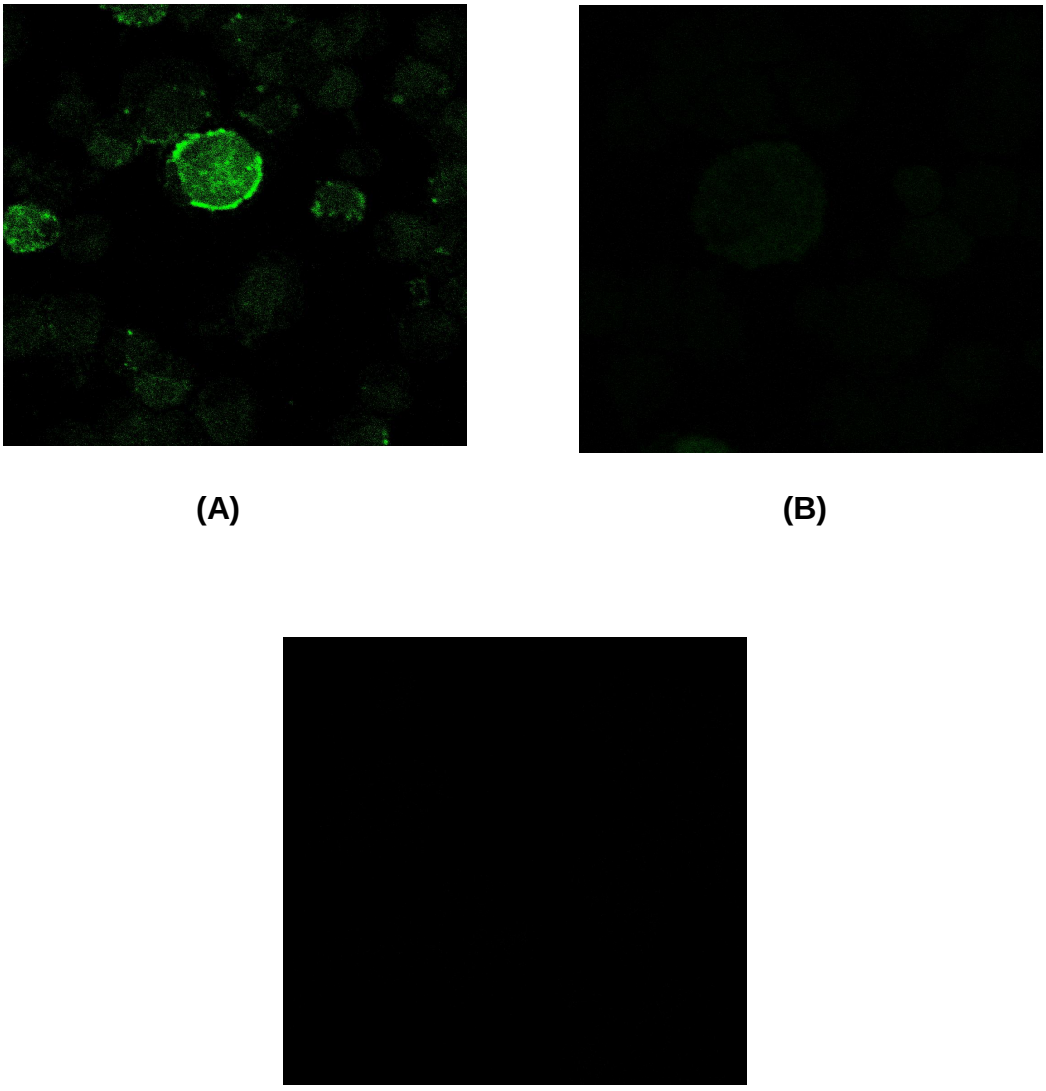


Figure 6. **Confocal microscopy images of Vero cells exhibiting surface expression of bovine CD18.** Vero cells were transfected with bovine CD18 recombinant vector alone and selected for stable expression with geneticin: **(A)** Stable cell surface expression of bovine CD18 detected with BAQ30A MAb. **(B)** No surface expression of bovine CD18 detected with non-specific mouse IgG (MOPC21). **(C)** Wild type Vero Cells showing no bovine CD18 expression as detected with BAQ30A MAb. Results of experiment with one representative clone out of three are shown.

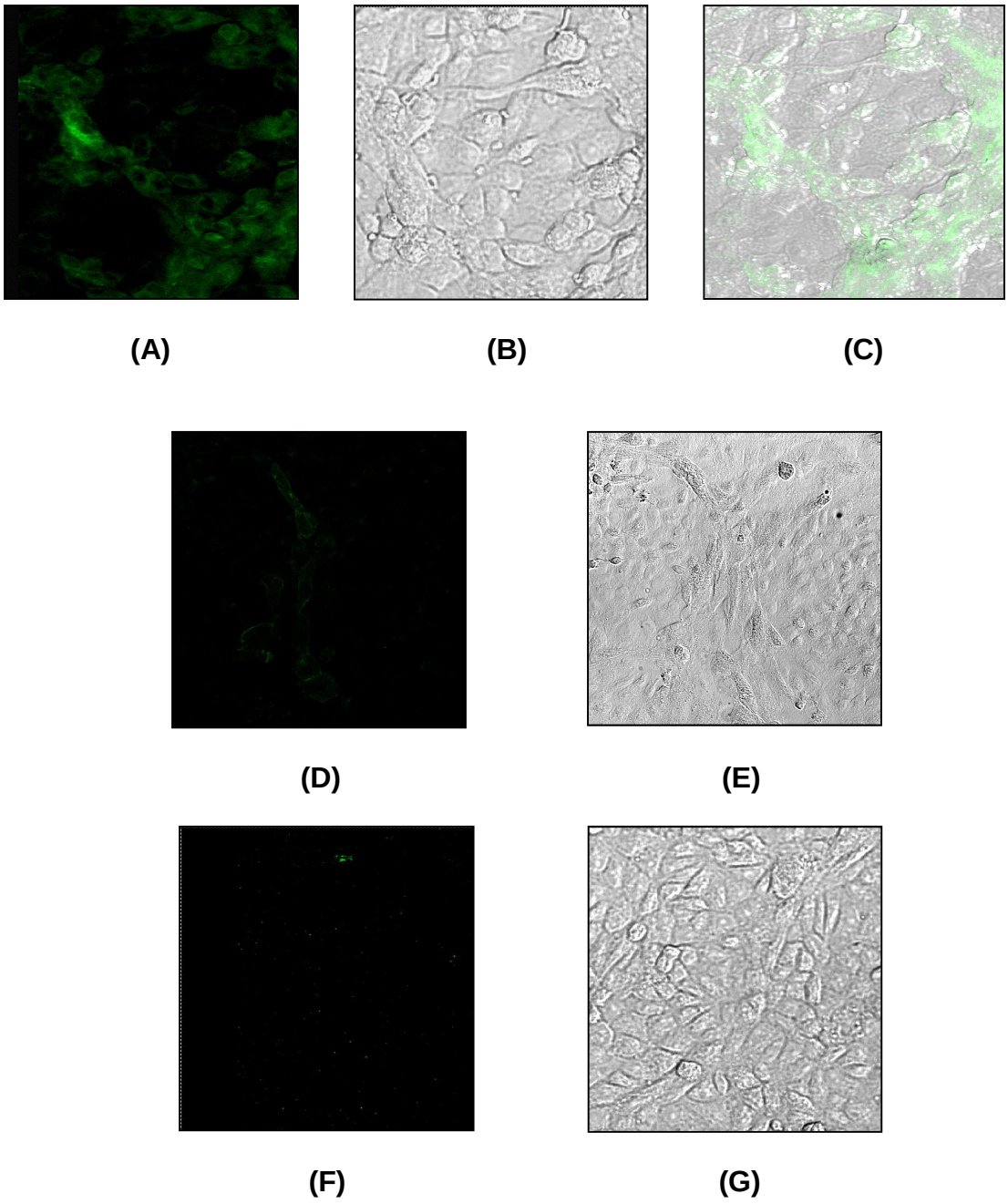


Figure 7. **Confocal microscopy images of Vero cells exhibiting surface expression of chimeric human CD11a/bovine CD18.** Vero cells clones expressing bovine CD18 were transfected with human CD11a recombinant vector to augment the surface expression of bovine 18. **(A), (B), and (C)** Confocal, transmitted, and overlay images of surface expression of bovine CD18 detected with BAQ30A MAb. **(D), and (E)** No surface expression of human CD11a on Vero cells transfected with human CD11a alone and detected with H1III MAb. **(F), and (G)** No surface expression of bovine CD18 on Vero cells transfected with bovine CD18 alone and detected with BAQ30A MAb.

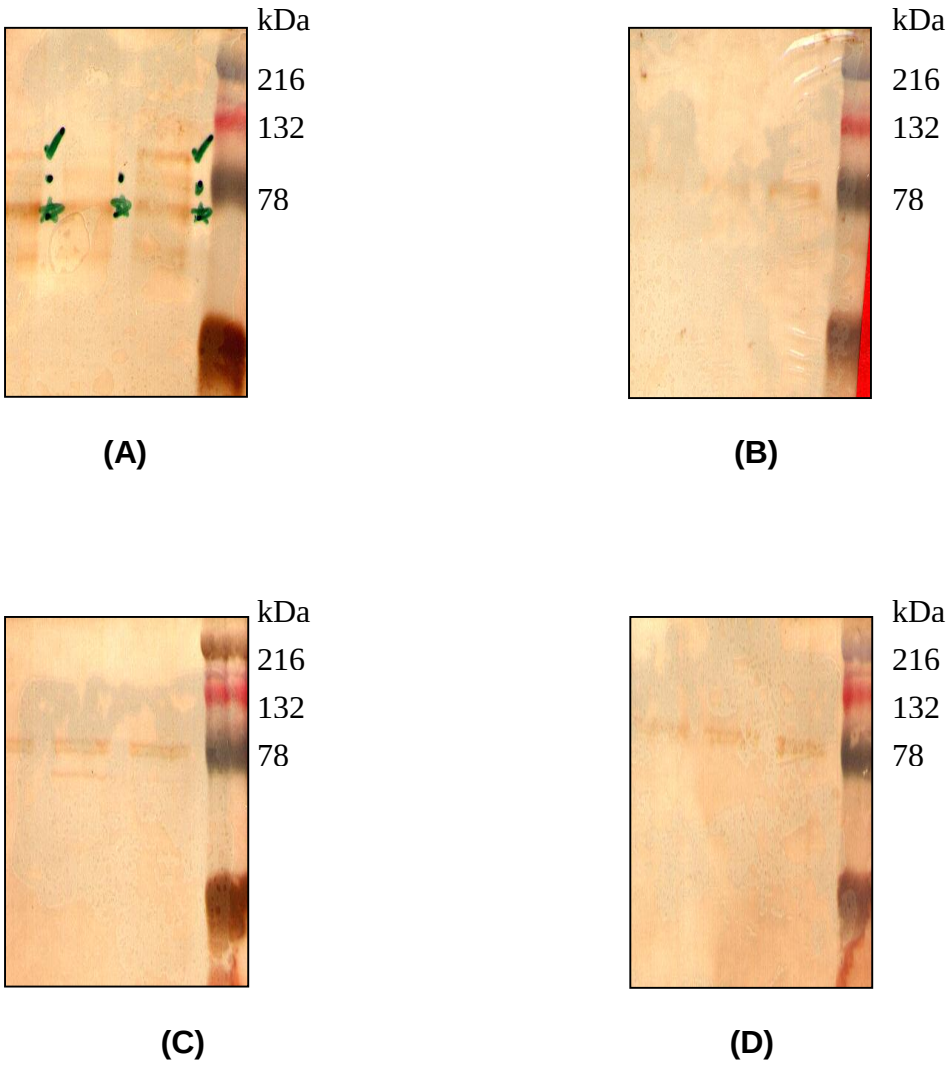


Figure 8. **Western blots showing the surface expression of bovine CD18 on transfected Vero cells.** The transfectants (Vero cells transfected with bovine CD18 alone, i.e., clones 1, 2 & 3) membrane extracts in Triton X 100 were mixed 1:5 (v/v) with 6X non-reducing SDS-PAGE sample buffer and subjected to SDS-PAGE on 8% gels. Following transfer to nitrocellulose membranes, the membranes were blocked with 3% non-fat dried milk, and sequentially incubated with 1:500 BAQ30A (Panel **A**), BAT75A (Panel **B**), MOPC-21 (Panel **C**) MAbs, and PBS (Panel **D**) for 3h, 1:1000 biotin conjugated rabbit anti-mouse IgG for 1h and 1:2000 freshly prepared peroxidase conjugate for 30 minutes at room temperature. Following incubations, the bound peroxidase was developed with 3, 3'-diaminobenzidine (DAB) substrate. Lanes 1, 2 and 3 in all the panels are membrane extracts of clones 1, 2 & 3, respectively. Lane 4 is the broad range protein molecular weight marker.

Treatment	% Viable Cells					
	BL3	Vero	Clone 1	Clone 2	Clone 3	
No LKT + No MAb	86 ±11	90 ± 4	86 ± 4	85 ± 2	93 ± 2	
LKT + No MAb	7 ± 2	87 ± 8	88 ± 6	89 ± 5	90 ± 1	
LKT + anti-bovineCD18 MAb (BAQ30A)	44 ±13	94 ± 3	92 ± 2	81 ± 6	87 ± 5	
LKT + murine irrelevant MAb (MOPC-21)	11 ± 3	85 ± 9	84 ± 4	82 ± 9	92 ± 2	

Table 8. **Inhibition of LKT-mediated cytolysis by anti-bovine CD18 MAb (BAQ30A) or murine irrelevant MAb.** BL3 cells (positive control), Vero cells (negative control) and Clones 1, 2 & 3 (Vero cells transfected with bovine CD18 recombinant vector alone) were incubated with 10 µg of the above mentioned MAb in 90 µl RPMI-1640 for 45 min at 37°C, prior to addition of 10 µl RPMI-1640 containing 0.1 µg LKT and an additional 45 min incubation. The exposed cells were mixed with trypan blue and number of viable cells enumerated by manual counting.

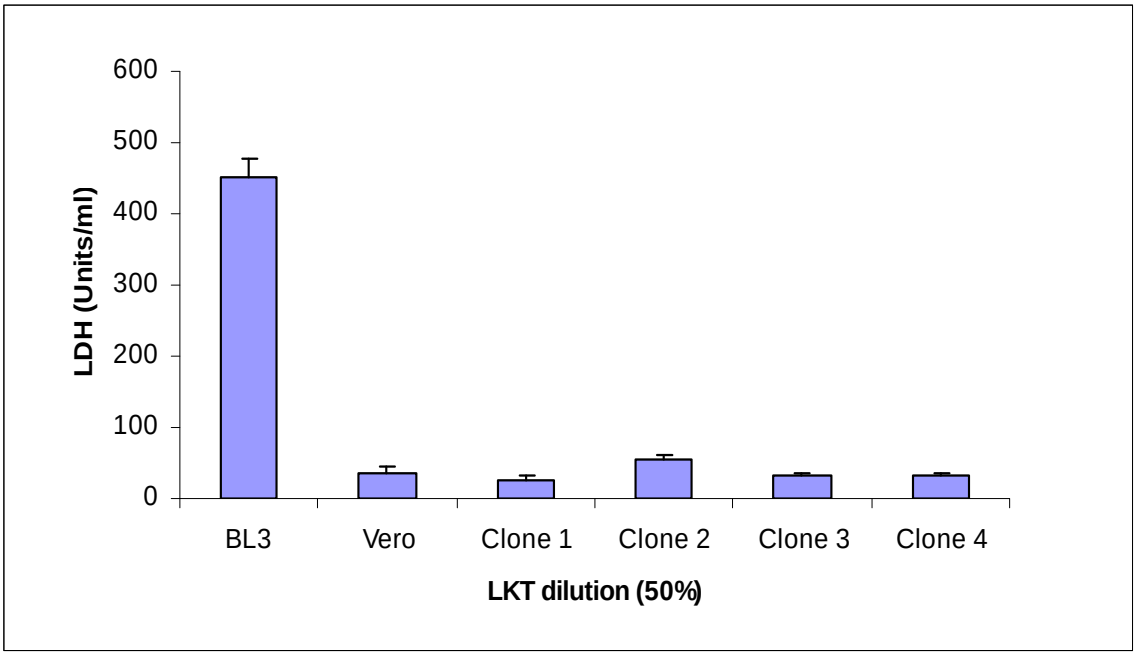
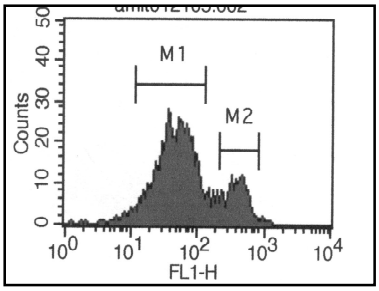
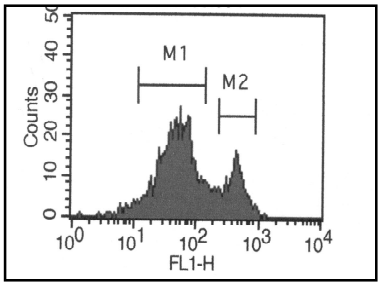


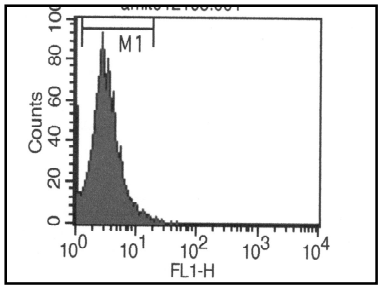
Figure 9. Leukotoxin-mediated lysis of BL3 cells (positive control), Vero cells (negative control) and Clones 1, 2, 3 & 4 (Vero cells transfected with bovine CD18 only) at 50% LKT dilution. The assay was done in triplicate and the error bars indicate standard deviations of the means.


M1
M2
Counts
FL1-H

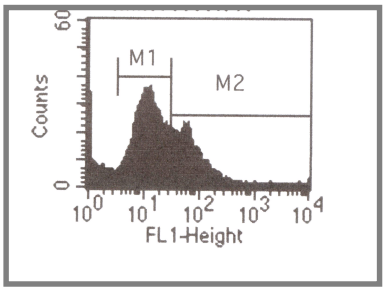
(A)


M1
M2
Counts
FL1-H

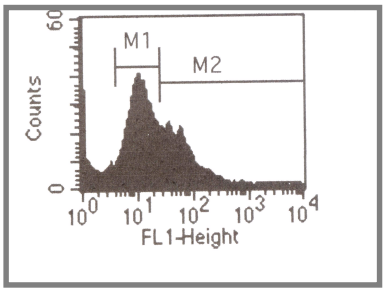
(B)


M1
Counts
FL1-H

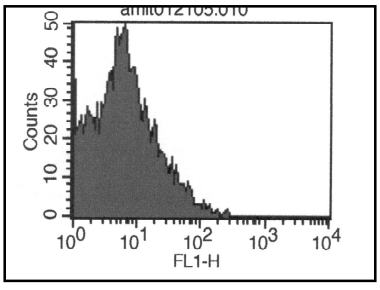
(C)


M1
M2
Counts
FL1-Height

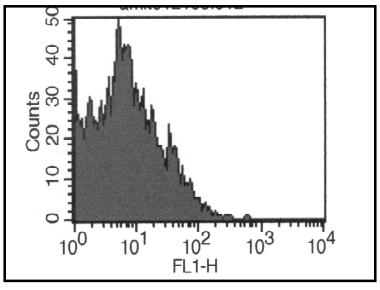
(D)


M1
M2
Counts
FL1-Height

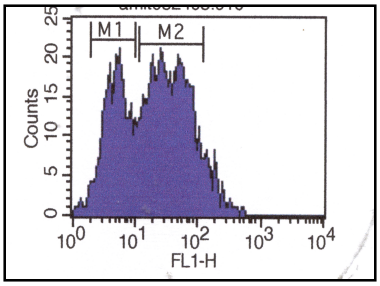
(E)


Counts
FL1-H

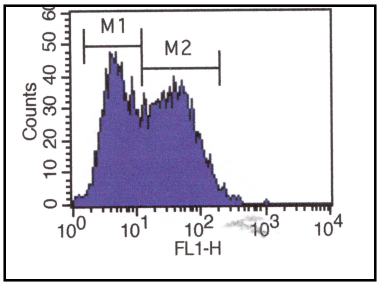
(F)


Counts
FL1-H

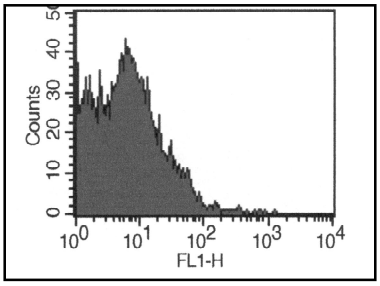
(G)



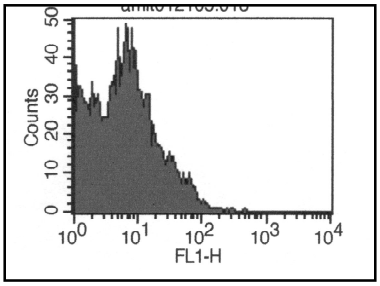
(H)



(I)



(J)



(K)

Figure 10. **Flow histograms showing the cell surface expression of bovine and human β_2 integrins in transfected K562 cells 48 hrs post-transfection. (A), and (B)** PBMCs (positive control) expressing bovine CD11a/CD18 as detected by BAT75A, and BAQ30A MAbs, respectively. **(C)** Wild type K562 negatively expressing CD11a/CD18 as detected by BAT75A MAb. **(D), and (E)** K562 cells transfected with bovine CD11a and CD18 expressing bovine CD11a/CD18 as detected by BAT75A, and BAQ30A MAbs, respectively. **(F)** K562 cells transfected with bovine CD11a alone depicting no expression as detected by H1III MAb. **(G)** K562 cells transfected with bovine CD18 alone depicting no expression as detected by BAQ30A MAb. **(H), and (I)** K562 cells transfected with human CD11a and CD18 expressing human CD11a/CD18 as detected by H20A, and H1III MAbs, respectively. **(J)** K562 cells transfected with human CD11a alone depicting no expression as detected by H1III MAb. **(K)** K562 cells transfected with human CD18 alone depicting no expression as detected by H20A MAb.

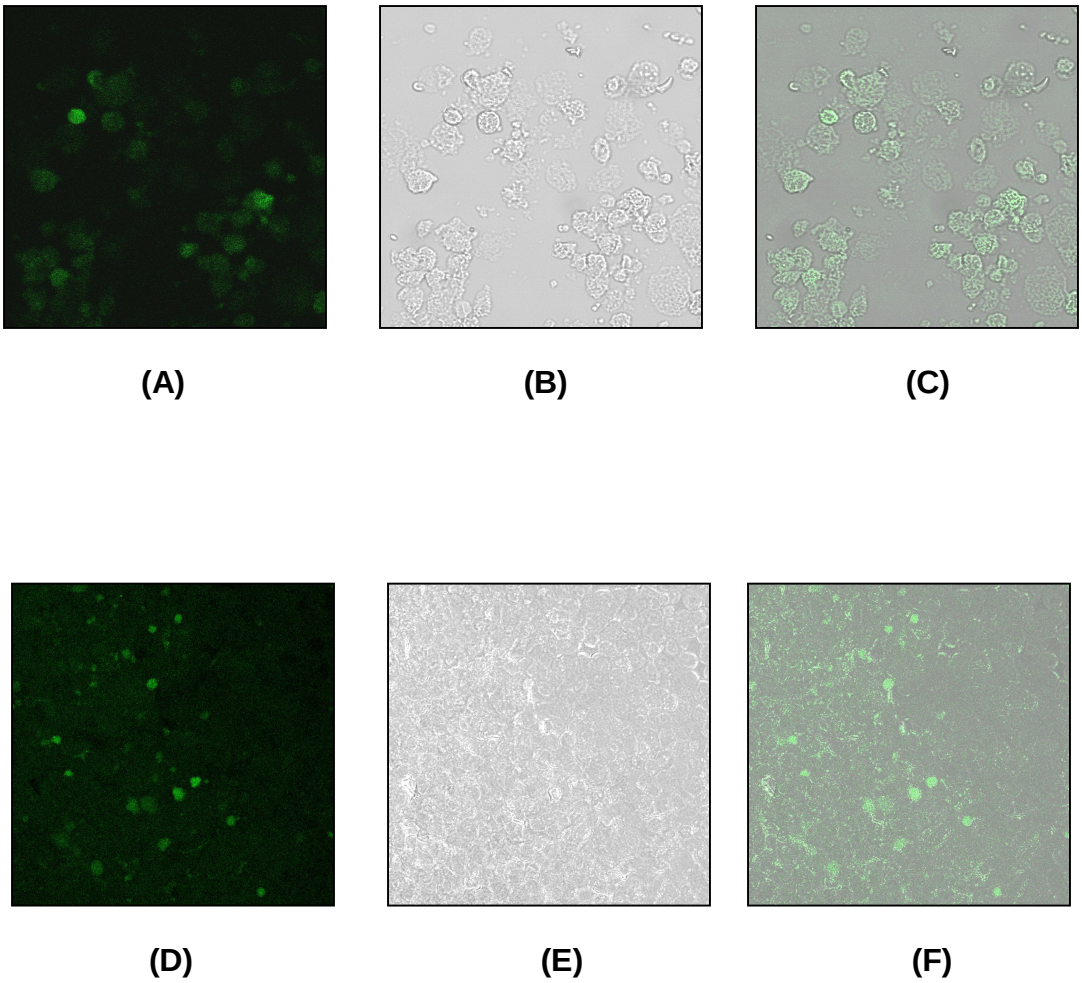


Figure 11. **Confocal microscopy images of K562 cells exhibiting rescued surface expression of bovine and human CD11a/bovine CD18 48 hrs posttransfection.** K562 cells clones living in the selective medium but not expressing bovine CD18 were transfected with bovine as well as human CD11a recombinant vector to rescue the surface expression of bovine CD18. **(A), (B), and (C)** Confocal, transmitted, and overlay images of surface expression of bovine CD18 rescued by bovine CD11a as detected with BAT75A MAb. **(D), (E), and (F)** Confocal, transmitted, and overlay images of surface expression of bovine CD18 rescued by human CD11a as detected with BAQ30A MAb.

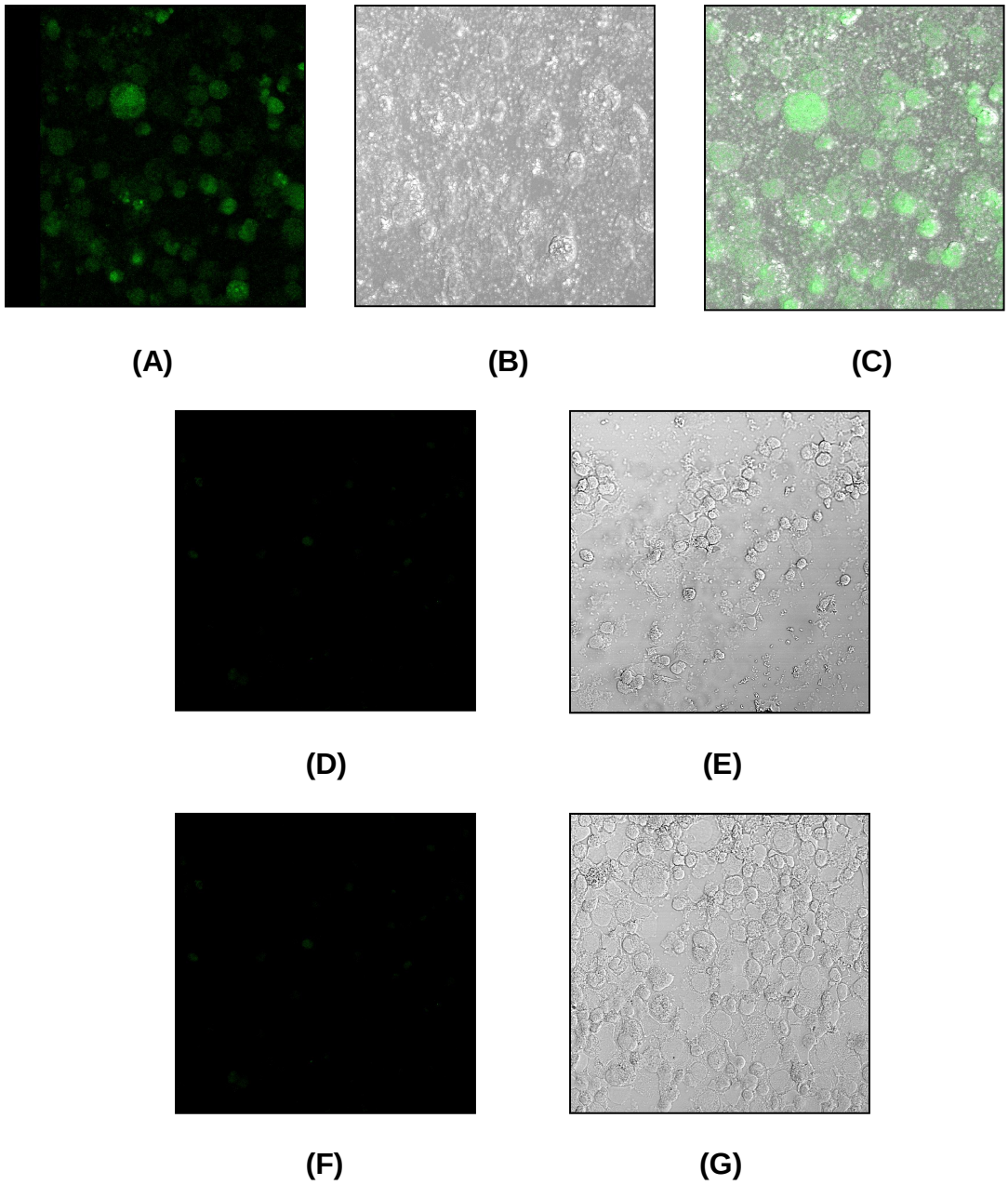


Figure 12. **Confocal microscopy images of K562 cells exhibiting co-expression of bovine CD11a/CD18.** K562 cells were co-transfected with bovine CD11a and CD18 recombinant vectors to detect the transient surface expression of bovine CD11a/CD18 48 hrs posttransfcetion. **(A), (B), and (C)** Confocal, transmitted, and overlay images of surface expression of bovine CD11a/CD18 detected with BAT75A MAb. **(D), and (E)** Confocal, and transmitted images of negative surface expression of bovine CD11a on K562 cells transfected with bovine CD11a alone and detected with H1III MAb. **(F), and (G)** Confocal, and transmitted images of negative surface expression of bovine CD18 on K562 cells transfected with bovine CD18 alone and detected with BAQ30A MAb.

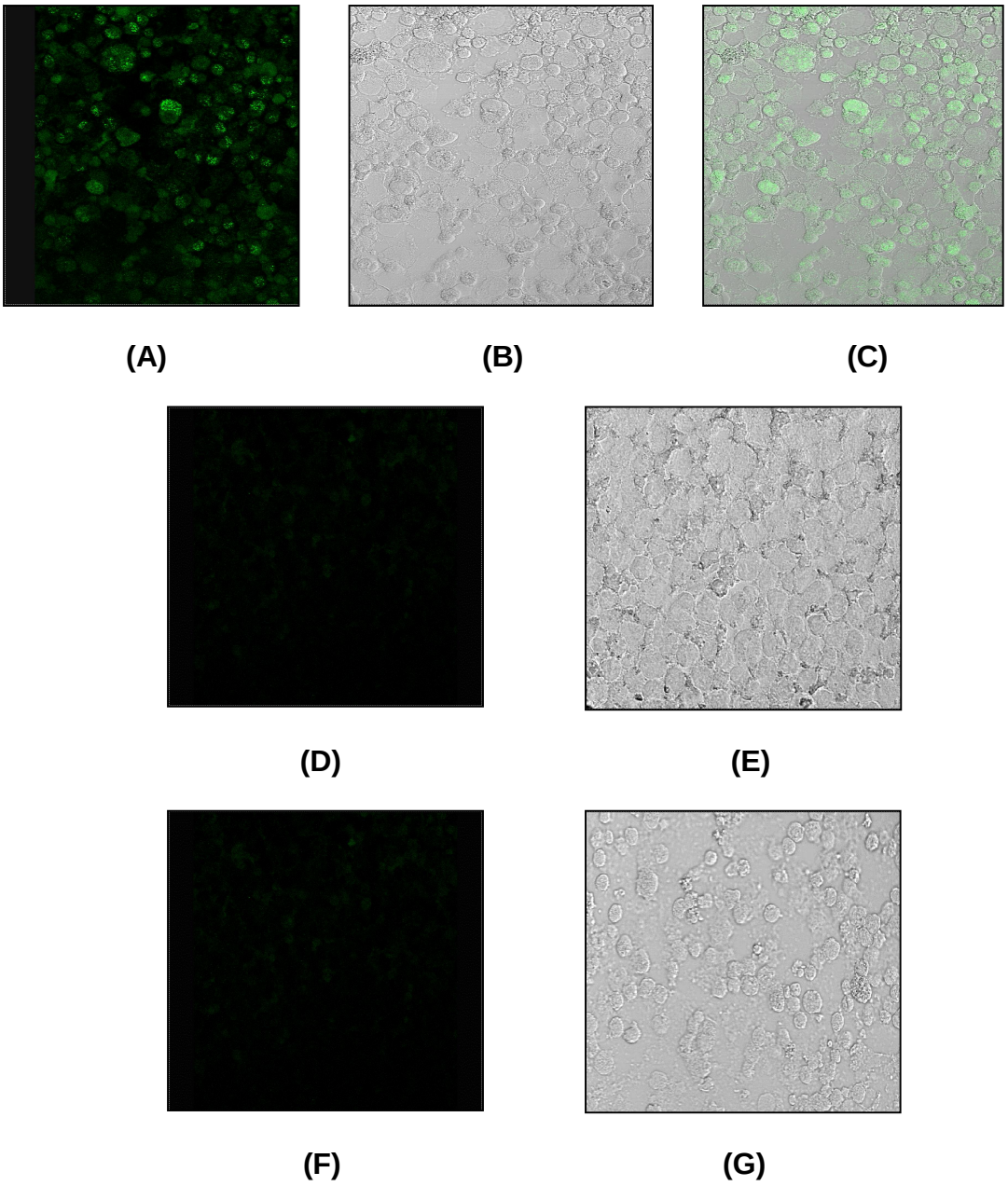


Figure 13. **Confocal microscopy images of K562 cells exhibiting co-expression of human CD11a/CD18.** K562 cells were co-transfected with human CD11a and CD18 recombinant vectors to detect the transient surface expression of human CD11a/CD18 48 hrs posttransfcetion. **(A), (B), and (C)** Confocal, transmitted, and overlay images of surface expression of human CD11a/CD18 detected with H20A MAb. **(D), and (E)** Confocal, and transmitted images of negative surface expression of human CD11a on K562 cells transfected with human CD11a alone and detected with H1III MAb. **(F), and (G)** Confocal, and transmitted images of negative surface expression of human CD18 on K562 cells transfected with human CD18 alone and detected with H20A MAb.

Chapter 4

General Discussion and Conclusions

Mannheimia hemolytica is the etiological agent for an economically important pneumonic disease of cattle that causes almost a billion dollars in annual losses to the beef cattle industry in North America (Whiteley *et al*., 1992). A critically important virulence factor for this organism is leukotoxin, an exotoxin elaborated by *M. hemolytica* that destroys bovine immune cells sent to protect the host. Data generated by several investigators strongly suggests an important role of $ß_2$ integrins as receptor for binding and subsequent toxicity secondary to *M. hemolytica* LKT (Ambagala *et al*., 1999; Jeyaseelan *et al*., 2000; Li *et al*., 1999; Wang *et al*., 1998). Based on their observation that MAbs specific for CD11a/CD18 and CD18 inhibited LKT-mediated apoptosis, Wang et al. (1998) were the first to report identification of $ß_2$ integrins as a receptor for the LKT. Identification of a 95-100 kDa protein by a MAb specific for bovine CD18 in the eluant from LKT-labelled beads, preincubated with lysate from BL3 (bovine lymphoma 3) cells, prompted these workers to suggest that bovine CD18 is involved in LKT binding to bovine leukocytes. Binding of bovine CD18 isolated from BL3 cells by LKT in ligand blotting experiments, and partial inhibition of LKT-induced cytolysis by anti-CD18 (BAQ30A) or anti-CD11a/CD18 (BAT75A) MAbs, prompted Li *et al*. (1999) to propose bovine CD18 as a species-specific receptor for *M. hemolytica* LKT.

In other studies (Ambagala *et al*., 1999), four proteins were isolated from polymorphonuclear (PMN) cell lysates run over LKT-affinity columns. The proteins had relative molecular masses of 180, 170, 150, and 95 kDa. The amino acid sequence data clearly identified the 170-kDa band as CD11b. Furthermore, the PMN proteins eluted from the LKT-bound column reacted with MAbs specific for CD11a, CD11b, CD11c, and CD18 in a radioimmunoassay indicating that the proteins contained all the three α subunits (CD11a, CD11b, and CD11c) and the ß subunit (CD18) of $ß_2$ integrins. These results suggested that the CD18 subunit brought down all the three α subunits along with it when the cell lysate was passed through a column prebound with LKT. In addition, in a cytotoxicity inhibition assay, an anti-CD18 MAb reduced LKT-induced cytotoxicity of bovine PMNs by more than 50%. Taken together, these results indicated that *M. hemolytica* LKT binds to $ß_2$ integrins, most likely via CD18 (Ambagala *et al*., 1999; Li *et al*., 1999). However, a later study by Jeyaseelan *et al*. (2000), based on western blot analysis of proteins eluted from LKT-labelled beads and blocking experiments with integrin subunit-specific MAbs, concluded that LKT binds to CD11a and not to CD18.

This study was designed to resolve the discrepancy on the role of bovine CD18 in leukotoxin-induced cytolysis as reported in the literature. It was reasoned that the recombinant expression of an immunobiologically-active bovine CD18 with human or bovine CD11a on LKT-resistant cells and examination of the susceptibility to LKT-induced cytolysis would unequivocally determine the role of

bovine CD18 in *M. hemolytica* LKT-induced cytotoxicity. Examination of LKT-induced lysis of cells rather than binding of LKT to the cells was chosen for analysis since binding of LKT to bovine leukocytes is not specific. It has been demonstrated that leukotoxin also binds to some non-ruminant leukocytes without eliciting any effects (Jeyaseelan *et al*, 2000).

Toward the goal of characterizing the interaction between LKT and the putative host cell receptor CD18, the strategy was to establish stable transfected cell lines expressing bovine CD11a/CD18, human CD11a/CD18, or hybrid $ß_2$ integrins such as human CD11a/bovine CD18, bovine CD11a/human CD18 and to characterize their sensitivity to LKT. Coding sequences for bovine CD18, and human CD11a and CD18 were readily generated utilizing primers generated from published sequences. Sequence information for bovine CD11a initially was not available and obtaining the full length coding sequence was difficult and time-consuming. Although the bovine CD11a coding sequence was eventually generated, the probable pitfall apart from the ones mentioned in the results section in generating the bovine CD11a subunit might have been selection of incorrect primer combinations from the consensus sequences. Although consensus sequences are largely utilized for designing primers using the regions that are conserved across different species, it is not always certain that the designed consensus primers would yield the desired result. Atleast in this case, even three sets of consensus primers did not generate a full length bovine CD11a sequence that had reasonable homology with CD11a sequences from

other species. Furthermore, when alignment of the consensus primers with the full length coding sequence of bovine CD11a published by Fett *et al*. (2004) was attempted, none of the consensus primer sets aligned. This clearly implied that none of the consensus primer sets hybridized with bovine cDNA in the first place. Ultimately, the bovine CD11a coding sequence published by Fett *et al*. (2004) was used to design the primers with incorporation of *HindIII* & *XbaI* in the sense and antisense primers, respectively. This primer set finally yielded a product (~3500 b.p.) that matched the full length bovine CD11a coding sequence.

While working to acquire bovine CD11a coding sequence, the three coding sequences already in hand (bovine CD18, and human CD11a and CD18) were cloned in pUC19 and ligated into a pcDNA3.1 expression vector with either a Neomycin^R or Zeocin^R resistance cassette. According to the original plan, LKT-resistant K562 cells were selected to be transfected with different combinations of recombinant vectors on account of the relative ease of testing these cells with flow cytometric analysis and previous documentation of recombinant human CD11a/CD18 surface expression by Lally *et al*. (1997). However, multiple attempts to optimize transfection conditions in K562 cells with bovine CD18 recombinant vector alone failed. Therefore, Vero cells were chosen for expression on account of their known high stable recombinant protein expression characteristics. Thus, Vero cells were transfected with bovine and human CD18 recombinant vectors. Three Vero cell clones transfected with bovine CD18 survived in the selective (Geneticin^R) media and exhibited low levels of bovine

CD18 expression as determined by immunostaining and fluorescent confocal microscopy. None of the human CD18 clones isolated from the selective media exhibited any surface expression of CD18. As a matter of fact, any CD18 expression at this juncture was unexpected as CD18 is reportedly only expressed as a heterodimer with CD11 (Marlin *et al*., 1986), which was not co-transfected in this experiment. Therefore, the low levels of bovine CD18 expression as determined by immunostaining and fluorescent confocal microscopy was surprising.

The expression of bovine CD18 by Vero cells when transfected alone was paradoxical. Did bovine CD18 rescue a CD11 subunit? Did bovine CD18 heterodimerize with some other subunit in the endoplasmic reticulum of Vero cells, got transported and expressed on the cell surface? To answer these questions, we extracted the cell membranes from the three bovine CD18-expressing clones and tested the membrane extracts with anti-bovine CD18 (BAQ30A), anti-bovine CD11a/CD18 (BAT75A), and murine non-specific (MOPC-21) MAbs for the expression of bovine CD18 via western blotting. Two out of the three clones exhibited a band in the range of bovine CD18 (95-100 kDa) when detected with anti-bovine CD18 MAb (BAQ30A). Anti-CD11a/CD18 MAb (BAT75A) did not label the CD18-expressing clones. However, this might have been due to lack of specificity or CD11a expression. All subsequent attempts to reproduce the bovine CD18 product from these three Vero cell clones following passage were failures.

Although the bovine CD18 expression was certainly low, we tested the Vero cell clones for LKT-sensitivity with a trypan blue dye exclusion test and LDH release assay. Trypan blue dye exclusion test was conducted to assess lysis of the Vero cell clones when challenged with the LKT. To prove that the lysis was mediated through engagement of CD18, the cells were protected before LKT challenge with anti-bovine CD18 antibodies. None of the clones exhibited cell death that was significantly different from the parent Vero cells when incubated with the LKT alone. The anti-bovine CD18 MAb inhibited around 45-50% cytolysisof BL3 cells, whereas the cell viability among clones and parent Vero cells was no different from cells incubated with LKT alone.

LDH release assay is regarded as the 'gold standard' for testing the biological activity of bovine CD18 expressed by the Vero cells because it has been used for this purpose by other investigators (Sun et al., 2000; Murakami et al., 2002). Also, the LDH release assay is more sensitive to cell injury that does not result in cell death. This assay was utilized to elucidate the impact of LKT-induced cytolysis on CD18 expressing cells following incubation with 0.5 U/ml of the LKT preparation by measurement of intracellular lactate dehydrogenase (LDH) leakage. The amount of LDH leakage was directly proportional to concentration of LKT used and the expression of bovine CD18 on target cells. Sun *et al*. (2000) showed the lytic effects of 0.1 and 1.0 U/ml of a LKT preparation on bovine lymphocytes. We chose 0.5 U/ml concentration of the LKT to enable the LKT to bind and lyse the bovine CD18 expressing Vero cell clones. Except for the

positive control BL3 cells, none of the clones and/or the parent Vero cells exhibited LDH leakage due to LKT treatment .

The results of the trypan blue dye exclusion test and LDH release assay suggested several possibilities. First, the equivocal CD18 expression raised the possibility of LKT not binding to the cloned CD18 molecule or that CD18 expression with CD11 as a heterodimer is critical for LKT: receptor interaction that results in cell death. Furthermore, we could not rule out factors independent of CD18 expression in the resistance of Vero cells to the LKT. One such factor could be the sturdiness of these cells that makes them inherently resistant to the LKT even though they are forced to express the CD18 molecule. At the end of studies conducted with the Vero cells, there were still many unanswered questions such as why was bovine CD18 apparently expressed alone? Did the bovine CD18 subunit rescue a monkey CD11 subunit in the Vero cells? Although Vero cells are derived from the kidneys of African green monkeys, these cells have been documented to be devoid of any monkey CD11/CD18 expression on their surface.

Subsequently, attempts to rescue or augment the low expression of CD18 by transfecting the Vero cell clones with human CD11a recombinant vector as a full length bovine CD11a sequence had not yet been acquired. The Vero cell clones transfected with human CD11a subunit showed an increased transient expression of the chimeric heterodimer human CD11a/bovine CD18 48 hrs

following transfection as detected by immunostaining and confocal microscopy. The clones selected 48 hrs post-transfection for stable expression of human CD11a/bovine CD18 with Geneticin^{R} and Zeocin^{R} did not survive long enough in the selective media to be tested. The death of the clones in the selective media might have been due to the coupled toxic effect of the antibiotics. Another possibility might have been that some Vero cell clones may have not received the human CD11a recombinant vector cells, and would have ultimate died due to the toxicity conferred by the other antibiotic.

The probable implications of LKT-sensitivity assessment conducted on low bovine-CD18 expressing Vero clones coupled with immunostaining and confocal microscopy results from the same Vero clones rescued with human CD11a subunit are as follows:

1) There was no real bovine CD18 surface expression on the Vero cell clones.
2) Bovine CD18 expression alone might not have been recognized by the LKT. Even if the CD18 expression was recognized, it was not sufficient for LKT-mediated cytolysis.
3) Expression of CD11 subunit with CD18 as a heterodimer is probably required for LKT binding and subsequent cytotoxicity. This further implies that the CD11 subunit might be directly or indirectly involved in the mediation of toxicity, but CD11 is necessary for the proper conformation

and recognition of the heterodimer by the LKT, for binding and following cytotoxicity.

4) Even though the LKT bound to bovine CD18, the probable inherent vitality of the Vero cells would have made them resistant to the cytolytic effects of the LKT.

The Vero clones were not tested any further and were frozen. For all the subsequent transfections, K562 cells were chosen for their non-adherent nature and the relative ease of testing them with flow cytometric analysis. Also, there was documented evidence of these cells being invoved in the recombinant expression of human CD11a/CD18 (Lally et al., 1997). *As per* expectation and the published literature, K562 cells transfected with the bovine CD18 subunit alone and stably selected with Geneticin^{R} did not show any bovine CD18 using flow cytometry. By this time, successful generation of the full length coding sequence of bovine CD11a was accomplished. These non-bovine CD18 expressing clones when rescued with bovine and human CD11a subunits exhibited transient expression of the heterodimer as detected by immunostaining and confocal microscopy. Whereas these results were promising, the transient expression technique did not reliably produce enough cells for the necessary downstream experiments of trypan blue dye exclusion test, LDH release assay or any other cell viability/cytotoxicity assays.

Therefore, in continuing attempts to generate stably expressing clones, the K562 cells were co-transfected with bovine CD11a and CD18 as well as human CD11a and CD18 subunits as originally planned. The transient expression experiments consistently showed some expression of the expected heterodimers, whereas the generation of stable expressing clones still remained elusive. While it is acknowledged that the stable expression of CD11a/CD18 by the co-transfected cells might not have been achieved due to their limited survival in the selection antibiotics, the low level of the transient heterodimeric expression was confusing. Since the co-transfected cells were propagated in antibiotic-free media and were tested for expression 48 hrs following transfection, there was no obvious confounding factor(s) that would corroborate the low levels of transient CD11a/CD18 expression. The probable reasons may have been the differences in copy number of the recombinant vectors in the transfected cell, and/or the presence of a similar CMV promoter in both pcDNA3.1/Neo and pcDNA3.1/Zeo. CMV, being a strong promoter competes for driving gene expression. This means that the CMV promoters of pcDNA3.1/Neo and pcDNA3.1/Zeo would have competed with one another resulting in decreasing or completely shutting of each other's capability to drive their specific gene expressions, and ultimately, the expression of the heterodimer.

To augment the transient expression of CD11a/CD18, pBUDCE4.1 (Catalog # V532-20, Invitrogen Inc.) was acquired. The pBudCE4.1 vector was specifically designed for the transient expression of two genes from a single plasmid in

mammalian cells. Using pBudCE4.1 to generate transient mammalian expression cell lines would have ensured that there was an equivalent copy number of each gene in the transfected cell. This could have eliminated the variable expression due to differences in gene copy number and the presence of non-competing promotors. pBudCE4.1 had built-in expression cassettes with the following features:

- The CMV promoter for high-level transcription of genes.
- The human EF-1α promoter for high-level expression of genes.
- The *Sh ble* (ZeoR) gene for efficient selection in mammalian cells with the selection agent Zeocin^{R}.
- Two multiple cloning regions with different sets of restriction sites for the directional cloning of two genes.

When recombinant pBUDCE4.1 was being constructed, the process of generating bovine CD11a coding sequence was still incomplete. As a result, a human CD11a sequence was incorporated using *HindIII* and *XbaI* restriction sites into one of the multiple cloning regions and human CD11a expression was supposed to be driven by CMV promoter. Further, the same primer set that was designed for bovine CD18 earlier was used, except that instead of *HindIII* and *XbaI*, *Csp45 I* and *Sfi I* restriction sites were incorporated into the sense and antisense primers, respectively for directional cloning. The PCR generation of

bovine CD18 with upstream *Csp45 I* and downstream *Sfi I* went smoothly. Extreme difficulty in incorporating this newly generated gene into the other multiple cloning region of pBUDCe4.1 vector that already had human CD11a was encountered. Multiple efforts to accomplish this met with failure.

As an alternate strategy, we tried to incorporate bovine CD18 first followed by human CD11a. All such efforts failed. Bovine CD18 apparently would not insert into pBUDCE4.1. The possibility of using restriction sites other than *Csp45 I* and *Sfi I*, or using *HindIII* and *XbaI* for bovine CD18 and some other restriction sites for human CD11a was explored. Due to limited options of the restriction sites present in the two multiple cloning sites, all such possibilities were thwarted. Therefore, no further attempts to generate a recombinant pBUDCE4.1 vector were made. In the mean time, some more unsuccessful efforts and time was spent in generating stable CD11a/CD18 expressing clones using bovine and human CD11a and CD18 recombinant vectors.

Future Directions:

Following generation and testing of the K562 clones that stably express an immunobiologically active heterodimer (bovine CD11a/CD18, and/or human CD11a/bovine CD18), the extracellular domain of LKT-sensitive bovine CD18 that mediates LKT binding and subsequent cytotoxicity can be swapped with the

extracellular domain of LKT-resistant human CD18. This chimeric CD18 subunit could again be expressed in K562 cells as a heterodimer with bovine or human CD11a. The expressed heterodimer would supposedly be immunologically active, *i.e.*, its expression would be detectable by monoclonal antibodies, but would be resistant to the LKT-mediated cytolysis. The coding sequence of such LKT-resistant hybrid CD18 molecule could further be used to transfect bovine primordial stem cells in preparation for cloning an animal that is genetically-resistant to the leukolytic action of *M. hemolytica* LKT.

An alternative approach can be to resolve the crystal structure of the LKT-binding region on the extracellular domain of bovine CD18. This would enable elucidation of the pertinent amino acids or more precisely the nucleotides in the bovine CD18 extracellular domain that might be involved in LKT binding. These amino acids or nucleotides can further be used as potential drug targets to prevent LKT binding, cytolysis and ultimately the animal mortality.

References

1. **Ackermann M.R., and K.A. Brogden.** 2000. Response of the ruminant respiratory tract to *Mannheimia* (*Pasteurella*) *hemolytica*. Microbes Infect. **2**(9): 1079-88.
2. **Ackermann M.R., K.A. Brogden, A.F. Florance, and M.E. Kehrli Jr.** 1999. Induction of CD18-mediated passage of neutrophils by Pasteurella haemolytica in pulmonary bronchi and bronchioles. Infect Immun. **67**(2): 659-63.
3. **Ackermann M.R., M.E. Kehrli Jr., and K.A. Brogden.** 1996. Passage of CD18- and CD18+ bovine neutrophils into pulmonary alveoli during acute Pasteurella haemolytica pneumonia. Vet Pathol. **33**(6): 639-46.
4. **Allen J.W., L. Viel, K.G. Bateman, S. Rosendal, and P.E. Shewen.** 1992. Cytological findings in bronchoalveolar lavage fluid from feedlot calves: associations with pulmonary microbial flora. Can J Vet Res. **56**(2): 122-6.
5. **Allen J.W., L. Viel, K.G. Bateman, and S. Rosendal.** 1992. Changes in the bacterial flora of the upper and lower respiratory tracts and bronchoalveolar lavage differential cell counts in feedlot calves treated for respiratory diseases. Can J Vet Res. **56**(3): 177-83.
6. **Ambagala T.C., A.P. Ambagala, and S. Srikumaran.** 1999. The leukotoxin of *Pasteurella hemolytica* binds to beta (2) integrins on bovine leukocytes. FEMS Microbiol Lett. **179**(1): 161-7.

7. **Ames T.R., R.J. Markham, J. Opuda-Asibo, J.R. Leininger, and S.K. Maheswaran.** 1985. Pulmonary response to intratracheal challenge with *Pasteurella hemolytica* and *Pasteurella multocida*. Can J Comp Med. **49**(4): 395-400.
8. **Angen O., R. Mutters, D.A. Caugant, J.E. Olsen, and M. Bisgaard.** 1999. Taxonomic relationships of the [*Pasteurella] hemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia hemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov. Int J Syst Bacteriol. **49** Pt 1: 67-86.
9. **Arnaout M.A.** 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood. **75**(5): 1037-50.
10. **Arnaout M.A.** 1990. Leukocyte adhesion molecules deficiency: its structural basis, pathophysiology and implications for modulating the inflammatory response. Immunol Rev. **114**: 145-80.
11. **Atwal O.S., K.J. Minhas, B.G. Ferenczy, D.S. Jassal, D. Milton, and V.G. Mahadevappa.** 1989. Morphology of pulmonary intravascular macrophages (PIMs) in ruminants: ultrastructural and cytochemical behavior of dense surface coat. Am J Anat. **186**(3): 285-99.
12. **Ayalew S., A.W. Confer, and E.R. Blackwood.** 2003. Characterization of immunodominant and potentially protective epitopes of Mannheimia

haemolytica serotype 1 outer membrane lipoprotein PlpE. Infect Immun. **72**(12): 7265-74.

13. **Babiuk L.A., P.J. Lewis, G. Cox, S. van Drunen Little-van den Hurk, M. Baca-Estrada, and S.K. Tikoo.** 1995. DNA immunization with bovine herpesvirus-1 genes. Ann N Y Acad Sci. **27; 772: 47**-63.
14. **Baluyut C.S., R.R. Simonson, J.J. Bemrick, and S.K. Maheswaran.** 1981. Interaction of *Pasteurella hemolytica* with bovine neutrophils: identification and partial characterization of a cytotoxin. Am J Vet Res. **42**(11): 1920-6.
15. **Billingham M.E.** 1987. Cytokines as inflammatory mediators. Br Med Bull. **43**(2): 350-70.
16. **Bolin S.R., and J.F. Ridpath.** 1992. Differences in virulence between two noncytopathic bovine viral diahhrea viruses in calves. Am J Vet Res. **53**(11): 2157-63.
17. **Botcher L., I. Stoter, and E. Hellmann.** 1993. In vitro adherence of *Pasteurella hemolytica* to tracheal mucin and a tracheal epithelial cell preparation from cattle. Berl Munch Tierarztl Wochenschr. **106**(10): 333-6.
18. **Breider M.A., and Z. Yang.** 1994. Tissue factor expression in bovine endothelial cells induced by Pasteurella haemolytica lipopolysaccharide and interleukin-1. Vet Pathol.**31** (1): 55-60.
19. **Brennan R.E., R.E. Corstvet, and D.B. Paulson.** 1998. Antibody responses to Pasteurella haemolytica 1:A and three of its outer membrane

proteins in serum, nasal secretions, and bronchoalveolar lavage fluid from calves. Am J Vet Res. **59**(6): 727-32.

20. **Brogden K.A., C. Adlam, H.D. Lehmkuhl, R.C. Cutlip, J.M. Knights, and R.L. Engen.** 1989. Effect of *Pasteurella hemolytica* (A1) capsular polysaccharide on sheep lung in vivo and on pulmonary surfactant in vitro. Am J Vet Res. **50**(4): 555-9.
21. **Brown J.F., F. Leite, and C.J. Czuprynski.** 1997. Binding of *Pasteurella hemolytica* leukotoxin to bovine leukocytes. Infect Immun. **65**(9): 3719-24.
22. **Bryson D.G., M.S. Mcnulty, H.J. Ball, S.D. Neill, T.J. Connor, and P.F. Cush.** 1979. The experimental production of pneumonia in calves by intranasal inoculation of parainfluenza type III virus. Vet Rec. **105**(25-26): 566-73.
23. **Burriel A.R.** 1997. Isolation of *Pasteurella hemolytica* from grass, drinking water, straw bedding used by sheep. Curr Microbiol. **35**(5): 316-8.
24. **Chang Y.F., R. Young, D. Post, and D.K. Struck.** 1987. Identification and characterization of the *Pasteurella hemolytica* leukotoxin. Infect Immun. **55**(10): 2348-54.
25. **Clarke C.R., A.K. Lauer, S.J. Barron, and J.H. Wyckoff 3rd.** 1994. The role of eicosanoids in the chemotactic response to Pasteurella haemolytica infection. Zentralbl Veterinarmed B. **41**(7-8): 483-91.
26. **Clinkenbeard K.D., D.A. Mosier, A.L. Timko, and A.W. Confer.** 1989. Effects of *Pasteurella hemolytica* leukotoxin on cultured bovine lymphoma cells. Am J Vet Res. **50**(2): 271-75.

27. **Clinkenbeard K.D., D.A. Mosier, and A.W. Confer.** 1989. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella hemolytica* leukotoxin. Infect Immun. **57**(2): 420-5.
28. **Clinkenbeard K.D., and M.L. Upton.** 1991. Lysis of bovine platelets by *Pasteurella hemolytica* leukotoxin. Am J Vet Res. **52**(3): 453-7.
29. **Confer A.W., R.J. Panciera, and D.A. Mosier.** 1988. Bovine pneumonic pasteurellosis: immunity to *Pasteurella hemolytica*. J Am Vet Ned Assoc. **193**(10): 1308-16.
30. **Confer A.W., K.R. Simons, R.J. Panciera, A.J. Mort, and D.A. Mosier.** 1989. Serum antibody response to carbohydrate antigens of *Pasteurella hemolytica* serotype 1: relation to experimentally induced bovine pneumonic pasteurellosis. Am J Vet Res. **50**(1): 98 105.
31. **Confer A.W., K.R. Simons, M.T. Barrie, and K.D. Clinkenbeard.** 1990. Effects of *Pasteurella hemolytica* leukotoxin on neutrophils from white-tailed deer and several exotic ruminant species. Vet Res Commun. **14**(3): 175-180.
32. **Confer AW.** 1993. Immunogens of *Pasteurella*. Vet Microbiol. **37**(3-4): 353-68.
33. **Confer A.W., R.D. McCraw, J.A. Durham, R.J. Morton, and R.J. Panciera.** 1995. Serum antibody responses of cattle to iron-regulated outer membrane proteins of *Pasteurella hemolytica* A1. Vet Immunol immunopathol. **47**(1-2): 101-10.

34. **Confer A.W., K.D. Clinkenbeard, D.M. Gatewood, B.A. Driskel, and M. Montelongo.** 1997. Serum antibody responses of cattle vaccinated with partially purified native *Pasteurella hemolytica* leukotoxin. Vaccine. **15**(12-13): 1423-9.

35. **Cruz W.T., R. Young, Y.F. Chang, and D.K. Struck.** 1990. Deletion analysis resolves cell-binding and lytic domains of the *Pasteurella* leukotoxin. Mol Microbiol. **4**(11): 1933-9.

36. **Cudd L., C. Clarke, K. Clinkenbeard, M. Shelton, P. Clinkenbeard, and G. Murphy.** 1999. Role of intracellular calcium in *Pasteurella hemolytica* leukotoxin-induced bovine neutrophil leukotriene B4 production and plasma membrane damage. FEMS Microbiol Lett. **172**(2): 123-9.

37. **Dustin M.L., R. Rothlein, A.K. Bhan, C.A. Dinarello, and T.A. Springer.** 1986. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J Immunol. **137**(1): 245-54.

38. **Dyer R.M., C.E. Benson, and M.G. Boy.** 1985. Production of superoxide anion by bovine pulmonary macrophages challenged with soluble and particulate stimuli. Am J Vet Res. **46**(2): 336-41.

39. **Emau P., S.N. Giri, and M.L. Bruss. 1987.** Effects of smooth and rough *Pasteurella hemolytica* lipopolysaccharides on plasma cyclic-nucleotides and free fatty acids in calves. Vet Microbiol. **15**(4): 279-92.

40. **Fedorova N.D., and S.K. Highlander.** 1997. Generation of targeted nonpolar gene insertions and operon fusions in *Pasteurella hemolytica*

and creation of a strain that produces and secretes inactive leukotoxin. Infect Immun. **65**(7): 2593-8.

41. **Fett T., L. Zecchinon, E. Baise, and D. Desmecht.** 2004. The bovine (*Bos taurus*) CD11a-encoding cDNA: molecular cloning, characterization and comparison with the human and murine glycoproteins. Gene. **21**(325): 97-101.
42. **Filion L.G., P.J. Willson, H. Bielefeldt-Ohmann, L.A. Babiuk, and R.G. Thomson.** 1984. The possible role of stress in the induction of pneumonic pasteurellosis. Can J Comp Med. **48**(3): 268-74.
43. **Forsyth C.B., E.F. Plow, and L. Zhang.** 1998. Interaction of the fungal pathogen Candida albicans with integrin CD11b/CD18: recognition by the I domain is modulated by the lectin-like domain and the CD18 subunit. J Immunol. **161**(11): 6198-205.
44. **Frank G.H., and L.B. Tabatabai.** 1981. Neuraminidase activity of *Pasteurella hemolytica* isolates. Infect Immun.**32** : 1119 1122.
45. **Frank G.** 1988. When *Pasteurella hemolytica* colonizes the nasal passages of cattle. Vet Med. **83**: 1060 64.
46. **Frank G.H.** 1986. The role of *Pasteurella hemolytica* in the bovine respiratory disease complex. Vet Med. **81**: 838-46.
47. **Gaddum R.M., S.A. Ellis, A.C. Willis, R.S. Cook, K.A. Staines, L.H. Thomas, and G. Taylor.** 1996. Identification of potential CTL epitopes of bovine RSV using allele-specific peptide motifs from bovine MHC class I molecules. Vet Immunol Immunopathol. **54**(1-4): 211-9.

48. **Gahmberg C.G., L. Valmu, S. Fagerholm, P. Kotovuori, E. Ihanus, L. Tian, and T. Pessa-Morikawa.** 1998. Leukocyte integrins and inflammation. Cell Mol Life Sci. **54**(6): 549-55.
49. **Gartner S.L., D.G. Siekmann, and Y.H. Kang.** 1988. Effects of lipopolysaccharide, lipid A, lipid X and phorbol esters on cultured bovine endothelial cells. Lab Invest. **59**: 181-191.
50. **Gentry M. J., A.W. Confer, E.D. Weinberg, and J. T. Homer.** 1986. Cytotoxin (leukotoxin) production by *Pasteurella hemolytica*: Requirement for an iron containing compound. Am. J. Vet. Res. **47**: 1919-1923.
51. **Gerbig D.G. Jr., R.D. Walker, J.D. Baker, J.S. Foster, and R.N. Moore.** 1989. Calcium ion involvement in the action of *Pasteurella hemolytica* leukotoxin. Vet Microbiol. **19**(4): 325-35.
52. **Gonzalez C.T., and S.K. Maheswaran.** 1993. The role of induced virulence factors produced by *Pasteurella hemolytica* in the pathogenesis of bovine pneumonic pasteurellosis: review and hypotheses. Br Vet J. **149**(2): 183-93.
53. **Gottschalk A.** 1960. Sialic acids: their molecular structure and characteristic reactions. Arzneimittelforschung. **10**: 355-7.
54. **Guo Y., Y. Wu, W. Chen, and J. Lin.** 1994. Endotoxic damage to the stria vascularis: the pathogenesis of sensorineural hearing loss secondary to otitis media. J Laryngol Otol. **108**(4): 310-3.

55. **Haeffner-Cavaillon N., J.M. Cavaillon, M. Etievant, S. Lebbar, and L. Szabo.** 1985. Specific binding of endotoxin to human monocytes and mouse macrophages: serum requirement. Cell Immunol. **91**(1): 119-31.
56. **Howard C.J., K.R. Parsons, and L.H. Thomas.** 1986. Systemic and local immune responses of gnotobiotic calves to respiratory infection with Mycoplasma bovis. Vet Immunol Immunopathol. **11**(3): 291-300.
57. **Hsuan S.L., M.S. Kannan, S. Jeeyaseelan, Y.S. Prakash, G.C. Sieck, and S.K. Maheswaran.** 1998. *Pasteurella hemolytica* A1-derived leukotoxin and endotoxin induce intracellular calcium elevation in bovine alveolar macrophages by different signaling pathways. Infect Immun. **66**(6): 2836-44.
58. **Hsuan S.L., M.S. Kannan, S. Jeeyaseelan, Y.S. Prakash, C. Malazdrewich, M.S. Abrahamsen, G.C. Sieck, and S.K. Maheswaran.** 1999. *Pasteurella hemolytica* leukotoxin and endotoxin induce cytokine gene expression in bovine alveolar macrophages requires NF-KappaB activation and calcium elevation. Microb Pathog. **26**(5): 263-73.
59. **Inzana T.J., J. Ma, T. Workman, R.P. Gogolewski, and P. Anderson.** 1988. Virulence properties and protective efficacy of the capsular polymer of Hemophilus (Actinobacillus) pleuropneumoniae serotype 5. Infect Immun. **56**(8): 1880-9.
60. **Issekutz A.C., P. Megyeri, and T.B. Issekutz.** 1987. Role for macrophage products in endotoxin-induced polymorphonuclear leukocyte accumulation during inflammation. Lab Invest. **56**(1): 49 59.

61. **Jeyaseelan S., S.L. Hsuan, M.S. Kannan, B. Walcheck, J.F. Wang, M.E. Kehrli, E.T. Lally, G.C. Sieck, and S.K. Maheswaran.** 2000. Lymphocyte function-associated antigen 1 is a receptor for *Pasteurella hemolytica* leukotoxin in bovine leukocytes. Infect Immun. **68**(1): 72-9.
62. **Kapil S., and R.J. Basaraba.** 1997. Infectious bovine rhinotracheitis, parainfluenza-3, and respiratory coronavirus. Vet Clin North Am Food Anim Pract. **13**(3): 455-69.
63. **Keiss R.E., D.H. Will, and J.R. Collier.** 1964. Skin toxicity and hemodynamic properties of endotoxin derived from *Pasteurella hemolytica*. Am J Vet Res. 25: 935-42.
64. **Kimman T.G., G.M. Zimmer, F. Westenbrink, J. Mars, and E. van Leeuwen.** 1988. Epidemiological study of bovine respiratory syncytial virus infections in calves: influence of maternal antibodies on the outcome of disease. Vet Rec. **123**(4): 104-9.
65. **Kishimoto T.K., R.S. Larson, A.L. Corbi, M.L. Dustin, D.E. Staunton, and T.A. Springer.** 1989. The leukocyte integrins. Adv Immunol. **46**: 149-82.
66. **Kishimoto T.K., K. O'Conner, and T.A. Springer.** 1989. Leukocyte adhesion deficiency. Aberrant splicing of a conserved integrin sequence causes a moderate deficiency phenotype. J Biol Chem. **264**(6): 3588-95.
67. **Kooney J.M., and S. Falkow.** 1984. Nucleotide sequence homology between the immunoglobulin A1 protease of *Neisseria gonorrhoeae*,

Neisseria meningitides and *Hemophilus influenza*. Infect Immun. **43**: 101-107.

68. **Lally E.T., I.R. Kieba, D.R. Demuth, J. Rosenbloom, E.E. Golub, N.S. Taichman, and C.W. Gibson.** 1989. Identification and expression of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Biochem Biophys Res Commun. **159**(1): 256-62.
69. **Lally E.T., I.R. Kieba, A. Sato, C.L. Green, J. Rosenbloom, J. Korostoff, J.F. Wang , B.J. Shenker, S. Ortlepp, M.K. Robinson, and P.C. Billings.** 1997. RTX toxins recognize a beta2 integrin on the surface of human target cells. J Biol Chem. **272**(48): 30463-9.
70. **Li J., and K.D. Clinkenbeard.** 1999. Lipopolysaccharide complexes with *Pasteurella hemolytica* leukotoxin. Infect Immun. **67**(6): 2920-7.
71. **Li J., K.D. Clinkenbeard, and J.W. Ritchey.** 1999. Bovine CD18 identified as a species specific receptor for *Pasteurella hemolytica* leukotoxin. Vet Microbiol. **67**(2): 91 7.
72. **Lifely M.R., J.C. Lindon, J.M. Williams, and C. Moreno.** 1985. Structural and conformational features of the *Escherichia coli* K92 capsular polysaccharide. Carbohydr Res. **143**: 191-205.
73. **Lo R.Y., C.A. Strathdee, and P.E. Shewen. 1987.** Nucleotide sequence of the leukotoxin genes of *Pasteurella hemolytica* A1. Infect Immun. **55**(9): 1987-96.

74. **Lopez A., R.G. Thomson, and M. Savan. 1976.** The pulmonary clearance of *Pasteurella hemolytica* in calves infected with bovine parainfluenza-3 virus. Can J Comp Med. **40**(4): 385-91.
75. **Marlin S.D., C.C. Morton, D.C. Anderson, and T.A. Springer.** 1986. LFA-1 immunodeficiency disease. Definition of the genetic defect and chromosomal mapping of alpha and beta subunits of the lymphocyte function-associated antigen 1 (LFA-1) by complementation in hybrid cells. J Exp Med. **164**(3):855-67.
76. **Marlin S.D., and T.A. Springer.** 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell. **51**(5): 813-9.
77. **Maoyeri M., and R.A. Welch.** 1997. Prelytic and lytic conformations of erythrocyte-associated *Escherichia coli* hemolysin. Infect Immun. **65**(6): 2233-9.
78. **MartinS .W., K.G. Bateman, P.E. Shewen, S. Rosendal, and J.E. Bohac.** 1989. The frequency, distribution and effects of antibodies, to seven putative respiratory pathogens, on respiratory disease and weight gain in feedlot calves in Ontario. Can J Vet Res. **53**(3): 355-62.
79. **McBride J.W., R.E. Corstvet, D.B. Paulsen, J.R. McClure, and F.M. Enright.** 1992. Systemic and pulmonary antibody responses of calves to *Pasteurella hemolytica* after intrapulmonary inoculation. Am J Vet Res. **53**(10): 1889-94.

80. **Meyrick B.O., U.S. Ryan, and K.L. Brigham.** 1986. Direct effects of *Escherichia coli* endotoxin on structure and permeability of pulmonary endothelial monolayers and the endothelial layer of intimal explants. Am J Pathol. **122**: 140-151.

81. **Milligan T.W., C.J. Baker, and D.C. Straus.** 1978. Association of elevated levels of extracellular neuraminidase with clinical isolates of type III group B *Streptococci*. Infect Immun. **21**: 738-746.

82. **Morck D.W., T.J. Raybould, S.D. Acres, L.A. Babiuk, J. Nelligan and J. Costerton.** 1987. Electron microscop ic description of glycocalyx and fimbriae on surface of *Pasteurella hemolytica*-A1. Can J Vet Res. **51**(1): 83-8.

83. **Morck D.W., T.C. Watts, S.D. Acres, and J.W. Costerton.** 1988. Electron microscope examination of cells of *Pasteurella hemolytica* A1 experimentally infected cattle. Can J Vet Res. **52**(3): 343-8.

84. **Morck D.W., M.E. Olsen, S.D. Acres, P.Y. Daoust and J.W. Costerton.** 1989. Presence of bacterial glycocalyx and fimbriae on *Pasteurella hemolytica* in feedlot cattle with pneumonic pasteurellosis. Can J Vet Res. **53**(20): 167-71.

85. **Morrison D.C., L.F. Kline, Z.G. Oades, and P.M. Henson.** 1978. Mechanisms of lipopolysaccharide-initiated rabbit platelet responses: alternative complement pathway dependence of the lytic response. Infect Immun. **20**(3): 744-51.

86. **Morrison D.C., and J.A. Rudbach.** 1981. Endotoxin-cell membrane interactions leading to transmembrane signaling. Contemp Top Mol Immunol. **8**: 187-218.

87. **Morrison W.I., G. Taylor, R.M. Gaddum, and S.A. Ellis.** 1999. Contribution of advances in immunology to vaccine development. Advances in Veterinary Medicine. **41**: 181-195.

88. **Mosier D., J. Iandolo, D. Rogers, G. Uhlich, and S. Crupper.** 1998. Characterization of a 54-kDa heat-shock-inducible protein of *Pasteurella hemolytica.* Vet Microbiol. **60**(1): 67-73.

89. **Mosier D.A., R.J. Panciera, D.P. Rogers, G.A. Uhlich, M.D. Butine, A.W. Confer, and R.J. Basaraba.** 1998. Comparison of serologic and protective responses induced by two *Pasteurella* vaccines. Can J Vet Res. **62**(3): 178-82.

90. **Murakami Y., T. Xu, E.J. Helmerhorst, G. Ori, R.F. Troxler, E.T. Lally, F.G. Oppenheim.** 2002. Inhibitory effect of synthetic histatin 5 on leukotoxin from Actinobacillus actinomycetemcomitans. Oral Microbiol Immunol. **17**(3): 143-9.

91. **Murphy G.L., L.C. Whitworth, K.D. Clinkenbeard, and P.A. Clinkenbeard.** 1995. Hemolytic activity of the *Pasteurella hemolytica* leukotoxin. Infect Immun. **63**(8): 3209-12.

92. **Nakstad B., N.P. Boye, and T. Lyberg.** 1987. Procoagulant activities in human alveolar macrophages. Eur J Respir Dis. **71**(5): 459-71.

93. **Odendaal M.W., and C.E. Ellis.** 1999. The production and evaluation of *Pasteurella hemolytica* leukotoxin in the supernatant of submerged cultures in fermenters. Onderstepoort J Vet Res. **66**(4): 265-72.
94. **Olchowy T.W., T.R. Ames, and T.W. Molitor.** 1994. Interaction of bovine respiratory syncytial virus with bovine alveolar macrophages in vivo: effects of virus infection upon selected cell functions. Can J Vet Res. **58**(1): 42-8.
95. **Otulakowski G.L., P.E. Shewen, and A.E. Udoh.** 1983. Proteolysis of sialoglycoprotein by *Pasteurella hemolytica* cytotoxic culture supernate. Infect Immun. **42**: 64-70.
96. **Panciera R.J., R.E. Corstvet.** 1984. Bovine pneumonic pasteurellosis: model for *Pasteurella hemolytica*- and *Pasteurella multocida*-induced pneumonia in cattle. Am J Vet Res. **45**(12): 2532-7.
97. **Pandher K., G.L. Murphy, and A.W. Confer.** 1999. Identification of immunogenic, surface-exposed outer membrane proteins of Pasteurella haemolytica serotype 1. Vet Microbiol. **65**(3): 215-26.
98. **Pavia D.** 1987. Acute respiratory infections and mucociliary clearance. Eur J Respir Dis. **71**(4): 219-26.
99. **Pavia D., M.T. Lopez-Vidriero, and S.W. Clarke.** 1987. Mediators and mucociliary clearance in asthma. Bull Eur Physiopathol Respir. **10**: 89s-94s.

100. **Porter P., and M.A. Linggood.** 1983. Novel mucosal anti-microbial functions interfering with the plasmid-mediated virulence determinants of adherence and drug resistance. Ann N Y Acad Sci. **409**: 564-79.

101. **Potgieter L.N.** 1997. Bovine respiratory tract disease caused by bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract. **13**(3): 471-81.

102. **Potter A.A, K. Ready, and J. Gilchrist.** 1988. Purification of fimbriae from *Pasteurella hemolytica* A-1. Microb Pathog. **4**(4): 311-6.

103. **Proctor R.A.** 1987. Fibronectin: A brief review of its structure, function and physiology. Rev Infect Dis. **9**(Supple 4): 317-321.

104. **Radi Z.A., K.B. Register, E.K. Lee, M.E. Kehrli Jr., K.A. Brogden, J.M. Gallup, and M.R. Ackermann.** 1999. In situ expression of intercellular adhesion molecule-1 (ICAM-1) mRNA in calves with acute Pasteurella haemolytica pneumonia. Vet Pathol. **36**(5): 437-44.

105. **Ray P.K.**1977. Bacterial neuraminidase and altered immunological behavior of treated mammalian cells. Adv Appl Microb. **21**: 227-267.

106. **Rashid J., D.J. Weiss, R. Bach, and M. Murtaugh.** 1997. Role of tissue factor in intra-alveolar fibrin deposition and coagulopathy associated with pneumonic pasteurellosis in cattle. Am J Vet Res. **58**(1): 28-33.

107. **Rimsey R.L., J.E. Coyle-Dennis, L.H. Lauerman, and P.G. Squire.** 1981. Purification and biological characterization of endotoxin fractions from *Pasteurella hemolytica*. Am J Vet Res.**42** (12): 2134-8.

108. **Roth J.A., M.L. Kaeberlae, and R.D. Hubbard.** 1984. Attempts to use thiabendazole to improve the immune response dexamethasone-treated or stressed cattle. Immunopharmacology. **8**(3-4): 121-8.

109. **Rothlein R., M.L. Dustin, S.D. Marlin, and T.A. Springer.** 1986. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J Immunol. **137**(4): 1270-4.

110. **Roubin R., and J. Benveniste.** 1985. Formation of prostaglandins, leukotrienes and paf-acether by macrophages. Comp Immunol Microbiol Infect Dis. **8**(2): 109-18.

111. **Scott P.R.** 1994. Field study of undifferentiated respiratory disease in housed beef calves. Vet Rec. **134**(13): 325-7.

112. **Sharma S.A., T.W. Olchowy, and M.A. Breider.** 1992. Alveolar macrophage and neutrophil interactions in *Pasteurella hemolytica*-induced endothelial cell injury. J Infect Dis. **165**(4): 651-7.

113. **Shewen P.E., and B.N. Wilkie.** 1982. Cytotoxin of *Pasteurella hemolytica* acting on bovine leukocytes. Infect Immun. **35**(1): 91 94.

114. **Shewen P.E., and B.N. Wilkie.** 1988. Vaccination of calves with leukotoxic culture supernatant from *Pasteurella hemolytica*. Can J Vet Res. **52**: 30–36.

115. **Shuster D.E., M.E. Kehrli Jr., M.R Ackermann, and R.O. Gilbert.** 1992. Identification and prevalence of a genetic defect that causes leukocyte adhesion deficiency in Holstein cattle. Proc Natl Acad Sci U S A. **89**(19): 9225-9.

116. **Spitzer J.A., and I.V. Deaciuc.** 1986. Effect of endotoxicosis and sepsis on intracellular calcium homeostasis in rat liver. Mitochondrial and microsomal calcium uptake. Circ Shock. **18**(2): 81 93.

117. **Springer T.A.** 1990. Adhesion receptors of the immune system. Nature. **346**(6283): 425-34.

118. **Stevens P.K., and C.J. Czuprynski.** 1996. *Pasteurella hemolytica* leukotoxin induces bovine leukocytes to undergo morphologic changes consistent with apoptosis in vitro. Infect Immun. **64**(7): 2687-94.

119. **Sun Y., K.D. Clinkenbeard, L.A. Cudd, C.R. Clarke, and P.A. Clinkenbeard.** 1999. Correlation of *Pasteurella hemolytica* leukotoxin binding with susceptibility to intoxication of lymphoid cells from various species. Infect Immun. **67**(12): 6264-9.

120. **Sun Y., K.D. Clinkenbeard, C. Clarke, L. Cudd, S.K. Highlander, and S.M. Dabo.** 1999. *Pasteurella hemolytica* leukotoxin induced apoptosis of bovine lymphocytes involves DNA fragmentation. Vet Microbiol. **65**(2): 153-66.

121. **Sun Y., K.D. Clinkenbeard, C.L. Ownby, L. Cudd, C.R. Clarke, and S.K. Highlander.** 2000. Ultrastructural characterization of apoptosis in bovine lymphocytes exposed to *Pasteurella hemolytica* leukotoxin. Am J Vet Res. **61**(1): 51-6.

122. **Tabatabai L.B., and G.H. Frank.** 1981. Neuraminidase from *Pasteurella hemolytica*. Curr Microbiol. **5**: 203-206.

123. **Tajima M., M. Irie, R. Kirisawa, K. Hagiwara, T. Kurosawa, and K. Takahashi.** 1993. The detection of a mutation of CD18 gene in bovine leukocyte adhesion deficiency (BLAD). J Vet Med Sci. **55**(1): 145-6.

124. **Taylor G., L.H. Thomas, S.G. Wyld, J. Furze, P. Sopp, and C.J. Howard.** 1995. Role of T-lymphocyte subsets in recovery from respiratory syncytial virus infection in calves. J Virol. **69**(11): 6658-64.

125. **Tipping P.G., D.A. Campbell, N.W. Boyce, and S.R. Holdsworth.** 1988. Alveolar macrophage procoagulant activity is increased in acute hyperoxic lung injury. Am J Pathol. **131**(2): 206-12.

126. **Tjornehoj K., A. Uttenthal, B. Viuff, L.E. Larsen, C. Rontved, and L. Ronsholt.** 2003. An experimental infection model for reproduction of calf pneumonia with bovine respiratory syncytial virus (BRSV) based on one combined exposure of calves. Res Vet Sci.**74** (1): 55-65.

127. **Tobias P.S., K.P. McAdam, K. Soldau, and R.J. Ulevitch.** 1985. Control of lipopolysaccharide-high density lipoprotein interactions by an acute-phase reactant in human serum. Infect Immun. **50**(1): 73 6.

128. **Toth T.E., and G.H. Frank.** 1988. Bovine parainfluenza-3 virus proteins recognized by antibodies of aerosol-exposed calves. Am J Vet Res. **49**(11): 1945-9.

129. **Viuff B., K.Tjornehoj, L.E. Larsen, C.M. Rontved, A. Uttenthal, L. Ronsholt, and S. Alexandersen.** 2002. Replication and clearance of respiratory syncytial virus: apoptosis is an important pathway of virus

clearance after experimental infection with bovine respiratory syncytial virus. Am J Pathol. **161**(6): 2195-207.

130. **Wadstrom T.** Bacterial neuraminidase. In: **Jeljaszewicz J., and T. Wadstrom.** Bacterial Toxins and Cell Membranes. 1978. Academic Press, London, England. 27-33.

131. **Walzog B., F. Jeblonski, A. Zakrzewicz, and P. Gaehtgens.** 1997. Beta2 integrins (CD11/CD18) promote apoptosis of human neutrophils. FASEB J. **11**(13): 1177-86.

132. **Wang J.F., I.R. Kieba, J. Korostoff, T.L. Guo, N. Yamaguchi, H. Rozmiarek, P.C. Billings, B.J. Shenker, and E.T. Lally.** 1998. Molecular and biochemical mechanisms of *Pasteurella hemolytica* leukotoxin-induced cell death. Microb Pathog. **25**(6): 317-31.

133. **Werb Z., and J.R. Chin.** 1983. Endotoxin suppresses expression of apoprotein E by mouse macrophages in vivo and in culture. A biochemical and genetic study. J Biol Chem. **258**(17): 10642-8.

134. **Whiteley L.O., S.K. Maheswaran, D.J. Weiss, and T.R. Ames.** 1990. Immunohistochemical localization of *Pasteurella hemolytica* A1 - derived Endotoxin, leukotoxin and capsular polysaccharide in experimental bovine *Pasteurella* pneumonia. Vet Pathol. **27**(3): 150-61.

135. **Whiteley L.O., S.K. Maheswaran, D.J. Weiss, and T.R. Ames.** 1991. Alterations in pulmonary morphology and peripheral coagulation profiles caused by intratracheal inoculation of live and ultraviolet light-killed *Pasteurella hemolytica* A1 in calves. Vet Pathol. **28**(4): 275-85.

136. **Whiteley L.O., S.K. Maheswaran, D.J. Weiss, and T.R. Ames.** 1991. Morphological and morphometrical analysis of the acute response of the bovine alveolar wall to *Pasteurella hemolytica* A1-derived endotoxin and leukotoxin. J Comp Pathol. **104**(1): 23-32.

137. **Whiteley L.O., S.K. Maheswaran, D.J. Weiss, T.R. Ames and M.S. Kannan.** 1992. *Pasteurella hemolytica* A1 and bovine respiratory disease: pathogenesis. J Vet Intern Med. **6**(1): 11 22.

138. **Wilson C.F., A.D. Sutherland, L. Inglis, and W. Donachie.** 1992. Characterization and biological activity of monoclonal antibodies specific for *Pasteurella hemolytica* A1 capsule and lipopolysaccharide. Vet Microbiol. **31**(2-3): 161-8.

139. **Woods D.**E. 1987. Role of fibronectin in the pathogenesis of gram negative pneumonia. Rev Infect Dis. **9**(Supple 4): 386-390.

140. **Yoo H.S., S.K. Maheswaran, G. Lin, E.L. Townsend, and T.R. Ames.** 1995. Induction of inflammatory cytokines in bovine alveolar macrophages following stimulation with *Pasteurella hemolytica* lipopolysaccharide. Infect Immun. **63**(20): 381-8.

VITA

Amit Dewan

Candidate for the Degree of

Master of Science

Thesis: EXPRESSION OF ß$_2$ INTEGRINS (CD11a/CD18) IN K562 CELLS

Major Field: Veterinary Biomedical Sciences

Biographical:

Personal Information: Born in New Delhi, India, on December 12, 1977, the son of B.P. Dewan and Yogita Dewan.

Education: Graduated from Salwan Public School, New Delhi, India in July 1995; received Bachelor of Veterinary Sciences & Animal Husbandry from G.B. Pant University of Agriculture & Technology, Pantnagar, India in July 2001. Completed the requirements for the Master of Science degree with a major in Veterinary Biomedical Sciences at Oklahoma State University, Stillwater, Oklahoma, in May 2005.

Experience: Veterinary intern at G.B. Pant University of Agriculture & Technology, Pantnagar, India. Graduate Teaching Assistant, Department of Physiological Sciences, 2001 2002, Department of Veterinary Pathobiology, 2002-2004; Graduate Research Assistant, 2004-present at Oklahoma State University, Stillwater, Oklahoma.

Name: Amit Dewan Date of Degree: May, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EXPRESSION OF ß$_2$ INTEGRINS (CD11a/CD18) IN K562 CELLS

Pages in Study: 116 Candidate for the Degree of Master of Science

Major Field: Veterinary Biomedical Sciences

Scope and Method of Study: *Mannheimia haemolytica* is the etiological agent for an economically important pneumonic disease of cattle. A critically important virulence factor for this organism is leukotoxin (LKT), an exotoxin elaborated by *M. haemolytica* that destroys bovine immune cells sent to protect the host. The overall goal of this project was to characterize the interaction between LKT and the putative host cell receptor bovine CD18. Toward this goal, the strategy was to establish transfected cell lines that stably express bovine CD11a/CD18, human CD11a/CD18, or inter-species hybrids (human CD11a/bovine CD18 and bovine CD11a/human CD18). Inserts were generated by PCR of either bovine or human cDNA with CD11a or CD18 specific primers that flanked the coding sequence and included restriction sites to facilitate directional cloning and subcloning. Following generation of recombinant vectors, transfections were performed in both Vero (adherent) and K562 (non-adherent) cells. Initially, Vero cells were transfected with bovine and human CD18 recombinant vectors alone.

Findings and Conclusions: For bovine CD18, 3 out of 15 clones that survived in the Geneticin^R selection medium exhibited CD18 expression as determined by immunostaining and confocal microscopy. Similarly, 5 human CD18 transfected Vero clones were isolated from the selection medium; however none of these exhibited surface CD18 expression. When tested with immunoblotting, 2 out of the 3 bovine CD18 expressing clones exhibited a band of the appropriate size for bovine CD18. Antibodies for the CD11a/CD18 heterodimer did not label CD18 expressing clones; however this might have been due to the lack of specificity. Further, bovine CD18-expressing Vero clones were not sensitive to *in vitro* LKT challenge. When K562 cells were sequentially as well as co-transfected with different combinations of bovine and human CD11a and CD18, there was usually transient expression of the species as well as inter-species heterodimers as detected by immunostaining and confocal microscopy or flow cytometry. The major drawback of conducting transient expression studies in K562 cells was generating enough cells that could be simultaneously tested for heterodimeric expression and LKT sensitivity.

ADVISER'S APPROVAL: Dr. Jerry Ritchey