EFFECTS OF BOVINE RESPIRATORY SYNCYTIAL VIRUS OR BOVINE VIRAL DIARRHEA VIRUS INFECTION AND N-ACETYL CYSTEINE SUPPLEMENTATION ON INTRACELLULAR GLUTATHIONE LEVELS, PROLIFERATION AND INTERFERON-γ TRANSCRIPTION BY BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS AND NATURAL KILLER CELLS

By

Laurice A. Matulka

A DISSERTATION

Presented to the faculty of The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Doctor of Philosophy

Major: Animal Science

Under the Supervision of Professors Dennis R. Brink and Clayton L. Kelling

Lincoln, Nebraska

May, 2004

DISSERTATION TITLE

Effects of Bovine Respiratory Syncytial Virus or Bovine Viral Diarrhea Virus Infection and N-Acetyl Cysteine Supplementation on Intracellular Glutathione Levels, Proliferation and Interferon-gamma Transcription by Bovine Peripheral Blood Mononuclear ~~Cells and Natural Killer Cells~~

BY

Laurice A. Matulka

SUPERVISORY COMMITTEE:

	Approved	Date
Signature	Glenn R Brink	4-26-04
Typed Name	Dennis R. Brink	
Signature	Clayton L Kelling	4-26-04
Typed Name	Clayton L. Kelling	
Signature	S Srikumaran	4-20-04
Typed Name	S. Srikumaran	
Signature	John S Weber	4-20-04
Typed Name	J. Weber	
Signature		
Typed Name		
Signature		
Typed Name		

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EFFECTS OF BOVINE RESPIRATORY SYNCYTIAL VIRUS OR BOVINE VIRAL DIARRHEA VIRUS INFECTION AND N-ACETYL CYSTEINE SUPPLEMENTATION ON INTRACELLULAR GLUTATHIONE LEVELS, PROLIFERATION AND IFN-γ TRANSCRIPTION BY BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS AND NATURAL KILLER CELLS

Laurice Annette Matulka, Ph.D.
University of Nebraska, 2004

Advisors: Dennis R. Brink & Clayton L. Kelling

Cell proliferation and lytic activity have been shown to be influenced by intracellular glutathione (GSH) levels in peripheral blood mononuclear cells (PBMCs). Since both functions are important for effective elimination of viral infections, viruses that infect PBMCs could potentially alter cellular GSH levels. The central hypothesis of this study was bovine respiratory syncytial virus (BRSV) and bovine viral diarrhea virus (BVDV) infection lower GSH levels in PBMCs disrupting the anti-viral responses to these viruses. It was further hypothesized that infected cells augmented with N-acetyl cysteine (NAC) have increased GSH levels, changing the ability of infected cells to respond to infection. Mononuclear cells from calf peripheral blood were non-infected or infected with BRSV or BVDV, in the presence or absence of IL-2. Intracellular GSH levels, measured by flow cytometry, were decreased in PBMCs infected with BRSV or BVDV. Proliferation was increased in BRSV- or BVDV-infected PBMCs, in the presence of IL-2 compared to infected PBMCs in the absence of IL-2. IFN-γ transcription was decreased in BRSV- and BVDV-infected PBMCs in the absence of IL-2. N-acetyl cysteine supplementation increased IFN-γ expression in PBMCs infected with BRSV compared to BRSV-infected PBMCs without NAC. Given the importance of

NK cells in innate immunity and attenuating viral infections, the NK cell subset was analyzed. GSH levels decreased in NK cells infected with BRSV and BVDV. NAC supplementation resulted in restoration of GSH levels. Flow cytometry revealed IL-2 enhanced proliferation of NK cells infected with BRSV or BVDV compared to infected cells in the absence of IL-2. BRSV or BVDV infection, in the absence of IL-2 caused decreased expression of IFN-γ compared to infected cells in the presence of IL-2. The results of this study revealed BRSV or BVDV infection reduced intracellular GSH levels in PBMCs and NK cells and are capable of suppressing immune responses.

Acknowledgements

I am so excited to be finished with this degree and moving on in my career. This was definitely an experience I have no wish to repeat. I would like to thank Dennis Brink and Clayton Kelling for serving as co-chairs of my committee. I would also like to thank S. Srikumaran and Jack Weber for serving on my committee and for their time and input as my reading committee. Dwane Wylie began as a member of my committee but was unable to finish in this role when he moved out of state. However, I need to sincerely thank him for all of his support and guidance. I believe it was his teaching and intellect that led me to realize I truly wanted a career in science. When I speak of my mentor during my Ph.D. career it will be Dwane Wylie that I am referring to. I also wish to thank other students; Jutta, Anna, Holly and Melissa, who I now consider my friends that provided me with stimulating science conversations and more importantly, friendships that I will value forever. My first semester here I was fortunate enough to meet one of my future best friends, Jay, and I want to thank him for all his support, encouragement and advice throughout the years. I want to thank my parents and family for all of their support throughout this program. It took strength, determination and hard work to complete this degree and I am very lucky to have had two wonderful teachers to instill those characteristics in me, thank you Mom and Dad. Finally, it has not always been easy for him, but I want to thank my son, Jace, for being the ultimate "trooper" throughout my program. He makes me want to be the very best I can be and I am so blessed to have him.

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Introduction

Immunity comprises both nonspecific and specific components. The innate arm of the immune response, which includes natural killer (NK) cells and macrophages, is thought to play a significant role in limiting early progression of a viral infection. It is not specific to a particular pathogen but rather provides signals capable of stimulating and directing the adaptive immune response. Innate and adaptive immunity cooperate to provide more efficient immunity. The adaptive response is capable of recognizing specific pathogens and exhibits "memory" to that particular pathogen when exposed later. The secondary immune exposure results in a quicker, stronger response with accelerated clearance of the pathogen (1). The adaptive response involves lymphocytes, such as T and B cells as well as the cytokines and antibodies that they produce.

Bovine respiratory disease (BRD) complex is caused by a medley of viral, bacterial and fungal pathogens. Viruses commonly involved in BRD are infectious bovine rhinotracheitis virus (IBRV), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV) and parainfluenza-3 (PI-3) virus. Mortality, impaired performance caused by irreversible pulmonary lesions and high costs of therapeutic interventions impart the important economic impact of BRD complex, with the overall cost of BRD estimated at $14.71 per calf year (2).

The glutathione (GSH) level of lymphocytes, in particular NK cells, has been shown to affect proliferation (3) and lytic activity (4, 5), necessary to halt the invading viral pathogen. HIV infection results in a decrease of lymphocyte GSH level (6, 7).

Cysteine supplementation with N-acetyl cysteine (NAC) restored GSH in lymphocytes and tissues of infected subjects (8, 9). These studies will characterize the role of GSH, a ubiquitous intracellular reducing agent, in the peripheral blood mononuclear cell (PBMC) and NK cell subset responses after a BRSV or BVDV infection and following NAC supplementation.

Mononuclear Cells and Natural Killer Cells

White blood cells, also called leukocytes, are cells carried within the blood and lymphatic system important in the immune response. The leukocytes consist of subsets of cell groups which are lymphocytes, neutrophils, monocytes, eosinophils and basophils. The lymphocyte subset contains T, B and NK cells. PBMCs, which are discussed throughout this research are mononuclear leukocytes and consist of T cells, B cells, NK cells and monocytes.

NK cells are a small subset of lymphocytes, thought to play an important role in innate immunity, especially against viruses and tumors. They differ from T or B lymphocytes by their morphology, phenotype and functional capability. They are defined as effector cells that arbitrate innate immunity and are involved in non-MHC-restricted cytotoxicity against tumor, virus-infected and other target cells (10). In contrast to T and B cells, NK cells do not rearrange TCR or Ig genes, respectively, and are able to mediate their effector function against target cells *in vitro* or *in vivo* without prior activation. Moreover, NK cells can mature in the absence of a functional thymus. Despite differences, NK and T cells share progenitors, surface markers and effector functions. A 14.5 day fetal thymocyte population lacking TCR CD4 and CD8 but expressing

FcγRII/III matured inside or outside of the thymus resulted in differentiation into T cells or NK cells, respectively (11). Thus, a population of immature thymocytes has the ability to differentiate into either T or NK cells.

NK cells are typically not in cell cycle, except during response to infection with particular pathogens or stimulation with high doses of specific cytokines. Although not continuously cycling, mature NK cells are not end stage cells with a permanent life span. They do respond to endogenous cues, which can lead to proliferation, survival or loss (12, 13). For example, IL-15 appears to be necessary for mature NK cell survival, but not for homeostatic proliferation (13).

NK cells' ability to lyse virus-infected cells suggests they are important in controlling viruses. For example, NK cells from HIV infected patients exhibit a reduced capability to lyse target cells and produce lower levels of IFN-γ and TNF-α compared to control cells from healthy donors (14). HIV-infected individuals receiving highly active anti-retroviral therapy (HAART) can experience successful virus suppression and restoration of impaired NK function (15). Moreover, it appears that IL-15 is the cytokine specifically responsible for the up-regulation of NK cell cytoxicity following multiple viral infections, including RSV (16). Some viral infections will induce the expression of IL-12 which results in IFN-γ production by the NK cells, which in turn fosters an antiviral state (17).

Natural Killer Cells and Their Receptors – The receptors of NK cells play a pivotal role in activation or inhibition of the cell. It is important to understand the process of initiation of an immune response and the differences between NK cells and lymphocytes. Receptor response has been linked to GSH levels of the cell. The effect of

GSH varies with the state of activation of the killer cells. Addition of GSH during first 24 h of activation decreased generation of killer cell activity in resting cells however, after 48 h of activation the addition of GSH increased the killer cell activity (18). It is important to understand the role of NK cells in the immune response, the role of key receptor pathways controlling the NK cell-mediated response and the importance of GSH levels in modulating the pathways. NK cells differ from B and T lymphocytes in that they do not rearrange known receptors for antigen. NK cells express a low affinity receptor of IgG, FcγRIII. NK cells use two categories of receptors to regulate lysis of infected cells. One category leads to lysis, while the other inhibits lysis. This is known as the "opposing-signals model (Appendix I)." NK cell effector functions are triggered when activation (pro-lysis) receptors bind target cell ligands at the same time inhibitory receptors detect no or low levels of MHC class I (1).

Multiple receptors activate the pro-lysis pathways. FcγRIII, the first major NK cell receptor to be characterized and the only Fc receptor identified on NK cells, mediates antibody-dependent cellular cytotoxicity (ADCC). NK cells also express the immunoreceptor tyrosine based activation motif (ITAM)-containing DAP12 molecule, an adapter protein. Ligand binding results in tyrosine phosphorylation of the ITAMs of the adapter proteins, which then leads to recruitment and activation of Syk family tyrosine kinases ZAP-70 and Syk (19). In THP-1 cells, pretreatment of cells with N-acetyl cysteine (NAC) almost completely blocked tyrosine phosphorylation of Syk (20). Thus it appears increasing GSH levels at the inappropriate time of activation will result in a block of receptor activity and thus inhibition of further activation of the cell. Other activation receptors, which are members of the C-type lectin superfamily, are Ly49D,

Ly49H and CD94/NKG2C (19). These activation receptors are related to the inhibitory receptors; however, they do not exhibit cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Instead, the activation receptors contain charged residues in their transmembrane domain that assist interaction with the ITAM-containing signaling chains, including CD3ξ, FcεRIγ and DAP12 (19). The recruitment of the Syk family tyrosine kinases and downstream events leads to target killing by exocytosis of granules containing perforin, granzymes and cytokine secretion. In addition to lectins, other molecules on NK cells, such as CD2, CD16 and CD28 may be involved in activation, rather than direct stimulation of NK cells (21). Additionally, partial transcripts of the homologues of the genes encoding the activating receptors NKR-P1 and NKG2-D have been discovered in cattle. The nucleotide sequence exhibited 90 and 75% identity, respectively, to the human sequences (22). The human NKRP1 and NKG2 gene families have been mapped to chromosome 12 within the natural killer gene complex (NKC) (23). An elevated level of conservation has been displayed between human chromosome 12 and a linkage segment on bovine chromosome 5 (24). This would suggest the presence of a bovine NKC targeted to chromosome 5.

NK cell cytotoxic activity is also regulated by inhibitory receptors on the surface of the cell. The ligands for many inhibitory receptors have been identified as class I MHC molecules. There are three discrete families of class I MHC-recognizing NK cell receptors; Ly49, CD94/NKG2 and killer cell immunoglobulin-like receptors (KIR). Ly49 and CD94/NKG2, found in mice and humans, are members of C-type lectin-like receptor superfamily. In humans these receptors recognize HLA-E on potential target cells. HLA-E is not transported to the surface of the cell unless it has bound a leader

peptide derived from HLA-A, HLA-B or HLA-C. Therefore, the amount of HLA-E on the surface serves as a measure of the overall biosynthesis of class I MHC within the cell. These receptors are not specific for a particular HLA allele and will inhibit NK cell killing if adequate levels of class I MHC are being expressed on the target cell (25).

The KIR, found only in humans, are type 1 transmembrane glycoproteins with two or three extracellular C2-type immunoglobulin domains. The inhibitory forms of these receptors contain ITIMs in their cytoplasmic tails (25). Upon tyrosine phosphorylation, the src homolgy-2 containing tyrosine phosphatases (SHP)-1 and SHP-2 are recruited to these receptors (21). The precise sequence of biochemical actions resulting in inhibition of NK cell function remains to be identified.

The regulation of NK cells to kill only target cells and not normal cells is accomplished through a balance of both activating and inhibitory signals mediated through distinct classes of receptors, as described above. The dominant signal received by an NK cell through its interaction with normal levels of self-MHC class I is inhibitory. When the quantitative level of MHC class I is reduced through infection or a tumor, this inhibitory signal is released and the NK cell is more likely to be activated (26).

Unlike T and B cells NK cells may express multiple inhibitory and stimulatory receptors simultaneously. The wide expression of receptors on NK cells results in their broader specificity than CTLs. It is possible that an individual NK cell could be fully triggered by any one of its activation receptors. Therefore, pathogens may express adequate levels of only one ligand but could still trigger most of the NK cells because of widespread expression of the cognate activation receptor (19). This would result in rapid

amplification of the lytic response, allowing NK cells to contribute to early, innate immunity.

Role of GSH in Mononuclear Cells, Natural Killer Cells and Redox Modulation - Mononuclear cells depend on synthesis of GSH, as they are not able to directly import GSH (27). GSH is synthesized in a two step pathway, involving γ-glutamylcysteine synthetase in the first reaction and glutathione synthetase in the second reaction. The first step of the reaction is rate-limiting for GSH synthesis and under feedback inhibition by GSH (28). The greater initial level of GSH in NK cells compared to CTLs (29) may be due to the cysteine/cystine transport system. NK cells, but not T cells, have the capacity to take up cystine from the medium (29), thus amplifying the sum of compounds necessary for GSH synthesis. Studies indicate treatment with ifosfamide, a cytostatic agent frequently used in therapy for solid tumors, resulted in a depletion of intracellular GSH levels and a significant inhibition of proliferation and lytic activity in CTLs (5). Conversely, the lytic activity in NK cells was not extensively affected, and this correlates with the findings that NK cells have a higher initial GSH level and a higher rate of GSH synthesis (5).

Thiol-related compounds, such as L-cystine, 2-ME (β-mercaptoethanol) or reduced GSH, are important in many lymphoid cell activation pathways. Mononuclear cells required GSH for progression from G1 into S phase (30). Depletion of GSH and L-cystine from media used in interleukin-2 (IL-2) activated NK cell cultures resulted in inhibition of proliferation (3). Thiol deprivation increases Fas-induced apoptosis in NK cells, while pretreatment of cells with N-acetyl cysteine (NAC), reduced apoptosis and reversed the effect of thiol-deficient medium (31).

Proliferation, apoptosis and lytic activity of NK cells have been shown to be regulated by cellular redox status (3,4,5,31). For example, suppression of calcineurin and NFAT (Nuclear factors of activated T cells) activation due to oxidative stress, induced by thiol deprivation, resulted in inhibition of FasL induction in activated NK cells and thus inhibition of apoptosis (32). Redox regulation has been implicated in various biological processes and appears to be keenly involved in the modulation of optimal NK cell function.

Reactive oxygen intermediates (ROI) are side products continuously produced during redox reactions within the cell. Studies indicate that ROI may play a significant role in generation of an NK cell cytotoxic response (4).

As presented, GSH levels are critically important for ideal function of mononuclear cells and NK cells. Redox status, which GSH is a part of, is involved in a medley of cellular activities, such as, cytotoxicity, cytokine production, and apoptosis of the cell. Research to examine how GSH level affects activities of NK cells after a BRSV or BVDV infection, as well as enhancement of these activities after supplementation with NAC will be beneficial.

Bovine Respiratory Syncytial Virus

BRSV was first isolated in Switzerland in 1967. An outbreak of a respiratory disease in cattle led to isolation of an agent antigenically related to the human RS virus (33). Bovine strains of RSV were isolated from nasal exudates of calves with acute respiratory disease in 1972 in the United States (34). BRSV is a nonhemagglutinating pneumovirus of the paramyxovirus family. It is an enveloped, single-stranded, negative-

sense RNA virus. The BRSV genome encodes at least ten proteins. They include two nonstructural proteins (NS1 and NS2); four RNA-associated proteins, the nucleoprotein N, the phosphoprotein P, the large, catalytic subunit L and a transcription elongation factor encoded by the M2 gene. There are three envelope-associated proteins, the fusion protein F, the attachment protein G, and the small hydrophobic protein SH (35). Little is known about the function of the NS1 and NS2 genes; however, their presence differentiates the members of the genus *Pneumovirus* from all other paramyxoviruses. The F protein consists of two subunits, F1 and F2, with the F2 sub-unit determining RSV infection in a species specific manner (36). The G attachment glycoprotein facilitates infection, however, in a non-species specific manner (36). Disease is characterized by clinical signs such as incidence of cough, harsh lung sounds, adventitious sounds and dyspnea and increases in rectal temperature and respiratory rate. Post-mortem signs of disease include varying degrees of necrotizing and proliferative bronchiolitis and alveolitis with syncytial formation. In tissue culture the virus generates a cytopathic effect evidenced by the formation of syncytium.

The mechanism by which BRSV causes disease remains inadequately defined. Outbreaks of BRSV infection are commonly associated with secondary bacterial pneumonia, which supports the hypothesis of a deleterious effect of this virus on the host's immune defense mechanism (37). It has been speculated that BRSV infection may promote infections by other pathogens. An experimental BRSV infection in lambs resulted in bronchiolitis lesions with destruction of the mucociliary apparatus, the presence of syncytial cells in alveoli and a progressive interstitial reaction (38). In vitro BRSV infection of ovine lymphocytes resulted in a depressive effect on the proliferative

response of the lymphocytes to PHA, Con A and PWM (39). Lymphocyte subsets have been shown to change during BRSV infection in calves (40, 41). There were approximately equal numbers of CD8+ and CD4+ T cells in the lung and trachea of uninfected calves; however, after day 10 of a BRSV infection, CD8+ T cells outnumbered CD4+ T cells by 3:1 in the lungs and 6:1 in the trachea of infected calves (40). This suggests CD8+ T cells compose the primary lymphocyte sub-population involved during recovery and clearance of a BRSV infection. Experimental infection in calves resulted in a severe respiratory tract disease. Histopathology revealed severe lesions in small bronchi, bronchioli and alveoli as well as necrosis, epithelial loss, hyperplasia and metaplasia in the epithelium of small bronchi and bronchioli. BRSV was isolated from the lower respiratory tract of infected calves, distinguishing it as an important pathogen of the lower respiratory tract (42).

Bovine Respiratory Syncytial Virus in Lymphocytes - BRSV replicates in ovine and bovine lymphocytes (43). Experimental infection of lambs with BRSV resulted in neutropenia and lymphocytopenia at 3 to 7 days post infection (44). The percentage of lymphocytes positive to BRSV antigens in the peripheral blood increased from day 3 to 7 (44). It appears that peripheral lymphocytes support a low degree of viral replication compared to other locations and cell types within the host (43). However, lymphocytes may harbor BRSV and transport the virus throughout the body. Additionally, since peripheral lymphocytes are susceptible to infection and are capable of supporting some viral replication this may result in induction of an inappropriate immune response for a viral infection. In contrast, bovine alveolar macrophages (BAMs) appear to be resistant to infection with BRSV and not play a dominant role in replication of the virus (45).

However, BAMs did shelter virus antigen for several days and this may act to alter function of the cells and allow for an extended BRSV infection (45). There was no evidence that bovine monocyte-derived dendritic cells supported replication of BRSV. However, it appears the virus did increase apoptosis/necrosis of the dendritic cells (46).

Major histocompatability complex (MHC) class-I-restricted CD8+ CTLs have been implicated in recovery from BRSV infection in calves. CD8+ CTLs appear 7 to 10 days after infection with BRSV. A CTL response was detected and was shown to be MHC-restricted in peripheral blood of calves following a BRSV infection (47). However, in RSV-infected mice CD8+ T cells have been shown to be essential to the influx of eosinophils into the lung and the evolvement of airway hyperresponsiveness (AHR) (48). A study comparing RSV infected mice in the presence or absence of CD8+ T cells showed removal of CD8+ T cells resulted in no lung eosinophilia or AHR (48). Interleukin-5 is involved in recruitment of eosinophils to the lung and development of AHR, suggesting that CD8+ T cells are acting as noncytotoxic, IL-5 producing cells. In contrast, immunodeficient humans have presented with severe and often fatal RSV infections (49). This would indicate two distinct roles for T cells in mice and in humans during infection. However, there are no reports of bovine CD8+ T cells acting as IL-5 producers that exacerbate disease. In contrast to mouse and human, depletion of CD8+ T cells in cattle confirmed their significance for virus clearance from the lung (50). BRSV infection of lambs is accompanied by modifications in lymphocyte subpopulations in the peripheral blood as well as the presence of viral antigens in the lymphocytes (44). RSV compromises host immune responses against bacterial infections (51) of the respiratory tract, which may be due to reduction in intracellular GSH of NK cells.

Bovine Respiratory Syncytial Virus and Cytokine Production - Bovine studies tend to present a mixed pattern of Th1 and Th2 cytokine expression following BRSV infection. Interferon-γ, IL-2, IL-4 and IL-10 mRNA have been detected in pulmonary and peripheral blood mononuclear cells from RSV-infected calves. Interferon-γ mRNA was detected in pulmonary mononuclear cells (MNC) of uninfected calves; however, neither IL-2, IL-4, nor IL-10 mRNAs were detected in pulmonary MNCs from non-infected calves. Interleukin-4 and IL-10 were discovered in only the lungs and peripheral blood of calves with extensive lung consolidation (52). This suggests a role for Th2 cytokines in more severe infections. A Th1 or Th2 cytokine profile alters the outcome of a BRSV infection. Often a Th1 profile will lead to recovery and clearance of the virus whereas a Th2 profile leads to enhancement of disease. The vaccine enhanced illness that occurred in infants in the 1960s associated with RSV and a formalin-inactivated vaccine (53) has advanced the need for vaccine studies and development of a safe and effective vaccine. Calves challenged with BRSV after vaccination with a formalin-inactivated BRSV had significantly higher clinical scores than mock-vaccinated controls. The PBMCs from vaccinated calves produced significantly less IFN-γ (54) suggesting a Th-2 bias in response to the infection. The decrease in IFN-γ, a cytokine necessary for viral clearance, demonstrated the inadequacy of a formalin-inactivated vaccine in halting an RSV infection. Utilizing a murine model it has been shown that priming of mice with inactivated virus induced a dominant Th2 cytokine response, after challenge. In contrast, challenge of mice primed with live RSV induced a Th1 cytokine response (55). Priming mice with inactivated virus versus live virus resulted in an increase in IL-4 mRNA expression compared to IFN-γ mRNA expression. The reciprocal result was observed

when priming with live virus versus inactivated virus (55). A primary RSV infection in mice results in the activation of T cells. The cytokines produced play roles in causing lesion formation. Interferon-γ governs the immune response to a primary RSV infection in mice, implying a Th-1 type response (56).

Mutant RSVs lacking the G and SH proteins have been utilized to study their role in the development of Th2 type cytokines. Interestingly, deletion of G and SH resulted in a significant increase in NK cells from bronchoalveolar lavage (BAL) of infected mice (57). Additionally, higher IFN-γ and IL-12 expression was visible during infection with this mutant versus a virus with the G and SH proteins intact (57). The rise in Th1 cytokine expression may be a factor of the increased NK cells present, and would hint to a significant role for the G and/or SH proteins in directing a Th2 type cytokine response during RSV infection.

Additional cytokines and their roles during RSV infection have been studied. Interleukin-13, a Th2-type cytokine can contribute to severe allergic asthmatic responses during a primary RSV challenge (58). This relates well with IL-5 in allergic airway responses when induced by a RSV infection (48). Persistently infected murine macrophages secreted more IL-1β and IL-6 compared to non-infected cells (59). In mice, IL-12 treatment down-regulates type 2 cytokine responses to G protein (60). When CD8+ T cells produce IFN-γ a reduction in lung eosinophilia occurs but with further augmentation of illness whereas IL-12 activated NK cells expressing IFN-γ also reduce lung eosinophilia but without causing extended illness (60). Bovine studies have not been reported that assessed change in lung eosinophilia or disease after IL-12 treatment. However, the presence of IL-12 and BRSV markedly increased IFN-γ production in

PBMCs and analysis of the T cell subsets identified CD4+ T cells as contributing the most IFN-γ (61). Cytokine generation occurs in a complex pattern and is dictated on a cell to cell basis, by time as well as by type of viral infection.

There are no reports of recent studies directed towards characterizing the effect of BRSV infection on bovine NK cells or on GSH level. BRSV infection does alter and may cause the wrong immune response for a viral infection, enhancing the susceptibility of the host to secondary infections. The goal of the present study was to characterize the effect of BRSV infection on NK cell function and GSH level. A correlation has been shown between disease and reduced GSH, however, this has not been studied in BRSV infection. The findings in the present study will demonstrate the connection between BRSV infection and GSH levels in NK cells.

Bovine Viral Diarrhea Virus

BVDV was first identified in cattle in the United States in 1946. Consequences of BVDV infections include: diarrhea, immunosuppression, repeat breeding problems, abortion, mummification, congenital defects, immunotolerance and persistent infection, and acute and chronic mucosal disease (62). BVDV is a positive-stranded RNA virus belonging to the genus *Pestivirus*, of the family *Flaviviridae*. BVDV infection occurs primarily in lymph nodes, but can also be detected in lymphocytes and various cell lines during an *in vitro* infection. Virions are assembled by budding into the endoplasmic reticulum (ER) whereupon they obtain their envelope. The envelope contains the E1 and E2 glycoproteins, disulfide linked into homo- and heterodimers. E2 is vital for virus infectivity in cell culture (63) however the function of E1 is unknown. The genomic

RNAs contain continuous long open reading frames (ORFs), which are translated into polyproteins. Processing that occurs co- and posttranslationally by cellular and viral proteases results in the mature viral proteins. Upstream of the large ORF is an untranslated region (UTR). The 5' UTR, highly conserved among pestivirus species is used to differentiate among member viruses (64). Two subgroups, BVDV I and BVDV II are distinguished by nucleotide sequences in the 5' UTR. The two subgroups are as distinct from each other as they are from other pestiviruses such as hog cholera virus (65). BVDV II was isolated principally from fetal bovine sera, persistently infected calves and cattle that had died from an acute form of BVDV (65). Type I BVDV is usually associated with mild, subclinical infections whereas type II is competent of causing severe clinical disease in cattle. BVDV infection can cross species and infect pigs. Pigs became infected with type I BVDV at lower infectious doses than with type II, while type II did not result in clinically evident disease (66).

Besides subgroup, BVDV pathogenesis also relies on biotype. BVDV can be divided into two biotypes. The cytopathic biotype (cp) results in cell death and is associated with the development of mucosal disease. Noncytopathic (ncp) BVDV infection does not result in host cell death or visible infection in cell culture. However, it is important in causing immunosuppression in post natal infections.

Both biotypes can be identified in cell culture and infected tissues by immunoperoxidase staining and protein expression patterns, respectively. Staining patterns are similar 3 days post infection (p.i.), yet at 6 days p.i. there is a decrease in the staining intensity with the ncp strain (67), which may be due to a decrease in production of viral proteins. This decrease could enable the ncpBVDV to go undetected by the

immune system and thus establish persistent infection. In infected tissues, the non-structural protein NS3 is expressed only in cp-infected cells whereas ncp-infected cells display NS2-3 (68, 69). A 27-nucleotide insertion is present in RNA of cp viruses but not in ncp counterparts (70). This insertion is responsible for the cleavage of the NS2-3 and generation of the NS3 protein (71). Cytopathic and ncp biotypes have been recovered in animals exhibiting either mild or severe disease. Moreover, infection with BVDV that results in mucosal disease resolves in isolation of both ncp and cpBVDV. Evidence indicates that mucosal disease occurs when animals persistently infected with an ncp virus produce cp virus after recombination of the ncp form (72). The interactions of subgroups and biotype are keys in the dissemination of virus as well as severity of disease.

Bovine Viral Diarrhea Virus in Lymphocytes - Bovine PBMCs are susceptible to infection with BVDV both *in vivo* and *in vitro*. BVDV infection appears to interfere with PBMC function through depressed response to mitogen stimulation (72) as well by causing reduced synthesis of IgG and IgM (73). Although ncpBVDV infection often results in mild or no clinical symptoms, it can result in immunosuppression. Cattle with an established immune response to *Mycobacterium bovis* or *Mycobacterium avium* exhibit transient suppression of the immune response after acute infection with ncpBVDV (74, 75). Cattle seronegative to BVDV can be persistently infected, and thus immunotolerant to the virus. PBMCs obtained from persistently infected cattle (seronegative) fail to induce a proliferative response to BVDV (76). Nevertheless, cells retained the ability to proliferate after stimulation with ConA. Moreover, PBMCs from