THE ROLE OF INTERFERON AND NON-CONVENTIONAL T-CELLS IN THE CLEARANCE OF PRIMARY BHV-1 RESPIRATORY INFECTION

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ABSTRACT

Bovine herpesvirus-1 (BHV-1) is prevalent globally and is an important cause of bovine disease. Humoral and cell-mediated immune responses contribute to viral clearance following a primary BHV-1 respiratory infection but innate immune mechanisms contributing to early control of BHV-1 infection are not known. Gene expression analyses and immunohistochemical (IHC) studies were used to investigate the role of interferons (IFNs) and innate immune cells during a primary BHV-1 infection. There was significant ($P < 0.05$) induction of type I IFN and IFN-stimulated genes (ISGs) in the URT within 3 days post-infection (pi) but virus shedding continued for another 4 to 7 days. *In vitro* studies demonstrated that both type I and II IFNs had limited capacity to inhibit BHV-1 replication and BHV-1 infection did not block ISG transcription. Consequently, immune cell recruitment to the URT was analyzed following BHV-1 infection to determine if alternative defence mechanisms were activated. Morphometric analyses of lymphocytes in URT tissues before and after BHV-1 infection revealed significant ($P < 0.05$) increases in cell populations expressing $CD335^+$ (NKp46 natural cytotoxicity receptor), $CD3^+$ (T-cell lineage marker) and $CD8^+$ (cytotoxic T-cell marker) on day 5 pi. Confocal microscopy confirmed that approximately 90% of lymphocytes present on day 5 pi were T-cells co-expressing CD335 and CD8. This bovine T-cell subpopulation is known as non-conventional T cells, which may be innate lymphocytes. Specific recruitment of non-conventional T-cells to the site of BHV-1 infection in the URT raised questions regarding what chemokines may be involved in regulating this selective cell migration. Chemokine(s) involved in non-conventional T-cell recruitment in cattle are currently not known. qRT-PCR analysis of known bovine chemokine genes revealed that the expression of *CCL4*, *CCL5* and *CXCL9* genes were significantly ($P < 0.05$) up-regulated following BHV-1 infection in nasal turbinates. Thus, one or

more of these chemokines may be involved in the selective recruitment of bovine non-conventional T-cells to the site of BHV-1 infection. The specific recruitment of non-conventional T cells to infected tissue suggests these cells may play an important role in either viral clearance or regulating host responses during BHV-1 infection.

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Cheers to Research…we truly stand and build on the shoulders of Giants. Thanks to my committee members, I have built on their excellent contributions.
And a lasting thanks to the 30 calves that gave life to this work, may their descendants forever be aware and BHV-1-free.

DEDICATION

To My Parents, Ahmed Osman and Aster Abraham,
I'm forever fortunate to be your daughter
&
My loving husband, Patrick Okoye

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ABBREVIATIONS

ADCC: Antibody-dependent cellular cytotoxicity

ANOVA: Analysis of Variance

AP: Antimicrobial anionic peptide

APC: Antigen presenting cells

BHV-1: Bovine herpesvirus-1

bICP0: Bovine infected cell protein-0

BRD: Bovine respiratory disease

BRDC: Bovine respiratory disease complex

BRSV: Bovine respiratory syncytia virus

BRSV: Bovine respiratory syncytial virus

BST-2: Bone marrow stromal antigen-2

BVDV: Bovine viral diarrhea virus

CD: Cluster of differentiation

CLU: Cryptolymphatic units

COPD: Chronic obstructive pulmonary disease

CTL: Cytotoxic T-lymphocytes

DAMPs: Danger-associated molecular patterns

DCs: Dendritic cells

eIF2-α: Eukaryotic initiation factor-2-alpha

ELISA: Enzyme-Linked Immunosorbent Assay

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HCF-1: Host-cell factor -1

HEV: High endothelial venules

HSV-1: Herpes simplex virus-1

IBR: Infectious Bovine Rhinotracheitis

ICAM-1: Intercellular adhesion molecule -1

IE: Immediate early

IF: Immunofluorescence

IF: Inter-follicular zone

IFN: Interferons

IFNAR: Interferon alpha receptor

IFNGR: Interferon gamma receptor

IHC: Immunohistochemistry

IKKe: IκB kinase epsilon

ILF: Isolated lymphoid follicles

IN: Intranasal

IPB: Infectious Pustular Balanoposthitis

IPV: Infectious Pustular Vulvovaginitis

IRF: Interferon regulatory factor

ISG: IFN-stimulated genes

JAK/STAT: Janus kinase/signal transducers and activators of transcription

JAM: Junctional adhesion molecules

KIR: Killer-cell immunoglobulin-like receptors

LAP: Lingual (tongue) antimicrobial peptide

LAP: Lingual antimicrobial peptide

LFA-1: Lymphocyte function associated antigen -1

LN: Lymph node

LR: Latency-Related

mAb: Monoclonal antibodies

Mac-1: Macrophage antigen-1

MadCAM-1 : Mucosal vascular addressin cell adhesion molecule 1

MAIT: Mucosal associated invariant T-cells

MALT: Mucosa-associated lymphoid tissue

MDBK: Madin-Darby bovine kidney

MHC: Major histocompatibility complex

MLV: Modified live vaccines

MUC: Mucins

Mx: Myxovirus resistance

NALT: Nasal-associated lymphoid tissue

NCR: Natural cytotoxicity receptors

NK: Natural Killer

NKT: Natural Killer T-cell

NLRs: NOD-like receptors (NLRs)

OAS: Oligoadenylate synthetase

PAMPs: Pathogen - associated molecular patterns

PBMC: Peripheral blood mononuclear cells

PI3: Parainfluenza-3

PKR: Protein kinase R

PRRs: Pattern recognition receptors

PSGL-1: P-selectin glycoprotein ligand -1

rBoIFN: Recombinant bovine interferon

RFLP: Restriction fragment length positioning

RIG-I: Retinoid acid-inducible gene I

RPS9: Ribosomal protein S9

sIg: Surface immunoglobulin

sIgA: Secretory IgA

SLAMF9: Signaling lymphocytic activation molecule family member 9

TAP: Tracheal antimicrobial peptide

TAP*: Transporter associated with antigen presenting

TBK1: TANK- binding kinase 1

TCR:T-cell receptor

TLR: Toll-like receptors

URT: Upper respiratory tract

VCAM-1: Vascular cell adhesion molecules

Vhs: Virus host shutoff

VLA: Very late antigen

VN: Virus neutralizing

VSV: Vesicular Stomatitis Virus

VZV: Varicella Zoster Virus

WC: Workshop cluster

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 THE MUCOSAL IMMUNE SYSTEM IN THE UPPER RESPIRATORY TRACT OF CALVES

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I wrote all sections of the manuscript included in my thesis with editing by Dr. PJ Griebel.

1.1.1 ABSTRACT

Respiratory infections remain the second most common cause of clinical disease and mortality in young calves, which has led to increased interest in using vaccines early in life to mitigate this risk. Intranasal (IN) vaccination of neonatal calves can be an effective strategy to circumvent vaccine interference by maternal antibody but this raises questions regarding onset of immune competence in the upper respiratory tract (URT) following birth. Little is known, however, about the development and function of mucosal-associated lymphoid tissue (MALT) in the URT of young calves and what factors, including the commensal microbiome, contribute to this early development. We review the structure, development, and function of MALT in the bovine URT during the first six weeks of life and identify knowledge gaps regarding this early development. This information is critical when designing vaccination programs for young calves, especially when targeting respiratory pathogens that may reside within the commensal microbiome.

1.1.2 INTRODUCTION

The upper respiratory tract (URT) is a point of entry for many pathogens that cause either respiratory or enteric infections. The URT consists of nostrils, nasal turbinates, the

nasopharyngeal tonsil, the retropharyngeal lymph node, and the pharynx (Figure 1.1). It is also an important route of transmission for pathogens that are shed in oral or nasal secretions. The URT is covered by mucosal epithelium that provides an interface between the host and the diverse bacterial and viral populations, which constitute both "resident" microbiota and potential pathogens. Resident microbiota refers to microorganisms, which persist over prolonged periods in close association with the mucosal epithelium of the URT. In contrast, the transitory microbiota includes microorganisms that are either inhaled or ingested but persist for a brief time in the URT [1]. The mucosal surface forms the host's physical and immunological barrier to resident and transitory microbiota and potential pathogens. Maintenance of this barrier is critical to the health of an animal and a variety of innate and acquired immune responses contribute to barrier maintenance. Rapid development of this barrier is crucial as the newborn calf emerges from the sterile uterine environment to an environment populated with microorganisms.

1.1.3 INNATE IMMUNE RESPONSES

Mucosal surfaces in the URT provide protective physical and immunological barriers against pathogens and toxic substances in the environment. The local innate immune system is comprised of the mucosal epithelium, mucins, pattern-recognition receptors (PRRs), antimicrobial peptides, and a variety of effector cells.

1.1.3.1 Physical barriers

The first barrier to pathogens and large particulate matter in the bovine URT is hair, which filters large airborne material as it enters the nasal cavity [2]. The next physical barrier is

the pseudostratified columnar mucosal epithelium on the surface of the nasal conchae. The epithelial barrier involves tight junctions with lateral cell-to-cell contact to prevent invasion by pathogens or resident microbiota. The mucosal epithelium also includes specialized cells such as ciliated epithelial cells and goblet cells. Goblet cells produce mucus and ciliated epithelial cells are involved in mucus clearance. Mucus is a viscous fluid, which covers mucosal surfaces. It forms a thick layer on the mucosal epithelial surface and traps foreign substances and microbial pathogens that are inhaled. This trapped material is then cleared through mucociliary clearance. The composition of mucus in the bovine URT has not yet been characterized. In humans, however, it is primarily composed of a complex mix of polysaccharides, including mucins (MUC) [3], with MUC2 and MUC3 being the most abundant. MUC 1, 4 and 16 are membrane bound while MUC2, 5AC, 5B and 19 are secreted into the human respiratory tract [4]. Although mucus has a protective role at mucosal surfaces, unregulated or poorly regulated mucin production is associated with diseases such as cystic fibrosis and chronic obstructive pulmonary disease (COPD) [3]. The production of mucins in the URT of newborn calves has not been investigated and it is not known at what age this defence mechanism is fully functional or what factors may delay or disrupt mucin production during this vulnerable period.

1.1.3.2 Pattern Recognition Receptors

If pathogens or other noxious substances breach the physical barrier of the mucosal epithelium, it is important that the host is able to sense this danger. Pattern recognition receptors (PRR) are expressed by mammalian mucosal epithelium and immune surveillance cells including dendritic cells (DCs), macrophages and neutrophils. These

PRRs include several families of molecules, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I). PRRs recognize pathogen-associated molecular patterns (PAMPs) of pathogens or commensal microbiota or danger associated molecular patterns (DAMPs) released during tissue damage. Although the expression of PRRs in the bovine URT has not been analyzed, it has been reported that the bovine TLR 1 through 10 genes are expressed in the tracheal mucosa [5]. It is not known, however, whether the expression of PRRs varies among regions of the URT, oral cavity, or the lung. Further work is also required to determine which PRRs are expressed by the mucosal epithelium and resident immune cells in the mucosa and submucosa of the URT. PRRs play an important role in not only recognizing invading pathogens but also provide signals that regulate the induction of the acquired immune responses to vaccines. Determining if there are significant regional and age-dependent differences in the expression of PRRs in the bovine URT may inform the formulation and delivery of intranasal (IN) vaccines in young calves.

1.1.3.3 Antimicrobial peptides

Another component of the innate immune defence provided by the mucosal epithelium is the production of host defence peptides, also known as antimicrobial peptides. These small peptides have many functions, including cytotoxic effects on bacteria and viruses, anti-inflammatory activity, stimulation of epithelial cell growth, tissue damage repair, and immune cell activation and recruitment. These secreted peptides are often retained within the mucus layer. In sheep, antimicrobial anionic peptide (AP) is produced by alveolar epithelium and has been shown to bind to and inactivate *Mannheimia haemolytica* [6]. In

cattle, a number of beta-defensins have been identified in the respiratory tract. Tracheal antimicrobial peptide (TAP) was the first bovine beta-defensin discovered in bovine tracheal mucosa and shown to have antimicrobial activity against Gram-positive and negative bacteria. Lingual (tongue) antimicrobial peptide (LAP) was the second beta-defensin discovered and is expressed in bovine nasal, buccal, conjunctival and tracheal mucosa. The same study showed that LAP expression is present in young and adult calves but absent in fetal tongue, suggesting exposure to microbes may contribute to antimicrobial peptide expression [7]. Another bovine beta-defensin, DEFB103 was shown to be highly expressed in epithelial cells located in the oral cavity, and tracheal epithelium of young calves. The level of DEFB103 expression in neonatal calves was similar to that observed in older calves [8]. Additionally, DEFB103 expression was up-regulated in the oral epithelium of 6 to 8 months old calves following an URT infection by bovine herpesvirus-1 (BHV-1). Further studies are required to fully characterize the range of host defence peptides expressed throughout the bovine URT and better define the many roles they may play in regulating microbial infections [8].

1.1.3.4 Effector cells

Innate immune effector cells in the bovine respiratory tract include phagocytic dendritic cells (DCs), macrophages, natural killer (NK) cells and granulocytes [2, 9]. It has been shown that the abundance of bovine NK cells and neutrophils increases in blood during the first 3 months of life [10]. Studies of bovine mucosal tissues in the gut have shown that macrophages and DCs are present in neonatal calves at 21 days of age [11]. However, the abundance and distribution of these cells has yet to be analyzed in the URT of young

calves. Thus, it is not known if there is a paucity of innate immune effector cells present at mucosal surfaces of the newborn URT and at what age these defences may become fully functional. This information is critical for understanding not only innate immune defences but also for understanding the capacity of mucosal immune tissues to sample foreign antigens and initiate the induction of an acquired immune response.

1.1.4 ACQUIRED IMMUNE RESPONSES

1.1.4.1 Immune Induction Sites

Mucosa-associated lymphoid tissue (MALT) in the URT consists of both inductive and effector sites. The inductive sites consist of organized lymphoid tissues, with aggregated or isolated lymphoid follicles (ILF) distributed within T-cell rich regions, known as the inter-follicular zone (IF). Specialized epithelium overlying this organized lymphoid tissue contains microfold (M)-cells that play a key role in antigen sampling. DCs are also distributed throughout the mucosal epithelium and play an important role in immune surveillance. Antigen taken up by DCs is then transported through the lymphatic system to draining lymph nodes associated with the URT. The primary function of MALT inductive sites is the generation of antigen-specific, Th2-dependent immunoglobulin A (IgA) plasma cells.

1.1.4.2 Mucosa-Associated Lymphoid Tissue (MALT) in the Bovine URT

The MALT in the bovine URT includes nasal-associated lymphoid tissue (NALT) and the lymphoid tissues of Waldeyer's ring, which includes the lingual, palatine, pharyngeal and tubal tonsils. NALT has been most extensively studied in mice and humans, with few

studies in domestic animals, including ruminants. Murine NALT is characterized by a pair of aggregated lymphoid follicles located at the base of the nasal cavity near the entry of the pharyngeal duct [12]. In sheep, ILFs have been shown to be distributed across the nasopharyngeal region and contain numerous well-developed germinal centres in the region of the Eustachian tube [13]. Sheep nasal lymphoid tissues are covered by ciliated pseudostratified columnar (epithelial) and non-ciliated M-cells [14]. Previous studies have also shown that the equine ILFs are distributed in 9 different locations with varying density within the nasal cavity [15]. Although a higher density and number of inductive sites may indicate a greater capacity for induction of mucosal immune responses, this hypothesis has not been tested. Alternatively, these ILFs may represent antigen-induced MALT as has been reported for bronchus-associated lymphoid tissue in the lung [16].

Waldeyer's ring consists of several aggregated lymphoid nodules known as tonsils. In cattle, this includes the lingual, palatine, pharyngeal, tubal tonsils and the tonsil of the soft palate [16]. The palatine, lingual and soft palate tonsils protect the oropharynx, while the pharyngeal tonsil protects the nasopharynx. The lingual tonsil consists of many cryptolymphatic units (CLU), which extend from the base of the tongue along the lateral pharynx to the epiglottis. CLU consists of epithelial crypts, which are encircled by lymphoid follicles and interfollicular spaces. The palatine tonsil is set in the wall of the oropharynx (Figure 1.1), while the tonsil of the soft palate is made up of CLUs, which are exposed to the oral cavity.

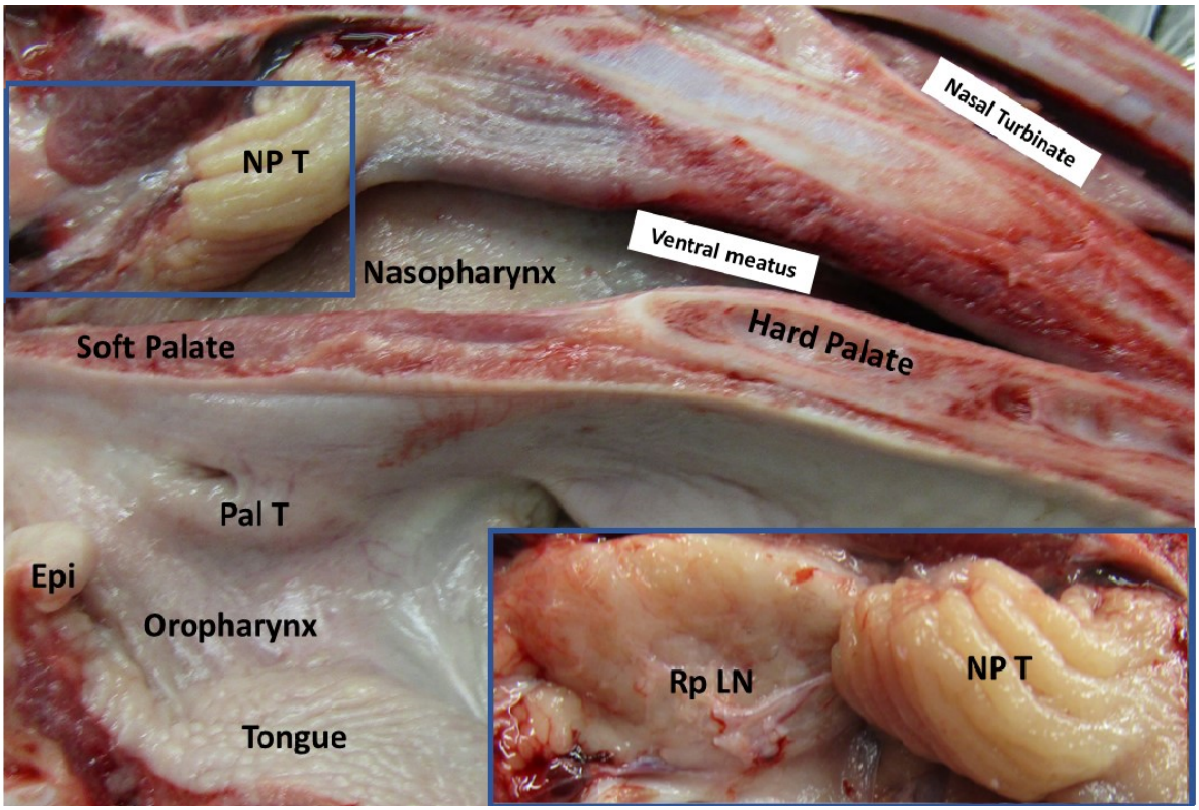

Figure 1.1 Mucosa-associated lymphoid tissue in the upper respiratory tract of a young calf. A midsagittal section of the head reveals organized lymphoid tissues present in the nasopharynx and oropharynx of a young calf. Located posterior to the nasal passages and protruding into the nasopharynx is the nasopharyngeal tonsil (NP T). The NP T is characterized by numerous rugae separated by folds. The palatine tonsil (Pal T) is located in the submucosa of the oropharynx lateral to the base of tongue. The Pal T is only visible as the crypt that communicates with the oropharynx. **Inset:** Mucosal epithelium overlying the retropharyngeal lymph node (Rp LN) was removed to reveal the Rp LN located in the submucosa adjacent to the NP T.

The pharyngeal and palatine tonsils are the major components of the Waldeyer's ring in cattle (Figure 1.1). The pharyngeal tonsil is first recognizable at 95 days of gestation. This tonsil consists of parallel "rugae", which form a series of shallow folds and furrows with the number of rugae ranging from 8 to 14 by 95 days of gestation [17]. The pharyngeal tonsil is not fully developed at birth but rapidly develops within the first two to three weeks postpartum (Figure 1.2). Post-natal development of rugae is characterized by increased infiltration of lymphocytes into the IF region and the formation of germinal centers is

evident within 3 weeks after birth (Figure 1.2). The size of this lymphoid tissue then decreases in animals older than 7 years due to a decrease in lymphoid follicle size and number [17]. A morphological analysis of pharyngeal tonsils in calves has shown that the pharyngeal tonsilar surface is composed of reticular and non-reticular epithelial cells, which are a mix of ciliated and non-ciliated pseudostratified columnar epithelial cells, rounded microvillus-covered cells and flat squamous cells harboring lymphocytes. These lymphocytes include T-cells ($CD4^+$, $CD8^+$, $\gamma\delta TCR^+$), macrophages ($CD14^+$), and DCs ($CD11c^+$ and CD $172a^+$) [18]. However, the abundance and distribution of these leukocyte populations during pharyngeal tonsil development in newborn calves is still unknown.

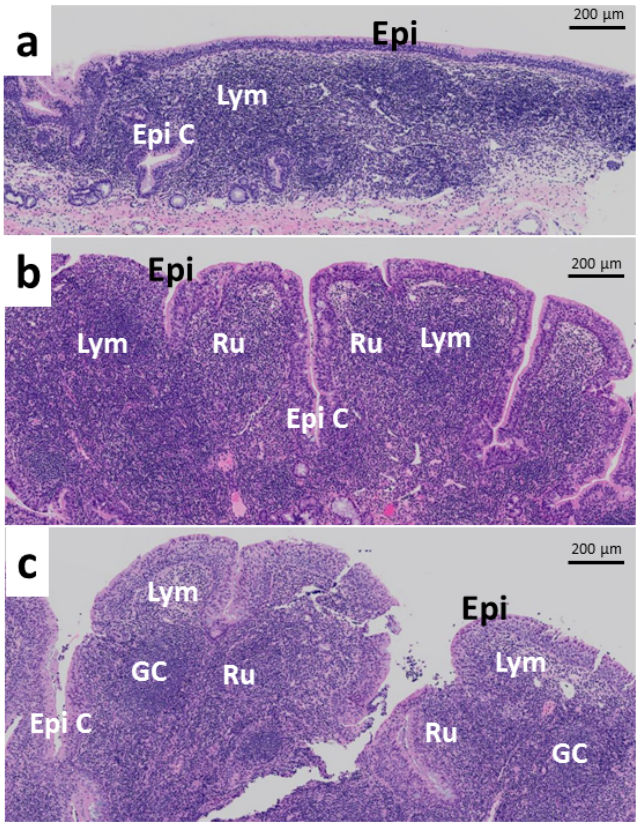

Figure 1.2. Rapid development of nasopharyngeal tonsil occurs in newborn calves. **(A)** The nasopharyngeal tonsil in the fetal calf (day 260 gestation) is characterized by few epithelial crypts (Epi C), no formation of rugae at the mucosal surface (Epi), and a diffuse lymphoid (Lym) infiltration in the submucosa. **(B)** In 7-day old calves, the nasopharyngeal tonsil is characterized by the formation of numerous rugae (Ru) separated by epithelial crypts. There is an extensive accumulation of lymphocytes within each ruga. **(C)** The nasopharyngeal tonsil in 21-day old calves has increased markedly in size and located within each ruga are numerous germinal centres (GC).

Similar to the pharyngeal tonsil, the bovine palatine tonsils are not fully developed at birth and may require as much as 2 months to be fully developed [19]. The number of secondary lymphoid follicles continue to increase until adulthood when distinct germinal centers and IF areas are clearly visible [20]. These observations are consistent with studies in mice that report NALT organogenesis is dependent on exposure to environmental antigens as well as the presence of chemokines such as CXCL13, CCL19 and CCL21 [21]. It is not known, however, whether exposure to commensal bacteria or pathogens contributes to the rapid development of bovine tonsils in the URT (Figure 1.2).

1.1.4.3 Lymphocenters and Lymph nodes

The bovine URT contains 3 lymphocenters, each consisting of multiple lymph nodes. The parotid lymphocenter drains the skin and anterior part of the nasal cavity. The mandibular lymphocenter includes the mandibular and pteryhoid lymph nodes, which also drain the anterior nasal cavity, the skin of the head and the oral and nasal cavities. The retropharyngeal lymphocenter includes the medial and lateral retropharyngeal lymph node. These lymph nodes drain the nasopharynx and also collect lymph from the mandibular and parotid lymphocenters, in addition to part of the thymus, buccal mucosa, the larynx and pharynx [22]

1.1.5 IMMUNE SYSTEM FUNCTION IN THE URT OF YOUNG CALVES

The functional capacity of the mucosal immune system in URT of newborn calves has been a point of interest due to frequent respiratory disease and an increased interest in early use of intranasal (IN) vaccines. The high incidence of respiratory disease in young calves has been attributed, in part, to the immaturity of the neonatal mucosal immune system and a rapid decline in protective maternal IgA levels within 5 to 7 days after birth [23]. A Th2-biased neonatal immune response makes newborn calves susceptible to intracellular pathogens like viruses. One explanation for a Th2-biased response may be a reduced number and functional capacity of accessory cells like dendritic cells [24]. Another explanation may be maternal supression of the neonatal systemic immune system as a consequence of the Th2-bias that develops during pregnancy to maintain immune tolerance of the fetus. This Th2-bias in newborn calves can be overcome to some extent when parenteral vaccines are adjuvanted with immune stimulatory complexes [25]. However, an increasing number of IN vaccination and disease challenge studies are providing evidence that MALT in the URT has the capacity to respond to foreign antigens within the first week of life .

1.1.5.1 Mucosal immunoglobulin A (IgA) responses

Secretory IgA (sIgA) is the primary humoral immune response at mucosal surfaces of the bovine URT and plays an important role in neutralizing toxins and pathogens. sIgA also plays an important role in maintaining the barrier that excludes the dense population of commensal microorganisms at the mucosal surface. Antigen sampling by DCs present in mucosal epithelium and M-cells overlying tonsils captures inhaled and ingested antigens.

These antigens are then presented to either naive B-cells in the germinal centers where class switching to IgA can take place or DCs present processed antigens to naïve T-lymphocytes to support the generation of Th2-dependent IgA response [26].

Human infants are highly susceptible to infections in the first year of life [27] despite the protection provided by maternal antibody during this time. In the newborn calf, colostrum is the major source of passive immunity through the transfer of primarily IgG1 antibody but colostrum also includes minor amounts of IgM, IgA and IgG2. The level of maternal antibody in bovine milk decreases significantly within a few days of birth [23, 27]. Thus, the immaturity of the mucosal immune system makes newborn calves vulnerable to a variety of respiratory infections as the level of maternal IgA is rapidly depleted within the first week of life (23). Neonatal mucosal immunization strategies are now being explored as a strategy to rapidly increase immune protection during the neonatal period [23, 28]. High levels of maternal antibody circulating in the blood of newborn calves have, however, been reported to interfere with both killed and modified-live vaccines following parenteral injection [29]. Mucosal immunization targeting the URT may provide an effective strategy to circumvent vaccine interference by maternal antibody due to a rapid depletion of maternal IgA in mucosal secretions in the first week after birth [23].

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1.2 PRIMARY BOVINE HERPESVIRUS-1 INFECTION

1.2.1 BOVINE HERPESVIRUS -1

1.2.1.1 Herpesviruses: General Features

Herpesviridae is a family of large, double-stranded DNA viruses, with a wide range of hosts including mammals, birds, fish and reptiles [30]. Viruses were included in this family based on their unique virion architecture until the 1980s [30]. The virus structure consists of a core with a tightly packaged genomic DNA in an icosahedral capsid. The core is surrounded by a protein-rich matrix, known as tegument, and enclosed in a host-cell derived, lipid bilayer envelope, which anchors glycoproteins [31]. Although there is significant diversity among herpesviruses, all members of this family cause latent infections in neurons following a lytic infection and recrudesce periodically.

1.2.1.2 *Herpesviridae* Classification

Herpesviridae are further clustered based on biological properties into 3 subfamilies: *alpha- beta- and gamma-herpresvirinae*. The biological criteria consider host range, length of replication cycle, cytopathology and latency-related characteristics [32]. *Alphaherpresvirinae* includes herpesviruses with a broad host range and a short replication cycle in mucosal epithelial cells. Following a primary infection in epithelial cells, alphaherpesviruses establish latent infections primarily in host sensory nerves, such as the trigeminal ganglia [33]. Some of the well-documented members of this group include human herpesviruses, such as HSV-1 and Varicella zoster virus (VZV), porcine herpesviruses like Pseudorabies virus (PRV), equine herpesviruses including Equine

herpesvirus -1 (EHV-1), and ruminant herpesviruses such as bovine herpesvirus (BHV-1). *Betaherpesvirinae* include species-specific herpesviruses with long replication cycle resulting in enlarged infected cells followed by lysis and latent infections in secretory glands, macrophages and kidneys [31] Bovine and human cytomegalovirus (CMV) are examples of betaherpesviruses. Gammaherpesviruses, similar to betaherpesviruses, have a restricted host range and cause lytic infections in lymphocytes and macrophages and ultimately establish latent infections in lymphocytes [31]. Examples of gammaherpesviruses are Epstein-Barr Virus (EBV) in humans and Bovine herpesvirus–4 (BHV-4) in cattle [34].

1.2.1.3 BHV-1: Introduction

BHV-1 is a ruminant alphaherpesvirus, which is closely related to the human herpesvirus, HSV-1. Similar to other herpesviruses, BHV-1 is composed of double-stranded, linear genomic DNA tightly packaged in an approximately 100 nm icosahedral capsid, surrounded by a tegument, and enclosed in a lipid envelope anchoring viral glycoproteins.

1.2.1.4 Virus Structure

1.2.1.4.1 Genome

BHV-1 was first reported in 1953 in the US [35] and subsequently reported in 1958 in Ontario, Canada. BHV-1 infection in dairy herds was associated with sudden onset of severe inflammation in the upper respiratory tract (URT) and trachea and a rapid reduction in milk production [36]. The virus was isolated in 1956 [35], viral proteins were first identified and analyzed in 1983 [37], and the genome was sequenced in 1996 [38]. BHV-1 has a 135.3 kbp genome arranged as a Class D genome, which has two unique sequences:

unique long (U_L) and unique short (U_S). BHV-1 strains have been categorized, based on differences in genomic sequence, into BHV-1.1, 1.2a, and 1.2b subtypes ([37, 39]. The BHV-1 genome encodes for 73 proteins which have been classified on the basis of temporal regulation into Immediate Early (IE), Early (E) and Late (L) genes [40]. The BHV-1 genome also encodes 10 microRNA genes [41]. The IE proteins of BHV-1 are bovine infected cell protein (bICP)-0, bICP4, bICP22, and bICP27. These genes are named according to their homologues in HSV-1. IE genes are transcribed prior to DNA replication and do not require *do novo* viral protein synthesis for expression. IE gene expression is activated by a BHV-1 tegument protein, VP16, also known as α–trans-inducing factor (α-TIF), which forms a complex with host proteins HCF-1 and Oct-1 and binds to the promoter of bICP0 to initiate IE transcription [42]. Early genes code for DNA-binding proteins and enzymes involved in DNA replication [43]. Viral DNA replication is catalyzed by host RNA polymerase II. Late genes encode BHV-1 structural proteins contributing to formation of the capsid, tegument and glycoproteins.

1.2.1.4.2 Capsid

There is limited direct information regarding the structure of the BHV-1 capsid. The closely related alphaherpesvirus, HSV-1, has an icosahedral capsid with symmetry of T=16 composed of 162 capsomeres. The capsid is composed of two layers: the outer and intermediate layers, with the outer layer composed of four proteins [43]: VP5, VP19c, VP23, and VP26, in addition to proteins coded by UL6 and UL25 [44]. VP5 is the major capsid protein, which is conserved among alphaherpesviruses [45, 46].

1.2.1.4.3 Tegument

The tegument is a protein-rich, amorphous matrix surrounding the capsid of herpesviruses, which exists between the capsid and the envelope. There are over 15 tegument proteins identified in HSV-1[44] but only 4 tegument proteins have currently been identified in BHV-1. Known BHV-1 tegument proteins include VP8, VP16, VP22 and viral host cell shut off (Vhs) protein. VP8 is the most abundant tegument protein [40, 47] which was recently shown *in vitro* to prevent nuclear accumulation of the interferon-induced transcription factor, signal transducer and activator of transcription (STAT1) [48]. VP16 is a trans-inducing protein, which binds to host factors involved in protein synthesis to initiate transcription of immediate early viral proteins such as bICP0 [42]. BHV-1 VP22 binds to the transporter associated with antigen presenting (TAP*) heterodimer and causes a conformational arrest that results in inhibition of peptide transport and degradation of TAP* [49]. Through inhibition of TAP*, VP22 down-regulates expression of MHC class 1 which interferes with the detection of BHV-1 infected cells by cytotoxic T-lymphocytes. Vhs is encoded by UL41 gene and degrades pre-existing host mRNA and viral mRNA. Degradation of mRNA inhibits cellular protein synthesis and cellular defenses [50]. The tegument is a typical viral matrix in that it interacts with the capsid on one side and the cytoplasmic tails of the envelope-imbedded viral glycoproteins on the other side [44].

1.2.1.4.4 Envelope

Similar to other alphaherpesviruses, the BHV-1 envelope is derived from the host cell membrane and is composed of a lipid bilayer with viral proteins inserted within it [51]. BHV-1 has twelve glycosylated envelope proteins including gB, gC, gD, gE, gG, gH, gI,

gK, gL, gM, gN and US9 [52]. BHV-1 requires gB, gD and gH/gL for viral attachment, entry and viral-host envelope fusion during infection [53, 54].

1.2.1.5 Primary, Latent and Recrudescent BHV-1 infections

Primary BHV-1 respiratory infections are characterized by a mucopurulent nasal discharge, lacrimal discharge, high fever, loss of appetite, weight loss, a severe cough, excessive salivation, inflamed nose (hence named "red nose"), and reduced milk yield in lactating cows [55]. Additionally, the mucosal surfaces lining the nasal cavity are inflamed and develop a yellow diphtheritic membrane.

BHV-1 is the causal agent of Infectious Bovine Rhinotracheitis (IBR) in the respiratory tract and Infectious Pustular Vulvovaginitis (IPV in cows) and Infectious Pustular Balanoposthitis (IPB in bulls) in the genital tract. It is also one of the major pathogens associated with the respiratory disease syndrome, *shipping fever*. Systemic BHV-1 infection can cause abortion in pregnant cows, infertility in females, and conjunctivitis in cattle [52, 55]. Furthermore, BHV-1 infection can cause a fatal systemic infection and encephalitis in newborn calves [56]. Although most of the BHV-1 isolates from the respiratory tract belong to the BHV-1.1 type, subtypes 1.2a and 1.2b have also been associated with clinical respiratory disease. However, the initial classification of these subtypes was based on differences in restriction fragment length polymorphism (RFLP) in the viral DNA [37] and clinical syndromes caused by these isolates and may not reflect functional differences in viral gene expression or viral proteins.

A respiratory BHV-1 infection is acquired through direct nose-to nose contact between animals, viral transmission on fomites, and short distance aerosol transmission among animals [57]. Mucosal epithelium is the point of entry for BHV-1 in the upper respiratory tract, genital tract or conjunctiva [58]. Within 10 hours of infection, BHV-1 replicates and spreads cell to cell directly to adjacent, uninfected epithelial cells within the nasal mucosa. Subsequent spread of the virus to other cells can occur through an extracellular, paracrine manner, approximately three hours later [59]. The viral infection may be contained locally or develop into a systemic infection, which may lead to viremic disease and abortion in pregnant cows [60]. The primary sites of respiratory BHV-1 infection are nasal turbinates, pharyngeal tonsils and trachea [61]. Primary BHV-1 infection lasts 9-10 days and then spreads to the nervous system via cell-to-cell spread into sensory neurons that innervate the URT, and becomes latent in the trigeminal ganglia [55].

During viral latency, gene expression of immediate early genes, such as bICP0, are reduced [62] and viral gene expression is limited to latency related (LR) gene and open reading frame-E (ORF-E). Infectious virus particles are no longer detected [63]. Herpesviruses establish lifelong latency with no clinical symptoms until reactivated by external factors such as stress or the use of a synthetic corticosteroid, such as dexamethasone, in experimental situations [64] .

Upon reactivation, BHV-1 spreads cell-to-cell from neuronal cells to the mucosal epithelium of the URT and in this way is shielded from extracellular BHV-1

neutralizing antibodies. Recrudescent infections are characterized by the sudden appearance of detectable infectious BHV-1 particles in nasal or ocular secretions [65]. During recrudescence, there is increased expression of IE viral genes and reduced expression of LR genes within neurons [65].

1.2.1.6 BHV-1 and the Bovine Respiratory Disease (BRD) Complex

Bovine respiratory disease complex (BRDC), also known as *shipping fever* and *bovine pneumonia* occurs worldwide and has a significant economic impact in Canadian feedlots with an estimated cost of almost $12/animal [66]. BRDC is a multifactorial disease resulting from co-infection by multiple pathogens and infection is exacerbated by stressors like transportation and malnutrition [67, 68]. BRDC has been associated with multiple viruses, including BHV-1, bovine viral diarrhea virus (BVDV) type 1 and 2, parainfluenza-3 (PI-3) virus and bovine respiratory syncytia virus (BRSV) [69]. Numerous bacteria that are members of the commensal microbiome in the upper respiratory tract are also associated with BRDC. These bacteria include *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni, and Mycoplasma bovis [68, 70] .*

BRD is usually initiated by a primary viral infection followed by a secondary bacterial infection. Yates and colleagues described a BRDC viral- bacterial synergy following an initial BHV-1 infection. BHV-1 infection suppressed host immune responses, and increased the host's susceptibility to a fatal secondary bacterial infection [70]. Possible mechanisms mediating immunosuppression include:

- Lytic BHV-1 infection of ciliated epithelium and goblet cells lining the mucosal surfaces of the URT impairs mucin production and the mucosal clearance function of the epithelium. This increases the opportunity for bacterial colonization of the lung through a decreased capacity to remove bacteria [71].
- BHV-1 infection of activated T-helper cells (CD4+) leads to programmed death of CD4+ cells. This impairs cell-mediated immune response and host defense response against potential secondary bacterial infections [72].
- BHV-1 infection decreases neutrophil infiltration of the lungs in cattle by modifying pulmonary endothelial cells' response to pro-inflammatory cytokines like TNF-α and decreasing binding of neutrophils to endothelial cells. Impaired neutrophil recruitment may result in increased bacterial growth and colonization [73].

1.2.2 IMMUNE RESPONSE TO A PRIMARY BHV-1 INFECTION

1.2.2.1 INNATE IMMUNE RESPONSES

1.2.2.1.1 Non-immune barriers

Non-immune barriers refer to physical and biochemical host defense mechanisms orchestrated locally in tissues without the recruitment of immune cells. These mechanisms include mucus secretion by goblet cells, mucociliary clearance by ciliated epithelial cells, antimicrobial peptides produced by epithelial cells, coughing and physical barriers such as hairs in the external nares, and the tight junctions of epithelial cells.

1.2.2.1.2 Antimicrobial peptides

Antimicrobial peptides are short peptides or polypeptides less than 200 amino acids long and produced by cells within the epithelium. Bovine β-defensin DEF103, which is produced by basal epithelial cells in bovine buccal epithelium, is transcriptionally upregulated following a BHV-1 infection and may be an important host defense against BHV-1 infection [8]. Although other β-defensins such as tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP) have been shown in cattle to have microbial activity against gram positive and negative bacteria causing BRD [7, 74], their role in a BHV-1 infection remains unknown.

1.2.2.1.3 Mucopolysaccharides

There is limited information on the role of mucopolysaccarides during a BHV-1 infection. However, BHV-1 infection can increase glycoconjugate expression in the nasal mucosa which enhances adhesion and colonization by commensal bacteria involved in BRD [75]. Analysis of glycoconjugate composition in the bovine nasal mucosa revealed that three N-acetylgalactosamine specific lectins: *Dolichos biflorus, Glycine max, and Vicia villosa* were more reactive in bacterial adhesion following BHV-1 infection [75].

1.2.2.1.4 Interferons

Interferons (IFN) are an important component of innate immune defenses against viral infections and IFNs activate a variety of anti-viral defense mechanisms. There are three known types of IFNs: Type I, II, and III. Type I bovine IFNs include IFN-α, with at least 9 known subtypes, and IFN-β with 3 known subtypes [76]. IFN-γ is the sole member of type II interferons, while IFN-λ is the only member of type III bovine IFN. Type I

interferons are produced by all nucleated cells while type II interferons are produced primarily by Natural Killer (NK) cells, Natural Killer T (NKT) cells, and T-lymphocytes. It is currently not known which cells types produce type III IFNs. IFNs act in a two-step mechanism. IFN secretion is followed by IFN-activated receptor signaling that induces transcription of antiviral effector genes and translation of proteins, which interfere with viral replication. This interference with viral replication is the defining characteristic of interferon proteins which originally gave rise to the name, *Interferon* [77].

1.2.2.1.4.1 Interferon production

IFN production is induced following recognition of viral components by pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) and retinoic acid inducible gene -1 (RIG -1)-like receptors (RLRs). PRRs recognize virus components at the host cell surface and viral nucleic acids in endosomal compartment membranes. During a primary BHV-1 infection, bovine TLR 7 and 8 are transcriptionally up-regulated in nasal mucosa and lung epithelium but downregulated in tracheal epithelium [78]. In contrast, TLR 3 and 9 gene expression are not changed in the infected nasal mucosa and lung epithelium but downregulated in tracheal epithelium during a primary infection [78]. In contrast, during BHV-1 reactivation from latency, TLR 3 gene expression was up-regulated in nasal mucosa but no other tissues [78]. During reactivation, an upregulation of TLR7 and 8 gene expression was observed in tracheal and lung epithelium while expression of these genes did not change in the nasal mucosa [78]. These analyses indicate that TLR transcript levels during a BHV-1 infection vary based on tissue type and phase of BHV-1 infection. After engagement of TLRs, a signaling cascade is initiated with multiple transcriptional factors, such as interferon regulatory factors (IRFs) and NF-κB, leading to transcription of

interferons and other pro-inflammatory cytokines. Further, interferon transcripts are translated and ultimately secreted by infected cells and then act in either an autocrine or paracrine manner. BHV-1 is a potent inducer of IFN-α and -γ during acute respiratory infections [67]. Secreted Type I and II IFNs bind to the interferon receptors IFNAR and IFNGR, respectively, on the cell surface and initiate signalling pathways such as the JAK/STAT (as reviewed in [79]) which establishes an antiviral state through the induction of IFN-induced antiviral effector genes.

1.2.2.1.4.2 Interferon-induced antiviral immune responses

The antiviral state is established following activation of interferon-stimulated genes (ISG) and subsequent production of antiviral effector proteins such as double-stranded RNA-dependent protein kinase R (PKR), Myxovirus resistance (Mx) proteins, oligoadenylate synthetase-RNAase L (OAS-RNAse L) and BST-2 or Tetherin [80]. These antiviral proteins interact with different steps in the viral life cycle to inhibit replication, assembly, and release of infectious virions. PKR degrades dsRNA and RNase L degrades ssRNA, which inhibits viral protein synthesis. Mx1 binds to viral components in the cytosol and has the capacity to inhibit replication by binding to viral proteins during the uncoating process of the virus replication cycle [80]. Similarly, Mx1 also inhibits assembly of nascent virions by binding newly formed virus proteins and making them unavailable for assembly. It is not known however, if type I and II IFNs activate similar ISGs and antiviral effector proteins in bovine cells.

1.2.2.1.4.3 Interferon evasion mechanisms by *Alphaherpesvirinae*

Multiple alphaherpesviruses have been shown to replicate in the presence of interferons and it has been demonstrated *in vitro* that they can evade the activity of interferon-induced antiviral effector proteins.

Treatment of calves with recombinant bovine IFN-α_I did not reduce virus shedding following experimental BHV-1 challenge [81]. Similarly, infection of calves with a recombinant BHV-1 virus expressing IFN-γ did not reduce viral shedding compared to a wild-type strain [82]. Although BHV-1 is a potent inducer of type I and II interferon secretion during primary respiratory infections [67], BHV-1 ICP0 (bICP0) and bICP27 have been reported to induce degradation of IRF-3 and inhibit the activity of IFN-β promoters, respectively. Thus, both of these viral proteins can inhibit IFN-β transcription [83, 84]. BHV-1 bICP0 has also been reported to inhibit the intermediate kinases involved in the interferon-signalling pathway, including TANK-binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε), which can also result in inhibition of IFN-β promoter activity[85]. These studies were performed *in vitro* using a variety of cell types that may or may not reflect the biological responses of bovine mucosal epithelial cells. BHV-1 infection *in vivo* results in the production of both IFN-α and IFN-γ [67] even though BHV-1 encodes bICP0, which induces proteosome-dependent degradation of IRF-3 [84]. Therefore, one mechanism by which BHV-1 may evade IFN-induced antiviral effector proteins may be through inhibition of ISG transcription. However, it is not known whether BHV-1 evades IFN-α and -γ-induced antiviral effector proteins at the level of ISG transcription, translation, or post-translational modification.

Several other IFN evasion mechanisms have been identified for other members of the *Alphaherpesvirinae* subfamily. For example, HSV-1 encodes gene products that block IFN-α production and inhibits the antiviral activity of PKR through dephosphorylation of eIF-2 [86]. Varicella Zoster Virus (VZV) also inhibits PKR through phosphorylation of eIF-2α [87]. Therefore, BHV-1 may employ a variety of mechanisms to evade the antiviral activity of Type I and II IFNs and further investigations are required to fully understand the role of IFNs in the host innate immune response during an acute BHV-1 infection.

1.2.2.1.5 Natural Killer (NK) and NK-like cell responses

NK cells and NK-like cells are non-MHC- restricted lymphocytes, which kill infected cells by releasing cytotoxic granules containing perforin and granzyme B. Although similar to cytotoxic T-lymphocytes in their mechanism of action, NK cells are innate immune cells and not antigen-specific. NK cells isolated from the blood of calves following BHV-1 infection were first reported as non-adherent, non-B, non-T-cells which mediated the lysis of non-syngeneic BHV-1 infected cells [88]. The first analysis of cytotoxic activity during a primary BHV-1 respiratory infection reported an increase in non-MHC restricted killing of BHV-1 infected cells following viral infection. These non-MHC restricted leukocytes were isolated from the lung parenchyma but the phenotype of these cells was not further analyzed.

NK cells bear activation and inhibitory receptors and recognize target cells with reduced or no MHC expression [89]. NK cells also recognize and kill BHV-1 virions with antibody bound to their surface. $CD2^{+}CD4^{+}$ and CD8+ NK-like cells are another subset of innate

lymphoid cells shown to be non-MHC restricted and stimulated by cytokines to kill BHV-1 infected cells [90]. Although identified in humans and mice, it remains to be confirmed that cattle have functional natural killer T (NKT) cells, which in other species recognize lipid antigens in the context of a cell surface molecule, CD1d [91]. The CD1d gene is absent in cattle [91] but bovine NKT cells may be activated in a CD1d-independent manner [92]. It remains to be determined whether conventional NK cells or NKT cells play a role in the control of BHV-1 infections.

1.2.2.1.6 Macrophage responses

Macrophages are innate immune cells that phagocytose extracellular pathogens. During a primary BHV-1 infection, macrophages are stimulated by IFN-γ and can kill BHV-1-infected cells through a non-MHC restricted mechanism within 2 days after pulmonary infection [71, 90, 93]. Following BHV-1 infection, alveolar macrophages increase in number in the lungs with increased expression of MHC class II and display increased Fc-mediated phagocytosis [94]. It has also been shown that macrophages kill BHV-1 infected cells through antibody-dependent cellular cytotoxicity (ADCC) later during a primary infection [95]. BHV-1 also infects macrophages and can inhibit these antiviral functions [71, 96].

1.2.2.1.7 Polymorphonuclear (PMN) cell response

PMNs, including neutrophils and eosinophils, are phagocytes with PRRs and receptors for complement proteins. Neutrophils have been shown to mediate ADCC against BHV-1 infected cells in the presence of complement and low concentrations of

BHV-1 specific antibody [97]. Activated neutrophils can kill BHV-1 infected cells through the release of cytotoxic granules after binding to either activated complement proteins or antibody bound to infected cells [98]. BHV-1 infected cells and macrophages produce IL-8 which is a chemokine involved in recruitment of neutrophils. BHV-1 also expresses a glycoprotein (gC) that mimics the chemokine receptors on PMNs and reduces recruitment of these cells to the site of infection [99]. Neutrophils can also produce defensins in response to BHV-1 infection [2] but it is not known if these defensins have direct antiviral activity. Thus, neutrophils may be important innate immune effector cells following a primary BHV-1 infection.

1.2.2.2 ADAPTIVE IMMUNE RESPONSE

BHV-1 infections are thought to be controlled and cleared through the combined activity of both innate and adaptive immune responses. Adaptive immune responses are characterized by antigen specificity and immunological memory but this response takes days to weeks to develop. The adaptive immune response to BHV-1 infection includes both a humoral response, which involves virus neutralizing (VN) antibodies, and a cell-mediated immune response. VN antibodies are secreted by plasma cells, which differentiate from virus-specific B-cells, and cell-mediated immune responses are mediated by T-cells.

1.2.2.2.1 Humoral immune response

Three major BHV-1 glycoproteins, gB, gC and gD, have been identified as immunogenic and induce antibody responses during a primary BHV-1 infection [100]. Antibodies (Abs) specific to these viral glycoproteins are able to prevent viral infection of cells. IgA

antibodies against BHV-1 glycoproteins are induced in the URT [23] and secreted at mucosal surfaces while BHV-1 specific IgG antibodies can be detected in serum [23, 101]. Antibodies also bind and kill BHV-1 infected cells through ADCC mediated by PMNs, macrophages and NK cells in a non-MHC restricted manner [95]. BHV-1 has, however, developed mechanisms to evade the host humoral response by using intercellular egress across the tight junctions between epithelial cells to facilitate direct cell-to-cell spread. This mechanism of spreading viral infection avoids viral neutralization and opsonisation by extracellular antibodies [102]. This evasion mechanism highlights that the humoral response fails to restrict all modes of viral infection to adjacent cells and cell-mediated responses are thus essential in mediating clearance of BHV-1 infection.

1.2.2.2.2 Cell-mediated Immune Responses

Adaptive cell-mediated immune responses are MHC-restricted and mediated by T-cells. MHC-restriction refers to the immunological requirement of antigenic peptides to be presented complexed with "self" MHC molecules on the surface of antigen presenting cells (APCs) for recognition by T-cells through a T-cell receptor.

1.2.2.2.2.1 Antigen Presenting cells (APCs)

Dendritic cells and macrophages are immune cells that bridge the innate and adaptive arms of the host immune system through antigen presentation. Macrophages phagocytose pathogens and present antigens on MHC Class II but also present antigens through MHC Class I when infected. DCs are known as "professional APCs" which present antigens and have the capacity to activate naive T-cells [103]. DCs present peptides from BHV-1

proteins within the context of both MHC class I and II molecules and also co-express the co-stimulatory molecules required to activate naïve T-cells [103].

1.2.2.2.2.2 T-cell response

T-cells are characterized by the co-expression of variant T-cell receptors (TcR) with the invariant proteins that form the CD3 complex. Variant T-cell receptors (TcR) may be composed of heterodimer αβ or γδ chains. Each variant TcR protein is encoded by recombination of gene segments consisting of variable (V), junction (J), diversity (D), and constant (C) sequences. Most of the diversity exists in the complementarity-determining region 3 (CDR3), located at the centre of the TCR antigen binding site, which effectively increases the range of antigen recognition [104]. αβT-cells can be further divided into subsets based on the co-expression of other accessory proteins, such as CD4 and CD8.

1.2.2.2.2.2.1 T-helper (CD4+) T cells

CD4 T-cells recognize antigenic peptides bound to MHC class II complexes on APCs and secrete cytokines that can activate or regulate downstream cell-mediated immune responses [105]. CD4 T cells can be further subdivided into functional subsets on the basis of the cytokines that they secrete. Th1 cells secrete IL-2, IL-3 TNF-α, TNF-β and IFNγ and Th2 cells secrete IL-3, IL-4, IL-5, IL-6 and IL-10 [106]. Further, TH17 cells secrete IL-17, IL-17F, IL-21 and IL22 [107]. Th3 cells and regulatory T-cells produce TGF-β and IL-10 [107, 108]. $CD4^+$ T-cells have been shown to recognize a variety of BHV-1 proteins, including gB, gC, gD and VP8 [109, 110]. BHV-1 may evade $CD4^+$ T cell responses by infecting these cells when activated and inducing programmed death [72,

111]. BHV-1 may also prevent CD4 T cell activation by down-regulating MHC II expression on APCs which then disrupts antigen presentation [50].

1.2.2.2.2.2.2 Cytotoxic ($CD8^+$) T-cells

$CD8^+$ T- cells, also known as cytotoxic T-lymphocytes (CTLs), recognize antigenic peptides complexed with MHC Class I expressed on infected cells. Activated $CD8^+$T-cells can then produce IFN-γ and release cytotoxic granules. These cytotoxic granules contain perforin and granzyme B; proteins involved in forming membrane attack complexes and inducing programmed cell death in infected cells. Cytolytic $CD8^+$ T-cells can induce apoptotic death of BHV-1 infected macrophages through a perforin-independent mechanism [112]. BHV-1 gB protein induces a strong CTL response and elicits memory $CD8^+$cells [113]. $CD8^+$ T-cells have been shown to also target the BHV-1 tegument protein, UL49, and the immediate early proteins ICP4, ICP22, and the circ [114], an IE protein encoded by a *circ* gene located at the terminal repeats (TR) – UL junction of the circular BHV-1 genome [115]. BHV-1, in turn, evades $CD8^+$T-cell responses by down-regulating cell surface MHC Class I expression through inhibition of transporter associated antigen presentation (TAP) by the BHV-1 protein encoded by UL49.5.

1.2.2.2.2.2.3 γδT-cells

γδT-cells are the most abundant bovine T-cells in the blood of young animals, comprising up to 60% of bovine peripheral blood mononuclear cells (PBMCs) [116]. The majority of γδT-cells express the WC1 protein and co-express CD8 [117, 118]. $CD8^+$γδT-cells are

recruited to mucosal surfaces by the CCL19/CCL21 chemokines and bind to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) [119]. These $CD8^+$ γδT-cells may be important in host defenses against infections at mucosal surfaces. Bovine γδT-cell frequency has been shown to increase significantly in blood as early as 1 day after BHV-1 infection [120]. Previous studies have also shown that γδT-cells are activated following immunization with a modified-live BHV-1 vaccine and are re-activated *in vitro* by exposure to live BHV-1 [121]. Activated bovine γδT-cells spontaneously produce IL-10 and supress antigen-specific CD4 and CD8 T-cell proliferation. γδT-cells proliferate in response to IL-10 and TGF-β production by APCs which present peptides from Foot and Mouth disease Virus [116]. However, the role of γδT-cells during a primary BHV-1 infection remains uncertain.

1.2.3 LYMPHOCYTE MARKERS AND TRAFFICKING

1.2.3.1 LYMPHOCYTE MARKERS

This review highlights that immune responses to a primary BHV-1 infection are diverse and involve multiple immune cell populations. Monoclonal antibodies (mAbs) have been instrumental in the identification and characterization of these cells. Lymphocyte surface proteins have been used to identify both cell lineage and the state of cell activation or differentiation [122, 123]. Lymphocyte surface proteins may be receptors involved in antigen recognition or interactions with cytokines, cell adhesion molecules, and a variety of signal transduction functions [124].

1.2.3.1.1 History

Numerous immunological studies have been made possible through the collaborative work of a consortium of veterinary immunologists. Four international workshops have been organized to establish consistent nomenclature for ruminant leukocyte differentiation antigens [125-128]. The nomenclature used for these leukocyte antigens is based on comparison with human leukocyte antigens and individual molecules are designated as a cluster of differentiation (CD) [124]. CD refers to cell surface polypeptides expressed on immune cells that are recognized by monoclonal antibodies. Further, multiple mAbs recognizing the same cell surface protein were grouped to the same CD utilizing flow cytometry and statistical techniques [123]. Although much progress has been made in the past 30 years [123, 125], the limited availability of commercial mAbs against numerous bovine proteins continues to impede progress in bovine immunology. However, existing mAbs have allowed identification and quantification of multiple lymphocyte subpopulations, facilitated studies of lymphocyte distribution within tissues, and supported the analysis of lymphocyte trafficking.

1.2.3.1.2 Identification of cell subpopulations

Identification of lymphocyte surface proteins by mAbs makes possible the phenotypic analysis of multiple lymphocyte subpopulations. Common lymphocyte activation markers such as CD26 and CD50 have been reported to be expressed on activated bovine CD4, CD8, γδT-cells and NK cells [129]. Multiple lymphocyte proteins have been identified for each one of the major lymphocyte populations, including B-cells, T-cells and NK cells.

1.2.3.1.2.1 Identification of B-cell subpopulations

B-lymphocytes are located primarily in the follicular region and marginal areas of lymphoid tissues and organs [130]. Surface immunoglobulins (sIg) located on the membrane of B-cells were first demonstrated and quantified in the early 1970s by Rabellino and colleagues [131] using fluorescein-conjugated antisera. These analyses revealed that 45% of spleen and bone marrow lymphocytes and 7-14% of peripheral blood and lymph node cells expressed sIg. It was later shown that sIg could also be used to identify bovine B-cells and that sIg^+ B-cells constituted about 40% of bovine peripheral blood leukocytes [132, 133]. B cells expressing different antibody isotypes were also investigated and the majority of B-cells isolated from the blood of cattle were shown to express IgM [125, 134] while a small fraction expressed IgG and IgA[134]. A non-sIg protein, CD21, was also reported to be expressed by IgM and IgG-expressing mature B-cells in bovine peripheral blood [135]. Another bovine B-cell surface protein is BoWC4, which is a possible homolog of human CD19 and this protein has been shown to be expressed on B cells at mucosal surfaces [124, 136]. Other B-cell subsets have been defined by the co-expression of other surface proteins such as: BoWC5 [136] BoWC6 [137], WC10 [138], CD5 [139], and CD11b [139].

1.2.3.1.2.2 Identification of T-cell subpopulations

The original method for identifying bovine T-cells was through the binding of sheep erythrocytes to form rosettes, a phenomenon known as erythrocyte rosetting (E-rosetting) [140]. Selective binding of lectins was also used to identify most bovine T-lymphocytes but about 2% of B-cells also bind lectin [141, 142]. Due to the inconsistency in

reproducing results with these methods, significant effort was made to develop mAbs to detect T-cell lineage specific molecules [126, 128, 134]. A mAb detecting a common T-cell antigen expressed on all bovine T-cells was introduced by Rabinovsky and Yang as a T-cell surface molecule analogous to the human CD3 molecular complex [143]. CD3 is a multi-polypeptide complex associated with the T-cell receptor proteins and is involved in signal transduction during T-cell activation [144]. Bovine CD2, an orthologue of the human CD2, was also expressed on the surface of 60% of PBMCs and the majority of these cells were found to co-express either CD4 or CD8 [145]. The absence of CD2 expression on conventional NK cells has been used to differentiate between CTLs and cytotoxic NK cell populations [146]. Bovine T-helper (Th) cells were first identified as MHC Class II restricted $CD4^+$ cells, which modulate adaptive immune responses such as antibody production. These Th cells could comprise as much as 25 to 30% of the mononuclear cells isolated from bovine blood [147, 148]. Bovine $CD8^+$ T-cells were also shown to be cytotoxic T-cells that were MHC Class I restricted and represented approximately 20% of mononuclear cells present in bovine blood [149]. Another T-cell subpopulation identified in cattle was $CD2^-/CD4^-/CD8^-/WC1^+$ γδTcR-cells, which are abundant in young calves and constitute approximately 25% of blood mononuclear cells and under 5% of T-cells in lymphoid tissues [150]. A recent study demonstrated that activated bovine T-cell populations could be distinguished from resting T-cells [129]. CD25, CD45R0 and signaling lymphocytic activation molecule family member 9 (SLAMF9) expression is up-regulated on activated γδT-cells and CD25, CD44 and CD45R0 expression is up-regulated on activated $CD4^+$ and $CD8^+$ T-cells[129, 151]. Even

with the number of available mAbs for markers on T-cell subpopulations, CD3 and the T-cell receptor (TCR) remain the only lineage specific markers used to identify all T-cells.

1.2.3.1.2.3 Identification of NK cell populations

NK-cells were initially defined in mice and humans as large granular lymphocytes (LGL) with non-MHC restricted cytotoxicity [152]. Cells with similar morphological and functional characteristics were subsequently identified in cattle [153] but no lineage-specific cell surface proteins have been identified. Human NK-cells, were initially identified by their expression of CD56 and CD16 which divided NK-cells into $CD56^{high}/CD16^{+}$ and $CD56^{low}/CD16^{+/-}$ subpopulations. Murine NK-cell subpopulations were identified through their expression of CD27 and CD11b [154]. MAbs were not available for orthologous molecules on bovine NK-cells but recently a mAb was generated that specifically reacted with the natural cytotoxicity receptor (NCR), NKp46 [155]. Bovine NK cells are now identified as a separate lymphocyte lineage through the absence of T-cell lineage markers [153] such as CD2 [156] and CD3. Thus, bovine NK-cells have been identified as both $NKp46^{+}CD3^{-}$ [157] and $NKp46^{+}CD2^{-}$ [146]. A recent study also reported that expression of gp96, a peptide binding chaperone protein in the endoplasmic reticulum [158] and the absence of SLAMF9 can be used to identify activated bovine NK cells and distinguish them from T-cells [129].

Non-conventional T-lymphocyte populations which co-express the T-cell marker, CD3 and NKp46 have recently been identified in multiple species including bovine [157], porcine [159] and sheep [160] . The first non-conventional T-cells were discovered in reprogrammed human cytotoxic T-lymphocytes which co-express CD3 and NKp46 in

celiac disease [161]. Since then, other non-conventional T-cells including Natural killer T-cells (NKT) and mucosal associated invariant T-cells (MAIT) have been shown to co-express CD3 and NCR [162, 163]. Bovine non-conventional T-cells isolated from blood have also been shown to have non-MHC restricted cytotoxicity during a parasitic infection.

1.2.3.1.3 Cytoplasmic Proteins Present in Cytotoxic Lymphocytes

Cytotoxic lymphocytes are characterized by production and secretion of cytotoxic granules, which contain proteins such as perforin and granzyme B. Once perforin and cytotoxic lymphocyte lineages that can kill tumour cells and infected host cells. These cytotoxic granules are highly conserved and have been used to identify activated cytotoxic lymphocytes in multiple species, including humans [165, 166], pigs[167] and cattle[168].

1.2.3.2 LYMPHOCYTE RECRUITMENT

Naïve lymphocytes originate from primary lymphoid organs such as thymus and bone marrow and migrate to secondary lymphoid tissues, including lymph nodes, spleen and other organized lymphoid tissues. Naive lymphocytes can then recirculate between blood and secondary lymphoid tissues until they encounter foreign antigen or die [169]. Following an interaction with antigen, naïve lymphocytes are activated, and with appropriate co-signals proceed through clonal expansion before homing to the infected tissue as effector lymphocytes. Lymphocyte extravasation from blood vessels and into lymphoid or peripheral tissues involves specific and tightly regulated interactions between lymphocytes and adhesion molecules expressed on high endothelial venules (HEVs) [170]. Lymphocyte recruitment from immune inductive to effector sites during infection involves

chemokines produced within infected tissues and an interaction between adhesion molecules expressed on both leukocytes and high endothelial venules (HEVs). The interactions among these three components determine tissue specificity and the timing of recruitment. Thus, the stage of an inflammatory response which elicits specific lymphocyte recruitment is determined by the coordinated expression of chemokines and adhesion molecules [170].

Clearance of infections by cell-mediated immune responses requires recruitment of effector immune cells to the site of infection. The mechanism mediating leukocyte extravasation were first described over 25 years ago as a three-step process of leukocyte rolling, followed by leukocyte adhesion to endothelium, and subsequent migration of leukocytes through HEVs into lymphoid and peripheral tissues [170]. The definition of this mechanism has since been updated to include more steps in the leukocyte adhesion and transmigration cascade including leukocyte slow rolling, adhesion stabilization, intraluminal crawling, paracellular and transcellular migration and migration through the basal membrane of the endothelial cells into the inflamed tissue [171].

1.2.3.2.1 Adhesion Molecules

Adhesion molecules expressed on endothelial cells in mammalian tissues interact with their ligands on leukocytes and facilitate leukocyte rolling and attachment during leukocyte extravasation [170, 171]. Selectins and integrins are well-documented adhesion molecules expressed on endothelial cells and leukocytes in multiple species. [119, 151, 172, 173]. E-

selectin and P-selectin are expressed on endothelial cells and L-selectin is expressed mostly on leukocytes including lymphocytes [171]. Naïve T-cells recruited to the lymph nodes (LN) express L-selectin, which interacts with its carbohydrate ligand expressed on HEVs in human LN and tonsils [172] The expression of bovine L-selectin on lymphocytes also determines their tissue tropism as demonstrated by high L-selectin expression on lymphocytes in peripheral lymph nodes and a contrasting low L-selectin expression on lymphocytes in mucosal sites [174] . In cattle, P-selectin on bovine endothelial cells binds to LPS-activated peripheral blood leukocytes [175] and E-selectin is expressed on bovine aortic endothelial cells [176]. The P-selectin ligand, P-selectin glycoprotein ligand -1 (PSGL-1) was initially described as a ligand of P-selectin but has since been shown to bind to all three selectins [171, 177]. Increased expression of PSGL-1 on B, T and NK cells was observed in the presence of platelets indicating that platelets may promote lymphocyte adhesion to endothelial cells [178]. Furthermore, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecules (VCAM-1), which are members of the immunoglobulin supergene family, are also expressed on bovine endothelial cells following experimental exposure of these cells to lipopolysaccharide (LPS) [179]. ICAM-1 and VCAM-1 bind to the integrins lymphocyte function associated antigen -1 (LFA-1) and $\alpha_4\beta_1$ (also known as Very Late Antigen: VLA-4) expressed on lymphocytes [179]. This finding shows that ICAM-1 and VCAM-1 expression is induced during bacterial infection and may play an important role in lymphocyte recruitment during infection. Similarly, bovine mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1) is expressed on HEVs in mucosal sites including in the mesenteric lymph nodes (MLN) and the ileal mucosa and lamina propria [119]. Bovine $CD8^+$ γδT-cells have been shown to bind to

MadCAM-1 and accumulate in tissues with high MAdCAM-1 expression, while few bovine $CD8^+$ γδT-cells were recruited to tissues with low or no MAdCAM-1 expression [119]. Bovine $CD8^+$γδT-cells were also shown to express the MAdCAM-1 receptor, $\alpha_4\beta_7$ [119]. These findings are consistent with the current concept that expression of MAdCAM-1 regulates lymphocyte recruitment to mucosal sites.

Following leukocyte slow rolling and adhesion stabilization, leukocytes transmigrate through the HEVs or between the HEVs (paracellular migration) and basal membrane of the HEVs into the inflamed tissue and home to the site of infection. The interaction between adhesion molecules on HEVs and lymphocytes results in conformational change in the HEVs with increased expression of adhesion molecules in the tight junction between endothelial cells. This facilitates the migration of recruited cells from the lumen of the blood vessel, through the endothelial cells and into the inflamed tissue [171]. Junctional adhesion molecules (JAMs) are molecules expressed on the lateral membrane of endothelial cells, which regulate leukocyte transendothelial migration [180]. There are three known types of JAMs including JAM-A, JAM-B and JAM-C in human and mice [180-182] which bind to LFA-1[183] VLA-4 (α4β1) [182] and macrophage antigen -1 (Mac-1) [184] respectively. These JAM ligands are expressed on lymphocytes and are important for lymphocyte recruitment into tissues. The functional JAM orthologues in cattle are however still unknown. It is predicted, however, that bovine JAMs – A, B and C exist based on genetic similarity, computerized annotations and pathway reconstruction analysis in the database, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [185, 186].

1.2.3.2.2 The role of Chemokines

Chemokines are low molecular weight chemoattractant cytokines produced by multiple cell populations during inflammation. Chemokines increase adhesion molecule expression and direct circulating leukocytes to migrate up a chemokine gradient toward sites of inflammation. This chemotaxis is directed by chemokine binding to specific chemokine receptors differentially expressed on circulating leukocyte subpopulations [187].

Studies in chemokine-deficient animal models have been used to identify chemokines involved in lymphocyte recruitment in humans and mice [188]. CCL21 is a chemoattractant shown to recruit B and T-lymphocytes to lymphoid and non-lymphoid tissues including mesenteric lymph nodes, small and large intestine and lungs in mice [189]. *CCR6*-deficient mice experimentally treated with cockroach allergen (CA), were shown to produce CCL20 in murine lungs in an allergic asthmatic response and had over a 10-fold reduction in IgE production compared to wild–type mice. These observations support the conclusion that CCL20 and CCR6 are critical for recruitment of B-lymphocytes [190]. Similarly, CXCL13- expressing transgenic mice demonstrated that overexpression of CXCL13 in intestinal epithelial cells resulted in the recruitment of B-lymphocytes, NK- cells and lymphoid tissue-inducer cells (LTi) [191].

Non-MHC restricted lymphocytes were recruited to the lungs of calves following BHV-1 infection [192], but the identity of these cells has not been confirmed. In humans, Lande et al have shown that $CD4^+$ and $CD8^+$ T cells and conventional NK cells are recruited to CCL3, CCL4, CXCL9 and CXCL10-producing dendritic cells following a *Mycobacterium*

tuberculosis infection [193]. Furthermore, Type 1 interferons were shown to induce the expression of CXCL10 in *Mycobacterium tuberculosis*-infected dendritic cells, which led to selective recruitment of T cells, and NK cells [193]. In mice, invariant NKT cells have been shown to express multiple chemokine receptors, including CCR7, CXCR3, CXCR6, CCR4 and CCR6 [194]. Among these chemokine receptors CCR4 alone was important for pulmonary localization [195]. Human NKT cells also express multiple chemokine receptors, including CXCR6, CCR1, and CCR6 [196] but it is not known how the expression of these chemokine receptors may change in the face of infections. Further, *in vivo* studies with mice have shown that CCL28 promotes recruitment of CCR10-expressing IgA plasma cells to lactating mammary glands, where an upregulation of CCL28 transcription was observed [197] . Similarly, in healthy cattle, CCL28 and CCR10 gene expression was shown to be concurrently upregulated in mucosal tissues including mammary glands, parotid and mandibular salivary glands, and the small and large intestine, which suggests that B-lymphocytes are constitutively recruited to bovine mucosal tissues [198]. Multiple chemokines have been shown to direct bovine NK cell recruitment including the inflammatory chemokines CCL3, CCL4, CCL5, CCL20 and CXCL8, which are secreted by *Mycobacterium bovis*-infected dendritic cells [199]. Bovine $CD335^{+}CD2^{+}$ NK-cells express multiple chemokine receptors including CCR1, CCR8, CXCR6 and CX3CR1. In contrast, bovine $CD335^{+}CD2^{-}$ NK cells express CCR2, CCR5, CCR6, CCR7, CXCR3, CXCR4 and CXCR5 and have been shown by *in vitro* chemotaxis assay to migrate in response to relevant chemokines [199]. These studies have shown that multiple chemokines can play a role in the recruitment of lymphocytes. Furthermore, bovine γδT-cells and $CD4^{+}$ T-cells homing to skin have been reported to express CCR4 and CCR10

genes but the expression of the relevant ligands, CCL27 and CCL28, has yet to be determined for skin [200].

The lack of mAbs reacting with bovine chemokines and receptors has limited current knowledge in this area to a few transcriptional studies. Future development of specific mAbs reacting with bovine chemokines and chemokine receptors will enhance the study of leukocyte recruitment in the presence and absence of infection and may provide insight for the development of therapeutic tools to modulate inflammatory responses during infection and prevent immunopathology.

CHAPTER 2: STUDY RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1 RATIONALE

Primary Bovine herpesvirus-1 respiratory infection of cattle is an economically costly disease that is prevalent in Canada and worldwide. Multiple generations and types of BHV-1 vaccines have been developed and are used commercially but an ideal vaccine, which does not result in adverse events following immunization or does not establish a latent infection in the host, is not available. Killed viral vaccines or recombinant protein vaccines have been developed but they often provide limited protection from disease and do not induce long-term immune memory.

Furthermore, BHV-1 can cause abortions in pregnant cows and is a member of the Bovine Respiratory Disease Complex (BRDC), which causes fatal respiratory disease in young calves. BRDC includes multiple bovine viruses such as BHV-1 and BVDV, and bacteria, such as *Mannheimia haemolytica* and *Pasteurella multocida.* These pathogens interact and a virus-bacterial synergy that increases the severity of bacterial infections is commonly initiated by a primary BHV-1 respiratory infection. The prevention or rapid clearance of a BHV-1 respiratory infection is therefore important for the prevention of BRDC infections.

Decades of research related to BHV-1 infection has shown that humoral and cell-mediated immune responses are important for recovery from a primary BHV-1 infection. However, the immune mechanisms mediating early control of BHV-1 infection are still unknown. Further, although cell-mediated responses have been shown *in vitro* to be a component of

host responses to BHV-1 infection, the phenotype of the lymphocytes recruited to mucosal sites of a primary BHV-1 infection have yet to be determined.

2.2 HYPOTHESES:

1. BHV-1 is a potent inducer of IFN-α and –γ in the upper respiratory tract but IFN-induced antiviral effector mechanisms are not the primary mechanism limiting BHV-1 replication during an acute respiratory infection.

2. Innate lymphocytes recruited to the upper respiratory tract during an acute BHV-1 respiratory infection may provide an important effector mechanism for the control of viral infection

2.3 OBJECTIVE

The overall objective of this project was to identify innate mechanisms involved in the clearance of a primary Bovine herpesvirus-1 infection. To address this objective, we set out the following aims:

SPECIFIC AIMS:

1. Confirm that during an experimental BHV-1 infection of the bovine URT there was induction of type I and II IFNs, and relate the IFN response to virus replication in the URT.
2. Investigate the induction of interferon-induced antiviral effector genes during the primary BHV-1 infection in the URT of calves.
3. Analyze the direct antiviral effects of bovine type 1 and 2 interferons on BHV-1 replication in vitro, and induction of ISGs in bovine epithelial cells by bovine Type 1 and 2 interferons.
4. Analyze the phenotype and frequency of specific lymphocyte subpopulations recruited to nasal turbinates and trachea following a primary BHV-1 infection in calves.
5. Analyze gene expression of chemokines in BHV-1 infected tissues to determine whether specific chemokines may be implicated in the recruitment of specific lymphocyte subpopulations to the site of BHV-1 infection.

CHAPTER 3: INDUCTION OF INTERFERON AND INTERFERON-INDUCED ANTIVIRAL EFFECTOR GENES FOLLOWING A PRIMARY BOVINE HERPES VIRUS -1 (BHV-1) RESPIRATORY INFECTION

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I was responsible for conception, design of the studies and writing the manuscript. I also designed and validated primers, performed experiments, and analyzed the data.

Relevance of this study to the Thesis

These studies address questions posed within the first hypothesis of this thesis.

The induction and secretion of type I and II interferons and the transcription of interferon-induced antiviral effector genes were analyzed during a primary BHV-1 infection of the bovine upper respiratory tract.

3.1 Abstract

In vitro investigations have identified a variety of mechanisms by which herpesviruses evade interferon-stimulated antiviral effector mechanisms. However, these immune evasion mechanisms have not been evaluated during a bovine herpesvirus-1 (BHV-1) infection. This study investigated transcription and secretion of type I and II IFNs and transcription of IFN-stimulated genes (ISGs) during a primary BHV-1 infection of the upper respiratory tract (URT) in naïve calves. *IFN-α*, *-β,* and -γ transcription in nasal turbinates and protein levels in nasal secretions increased following infection. Increased IFN type I and II secretion was detected 3 days post-infection (pi) and IFN production increased in parallel with virus shedding. Expression of ISGs, including *Mx1*, *OAS* and

BST-2, also increased significantly ($P < 0.05$) in nasal turbinates on day 3 pi and elevated ISG expression persisted throughout the period of viral shedding. In contrast, *RNAase L* gene expression was not induced during the BHV-1 infection in the nasal turbinates but was induced on Day 10 pi in the trachea. *In vitro* studies confirmed that recombinant bovine (rBo)IFN-α, β and -γ induced expression of *Mx1*, *OAS*, and *BST-2* but decreased *RNAse L* transcript in bovine epithelial cells. Relative to Vesicular Stomatitis Virus (VSV), BHV-1 was resistant to the antiviral activity of rBoIFN-α and –γ, but treatment of epithelial cells with 10 ng rBoIFNβ/ml effected an 80% inhibition of BHV-1 replication and complete inhibition of VSV replication. These observations confirm that transcription and translation of type I and II IFNs increase during BHV-1 infection, and transcription of some ISGs was not inhibited.

3.2 Introduction

Interferons (IFN) are a diverse family of proteins that function as an important component of the innate immune defense against viral infections. There are three major types of IFNs, Type I, II, and III, known to have antiviral activity. Type I bovine IFNs include 9 subtypes of IFN-α and 3 subtypes of IFN-β [76]. Bovine type II IFN comprises only IFN-γ, encoded by a single gene [201] and Type III bovine IFN is currently represented by IFN-λ3 [202]. IFNs act in a biphasic manner, with increased IFN production activating IFN signaling pathways. IFN production is induced following detection of viral components by pattern recognition receptors (PRR), such as Toll-like receptors (TLRs). Both type I and II IFNs are known to establish an "antiviral state" when bound to appropriate cell surface receptors prior to viral infection [203].This antiviral state

involves activation of interferon-stimulated genes (ISG) and subsequent production of antiviral effector proteins, including double-stranded RNA-dependent protein kinase R (PKR), Myxovirus resistance (Mx) proteins, oligoadenylate synthetase-RNAase L (OAS-RNAse L) and BST-2 or Tetherin [204] . These antiviral effector proteins target different steps in the viral replication cycle to prevent both assembly and release of infectious virus particles [204]. It is not known, however, if both type I and II bovine IFNs activate similar ISGs and anti-viral effector proteins.

Bovine Herpes Virus-1 (BHV-1) is a member of the subfamily, *Alphaherpesvirinae*, characterized by lytic infection of epithelial cells at mucosal surfaces and the establishment of latent infections in peripheral sensory neurons [205]. BHV-1 was first described in 1928 as the cause of a venereal disease in cattle known as "Bläschenausschlag" by Reisinger and Reimann [206]. A respiratory form of BHV-1 infection was later discovered in 1953 and described as infection of the upper respiratory tract and named Infectious Bovine Rhinotracheitis (IBR) [36]. Primary BHV-1 respiratory infections may last 10 to 12 days, and bovine nasal turbinates, pharyngeal tonsils, and trachea are the primary sites of infection in the upper respiratory tract (URT) [61]. BHV-1 is a potent inducer of type I and II bovine interferons [67, 81, 82] and there is evidence that BHV-1 evades both type I and II IFN-induced antiviral effector mechanisms. Pre-treatment of calves with recombinant bovine IFN-α_1 did not significantly reduce virus shedding following an experimental BHV-1 challenge [81] and expression of bovine IFN-γ by a recombinant BHV-1 did not significantly reduce viral replication [82]. Previous studies have confirmed induction of ISGs, such as *OAS*, in blood leukocytes following BHV-1

infection [67], but no studies have evaluated the expression of ISGs at the site of viral infection in the URT. Thus, it is not known whether local production of type I and II IFNs results in increased ISG expression at the site of viral infection and whether the onset of ISG expression coincides with a decline in viral replication.

The mechanism(s) by which BHV-1 may evade IFN antiviral activity has been investigated *in vitro*. BHV-1 immediate early protein, bovine ICP0 (bICP0) was reported to induce degradation of IRF-3, resulting in decreased *IFN-β* transcription [84]. Similarly, bICP0 may also inhibit intermediate kinases in the interferon-signalling pathway, including TANK- binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε), resulting in an inhibition of IFN-β promoter activity and decreased ISG expression [85]. Another *in vitro* study reported that an immediate early BHV-1 protein, bICP27, inhibits the activity of the promoters of bovine IFN-β resulting in an inhibition of *IFN-β* transcription [83]. Other IFN evasion mechanisms have been identified for members of the *Alphaherpesvirinae* subfamily, such as Herpes simplex-1 (HSV-1). HSV-1 encodes gene products that block IFN-α production and inhibit the antiviral activity of PKR through dephosphorylation of eIF-2 [86]. Varicella Zoster Virus (VZV) also inhibits PKR through phosphorylation of eIF-2α [87]. Additionally, HSV-1 has been shown to block BST-2 function through Virion Host Shut off protein (Vhs)- mediated degradation of BST-2 mRNA[207]. Thus, *in vitro* studies provide evidence that Herpesviruses may inhibit transcription of IFN genes and inhibit IFN-induced antiviral effector proteins at both a transcriptional and post-transcriptional level. It has not been determined *in vivo*, however, whether BHV-1 evades the IFN response through an inhibition of IFN transcription or translation or through an inhibition of ISGs at a pre- or post-transcriptional level.

In this paper, we confirm that BHV-1 infection induces transcription and secretion of both IFN-α and –γ in the URT, and for the first time also demonstrate coincidental transcription and secretion of IFN- β. Production of both types I and II IFNs in the upper respiratory tract preceded peak levels of virus shedding in nasal secretions. To further test the hypothesis that BHV-1 evades IFN antiviral activity through an inhibition of ISG transcription, we also analyzed ISG transcription in URT tissues. This analysis supports the conclusion that BHV-1 evasion of IFN, a key innate immune defence, does not occur through an inhibition of either the transcription or translation of IFNs or a transcriptional inhibition of ISGs.

3.3 Methods and Materials

3.3.1 Animals:

Female and castrated male, 5 to 6 month old, crossbred (Angus X Hereford), suckling calves (n=30) were purchased from a single commercial herd. Calves were identified as seronegative for BHV-1 by screening with a recombinant, truncated glycoprotein D (tgD) antibody capture ELISA [208]. For this study, calves were weaned, transported to the VIDO research facility, and adapted for 2 weeks to a diet of free choice hay and 0.5 kg oats/day. Calves were housed in a single pen. The average weight of calves was 232 kg with body weights ranging between 174 and 242 kg. Tissue samples were collected from nasal turbinates and trachea of 6 animals on Days 0 (pre-infection), 3, 5, 7, and 10 post-infection (pi). All experiments were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care and the

experimental protocol was approved by the University of Saskatchewan Animal Care Committee.

3.3.2 **Experimental Infection and Sample Collection:**

All calves were aerosol challenged with BHV-1 isolate 108 (5 X 10^7pfu/animal) on Experimental Day 0 as previously described [67]. A clinical veterinarian, blinded to treatment group, examined calves daily and recorded body weight and temperature. Nasal swabs for the analysis of virus shedding and nasal secretions for the analysis of IFN were collected from all animals prior to BHV-1 challenge and on day 3, 5, 7, and 10 post-infection (pi). Nasal secretions for quantifying virus shedding were collected with sterile cotton swabs, which were then immersed in 1 ml of Minimum Essential Medium (MEM) and transported on ice to the lab. Swabs were stored at -20^{o}C prior to performing viral plaque assays to quantify infectious BHV-1 particles. Nasal secretions for assaying IFN secretion were collected by inserting a cotton tampon into the ventral meatus of the nostril for 20 minutes. The absorbed fluid was expressed from the tampon and stored at -80^{o}C until analyzed by ELISA. For tissue collection, cohorts of 6 calves were euthanized with an intravenous injection of Euthanyl (240 mg/ml; Bimeda-MTC, Canada) on Days 0, 3, 5 7, and 10 pi, immediately following the collection of nasal swabs for virus and nasal secretions for IFN analysis. Tissue samples were collected within 15-20 minutes after euthanasia and three replicate samples were immediately placed in RNAlater or 10% buffered formalin. Tissue samples from nasal turbinates were collected 10-12 cm from the external nares and tracheal mucosa was collected from the mid-trachea region of all calves. Formalin-fixed tissues were used for histology and immunohistochemical detection of

BHV-1. Tissues fixed with RNAlater were stored at -80^{o}C until RNA was extracted for qRT-PCR analysis of transcripts from IFN genes and ISGs.

3.3.3 Enzyme-Linked Immunosorbent Assay (ELISA):

Calves were screened for BHV-1-specific serum antibodies using an antibody-capture ELISA as previously described [209]. Briefly, recombinant BHV-1 truncated glycoprotein D (tgD) was used to coat ELISA plates for antibody capture following serial dilution of serum samples. Calves with serum antibody titres less than 1/100 were considered seronegative. IFN-α and -γ concentrations in nasal secretions were quantified by ELISA as previously described [82]. The IFN-α ELISA used in this study used a capture monoclonal antibody raised against the BoIFN-α_I1 protein expressed from the cloned sequence of the BoIFN-α_I1 gene shown in Supplemental Figure A [210]. IFN-β concentrations in nasal secretions were determined using a commercial bovine IFN-β ELISA kit (Biomatik), following the manufacturer's instructions.

3.3.4 Cells and Viruses:

A bovine mucosal epithelial cell line was established from fetal bovine intestine [209] and Madin-Darby bovine kidney (MDBK) cells were purchased from ATCC (ATCC # CCL22). The mucosal epithelial cells were cultured in DMEM with 5% FBS, 5μg/ml of Apo-transferin (Sigma), 0.025μg/ml of Epidermal Growth Factor (EGF) (Sigma), 0.1μg/ml of Hydrocortisone (Sigma), 10μg/ml of insulin (Sigma), and 50μg/ml Gentamicin (Gibco) per 500 ml Dulbecco's Modified Eagle Medium (DMEM). MDBK cells were cultured as

previously described [211]. The BHV-1 isolates used were 108, (Animal Disease Research Institute, Lethbridge, AB) and Cooper strain (ATCC# VR864). Vesicular Stomatitis Virus (ATCC# VR158) was used as a positive control when quantifying the antiviral activity of recombinant bovine (rBo) IFN-α, β and -γ.

3.3.5 BHV-1 Plaque Assay

MDBK cells were used to perform a plaque assay to quantify infectious BHV-1 shed in nasal secretions as described previously [211, 212].

3.3.6 Immunohistochemical (IHC) staining of bovine tissues for BHV-1 proteins

Tissues from the nasal turbinates, pharyngeal tonsils, and trachea were collected from 6 animals euthanized prior to BHV-1 infection and 6 animals each on days 3, 5, 7, and 10 pi. Tissues were fixed in 10% buffered formalin (HARLECO) prior to embedding in paraffin and 5 μm tissue sections were cut, treated for antigen recovery, and then stained with a polyclonal rabbit anti-BHV-1 antisera (Prairie Diagnostic Services [PDS], Saskatoon, SK).

3.3.7 RNA Extraction

The TRIzol method (Invitrogen), as described previously [8], was used to extract total RNA from nasal turbinates and trachea samples stabilized in RNAlater®. RNA was extracted from one million cultured mucosal epithelial cells seeded per well in *CellBind* 6-well plates (Corning). Cells were stimulated with 100 ng/ml of either rBoIFN-α, -β, or -γ for the indicated time intervals (Fig. 3.5). At each time point, culture supernatant was removed and one millilitre of TRIzol was added to each well, gently pipetted up and down

to lyse cells and the lysate was stored at -20^{o} C until RNA was extracted. RNA extraction was performed as described previously [8]. RNA quality and total RNA were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with an RNA Integrity Number (RIN) of 7 or higher were used to prepare cDNA.

3.3.8 cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA (1μg) was reverse-transcribed using qScript™ cDNA SuperMix (Quanta Biosciences™), following the manufacturer's protocol. cDNA samples were diluted in DNAse/RNAse free water for use as template in qRT-PCR reactions.

3.3.9 qRT-PCR primer design and validation

Primer pairs for interferon-stimulated gene (ISG) transcripts (Table 1) were designed using DNAMAN software (Lynnon Corp, Quebec) and primers for *IFN-α, β* and *-γ* transcripts were designed using Clone Manager 9.0 program (Sci-Ed Software). Primers for *IFN-α, -β* and *-γ* transcripts were designed to validate IFN-α, β and -γ protein levels detected by ELISA. The *BoIFN-α1* [210] nucleotide sequence was identified to be 99% homologous (Supplemental Fig. 3A (i)) to BoIFN-α-D and specific primers were designed for BoIFN-α-D. Sequences have been reported for 8 subtypes of bovine IFN-α and three subtypes of IFN-β [76]. Despite high homology among IFN-β subtypes (Supplemental Fig. 3B (ii)), there exist single nucleotide polymorphisms (SNPs) among the subtypes. To detect transcripts from all *IFN-β* subtypes, primer pairs designed for *IFN-β* genes were designed with IUPAC nucleotides whereby 50% of the primer(s) contains one nucleotide while the other 50% contains a different nucleotide at the same position (Table 3.1). Primer

specificity for individual IFN genes was confirmed through generation of a single peak in the melting curve and detecting a single band of expected size following gel electrophoresis of PCR products. Additionally, the *IFNα* and *–β* PCR products were cloned in the PCR 2.1 vector using the TA cloning kit (Invitrogen). Cloned PCR products were sequenced with a CEQ 200XL DNA Analysis system (Beckman Coulter) to confirm product identity and size. Sequencing of cloned *IFN-β* PCR products, *IFN-β* Clone A and B, identified the products as IFN-β2 and IFN-β1 (Supplemental Fig. 3A (iii) and (iv)), respectively, confirming that the primers identified multiple IFN-β subtypes. One *BoIFN-α-D* PCR product was cloned and sequenced and confirmed to be 99% homologous to *BoIFN-α-D* (Supplemental Figure 3A (ii)). Primer amplification efficiency was also determined by performing a standard curve as described previously [213].

3.3.10 qRT-PCR Analysis of ISG and IFN-α, IFN-β, and -γ Gene Expression:

Gene expression data for individual samples was normalized relative to *β-actin*, which was previously validated as suitable reference gene in bovine mucosal epithelial cells [214]. We also confirmed that expression of the reference gene did not change significantly over time. qRT-PCR was performed using the PerfeCTa® SYBR® Green FastMix for iQ (Quanta BioScience). Briefly, 9 μl of PerfeCTa® SYBR green master mix (2X), 3 μL of the primer pair at 3.3 μM, and 3 μL cDNA template were added to a 15 μL final volume. The reaction was performed in BioRad iCycler iQ PCR detection system using the following program: 1 cycle at 95 °C for 30 sec, 45 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. After the cycling, the temperature was increased starting from 56 °C at a rate of 1 °C

every 10 sec to build a melting curve (40 times). Amplification data obtained for individual genes was expressed as threshold cycle (Ct) and was subtracted from the Ct of the β-actin reference gene to obtain ΔCt (ΔCt = Ct gene – Ct β-actin). Fold-change was calculated by using the formula;

$$\text{Fold change} = \mathbf{2}^{-\Delta\Delta Ct}$$

ΔΔCt is calculated by subtracting ΔCt value of the control (untreated or uninfected sample) from the time- matched ΔCt value for the experimental group (treated or infected) [215]

3.3.11 Interferon Plaque-Inhibition Assay:

Monolayers of mucosal epithelial cells were prepared by seeding 12-well *CellBind* plates (VWR) with 2.5 x 10^4 cells/well. Cultures were 70-80% confluent within 3h of seeding and duplicate wells were then treated with IFN-α, -β and -γ doses ranging from 0.01 – 10,000 ng/ml. Duplicate wells without IFN treatment were infected with BHV-1 and used as positive controls for virus plaque-forming units (pfu). Confluent bovine mucosal epithelial cell cultures were incubated with the indicated type and concentration of interferon for 12 h before medium was aspirated. Fifty pfu BHV-1 (108) or VSV in 0.5ml serum-free DMEM were added to each well for 1h before overlaying the monolayer with bovine anti-BHV-1 antisera for BHV-1 infected cultures or 1% methylcellulose in complete media for VSV infected cultures. Cultures were then incubated at 37^o C for 40-45 hour before using 80% methanol and 0.5% Crystal Violet in to fix and stain cells. Viral plaques were enumerated under an inverted microscope.

3.3.12 Statistical Analysis:

Data were analyzed using GraphPad software (Version 7.0a). Virus shedding by infected calves was analyzed using a 2-way ANOVA to analyze the effect of days post-infection on BHV-1 shedding. A Mann-Whitney test was used to compare levels of *IFN*α , β or -γ detected in nasal secretions and nasal turbinate tissues following BHV-1 infection relative to uninfected tissues from Day 0. The student t-test was used to compare IFN inhibition of BHV-1 and VSV viral plaques. A Mann-Whitney test was used to analyze changes in the expression level of individual ISG in infected nasal turbinate and tracheal tissues relative to the uninfected controls.

Table 3.1. Primers For Amplification Of Bovine IFNs and ISGs

Gene		Primer Sequence*	Product size (bp)§	Primer efficiency‡
mx1	FWD	AGATGTCCTGTCTAGATCCC	127	**2.1**
	RV	GGCAAAGTCAGCTTCCTGG		
Oas	FWD	TCACAGAGTTCGGGTGTCC	160	**2.1**
	RV	CCTGATTTCTTCGATGAATTCT		
Rnasel	FWD	ATTGTGCCTCCTCATACTGG	111	**2.1**
	RV	TGTTATGGCTCTCAGTCTCC		
bst-2	FWD	TGCTTGTGCCCATGATCTAC	190	**2**
	RV	TCCCTCAGGGTCACCATAG		
IFN –α	FWD	ACACACACCTGGTTCAACAC	96	**1.97**
	RV	GATGACAGCAGAAATGAGTCTTCC		
IFN-β	FWD	RTCTGSAGCCAATCCARAAG	136	**1.98**
	RV	CAGGCACACCTGTYGTACTC		
IFN-γ	FWD	TGCAGATCCAGCGCAAAGC	122	**2.03**
	RV	CGTTGATGCTCTCCGGCC		

* Sequence of forward (FWD) and reverse (RV) primers;

§Size of amplified PCR product;

‡Efficiency of product amplification

3.4 Results

3.4.1 Virus shedding persists after induction of IFN secretion

BHV-1 shedding in nasal secretions of infected calves increased significantly (P=0.002) by Day 3 pi to a mean value exceeding one million pfu/ml of nasal secretion and remained at this level until Day 7 pi (Fig. 3.1a). Virus shedding then decreased approximately three logs on Day 10 pi to a mean value of approximately 1000 pfu/ml.

IHC staining of tissues collected from the URT with rabbit anti-BHV-1 antisera revealed a similar temporal pattern for virus replication (Fig. 3.1b). No visible staining for BHV-1 was observed in any of the tissues prior to BHV-1 infection (Fig. 3.1b). In contrast, nasopharyngeal tonsils from all animals (n = 6) had visible focal staining of epithelial cell for BHV-1 on days 3 and 5 pi but detectable staining then decreased to approximately 70% (4/6 animals) on day 7 pi and 50% (3/6 animals) on day 10 pi. A similar focal staining pattern was observed with nasal turbinates but visible staining was detected in tissue samples from all 6 animals only on day 5 pi (Fig. 3.1b). The frequency of animals with detectable staining for BHV-1 in tracheal tissue was much lower at all time points, with only 4 of 6 animals having visible foci on Day 3 pi and 50% of animals (3/6) positive on Days 5 and 7 pi (Fig. 3.1b) These observations support the conclusion that shedding of infectious BHV-1 in nasal secretions correlates with virus replication in URT tissues.

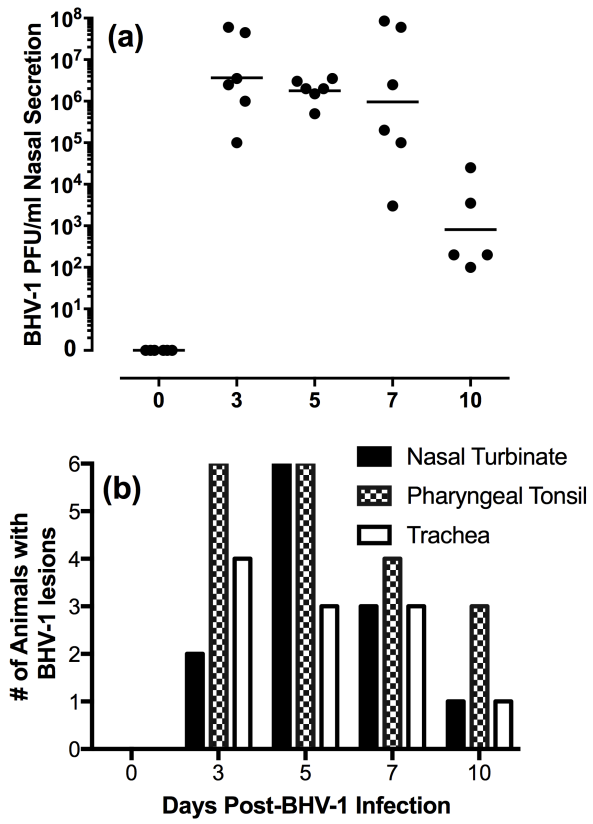

Figure 3.1. Viral replication in the upper respiratory tract following a primary BHV-1 infection. (a) Shedding of infectious BHV-1 particles in nasal secretions. Virus titres were determined by plaque assay and data presented are values for individual animals (n = 6/time point). **(b)** BHV-1 infection of nasal turbinate, nasopharyngeal tonsil, and tracheal mucosa was detected by IHC using a polyclonal rabbit anti-BHV-1 antisera. Data presented are the number of individual animals (n = 6/time point) from which tissue samples had detectable positive staining of BHV-1 antigens in mucosal epithelial cells.

Immunohistochemical staining of nasal turbinates revealed that BHV-1 replication occurred within discrete foci and was restricted primarily to the mucosal epithelial layer. The normal epithelial architecture of nasal turbinates is characterized by a pseudostratified columnar epithelium separated from the underlying lamina propria by a visible basement membrane (Fig. 3.2a). IHC staining with rabbit anti-BHV-1 antisera confirmed there was no visible staining of nasal turbinates (Fig. 3.2c) or nasopharyngeal tonsil (data not shown) collected on day 0, prior to BHV-1 infection. In contrast, IHC staining of tissue sections

collected following BHV-1 infection revealed infected foci within the mucosal epithelial layer, characterized by extensive disruption of normal tissue architecture and loss of epithelial cells (Fig. 3.2d). Staining for BHV-1 antigens was most intense in epithelial cells at the margins of these infected foci and there was no visible staining of adjacent mucosal epithelium. Thus, high levels of infectious viral particles in nasal secretions on days 3 to 7 pi did not result in a uniform infection of mucosal epithelial cells within the nasal turbinates or trachea but there was extensive loss of epithelium overlying the nasaopharyngeal tonsils (data not shown). This observation is important when considering qRT-PCR results for tissue samples collected from nasal turbinates since these samples included both BHV-1 infected and uninfected epithelial cells.

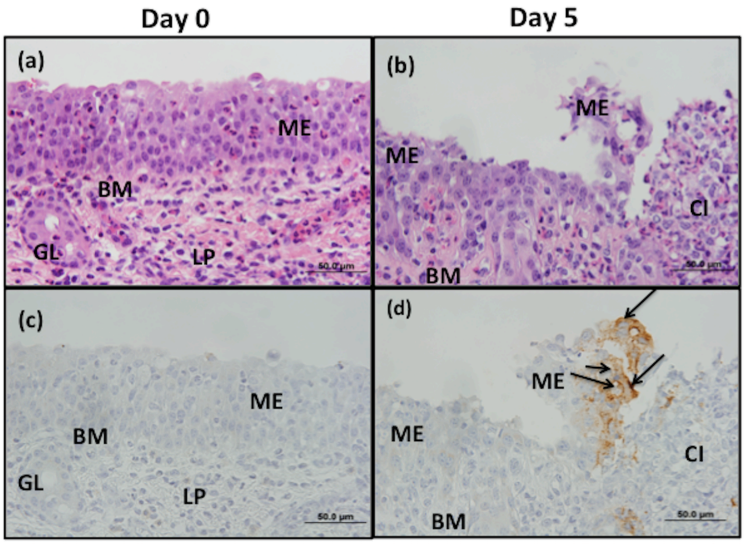

Figure 3.2. BHV-1 infection of mucosal epithelial cells in nasal turbinates. Mucosal tissue architecture was examined before and after primary BHV-1 infection. Tissue samples for histology were collected from the same animals used to quantify virus shedding in Figure 1. Nasal turbinate samples were collected in 10% buffered formalin on Day 0, prior to BHV-1 infection **(a)** and on Day 5 post-infection **(b)** and tissue sections were stained with haematoxylin and eosin (H&E). Serial tissue sections cut from the tissue blocks prepared for Day 0 **(c)** and Day 5 post-BHV-1 infection **(d)** were stained with polyclonal rabbit anti-BHV-1 antisera to visualize foci of BHV-1 infection. Small arrows indicate BHV-1 infected cells. GL = Glands, ME = Mucosal Epithelium, CI = Cell Infiltration. BM = Basement Membrane, LP = Lamina Propria.

High levels of infectious BHV-1 in nasal secretions on Day 3 pi coincided with a significant ($P< 0.01$) increase in IFN-α secretion (Figure 3.3a). Peak IFN-α levels were detected on Day 5 pi. IFN-α secretion declined significantly on Day 7 pi, even though virus shedding persisted at a high level. Thus, onset of IFN-α production in nasal secretions did not correlate with a decline in virus shedding and peak IFN-α levels did not coincide with a decline in virus production.

The commercial ELISA used to quantify bovine IFN-β in nasal secretions gave optical density reading consistent with IFN-β being present prior to viral infection (Fig. 3.3b). There was, however, a significant ($P < 0.05$) increase in IFN-β levels in nasal secretions on Day 5 pi (Fig. 3.3b). A high level of BHV-1 shedding was, therefore,

observed both before and after peak IFN-β production. Finally, a significant ($P < 0.0001$) increase in IFN-γ production was detected in nasal secretions on Day 3, 5 and 7 pi (Fig. 3.3c). Therefore, a high level of virus shedding persisted for at least 2 days after maximum levels of IFN-α, -β and -γ were detected in nasal secretions.

IFN levels in nasal secretions may not correlate directly with cytokine production within tissues. Therefore, qRT-PCR analysis of IFN gene expression in nasal turbinates was completed to determine whether IFN protein levels in nasal secretions reflected host responses at the site of viral infection. There was marked inter-animal variability in IFN-α transcript abundance when analyzing nasal turbinates collected prior to BHV-1 (Fig. 3.3d). Following BHV-1 infection, there was a consistent decrease in median ΔCt values of IFN-α transcript but this change was significant ($P < 0.05$) only on Day 7 pi. While protein levels in nasal secretions did not directly mirror IFN-α transcript abundance in nasal turbinates, the qRT-PCR analysis confirmed that *IFN-α* transcript increased early after viral infection. Furthermore, peak *IFN-α and β* transcript abundance and secretion both occurred during the period of peak virus shedding.

The qRT-PCR analysis also confirmed an early increase of *IFNβ* transcription following BHV-1 infection with significantly ($P < 0.05$) increased transcript abundance on days 3, 5 and 7 pi (Fig. 3.3e). *IFNβ* gene transcript abundance then declined on day 10 pi but did not return to pre-infection levels. Thus, qRT-PCR analysis revealed an early and sustained increase in transcript abundance for *IFNβ* genes in BHV-1 infected tissue, but increased transcription of *IFNβ* genes was not closely associated with a decrease in virus shedding.

The abundance of IFN-γ gene transcript also varied widely among individual animals prior to BHV-1 infection. High intra-group variance limited the capacity to detect

significant changes in transcript abundance when analyzing 6 animals/time point. There was, however, a consistent decrease in median ΔCt values for IFN-γ gene transcript on all days analyzed following BHV-1 infection (Fig. 3.3f). This decrease in ΔCt values was maximum on day 5 pi, coinciding with maximum IFN-γ protein levels in nasal secretions (Fig. 3.3c). Thus, qRT-PCR results were consistent with ELISA data (Fig. 3.3c).

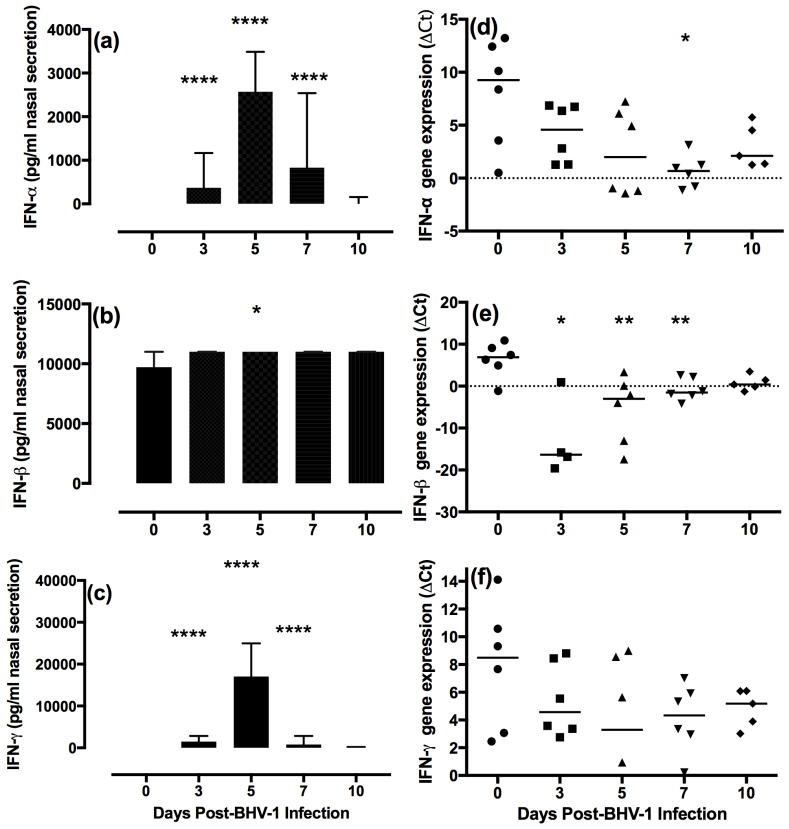

Figure 3.3. IFN secretion in nasal secretions and IFN gene expression in nasal turbinates following primary BHV-1 respiratory infection. The concentration of IFN-α **(a)**, IFN-β **(b)** and IFN-γ **(c)** in nasal secretions was determined with cytokine capture ELISAs. Nasal secretions were collected from animals (n = 6/time point) just prior to euthanasia and collection of tissue samples for the analysis of IFN gene expression. Data presented are the median and interquartile range of values for each cohort of animals. qRT-PCR analysis of *IFN-α* **(d)**, *IFN-β* **(e)**, and *IFN-γ* **(f)** gene expression was performed using primers that amplified multiple bovine *IFN-α* and all three known bovine *IFN-β* genes. Data are presented as ΔCt values relative to the β-actin gene and values for individual animals are presented. The horizontal bar represents the

median value at each time point. Significant changes in IFN protein levels or *IFN* gene expression relative to Day 0 are indicated: *P < 0.05; **P < 0.01; ***P < 0.001 and P ≤ 0.0001.

3.4.2 Expression of antiviral effector genes in nasal turbinates

Monitoring expression of known antiviral effector genes provides an independent confirmation that biologically active levels of IFN were present in infected tissues. qRT-PCR analysis revealed a highly significant (P < 0.001) increase in both *OAS* (Fig. 3.4a) and *Mx1* (Fig. 3.4b) transcripts within 3 days pi and this increase persisted throughout viral infection. A similar expression pattern was observed for *BST-2* (Fig. 3.4c), with a significant (P < 0.01) increase in *BST-2* transcript on days 3, 5 and 7 pi and a decline in transcript abundance on day 10 pi. In contrast, *RNAseL* transcript abundance (Fig. 3.4d) was significantly (P < 0.001) decreased on days 5 and 7 pi but approached pre-infection levels on days 10 pi.

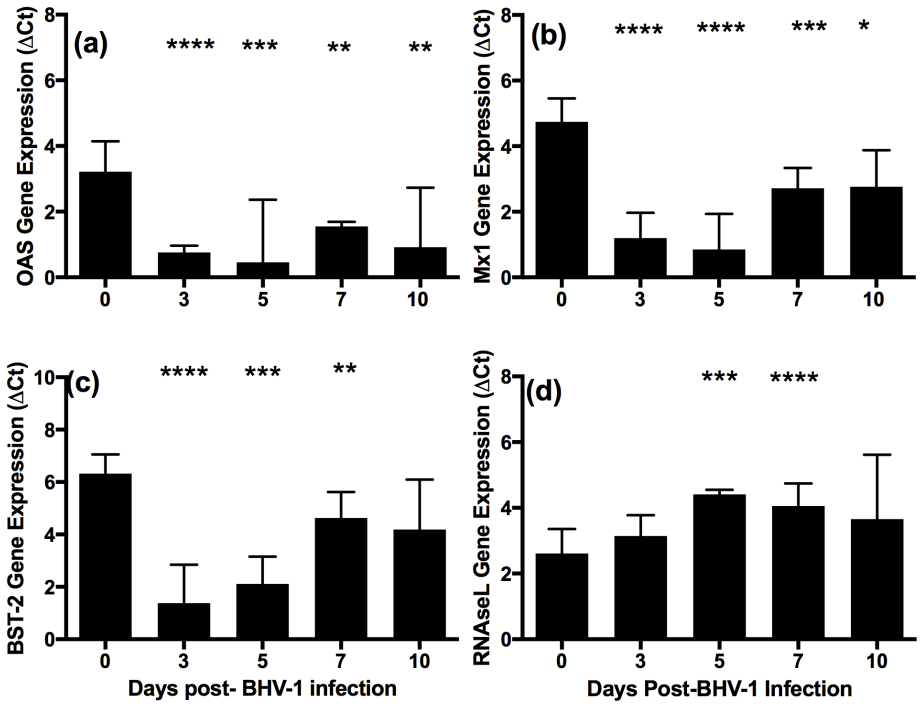

Figure 3.4. Interferon-induced antiviral effector gene expression in nasal turbinate tissue samples collected before and following BHV-1 infection.

qRT-PCR was used to quantify gene expression of *2'5'oligoadenylatesynthetase* (*OAS*) (**a**) *Myxovirus resistance 1* (*Mx1*) **(b)**, *Bone Marrow Stromal Cell Antigen 2* (*BST-2*) **(c)** and *Ribonuclease L (RNAse L)* **(d)** genes. Data are presented as ΔCt values relative to the β-actin gene and presented as median and interquartile range of values for tissue collected from 6 animals at each time point. Significant changes in gene expression relative to Day 0 are indicated: *P < 0.05; **P < 0.01; ***P < 0.001 and P ≤ 0.0001.

3.4.3 Expression of antiviral effector genes in trachea

Transcription of IFN-induced antiviral effector genes was also analyzed for tracheal tissue samples. A significant (P < 0.05) increase in *OAS* (Fig. 3.5a) and *Mx1* (Fig. 3.5b) transcripts was observed within 3 days pi with sustained increase throughout the period of viral infection. A significant increase in *BST-2* transcript level was not detected until Day 10 pi (Fig. 5c). As observed with nasal turbinates, there was a significant (P < 0.05)

decrease in *RNAse L* transcript on days 3 to 7 pi but this was reversed on day 10 pi when transcript abundance was significantly ($p < 0.01$) increased.

Thus, increased transcription of multiple ISGs was detected within 3 days pi for both nasal turbinates and trachea. While there were tissue-specific differences in ISG transcriptional responses, we consistently observed that the onset of increased ISG expression was not associated with a decline in virus shedding (Fig. 3.1a) or tissue infection (Fig 3.1b).

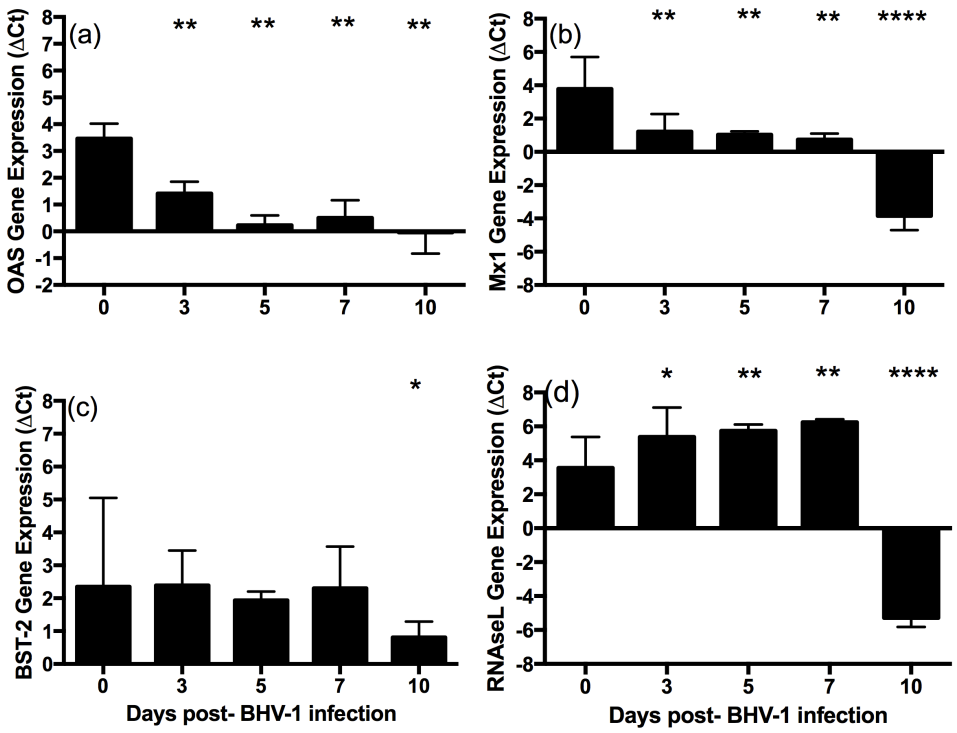

Figure 3.5. Interferon-induced antiviral effector gene expression in tracheal tissue samples collected before and following BHV-1 infection. qRT-PCR was used to quantify gene expression of *2'5'oligoadenylatesynthetase* (*OAS*) (**a**) *Myxovirus resistance 1* (*Mx1*) **(b)**, *Bone Marrow Stromal Cell Antigen 2* (*BST-2*) **(c)** and *Ribonuclease L* (*RNAse L*) **(d)** genes. Data are presented as ΔCt values relative to the β-actin gene and presented as median and interquartile range of values for tissue collected from 6 animals at each time point. Significant changes in gene expression relative to Day 0 are indicated: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ and $P \leq 0.0001$.

3.4.4 IFN induction of antiviral effector genes in bovine epithelial cell

qRT-PCR analysis revealed a significant induction of three of four IFN-induced effector genes analyzed in nasal turbinate samples following BHV-1 infection. Coincidental production of multiple IFN types (Fig. 3.3) suggested that more than one IFN type may be responsible for the observed increases in ISG transcripts. There is limited information regarding bovine ISG responses to IFN-α [216], and little is known regarding the induction of ISGs by bovine IFN-β and -γ. Therefore, we investigated whether rBoIFN-α , -β and –γ have the capacity to induce expression of the four antiviral effector genes analyzed following BHV-1 infection. *Mx1*, *OAS,* an*d BST-2* transcription increased 10- to 100-fold within 4 hours after bovine epithelial cells were exposed to rBoIFN-α and remained elevated for 12 hours (Fig 3.6a). Similarly, *Mx1, OAS,* and *BST-2* transcription increased within 4 hours after exposure to 100ng/ml of IFN-β but transcription then decreased to basal levels by 6 hours post-treatment (Fig. 3.6b). rBoIFN-γ also induced a sustained 10- to 100-fold increase *Mx1*, *OAS*, and *BST-2* transcription throughout the 12 hour period (Fig. 3.6c). Both rBoIFN-α and -γ induced increased *RNAse L* transcription at 4 hours post-treatment (Fig. 3.6a and 3.6c) but *RNAse L* transcription was not altered by rBoIFN-β treatment (Fig 3.6b). Thus, both type I and II rboIFNs have the capacity to induce expression of multiple ISGs in bovine epithelial cells.

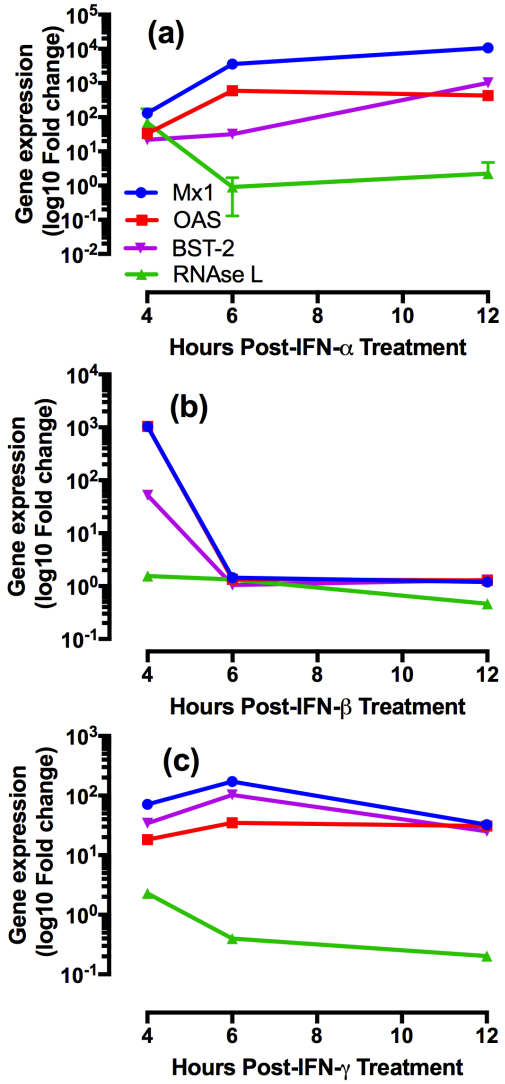

Figure 3.6. Induction of antiviral effector gene expression in bovine epithelial cells following treatment with rBoIFN-α, -β and -γ. qRT-PCR was used to quantify transcript abundance of *Mx1*, *OAS*, *RNAse L* and *BST-2* genes induced following treatment with 100 ng/ml of either rBoIFN-α_I **(a)**, rBoIFN-β **(b)**, or rBoIFN-γ **(c)**. Gene expression data are expressed as fold change relative to gene expression in time-matched cultures not treated with rBoIFN.

3.4.5 BHV-1 evasion of IFN antiviral activity

The persistence of a high level of BHV-1 shedding (Fig. 3.1a) following significant induction of IFN-α, -β, and -γ protein and gene expression in nasal turbinates (Fig. 3.3) suggested that BHV-1 effectively evades IFN-induced antiviral effector mechanisms. To address this possibility, a plaque inhibition assay was used to compare rBoIFN inhibition of BHV-1 replication relative to Vesicular Stomatitis Virus (VSV), a virus well-documented to be sensitive to IFN [217]. A 50% inhibition of VSV pfu was observed when cells were pre-treated with 0.005 ng rBoIFN-α/ml and complete inhibition of VSV replication was observed following treatment with 1 ng rBoIFN-α/ml (Fig. 3.7a). In contrast, rBoIFN-α inhibition of the BHV-1 isolate 108 (Fig. 3.7a) and Cooper isolate (data not shown) approached 50% only when cells were pre-treated with 10,000 ng rBoIFN-α/ml. This is an IFN-α concentration over 1000-fold higher than that detected in nasal secretions from infected animals (Fig. 3.3a). rBoIFN-β also displayed strong antiviral activity, with 50% inhibition of VSV pfu following pre-treatment of cells with 0.005 ng rBoIFN-β ml and complete inhibition of VSV replication with 10 ng rBoIFN-β ml (Fig. 3.7b). rBoIFN-β concentrations of 0.05 ng/ml and 10 ng/ml were able to effect a 50% and 80% inhibition, respectively, of BHV-1 108 (Fig. 3.7b) and Cooper isolates (data not shown). Thus, substantial inhibition of BHV-1 replication was observed with rBoIFN-β concentrations similar to those detected in nasal secretions on day 5 pi (Fig. 3.3b). rBoIFNγ also displayed substantial antiviral activity with 50% inhibition of VSV pfu when cells were pre-treated with 0.01ng rBoIFN-γ/ml and complete inhibition of VSV replication with 100 ng rBoIFN-γ/ml (Fig. 3.7c). When comparing the concentration of rBoIFN required to completely inhibit VSV replication then rBoIFNγ was 100-fold less

active than rBoIFNα and 10-fold less active than rBoIFN-β. Furthermore, the highest concentration of rBoIFN-γ tested *in vitro*, which was 500-fold higher than that detected *in vivo* (Fig. 3.3c), effected less than 30-40% inhibition of BHV-1 isolate 108 (Fig. 3.7c).

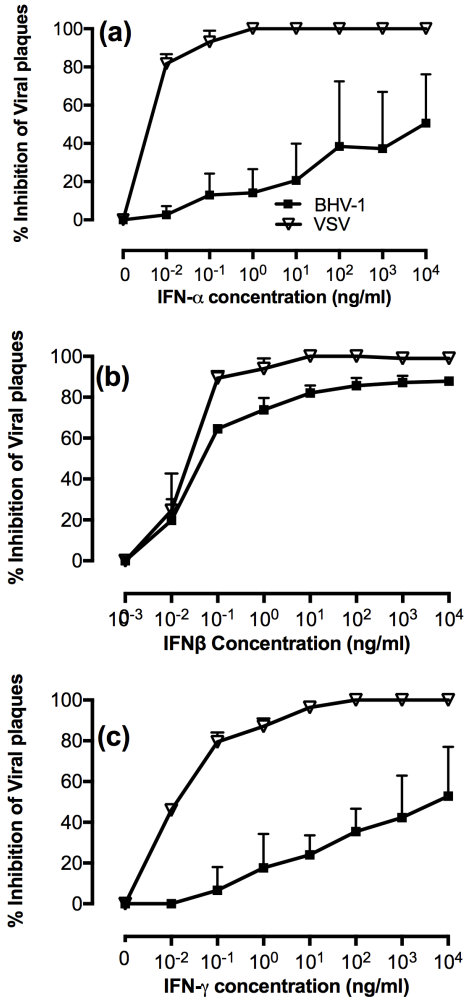

Figure 3.7. Inhibition of BHV-1 replication in bovine epithelial cells treated with rBoIFN. A plaque inhibition assay was used to quantify the capacity of individual rBoIFNs to inhibit replication of BHV-1 and Vesicular Stomatitis Virus (VSV). Bovine epithelial cells were infected with BHV-1 108 isolate 12 hours after treatment with varying concentrations of either recombinant bovine IFN-α **(a)**, -β **(b)**, or -γ **(c)**. Data presented are the mean + 1SD of values from three replicate assays performed with each IFN concentration and the percent inhibition of BHV-1 replication was calculated relative to epithelial cell cultures infected with 50 pfu of BHV-1 or VSV but not treated with rBoIFN.

3.5 Discussion

IFNs provide a key innate defence against viral infections but viral pathogens have developed a variety of strategies to prevent either the induction of IFNs or block the activity of IFN-induced antiviral effector proteins. *In vitro* studies have identified numerous mechanisms by which viruses block IFN induction, IFN signaling, or disrupt the activity of IFN-induced anti-viral effector proteins. Few studies have been completed, however, to determine whether these immune evasion strategies are relevant *in vivo* where multiple IFN types or subtypes can be simultaneously activated and multiple viral transcripts and proteins are being expressed. BHV-1 provides an interesting example of an acute respiratory infection known to induce both a robust IFN-α and -γ response [67], but high levels of IFN secretion coincide with sustained viral replication (Fig.3.1 & 3.3). For the first time, we also demonstrated that a primary BHV-1 infection is associated with significant ($P < 0.05$) induction of IFN-β transcript (Fig. 3.3e) and protein (Fig. 3.3b) in the bovine URT. Furthermore, induction of both type I (IFN-α and - β) and II (IFN-γ) responses occurred early during infection but viral replication persisted at a high level and for 10 days despite this multifaceted IFN response.

Previous in vitro observations led to the conclusion that BHV-1 evaded IFN-β antiviral activity by inhibiting IFN-β transcription [83, 84] and inhibiting transcription of ISGs [85] . Our *in vivo* observations do not support this conclusion since IFN-β transcription was significantly ($P < 0.05$) increased in infected tissues, beginning as early as day 3 pi (Fig. 3.3e). Although increased gene transcription may not result in increased protein production, ELISA results (Fig. 3.3b) confirmed bovine IFN-β production

increased significantly ($P < 0.05$) following BHV-1 infection. Contradictory results from *in vitro* studies [83, 84] and our *in vivo* study may be explained by several factors. Results obtained by Saira et al. [84] may be unique to the cell lines used and may not reflect the capacity of mucosal epithelial cells to respond to BHV-1 infection. We also designed qRT-PCR primers to detect transcripts from the three known bovine IFN-β genes (Supplemental Fig. 3B) and the transcriptional response we detected may represent transcripts from multiple IFN-β genes. Further studies may be warranted to determine whether bICP0 is equally effective in inhibiting the promoter activity of all bovine IFN-β genes. Finally, mucosal tissues in the URT represent a complex cell population (Fig. 3.3) with the potential to recruit immune cells. IFN transcript detected with RNA extracted from nasal turbinate and tracheal samples represents the collective response of both infected and uninfected cells (Fig. 3.3d-f). Other herpesvirus proteins, such as HSV-1 glycoprotein D, function as a potent activator of IFN responses in macrophages [218]. Thus, the IFN response detected at a tissue level may represent the response of a variety of cells to different viral proteins. The current observations highlight the importance of completing *in vivo* studies to confirm whether IFN evasion mechanisms identified in cultured cells or implicated by studies in an aberrant host [219] adequately explain viral immune evasion in the natural host.

Other respiratory viruses, such as influenza, have been reported to induce less than 0.15 ng IFN-α/ml in nasal secretions [220], a concentration 5 to 10-fold lower than what we detected following BHV-1 infection (Fig. 3.3a). Previous *in vivo* and *in vitro* investigations indicated that BHV-1 replication was not inhibited by either rBoIFN-α [81] or –γ [82]. There was, however, no previous analysis of ISG expression in URT tissues

following BHV-1 infection to determine if local IFN production activated local antiviral defence mechanisms. The current investigation confirmed BHV-1 evades the antiviral activity of both type I and II bovine interferons at a concentration that effectively induced transcription of key antiviral effector genes (Fig. 3.6). This IFN evasion was a conserved trait among plaque-purified BHV-1 isolates, 108 and Cooper's (data not shown) and *in vitro* studies support the previous conclusion that BHV-1 replication is not inhibited by either IFN-α or -γ (Fig. 3.7a & c). While IFN-β appeared to be a relatively weak inducer of antiviral effector gene transcription (Fig. 3.6b), it was the most potent inhibitor of BHV-1 replication (Fig. 3.7b). This *in vitro* observation suggests IFN-β might play a role in inhibiting BHV-1 replication *in vivo*. IFN-β may reduce the magnitude of BHV-1 replication but the early post-infection induction of IFN-β transcription and secretion suggests that it may have a limited effect on the duration of virus shedding (Fig. 3.3). A broader analysis of ISGs induced by bovine IFN-β is necessary to determine whether antiviral effector mechanism(s), other than those analyzed in the present study, play a role in limiting BHV-1 replication.

Previous studies of ISG expression following BHV-1 infection focused on OAS activity in blood leukocytes [221]. Subsequent studies confirmed that rBoIFN-α specifically induced OAS activity in lung macrophages and elevated OAS activity persisted in blood leukocytes after rBoIFN-α was no longer detectable in serum [222]. Current *in vitro* studies confirmed that rBoIFN-α is a potent inducer of *OAS* and other ISGs in bovine epithelial cells and IFN-α was at least 10-fold more effective in activating sustained expression of ISGs than rBoIFN–γ (Fig. 3.6). This observation is consistent with data reported by Liu et al. [223] and the proposal by Marié et al. [224] that IFN-α can

activate a positive feedback loop to amplify expression of a delayed set of *IFN-α* genes. rBoIFN-β also significantly increased the expression of *OAS*, *Mx1* and *BST-2* by 100 to1000-fold but this induction of ISG expression was transient.

Expression of *Mx1* and *OAS* genes in nasal turbinates and trachea increased significantly ($P < 0.05$) within 3 days pi and elevated ISG expression persisted throughout the period of viral shedding. It is not possible to conclude which IFNs were responsible for increased ISG expression in tissues since our *in vitro* analyses confirmed that all three rBoIFNs can induce antiviral effector gene expression in bovine epithelial cells (Fig. 3.6). This *in vitro* analysis also challenges whether the ISGs analyzed in the current study actually play a role in restricting BHV-1 replication. However, for a closely related herpesvirus, HSV-1, it has been reported that Mx1 and BST-2 can restrict HSV-1 replication [207, 225]. Both rBoIFN-*α* and -*γ* had strong antiviral activity against VSV replication and induced sustained ISG expression but had limited capacity to inhibit BHV-1 replication. Based on the plaque inhibition assay results, peak IFNα and -γ levels detected in nasal secretions (Fig. 3.3) would have the capacity to inhibit less than 20% of BHV-1 replication. Thus, *in vitro* results support the conclusion that IFN-α and -γ have limited capacity to directly inhibit BHV-1 replication despite their capacity to induce antiviral effector genes. In contrast, IFN-β was a more potent inhibitor of BHV-1 replication *in vitro* at concentrations observed *in vivo* (Fig. 3.3). Thus, the early induction of IFN-β following BHV-1 infection may indicate that it plays an important role in limiting the magnitude of BHV-1 replication.

In vitro studies confirmed that recombinant bovine rBoIFN-α and -γ induce sustained expression of *Mx1*, *OAS*, and *BST-2* but only transient *RNAse L* transcription in

bovine epithelial cells (Fig 3.6). A significant ($P < 0.01$) decrease in *RNAse L* transcript was observed in nasal turbinates (Fig. 3.4d) and trachea (Fig. 3.5d) following BHV-1 infection. These results cannot be interpreted as evidence that BHV-1 infection inhibited expression of this ISG if IFNs only induce transient increases in *RNAse L* transcript. Furthermore, the pattern of ISG expression observed may be tissue specific since there was significantly ($P < 0.01$) increased *RNAse L* transcript in trachea on day 10 pi (Fig 3.5d). RNAse L is potentially an important antiviral effector for limiting BHV-1 replication since it degrades single stranded mRNAs and inhibits translation of viral proteins [226]. However, while IFN-β was a potent inhibitor of BHV-1 replication *in vitro,* it did not induce increased expression of *RNAse L* in bovine epithelial cells (Fig 3.6b). Therefore, the present study provides no evidence to indicate that RNAse L may be an important antiviral effector protein for limiting BHV-1 replication.

BHV-1 evasion of IFN antiviral activity, especially for IFN-α and -γ raises questions regarding the potential value of this multifaceted IFN response for the host. The induction of this potent innate immune defence may not play a major role in limiting the duration of viral replication but IFN-α not only activates antiviral effector genes (Fig. 3.5) but also activates NK cells to produce IFN-γ [227]. IFN-γ may then have indirect antiviral effects through the induction of chemokines, such as CXCL10 and CXCL9, also known as monokines induced by IFN-γ (MIG). CXCLl0 may amplify NK-cell recruitment and MIG may recruit T-cells to the site of viral infection [228]. Thus, elevated IFN-γ levels at the site of viral infection may enhance recruitment of both innate immune cells and virus-specific effector T-cells. NK cells were recruited to the lung following BHV-1 infection and these cells had an enhanced capacity to kill BHV-1 infected cells [192]. An analysis of

immune cells recruited to the site of BHV-1 infection in the URT may clarify whether the IFN response indirectly benefits the host by enhancing viral clearance through the recruitment and activation of immune cells.

In conclusion, the present study confirmed that Type 1 and II rBoIFNs have the capacity to induce expression of several antiviral effector genes in bovine epithelial cells. At physiological concentrations, however, only rBoIFN-β was able to effect greater than 50% inhibition of BHV-1 replication. *In vivo* studies confirmed that transcription and secretion of both type I and II IFNs occurs within 3 days after a primary BHV-1 infection and this IFN response was associated with a significantly ($P < 0.05$) increased expression of multiple antiviral effector genes in the URT. However, viral replication in the URT continued for 10 days despite strong induction of both Type I and II IFN and onset of ISG expression early after viral infection. *In vivo* data supports the conclusion that BHV-1 evasion of the IFN response does not appear to occur through an inhibition of either IFN gene expression or ISG transcription.

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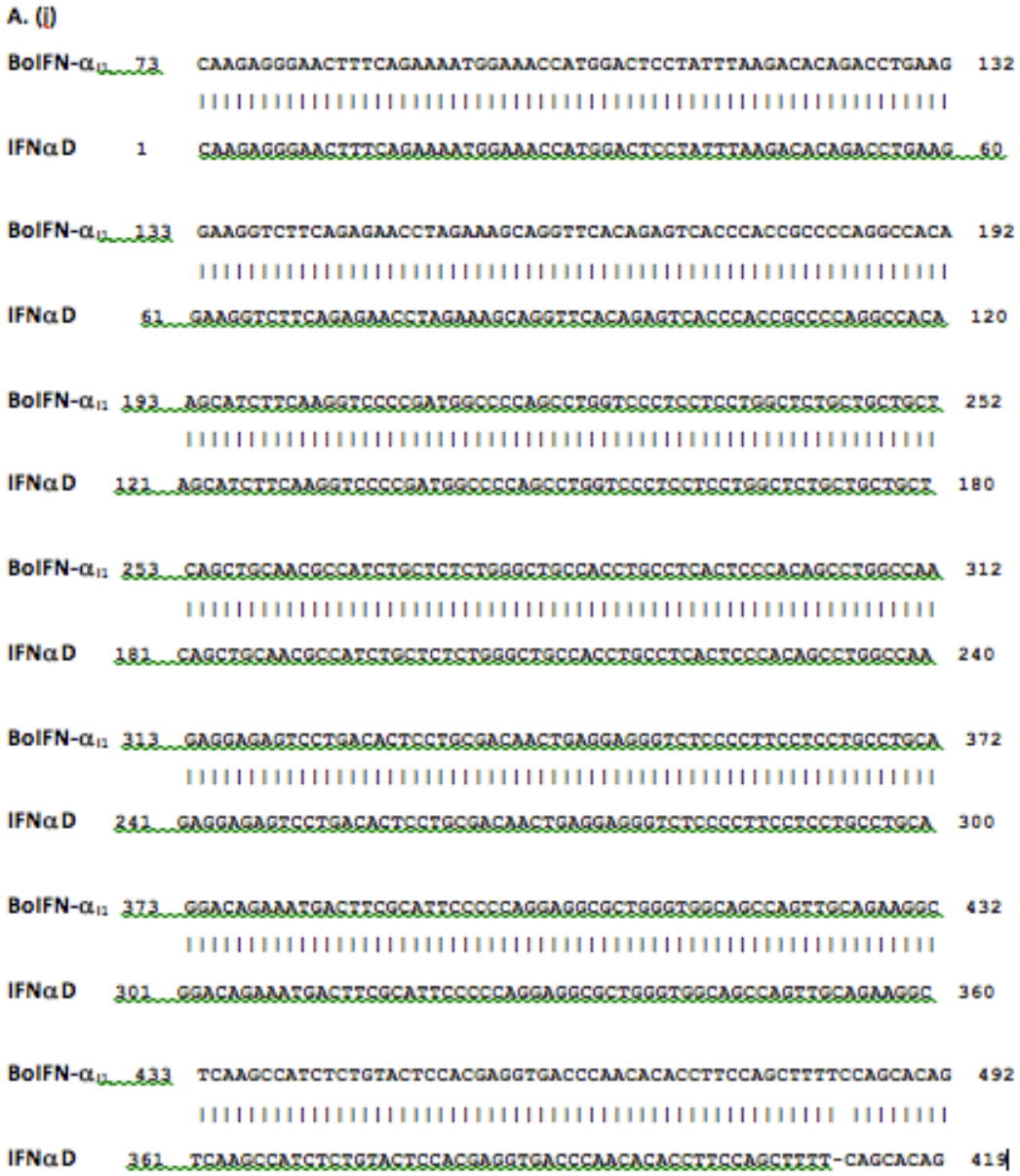
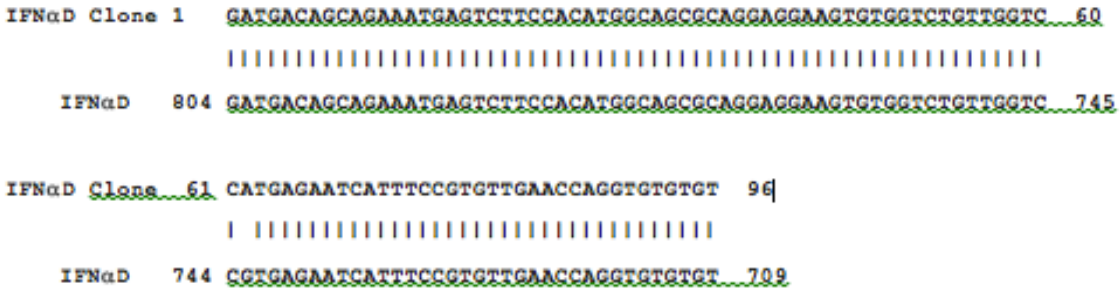
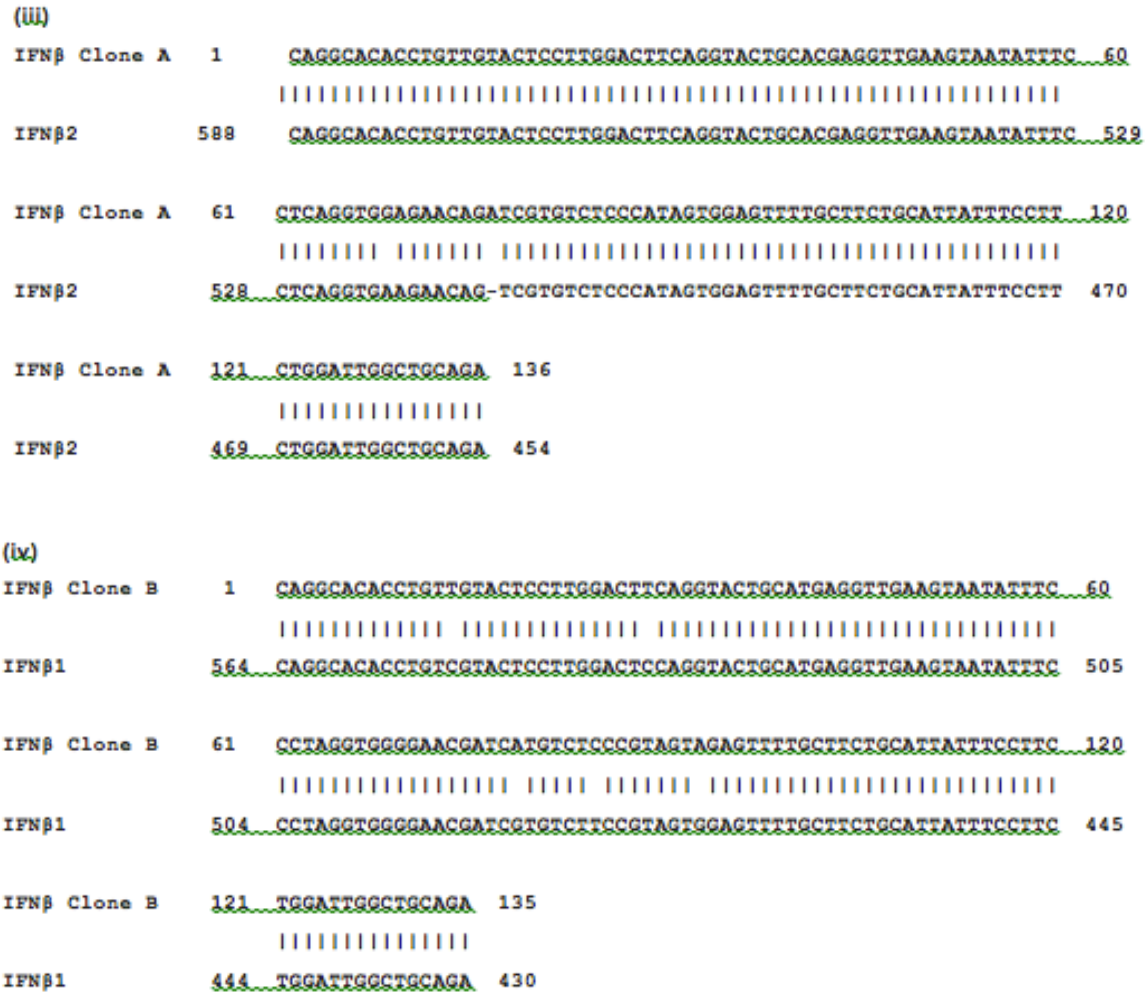
3.6 Supplemental Figures




BoIFN-α12 493 AGGGCTCGGCCGCTGTGTGGGATGAGAGCCTCCTGGACAGACTCCGCACTGCACTGGATC 552
IFNαD 420 AGGGCTCGGCCGCTGTGTGGGATGAGAGCCTCCTGGACAAGCTCCGCACTGCACTGGATC 479
BoIFN-α12 553 AGCAGCTCACTGACCTGCAAGCCTGTCTGAGGCAGGAGGAGGGGCTGCCAGGGGCTCCCC 612
IFNαD 480 AGCAGCTCACTGACCTGCAAGCCTGTCTGAGGCAGGAGGAGGGGCTGCCAGGGGCTCCCC 539
BoIFN-α12 613 TGCTCAAGGAGGACTCCAGCCTGGCTGTGAGGAAATACTTCCACAGACTCACTCTCTATC 672
IFNαD 540 TGCTCAAGGAGGACTCCAGCCTGGCTGTGAGGAAATACTTCCACAGACTCACTCTCTATC 599
BoIFN-α12 673 TGCAAGAGAAGAGACACAGCCCTTGTGCCTGGGAGGTTGTCAGAGCACAAGTCATGAGAG 732
IFNαD 600 TGCAAGAGAAGAGACACAGCCCTTGTGCCTGGGAGGTTGTCAGAGCACAAGTCATGAGAG 659
BoIFN-α12 733 CCTTCTCTTCCTCAACAAACTTGCAGGAGAGATTCAGGAGAAAGGACTGACACACACCTG 792
IFNαD 660 CCTTCTCTTCCTCAACAAACTTGCAGGAGAGATTCAGGAGAAAGGACTGACACACACCTG 719
BoIFN-α12 793 GTTCAACACGGAAATGATTCTCACGGACCAACAGACCACACTTCCTCCTGCGCTGCCATG 852
IFNαD 720 GTTCAACACGGAAATGATTCTCACGGACCAACAGACCACACTTCCTCCTGCGCTGCCATG 779
BoIFN-α12 853 TGGAAGACTCATTTCTGCTGTCATCAGGCACTGAACTGAA 892
IFNαD 780 TGGAAGACTCATTTCTGCTGTCATCAGGCACTGAACTGAA 819

(ii)


IFNαD Clone 1 GATGACAGCAGAAATGAGTCTTCCACATGGCAGCGCAGGAGGAAGTGTGGTCTGTTGGTC 60
IFNαD 804 GATGACAGCAGAAATGAGTCTTCCACATGGCAGCGCAGGAGGAAGTGTGGTCTGTTGGTC 745
IFNαD Clone 61 CATGAGAATCATTTCCGTGTTGAACCAGGTGTGTGT 96
IFNαD 744 CGTGAGAATCATTTCCGTGTTGAACCAGGTGTGTGT 709



Supplemental Figure 3A. Identification of IFN PCR products and alignment of BoIFN-α and -β gene sequences. **(i)** Clone manager was used to design primers targeting bovine *interferon alpha Class 1 (BoIFN-α$_1$1)* which was identified as *IFNαD* through NCBI-Blast search. *IFN* PCR products were cloned and DNA-sequenced to confirm the identity and expected size of the PCR products. NCBI-Blast search confirmed that the cloned *IFNαD* product **(ii)** was *IFNαD* (XM_005209896.1) with 99% homology. **(iii)** Cloned *IFNβ* product A was identified as *IFNβ2* (XM_603903.7) with 99% homology and **(iv)** cloned *IFNβ* product B was identified as *IFNβ1* (NM_174350.1) with 96% homology.

B. (i)

1 CAAGAGGGAA CTTTCAGAAA ATGGAAACCA TGGACTCCTA TTTAAGACAC AGACCTGAAG

GATGACAGCAGAAATGAGTCTTCC→
61 GAAGGTCTTC AGAGAACCTA GAAAGCAGGT TCACAGAGTC ACCCACCGCC CCAGGCCACA

121 AGCATCTTCA AGGTCCCCGA TGGCCCCAGC CTGGTCCCTC CTCCTGGCTC TGCTGCTGCT
<-ACACACACCTGGTTCAACAC

181 CAGCTGCAAC GCCATCTGCT CTCTGGGCTG CCACCTGCCT CACTCCCACA GCCTGGCCAA

241 GAGGAGAGTC CTGACACTCC TGCGACAACT GAGGAGGGTC TCCCCTTCCT CCTGCCTGCA

301 GGACAGAAAT GACTTCGCAT TCCCCCAGGA GGCGCTGGGT GGCAGCCAGT TGCAGAAGGC

361 TCAAGCCATC TCTGTACTCC ACGAGGTGAC CCAACACACC TTCCAGCTTT TCAGCACAGA

421 GGGCTCGGCC GCTGTGTGGG ATGAGAGCCT CCTGGACAAG CTCCGCACTG CACTGGATCA

481 GCAGCTCACT GACCTGCAAG CCTGTCTGAG GCAGGAGGAG GGGCTGCCAG GGGCTCCCCT

541 GCTCAAGGAG GACTCCAGCC TGGCTGTGAG GAAATACTTC CACAGACTCA CTCTCTATCT

601 GCAAGAGAAG AGACACAGCC CTTGTGCCTG GGAGGTTGTC AGAGCACAAG TCATGAGAGC

661 CTTCTCTTCC TCAACAAACT TGCAGGAGAG ATTCAGGAGA AAGGACTGAC ACACACCTGG

721 TTCAACACGG AAATGATTCT CACGGACCAA CAGACCACAC TTCCTCCTGC GCTGCCATGT

781 GGAAGACTCA TTTCTGCTGT CATCAGGCAC TGAACTGAAT CAATT

B(ii)


IFNβ1 1 -attcactctgcaaaccctgaagactcagcttcagcacctactagcaga
IFNβ2 1 -attcactctgcaaaccctgaagactcagcttcagcacctactagcaga
IFNβ3 1 cattcactctgcaagccctgaagactcagcgtcagcatctactagcaga
IFNβ1 50 acaggtagccctgtgcctgatttcatcatgacctaccggtgcctcctcca
IFNβ2 50 acaggcagccctgtgcctgatttcatcatgacccaccggtgcctcctcca
IFNβ3 51 acgggcagccctgtgcctgttttcatcatgacctaccggtgcctcctccc
IFNβ1 100 gatggttctcctgctgtgtttctccaccacagctctttccaggagctaca
IFNβ2 100 gatggttctcctgctgtgtttctccaccacagctctttccaggagctaca
IFNβ3 101 gatggttctcctgctgtgtttctccaccacagctctttccaggagctaca
IFNβ1 150 gcttgcttcgattccaacaacgtcagagccttaaagagtgtcagaaactc
IFNβ2 150 gcttgcttcgattccaacaaaggcgagccttgcgttatgtcagaaactc
IFNβ3 151 gcttgctcagattccagcaaaggcgagcgctgaggtgtgtcagaaactc
IFNβ1 200 ctggggcagttaccttcaacttctcaacattgcctcgaggccaggatgga
IFNβ2 200 ctgaggcagttaccttcaactcctcaacattgcctcgaggccaggatgga
IFNβ3 201 ctggggcagttacattcaacgcctcaacattgcctcgaggccaagatgga
IFNβ1 250 cttccagatgcctgaggagatgaagcaagaacagcagttccagaaggaag
IFNβ2 250 cttccagatgcctgaggagatgaagcaagcacagcagttccagaaggaag
IFNβ3 251 cttccaagtccctgaggagatgaaccaagcacagcagttccggaaggaag
IFNβ1 300 atgccatattggtcatgtatgaggtgctccagcacatcttcggcattctc
IFNβ2 300 atgccatattggtcatctatgagatgctccagcagatcttcaatattctc
IFNβ3 301 atgccatattggtcatctatgagatgctccagcagatcttcaatattctc
IFNβ1 350 accagagacttctccagcactggctggtctgagaccatcatcgaggacct
IFNβ2 350 accagagacttctccagcactggctggtctgagaccatcatcgaggacct
IFNβ3 351 accagagacttctccagcactggctggtctgagaccatcattgaggacct
FORWARD PRIMER
RTCTGSAGCCAATCCARAAG→
IFNβ1 400 ccttaaggaactctattggcagatgaatcgtctgcagccaatccagaagg
IFNβ2 400 ccttgaggaactctatgagcagatgaatcatctggagccaatccagaagg
IFNβ3 401 ccttgtggaactctatgggcagatgaatcgtctgcagccaatccagaagg
IFNβ1 450 aaataatgcagaagcaaaactccactacggaagacacgatcgttcccac
IFNβ2 450 aaataatgcagaagcaaaactccactatggagacacaaccgttcttcac
IFNβ3 451 aaataatgcaggagcaaaacttcaccatgcgggacacaaccgttcttcac
IFNβ1 500 ctagggaaatattacttcaacctcatgcagtacctggagtccaaggagta
IFNβ2 500 ctgaggaaatattacttcaacctcgtgcagtacctaaagtccaaggagta
IFNβ3 501 ctgaagaagtattacttcaacctcgtgcagtacctggagtccaaggagta
<-CAGGC
IFNβ1 550 cgacaggtgtgcctggacagtcgtgcaagtgcaaatactcacgaacgttt
IFNβ2 550 caacaggtgtgcctggacagtcgtgcgagtgcaaatactcaggaattttt
IFNβ3 551 caacaggtgtgcctggacagtcgtgcgagtgcaaatactcacgaacttttt
ACACCTGTYGTACTC
REVERSE PRIMER
IFNβ1 600 ctttcctgatgagactaacaggttacgtccgtgactga
IFNβ2 600 ctttcctgacgagactaacaggttacctccgtgaatga
IFNβ3 601 ctttcctgatgagactaacagcttccctccgtgactga

Supplemental Figure 3B. Alignment of the forward and reverse primers specific to boIFN type I genes. Clone manager was used to design primers targeting bovine interferon alpha Class 1 (*boIFN-αI1*) which was identified as *IFNαD* through NCBI-Blast Search. **(i)** Alignment of *IFNαD* (XM_005199122) primer binding sites with *IFNαD* sequence. *IFNβ* primers were designed with IUPAC nucleotides to target all three types of *boIFNβ*, *boIFNβ1* (NM_174350), *boIFNβ2* (NM_001015617.1) and *boIFNβ3* (NM_001114297.1)
(ii) Alignment of boIFNβ primers with boIFNβ1, boIFNβ2, and boIFNβ3 sequences.

Linking Statement

Data in the previous chapter provided evidence that the induction of interferon and interferon-induced antiviral effector genes occurred early during a primary BHV-1 infection but this multifaceted antiviral response was not sufficient to control a primary BHV-1 respiratory infection by reducing viral shedding and clinical disease. BHV-1 evasion of type I and II IFN induced antiviral activity *in vitro* and *in vivo* raised questions regarding the value of this IFN response for the host. IFN-γ may have indirect antiviral effects through the induction of chemokines, such as CXCL9 and CXCL10. CXCLl0 may amplify NK-cell recruitment and CXCL9 may recruit T-cells to the site of viral infection [228]. Thus, elevated IFN-γ levels at the site of viral infection may enhance recruitment of either innate immune cells or virus-specific adaptive effector T-cells. Non-MHC restricted cells were reported to be recruited to the lung following BHV-1 infection and these cells had an enhanced capacity to kill BHV-1 infected cells [192]. The phenotype and frequency of immune cells present in URT tissues following primary BHV-1 infection were then analyzed to determine whether specific innate or adaptive effector cells may be recruited early during infection. IFN responses may indirectly benefit the host by enhancing viral clearance through either the recruitment or activation of immune effector cells at the site of infection. Alternatively, if IFN production does not precede cell recruitment, then increased IFN-γ production at the site of viral infection may simply be a by-product of having an increased number of activated innate or adaptive effector cells to the site of infection. In the next chapter, I investigated lymphocyte recruitment to the URT following a primary BHV-1 respiratory infection.

CHAPTER 4: CD335 (NKP46)+ T-CELL RECRUITMENT TO THE BOVINE UPPER RESPIRATORY TRACT DURING A PRIMARY BOVINE HERPESVIRUS-1 INFECTION

This chapter has been published in Frontiers in Immunology as:
Osman RA, Griebel PJ. CD335 (NKp46)$^+$ T-Cell Recruitment to the Bovine Upper Respiratory Tract during a Primary Bovine Herpesvirus-1 Infection. Front Immunol. 2017 Oct 23;8:1393. doi: 10.3389/fimmu.2017.01393. eCollection 2017. PubMed PMID: 29114252; PubMed Central PMCID: PMC5660870.
I was co-responsible for conception, design of the study and writing the manuscript. I designed and validated primers, performed experiments, and analyzed the data.

Relevance of this study to the Thesis

This study addressed questions presented within the second hypothesis of this thesis. Lymphocyte recruitment to the upper respiratory tract during a primary BHV-1 infection was analyzed and CD335+ non-conventional T-cells were identified as the major lymphocyte subpopulation recruited during viral infection.

4.1. Abstract

Bovine Natural Killer (NK) cells were originally defined by the NK activation receptor, CD335 (NKp46) but following the discovery of NKp46 expression on human T-cells, the definition of conventional bovine NK cells was modified to $CD335^+CD3^-$ cells. Recently, a bovine T cell population co-expressing CD335 was also identified and these non-conventional T-cells were shown to produce IFN-γ and share functional properties with both conventional NK cells and T-cells. It is not known, however, if $CD335^+$ bovine T cells are recruited to mucosal surfaces and what chemokines may play a role in recruiting this unique T cell subpopulation. In this study, bovine herpesvirus -1 (BHV-1), which is closely related to herpes simplex virus-1, was used to investigate bovine lymphocyte cell populations recruited to the upper respiratory tract (URT) following a primary respiratory

infection in calves. Immunohistochemical staining with individual monoclonal antibodies revealed significant ($P < 0.05$) recruitment of $CD335^+$, $CD3^+$ and $CD8^+$ lymphocyte populations to the nasal turbinates on day 5 following primary BHV-1 infection. Analysis by dual-colour immunofluorescence revealed that the cells recruited to nasal turbinates were primarily T cells that co-expressed both CD335 and CD8α. This non-conventional T cell population represented 77.5% to 89.5% of the lymphocytes recruited to nasal turbinates on day 5 post-BHV-1 infection. However, due to diffuse IFN-γ staining of nasal turbinate tissue, it was not possible to directly link increased IFN-γ production following BHV-1 infection with the recruitment of non-conventional T-cells. Transcriptional analysis revealed CCL4, CCL5 and CXCL9 chemokine gene expression was significantly ($P < 0.05$) up-regulated in nasal turbinate tissue following BHV-1 infection. Therefore, no single chemokine was associated with recruitment of non-conventional T-cells. In conclusion, the specific recruitment of $CD335^+$ and $CD8^+$ non-conventional T cells to viral infected tissue suggests these cells may play an important role in either the clearance of a primary BHV-1 infection or regulating host responses during viral infection. The early recruitment of non-conventional T-cells following a primary viral infection may enable the host to recognize viral-infected cells through NKp46 while retaining the possibility of establishing T cell immune memory.

4.2. Introduction

Bovine herpes virus-1 (BHV-1), a member of *Alphaherpesvirinae* subfamily, is an important pathogen contributing to bovine respiratory disease (BRD) complex in young calves. BHV-1 causes a rhinotracheitis in the upper respiratory tract (URT) [71] and,

similar to other alphaherpesviruses in humans, pigs and horses, causes a lytic infection of mucosal epithelial cells [229] followed by a latent infection in the peripheral nervous system. The primary sites of BHV-1 infection in the URT include the nasal turbinates, pharyngeal tonsils and trachea [61]. Previous studies suggested that IFNs does not play a major role in the clearance of a primary BHV-1 infection [230] but cytotoxic cell-mediated immune responses mediated by macrophages, neutrophils, natural killer (NK) cells, and cytotoxic T-lymphocytes (CTLs) may contribute to viral clearance [192, 231, 232].

NK cells are non-antigen specific innate lymphocytes that respond rapidly to both infectious and non-infectious challenges. NK cells express both activation and inhibitory receptors. These heterologous receptors include killer-cell immunoglobulin-like receptors (KIR) and natural cytotoxicity receptors (NCR) such as NKp46 (NCR1 or CD335), NKp30 and NKp44 [233]. CD335 is the only NK receptor currently characterized for bovine NK cells [155] and is an activating receptor on NK cells which binds ligands and initiates signalling that activates cytotoxic responses. This process was demonstrated by activation of human NK cell cytotoxicity following NKp46 -binding of hemaglutinin (HA) of influenza viruses [234]. This activation signal results in the release of cytotoxic granules, which kill target cells through the combined action of perforin and granzyme [235].

CD335 was originally described as a bovine NK cell specific receptor [155] but a small subpopulation of bovine T-cells have also been identified that co-express $CD335^+$ [155, 157, 159, 160]. $CD335^+CD3^-$ cells are now defined as classical or conventional NK cells and lymphocytes that co-express CD3 with $CD335^+$ are described as non-conventional T

cells. Multiple non-conventional T-cells have been reported in several mammalian species, including humans[161], mice [236], pigs [159] and bovine [157]. The discovery of reprogrammed human cytotoxic T-lymphocytes, which co-express CD3 and NKp46 in celiac disease [161] highlighted the existence of T-cells acquiring natural cytotoxicity receptors (NCRs) previously associated with NK cells. Other non-conventional T-cells include Natural killer T-cells (NKT) and mucosal associated invariant T-cells (MAIT) that co-express CD3 and NCRs.

NKT cells were first discovered in mice [237] and characterized as a T cell subpopulation expressing NK1.1, and αβT-cell receptors [237, 238]. In other species that do not express NK1.1, the term NKT has been used to refer to T-cells which co-express NK cell receptors [239]. NKT cells studied in humans and mice were shown to express an invariant TCR and were termed invariant (i)NKT cells. This population recognized a limited repertoire of ligands relative to the extensive repertoire of conventional MHC-restricted T-cells. NKT cells also recognize lipid ligands complexed with the non-MHC surface molecule, CD1d, and were referred to as "CD1d-restricted" T-cells [162]. CD1d is absent in cattle [91] but bovine T cells co-expressing CD335 do recognize lipid ligands by a CD1d-independent mechanism [92].

MAIT cells were first reported in human blood by Porcelli et al [240] as unconventional αβT-cells with invariant TCRα chain and semi-invariant TCR repertoire. These non-conventional T-cells have since been identified in mice and found to be enriched at mucosal surfaces [163]. MAIT cells recognize antigens in the context of a non-classical-

MHC molecule, MR1 [163]. Recent studies have shown that MAIT cells have antimicrobial functions [241] and recognize vitamin B metabolite ligands [242]. MAIT cells have not been characterized in cattle but bovine non-conventional T-cells that co-express CD335 have been shown to have a cytotoxic effector function with parasite infected cells and secrete IFN-γ [157]. No information is available, however, regarding the role of these cells in controlling infections at mucosal surfaces.

Homing of innate and adaptive lymphocytes to sites of viral infection is crucial for effective cell-mediated immune responses and clearance of viral infected cells. Different non-conventional T-cell subsets home to specific tissues based on their expression of chemokine receptors and intrinsic tissue responses to pathogens or other danger signals[243]. Murine iNKT cells express CCR7, CXCR3, CXCR6, CCR4 and CCR6 chemokine receptors [194], of which CCR4 [195] is important for pulmonary localization. CXCR6, CCR1, and CCR6 are expressed by human NKT cells [196] but the chemokine receptors expressed by bovine non-conventional T-cells and the chemokines involved in their recruitment to specific tissues has yet to be determined. Previous studies demonstrated that non-MHC restricted lymphocytes were recruited to the lungs of calves following BHV-1 infection [192] but it was not determined whether these cells were conventional NK cells or non-conventional T cells. It is not known if non-conventional T-cells may be recruited to bovine mucosal surfaces since previous studies of bovine non-conventional T cells were limited to cells isolated from blood [91, 92, 157].

In this study we demonstrate that non-conventional T-cells co-expressing CD335 and CD8 are recruited to the bovine URT following an experimental BHV-1 infection and these non-conventional T-cells are the major effector cell population recruited within 5 days post-infection (pi). Our analysis of chemokine gene expression at the site of viral infection also identified multiple chemokines that may be involved in the highly specific recruitment of this non-conventional T-cell population. Thus, $CD335^{+}$ and $CD8^{+}$ bovine non-conventional T- cells appear to be the primary effector cell populations involved in the early immune response to a BHV-1 URT infection.

4.3. Materials and Methods

4.3.1. Animals:

Female and castrated male, 5 to 6 month old, crossbred (Angus X Hereford) calves (n=30) were purchased from a single commercial herd. Calves were identified as seronegative for BHV-1 by screening with a recombinant, truncated glycoprotein D (tgD) antibody capture ELISA [208]. Calves were weaned, transported to the research facility, and adapted for 2 weeks to a diet of free choice hay and 0.5 kg oats/day. During the adaptation period calves were housed as a single group and there was no contact with other cattle. The average weight of calves was 232 kg (range = 174 and 242 kg) and animals were housed in a single pen throughout the study. All experimental procedures were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care and the experimental protocols were approved by the University of Saskatchewan Animal Care Committee (Protocol #19940211 and 19940218). The animals used for tissue collection were the same animals used to analyze virus shedding, IFN gene

expression, and the expression of IFN-stimulated genes in a previous study [230]. In our previous study, we demonstrated that all infected animals shed greater than 10^5 infectious virus particles/ml of nasal secretion at the time tissues were collected between days 3 to 7 post-infection [230]. BHV-1 infection of nasal turbinate mucosal epithelium was also confirmed by immunohistochemical staining [230].

4.3.2. Experimental Infection and Sample Collection:

Six (6) of the 30 calves served as uninfected controls and tissue samples were collected from the control animals immediately prior to challenging the remaining 24 calves with BHV-1. This ensured there was no potential exposure of control calves to BHV-1. The 24 infected calves were aerosol challenged with BHV-1 isolate 108 (5 X 10^7pfu/animal) on Experimental Day 0 as previously described [67]. A clinical veterinarian, blinded to treatment group, examined calves daily and recorded body weight and temperature. For tissue collection, cohorts of 6 calves were randomly selected and euthanized with an intravenous injection of Euthanyl (240 mg/ml; Bimeda-MTC, Canada) on Days 3, 5 7, and 10 pi. Tissue samples were collected within 15-20 minutes after euthanasia and three replicate samples were immediately placed in RNAlater or 10% buffered formalin. Tissue samples from nasal turbinates were collected 10-12 cm from the external nares of all calves (Supplemental Figure 4A). A 4-5mm2 piece of nasal turbinate was placed on the surface of a 4-5 mm^2 and 1 mm thick slice of fresh liver tissue with the epithelial surface of the turbinate supported by the liver. This protected the mucosal epithelium from damage during cryosectioning. Tissue blocks were snap-frozen in liquid nitrogen and stored at-80^{o}C until tissues were used for cryosectioning and immunohistochemical (IHC) staining.

Formalin-fixed tissues were used for histology and IHC detection of BHV-1 proteins. Tissues fixed with RNAlater were stored at -80^{o}C until RNA was extracted for qRT-PCR analysis of bovine chemokine gene expression.

4.3.3. Monoclonal Antibodies

The monoclonal antibodies (mAbs) used for IHC and immunofluorescence (IF) and relevant isotype controls are listed in Table 1, which provides details on mAb specificity and the concentration of each mAb used for staining.

Table 4.1. Monoclonal Antibodies Used For Immunohistochemistry and Immunofluorescence

CD [a]	Target molecule	Target population	Isotype	mAb Clone	Conc.	Source
CD3	TCR[b] complex	Pan-T-cells	IgG1	MM1A	667ng/ml	VMRD
CD4	CD4	T_H cells [c], DC[d]	IgG1	CACT138A	10µg/ml	VMRD
CD8	CD8α	CTLs [e], DC	IgG1	CACT80C	5µg/ml	VMRD
CD335	NCR1[f], NKp46 [g]	Innate lymphoid cells	IgG1	AKS1	10µg/ml	Bio-Rad
sIgA	Surface IgA	IgA plasma cells	IgG1	BIG312D3	125ng/ml	VMRD
IFN-α	IFN-α	IFN-α	IgG1	Ascites	1:400 dilution	VIDO
IFN-γ	IFN-γ	IFN-γ	IgG1	7B6	2µg/ml	LSBio
Isotype Control			IgG1	MG100	5µg/ml	Life Tech.

[a] Cluster of differentiation;
[b] T-cell Receptor;
[c] T-helper cells;
[d] Dendritic cells;
[e] Cytotoxic T-lymphocytes;
[f] Natural cytotoxicity triggering receptor 1;
[g] Natural Killer cell p46-related protein.

4.3.4. Lymphocyte Phenotyping with Immunohistochemistry

We collected three 4-5mm^2 nasal turbinate tissue samples from each animal and the tissue block selected for cryosectioning was chosen randomly to avoid bias. The nasal turbinate tissue block was sectioned at-20^{o}C and serial 5μm sections were cut using a cryostat (DAMON/IEC Division, Microtome 3398) and mounted on Superfrost Plus slides (Fisherbrand#12550215, Ontario, Canada). The tissue sections selected for IHC staining were chosen on the basis of optimum tissue morphology. Tissue sections were fixed in chilled 100% acetone for 8 minutes and air-dried for 20 minutes prior to storage at 4^{o}C. An IgG1 isotype control was included at the same concentration as the primary antibody to control for non-specific binding of primary and secondary antibodies, and confirmed that non-specific staining due to endogenous expression of hydrogen peroxidase, avidin and biotin was adequately blocked. Indirect-immunolabelling was performed as described previously [244] to visualize the location and distribution of IFN-α and IFN-γ in tissue sections and to perform morphometric analyses of the frequency of CD335$^+$, CD3$^+$, CD8$^+$ and CD4$^+$ cells in nasal turbinates before infection (Day 0) and on Day 3, 5, 7 and 10 post-BHV-1 infection. Biotin-conjugated goat-anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA #BA-9200) and the Vectastain Elite Avidin-Biotin complex (ABC) Kit (Vector Laboratories, Burlingame, CA, USA #PK4000) were used to visualize bound mAbs. BHV-1 infection occurs primarily within mucosal epithelial cells of the nasal turbinate [230]**.** Thus boundaries set for morphometric analysis of individual lymphocyte populations included the mucosal epithelium and the underlying lamina propria region. Five contiguous microscopic fields (total area = 0.196 mm^2) were analyzed within each tissue section and cells with visible staining were manually counted using an Olympus

CX31 microscope (Olympus, Center Valley, PA, USA) with a 40X objective. The first field counted was located at the upper left corner of the cryosection and four contiguous fields were then counted. The top margin of each field was defined by the external surface of the mucosal epithelium and included the underlying lamina propria. An Olympus BX51 microscope & DP70 digital camera system (Olympus, Center Valley, PA, USA) were used to capture images of IHC stained cryosections.

4.3.5. Immunofluorescence

Dual-colour staining of tissue sections was performed using fluorochrome-conjugated mAbs that reacted with bovine CD3, CD8 and CD335. The mAbs (Table 1) were conjugated with fluorochromes using Molecular Probes® Antibody Labeling Kit (Thermofisher Scientific, Carlsbad, CA, USA), following the manufacturer's instructions. The anti-bovine CD3 mAb was conjugated with Alexa Fluor® 594. The anti-bovine CD8 mAb was conjugated with Alexa Fluor® 488 and Alexa Fluor® 594 so it could be used in appropriate combinations with the fluorochrome conjugated CD3 and CD335 mAbs. The anti-CD335 mAb was conjugated with Alexa Fluor® 488. IgG1 isotype control mAb (Table 4.1) was conjugated with Alexa Fluor® 594 to be used for confirmation of staining specificity of the three other fluorochrome-conjugated antibodies. Direct fluorochrome labeling was necessary since the three mAbs are all IgG1 isotype (Table 4.1) and mAb reactivity after fluorochrome conjugation was confirmed by flow cytometric analysis of labelled blood mononuclear cells. Serial nasal turbinate cryosections were cut and fixed as described previously and stored at 4 ^{o}C overnight. Acetone-fixed sections were brought to room temperature, air-dried for 30 minutes, and then rehydrated with phosphate buffered

saline (PBS). The PBS solution was changed three times at 5 min intervals before slides were blocked by incubating with 10% normal goal serum for 3 hours, followed by a 5 minute wash with PBS. Tissue sections were then flooded with 200 μl of PBS containing 5% normal goat serum and one of the following combinations of fluorochrome-conjugated mAbs: CD3 and CD335; CD335 and CD8; or CD3 and CD8. Cryosections were incubated in the dark at room temperature for 2.5 hours before washing twice for 5 minutes with PBS that was mixed with a magnetic stirrer. Tissue sections were counterstained by flooding with 200μl 4′, 6-diamidino-2-phenylindole (DAPI; 1μg/ml methanol) (Life Technologies, Camarillo, CA, USA) for 10 minutes at room temperature to visualize cell nuclei and then washed for 5 min with PBS. Tissue sections were cover slipped with Cytoseal 60 mounting media (Thermofisher Scientific, Carlsbad, CA, USA) and stored overnight at 4 ^{o}C before fluorescent imaging was completed. Confocal images were generated using a Leica TCS SP8 scanning confocal microscope (Leica Microsystems, Wetzlar, Germany), equipped with a 63X oil immersion objective and utilizing the 405 nm (UV) and 561 nm laser lines. Confocal images from a minimum of 15 microscopic fields were captured per sample and a minimum of 100 CD335+, CD3+ and CD8+cells were counted per tissue section. Tissue sections were analyzed for nasal turbinate samples collected from three animals on day 5 pi to determine the frequency of $CD335^{+}CD3^{+}$, $CD335^{+}CD8^{+}$ and $CD3^{+}CD8^{+}$ cells.

4.3.6. RNA Extraction

RNAlater® fixed nasal turbinate samples were homogenized as previously described [8] and total RNA was extracted using the RNeasy mini Kit (Qiagen Inc. Ontario, CA)

following the manufacturer's protocol. RNA quality and total RNA were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA Integrity Number (RIN) of 8 or higher were used to prepare cDNA.

4.3.7. cDNA synthesis

Total RNA (1μg) was reverse-transcribed using qScript™ cDNA SuperMix (Quanta Biosciences™ Beverly, MA, USA), following the manufacturer's protocol. cDNA samples were diluted in DNAse/RNAse free water for use as template in qRT-PCR reactions.

4.3.8. qRT-PCR primer design and validation

Primer pairs for chemokine transcripts (Table 4.2) were designed using Clone Manager 9.0 program (Sci-Ed Software). Primer specificity for individual chemokine genes was confirmed through generation of a single peak in the melting curve and detecting a single amplicon following gel electrophoresis of PCR products. PCR products were also cloned in the PCR 2.1 vector using the TA cloning kit (Life Technologies, Camarillo, CA, USA). Cloned products were sequenced with a CEQ 200XL DNA Analysis system (Beckman Coulter) to confirm product identity and size. Primer amplification efficiency (Table 4.2) was determined using standard curves as described previously [213].

Table 4.2 Primers For Amplification Of Bovine Chemokine Genes And Reference Genes

Gene		Primer Sequence*	Size §	Effic.‡
CCL2 (MCP-1)	FWD	TCGCTGCAACATGAAGGTCT	119	**2.02**
	RV	TATAGCAGCAGGCGACTTGG		
CCL3 (MIP-1α)	FWD	CGGCAGCTTTCTCGCAAAAT	166	**1.98**
	RV	CCTCTCAGGCATTCAGCTCC		
CCL4 (MIP-1β)	FWD	AGCTCTGCGTGACTGTCCTG	86	**2.15**
	RV	AGGGTCTGAGCCCATTGGTG		
CCL5 (RANTES)	FWD	GCTCCATGGCAGCAGTTGTC	128	**2.12**
	RV	AGGTTCAAGGCGTCCTCCAC		
CCL19 (MIP-3β)	FWD	AGGTGCCAACGACGCTGAAG	92	**2.17**
	RV	TGAGGAGCAGGTAGCGGTAG		
CCL20 (MIP-3α)	FWD	GACTGCTGTCTCCGATATAC	90	**1.98**
	RV	GATGTCACAGGCTTCATTGG		
CCL21	FWD	TGGTCCTGAGCATCCTTGTC	114	**2.07**
	RV	TGGCGGGAATCTTCTTTCGG		
CXCL8 (IL-8)	FWD	CGCTGGACAGCAGAGCTCACA	106	**2.06**
	RV	TGCCAAGAGAGCAACAGCCAGC		
CXCL9 (MIG)	FWD	AGTGGGAGAAACAGGTCAAC	129	**2.13**
	RV	AAGTGGGAGCTCATGTAGTC		
CXCL10 (IP-10)	FWD	AAGGGAAAGGGTGGCTCATC	114	**1.98**
	RV	GGCTGGGACTTAGCACATTG		
CXCL-11 (IP-9)	FWD	AGCAGCAACAAGCATGAGTG	100	**2.10**
	RV	CCGTCCGCCTTTGAACATAG		
β-Actin	FWD	CTAGGCACCAGGGCGTAATG	116	**2.00**
	RV	CCACACGGAGCTCGTTGG		
GAPDH	FWD	TGGAAAGGCCATCACCATCT		**2.7**
	RV	CCCACTTGATGTTGGCAG		
RPS9	FWD	CCTCGACCAAGAGCTGAAG		**2.7**
	RV	CCTCCAGACCTCACGTTTGTTC		

* Sequence of forward (FWD) and reverse (RV) primers;
§ Size of amplified PCR product;
‡ Efficiency of product amplification

4.3.9. qRT-PCR Analysis of Chemokine Gene Expression

Gene expression data for individual samples was normalized relative to *β-actin*, which was confirmed to be stable over multiple time points and not affected by BHV-1 infection in nasal turbinates (data not shown). We also analyzed the expression of *GAPDH* and *RPS9* reference genes. The expression of the three reference genes did not differ significantly when comparing samples collected at different time points following BHV-1 infection (data not shown), but *β-actin* transcription displayed the least variability among animals at each time point. The *β-actin* gene also had the lowest Cq value when comparing among the three reference genes and was therefore selected as the best reference gene for detection of genes with high transcriptional levels. qRT-PCR was performed using the PerfeCTa® SYBR® Green FastMix for iQ (Quanta BioScience, Beverly, MA, USA). Briefly, 9 μl of Perfecta SYBR green master mix (2X), 3 μL of the primer pair at 3.3 μM, and 3 μL cDNA template were added to a 15 μL final volume. The reaction was performed in BioRad iCycler iQ PCR detection system using the following program: 1 cycle at 95 °C for 30 sec, 45 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. After cycling, the temperature was increased starting from 56 °C at a rate of 1 °C every 10 sec to build a melting curve (40 times). Amplification data obtained for individual genes was expressed as quantification cycle (Cq) and was subtracted from the Cq of the β-actin reference gene to obtain ΔCq (ΔCq = Cq gene – Cq β-actin).

4.3.10. Statistical Analysis:

All statistical analyses in were performed using GraphPad software (Version 7.0a, La Jolla, CA, USA). A one-way ANOVA with Holm-Sidak's multiple comparisons test was used to compare the frequency of leukocyte subpopulations in nasal turbinates before BHV-1 infection (Day 0) with each day sampled pi. A Mann-Whitney test was used to analyze changes in the expression level of individual chemokine genes in nasal turbinates a following BHV-1 infection relative to pre-infection (Day 0) levels.

4.4. Results

4.4.1. Interferon production in nasal turbinate tissue

Previous studies confirmed IFN-α and -γ gene expression in nasal turbinates and the concentration of IFN in nasal secretions increased following a primary BHV-1 infection [67, 230]. IHC staining of nasal turbinate cryosections with mAbs specific for bovine IFN-α and -γ was performed to determine if the production of these cytokines could be localized to specific cells within the tissue. IHC staining revealed, however, a diffuse staining pattern with the most intense staining associated primarily with mucosal epithelial cells (Figure 4.1c & 4.1d). Diffuse staining was occasionally observed throughout the lamina propria but there was no consistent pattern and staining could not be localized to individual cells. Isotype-matched mAbs were used to confirm the specificity of the staining observed at the mucosal surface and in the lamina propria (data not shown). Due to the diffuse and inconsistent staining pattern observed for IFN in tissue sections, a qualitative analysis was used to determine the frequency of positive staining in nasal turbinates collected from animals before and after BHV-1 infection.

Some of the nasal turbinate samples collected from uninfected animals were positive for IFN-α (3/6 animals; 50%) and IFN-γ (4/6 animals; 60%) (Figure 4.1a & 4.1b). IHC staining of Day 5 tissue sections revealed more intense IFN-α staining of the epithelial cell layer in all animals but only 80% (5/6 animals) of the tissue samples were positively stained for IFN-γ. The staining reaction was darkest at the epithelial surface and generally less intense in the lamina propria (Figure 4.1c & 4.1d). Positive staining for Type I IFN on Day 0 may reflect environmental exposure to other pathogens or commensal microbes that maintain a basal level of innate immune activation in mucosal epithelium of the upper respiratory tract. IFN-α is a potent activator of NK cells, which may explain the concurrent expression of IFN-γ if these innate immune cells are present in the tissue. Therefore, we investigated the types of lymphocytes present in nasal turbinates before and after BHV-1 infection.

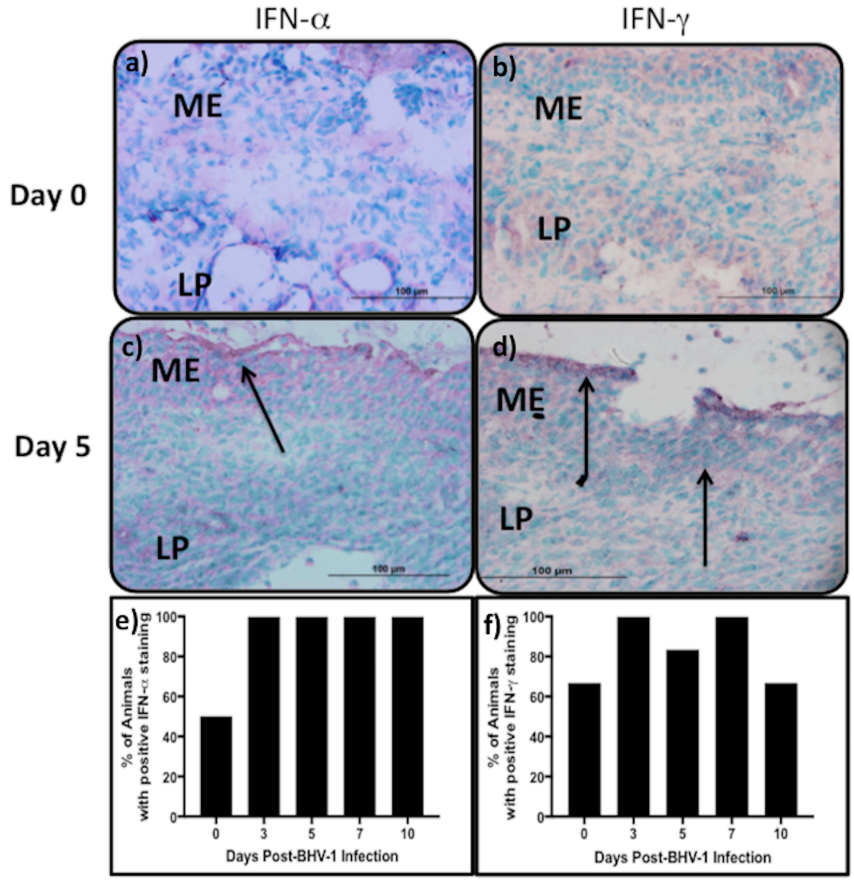


Figure 4.1. Type 1 and 2 interferon secretion in nasal turbinates following a primary respiratory BHV-1 infection of naïve calves. IHC staining of cryopreserved nasal turbinate tissues with mouse anti-bovine IFN-α and anti-bovine IFN-γ monoclonal antibodies was used to visualize the location of IFN-α (**a & c**) and IFN-γ (**b & d**) protein. Representative tissue sections are presented to demonstrate an absence of staining in the uninfected control (Day 0) and the pattern of staining 5 days (Day 5) after BHV-1 infection. Diffuse staining of nasal turbinate epithelial surfaces (arrows) was observed in both uninfected and BHV-1 infected calves and the percent animals (n = 6/time point) with positive IFN-α (**e**) and IFN-γ (**f**) staining in nasal turbinates is shown. One tissue section was stained per animal. Brown color shows positive staining.

4.4.2. Lymphocytes present in nasal turbinates following BHV-1 infection

We previously demonstrated that bovine IFN-γ had limited direct antiviral activity, despite high levels of IFN-γ production during a primary BHV-1 infection[230]. Thus, the role IFN-γ may play in clearing a primary BHV-1 respiratory infection is not

known. One hypothesis is that increased IFN-γ production may reflect increased recruitment of either NK cells or T-cells to the site of viral infection and subsequent activation of these cells by Type I IFNs. Nasal turbinates are a site of BHV-1 infection and replication and therefore, to test this hypothesis nasal turbinate tissue was collected from BHV-1 infected calves. IHC was used to analyze the phenotype and frequency of innate and adaptive lymphocytes present in the tissue.

Lymphoid populations present in nasal turbinates were first analyzed by staining for T cells (CD3) and both conventional NK cells and non-conventional T-cells, which express CD335. Tissue sections were also stained for CD4 and CD8α which are markers for subsets of α/βTcR T cells [11], and are also expressed on bovine NK cells and non-conventional T-cells [157]. Morphometric analysis of tissue sections revealed that both T cells ($CD3^+$) and either conventional NK cells or non-conventional T-cells ($CD335^+$) were present at low levels in nasal turbinates prior to BHV-1 infection (Figure 4.2). Cells staining for CD4 and CD8 were also present at low levels in the lamina propria prior to viral infection (Figure 4.2b & c; Figure 4.3c; CD4 staining not shown). The number of $CD335^+$, $CD8^+$, and $CD3^+$ cells present in the epithelial and lamina propria compartments increased significantly ($P < 0.05$) on Day 5 pi (Figure 4.2c and Figure 4.3) when compared to the number of cells present prior to infection (Figure 4.2). The increased number of cells present in nasal turbinates on Day 5 then declined to pre-infection levels on days 7 and 10 pi. In contrast, there was a significant ($P < 0.05$) decrease in the number of $CD4^+$ cells on days 3, 7, and 10 pi (Figure 4.2). Cells staining for CD3 were the most abundant on Day 5 pi, exceeding 100 cells per field (0.196 mm^2)

examined. The high frequency of CD3 cells on Day 5 pi could not be accounted for by adding the total number of $CD4^+$ and $CD8^+$ cells, suggesting that other CD3 co-expressing cell populations may be recruited to the nasal turbinates following viral infection. γδTCR T cells are a major T cell subpopulation present at mucosal surfaces of ruminants [245] but the mAbs evaluated for bovine γδTCR staining reacted strongly with the mucosal epithelium. This non-specific staining precluded an accurate enumeration of all known T cell subpopulations present in nasal turbinates following BHV-1 infection. IHC staining for IgA plasma cells also revealed consistently low numbers both before and after BHV-1 infection (data not shown).

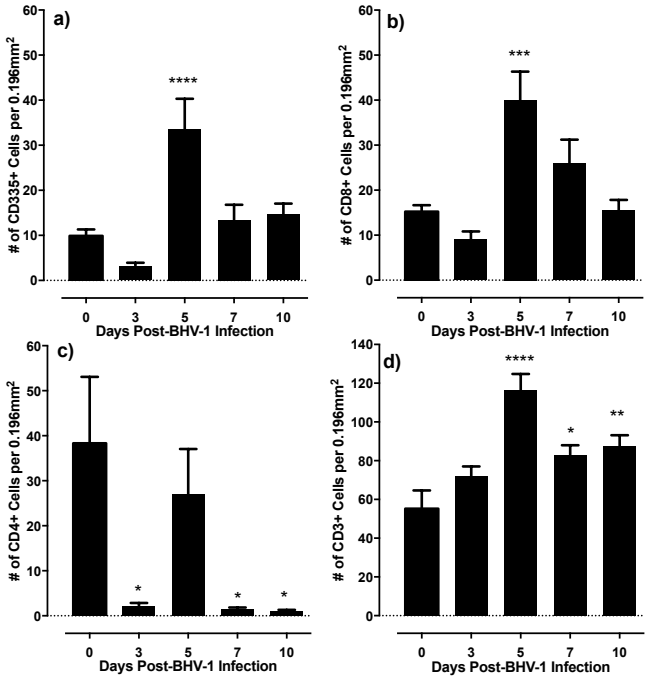


Figure 4.2. Frequency of lymphoid subpopulations present in nasal turbinates following a primary BHV-1 infection. Immunohistochemical staining was performed with monoclonal antibodies to identify CD335 (**a**) CD8 (**b**) CD4 (**c**), and CD3 positive cells (**d**). Morphometric analyses were used to quantify the frequency of each cell type located within the nasal turbinate epithelial and submucosal compartments. Data presented are the mean and standard error of the mean for values from tissues sections analyzed from 6 animals/time point following BHV-1 infection. One-way ANOVA was used to compare values relative to pre-infection (Day 0) levels and significant changes in frequency of leukocytes relative to Day 0 are indicated as $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), $P \leq 0.0001$ (****).

4.4.3. Non-conventional T-cells are recruited to nasal turbinates following BHV-1 infection

The CD335 mAb binds to the NKp46 receptor on innate immune cells and is involved in the activation of NK cells and target cell lysis [155]. The NKp46 receptor is, however, expressed on a variety of lymphocytes, which includes both conventional NK cells ($CD335^+CD3^-$) and non-conventional T-cells ($CD335^+CD3^+$) that play key roles in host immune defences and inflammation [157]. Therefore, we further investigated the phenotype of the CD335 cells recruited to nasal turbinates during BHV-1 infection to determine if this might account for the large increase in $CD3^+$ cells. A double staining protocol was optimized to determine if $CD335^+$ cells co-expressed CD8 (Figure 4.4a-c) or CD3 (Figure 4.4d-f). On average, 77.5% of $CD335^+$ cells present in nasal turbinates on day 5 pi co-expressed CD3 while 22.5% of the $CD335^+$ cells expressed markers typical for conventional NK cells ($CD335^+CD3^-$) (Figure 4.4g). Further, 89.7% of $CD335^+$ cells present on day 5 pi co-expressed CD8 (Figure 4.4g). Conversely, 78.5% of $CD3^+$ cells present in the nasal turbinates on day 5 pi co-expressed CD335 while 21.8% of the CD3+ cells co-expressed the markers of conventional T-cells ($CD3+CD335^-$) (Figure 4.4i). Furthermore, co-staining for CD8 and CD3 revealed 86.6% of $CD8^+$cells recruited to the nasal turbinates on day 5 pi co-expressed CD3 (data not shown). Double staining for CD8 and CD3 also indicated that only 73.4% of CD3 cells co-expressed CD8, which verified that other CD3-expressing cell populations were recruited to the nasal turbinates following BHV-1 infection. The specificity of the fluorescent signal observed when dual-staining cells was confirmed by the absence of detectable fluorescent signal in the mucosal epithelium and lamina propria when tissue sections

were staining with flourochrome-conjugated isotype control monoclonal antibodies (Supplemental Figure 4B). Thus, co-expression analysis revealed that the predominant population of lymphoid cells recruited to nasal turbinates on day 5 pi were characterized by co-expression of CD335 and CD3 and the majority of these non-conventional T cells also co-expressed CD8.

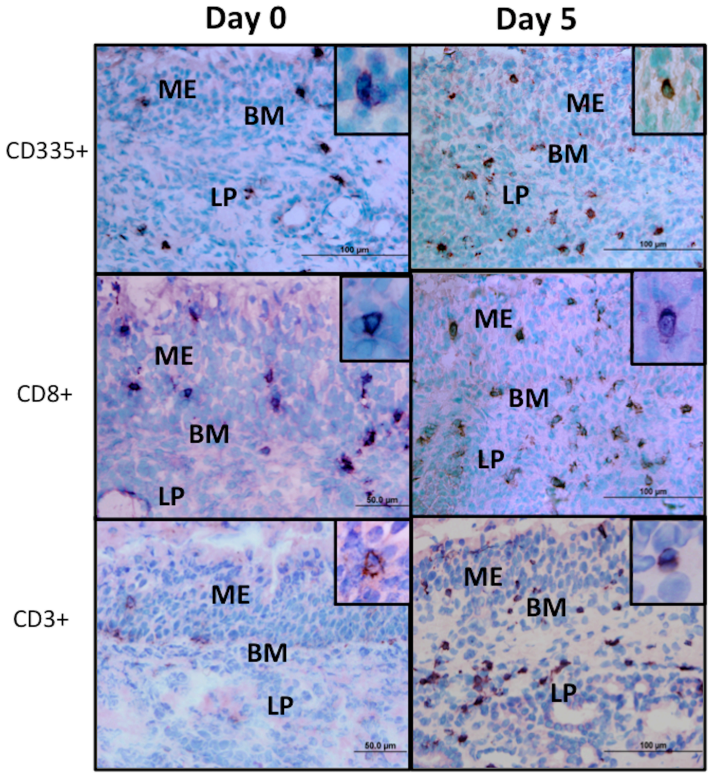


Figure 4.3. Immunohistochemical staining of lymphocyte subpopulations present in nasal turbinates following BHV-1 infection. Sections from cryopreserved nasal turbinate were stained with monoclonal antibodies and an immunoperoxidase conjugate to visualize the distribution of CD335 (**a & b**), CD8 (**c & d**) and CD3 (**e & f**) positive cells before (Day 0) and 5 days (Day 5) after BHV-1 infection. No specific cellular staining was observed when using an isotype-matched irrelevant monoclonal antibody. Positive cells stain brown and the inset in each panel shows the detail of cellular staining. Panel images were captured at 60X magnification and the inset images were captured at 100X magnification. ME = Mucosal Epithelium; LP = Lamina Propria; BM = Basal Membrane

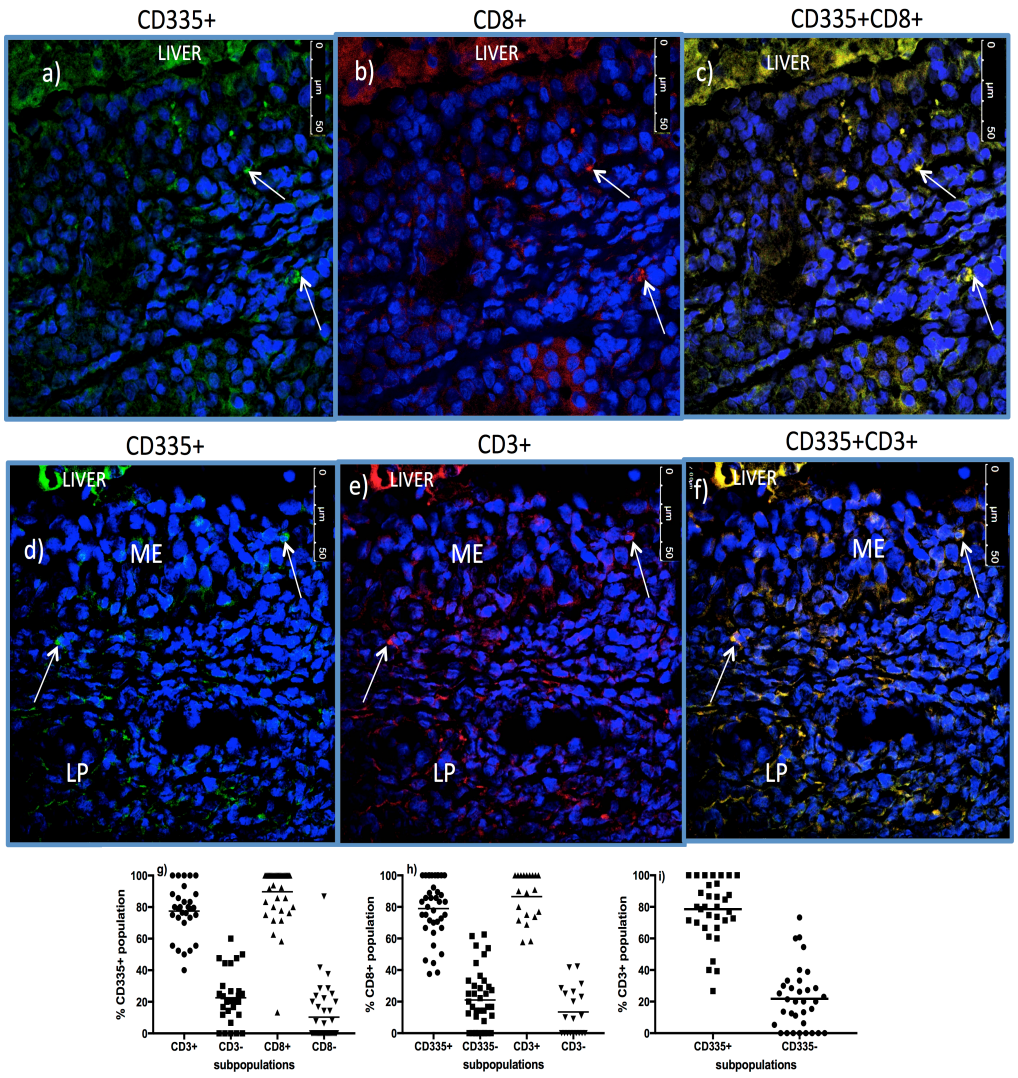


Figure 4.4. Co-expression of CD3 and CD8 on CD335$^+$cells present in nasal turbinates on day 5 after a primary BHV-1 infection. Nasal turbinate tissue was collected on day 5 pi and mounted on a piece of liver to maintain the morphology of the mucosal epithelium. Tissues samples were cryopreserved and tissue sections double stained by immunofluorescence. Staining for CD335 (Panel a - stained green) and CD8 (Panel b- red) was super-imposed to identify CD335$^+$CD8$^+$ cells (Panel c- yellow). Staining for CD335 (Panel d - stained green) and CD3 (Panel e- red) was super-imposed to identify CD335 cells co-expressing CD3 (Panel f- yellow). The liver portion of the cryosections is indicated (LIVER) and a white dashed line demarcates the separation between liver and nasal turbinate. DAPI was used as a counterstain to identify cell nuclei. Cell frequency data was generated through morphometric analyses. A minimum of 100 CD335$^+$, CD3$^+$ and CD8$^+$cells were counted per animal and tissue samples from three animals were used to analyze the frequency of CD335$^+$CD3$^+$, CD335$^+$CD8$^+$ and CD3$^+$CD8$^+$ cells. Each dot represents the % cells of co-expressing the surface molecules indicated on the y-axis/per image/animal. ME = Mucosal Epithelium, LP = Lamina Propria. The images were captured at 63X magnification.

4.4.4. Chemokine Induction Following BHV-1 Infection

Human and mouse NKT cells express many different chemokine receptors that are involved in the recruitment and localization of NKT cells at sites of inflammation [162]. Chemokine receptor expression has not been characterized for bovine NKT cells but the production of IFN-γ by human NKT has been shown to provide a positive feedback mechanism that enhances further NKT cell recruitment through induction of chemokines [246]. Peak IFN-γ production following BHV-1 infection occurs on day 5 pi and this coincides with maximum recruitment of non-conventional T-cells (Figure 4.2). Therefore, we designed and validated qRT-PCR primers for known bovine chemokine genes (Table 4.2) to investigate whether BHV-1 infection altered the expression of chemokines that may be involved in recruitment of non-conventional T-cells. Among the 10 bovine chemokine genes analyzed, transcript levels for *CCL4* (Figure 4.5a; $P < 0.05$), *CCL5* (Figure 4.5b; $P < 0.05$) and *CXCL9* (Figure 4.5c; $P < 0.01$) were significantly up-regulated on Day 7 pi. Thus, there did not appear to be a close temporal association between initial non-conventional T-cell recruitment to nasal turbinates and the onset of tissue expression of chemokine genes known to be involved in NKT cell recruitment.

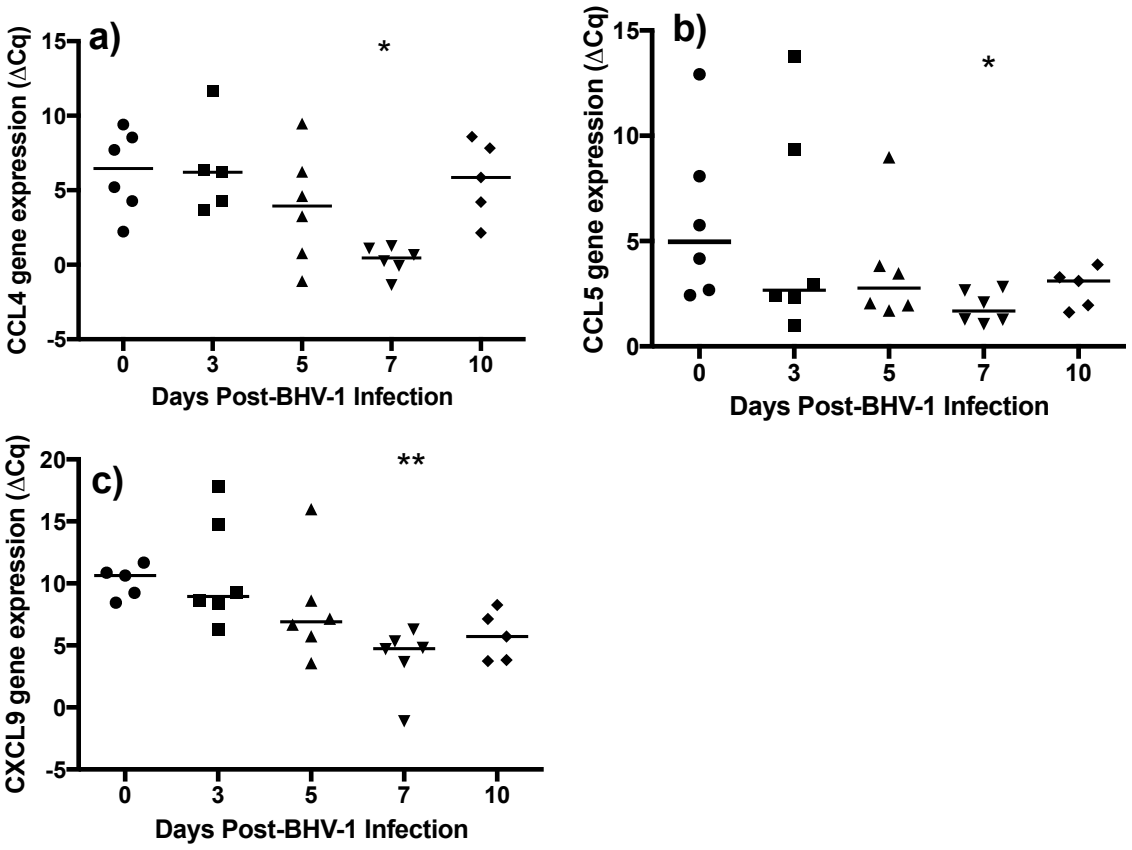


Figure 4.5. Expression of chemokines in nasal turbinate tissue following primary BHV-1 infection. Nasal turbinate samples were collected (n = 6 animals/time point) prior to and following BHV-1 infection and RNA extracted for qRT-PCR analysis abundance of CCL4 (a), CCL5 (b), CXCL9 (c) gene transcript abundance. Gene expression was calculated as the change in threshold cycle (ΔCq) relative to β-actin and data presented are values for individual animals. The horizontal bar represents the mean value for animals sampled at each time sampled. A Mann-Whitney test was used to compare values relative to pre-infection (Day 0) levels. Significant changes in gene expression relative to Day 0 are indicated: $P < 0.0332$ (*), $P \leq 0.0021$ (**), $P \leq 0.0002$ (***), $P \leq 0.0001$ (****)

4.5. Discussion

CD335 (NKp46, NCR1) is commonly used to identify NK cells in cattle and other mammalian species such as sheep [160] pigs [159], humans [247] and mice [236]. However, non-conventional T-cell subsets co-expressing CD335 have been reported in the blood, spleen, lungs and other tissues of both healthy and infected animals [157, 159, 248]. The current study provides the first demonstration that non-conventional T cells co-expressing both CD335 and CD8 are the predominant lymphocyte population recruited to the URT following a primary BHV-1 infection. The frequency of $CD335^{+}CD3^{+}$ T- cells in bovine blood ranges between 0.1 to 1.7 % of blood mononuclear cells and $CD3^{+}$ cells usually comprise less than 10% of all CD335+ lymphocytes in blood [157]. The frequency of $CD335^{+}$ T-cells recruited to the URT following a primary BHV-1 infection was 77.5% of all $CD335^{+}$ lymphocytes (Figure 4.4h) and 78.5% of all T cells (Figure 4.4i). This observation supports the conclusion that there was a highly selective recruitment of these non-conventional T-cells. Recruitment of CD335+ T cells to the lung of pigs was also reported following influenza infection [159], suggesting that CD335+ T cells may play an important role in the control of respiratory viral infections. Non-MHC restricted cytotoxic cells were previously reported to be recruited to the lungs of calves following a primary BHV-1 infection but the phenotype of these cells was not determined [192]. Thus, further phenotypic and functional analysis of immune cells recruited during primary viral infections may reveal that non-conventional T cells provide an important early response to infection.

The majority of non-conventional T-cells recruited to the URT also co-expressed CD8 (Figure 4.4g) and bovine $CD335^{+}CD8^{+}$ T-cells were shown to produce IFN-γ when stimulated through CD3 [157]. Recruitment of primarily $CD8^{+}$ non-conventional T cells to the URT is consistent with the previous observation that 75% of non-conventional T cells in blood co-express CD8 [157]. Maximum recruitment of non-conventional T cells to the nasal turbinates was observed on day 5 post-BHV-1 infection which coincides with maximum IFN-γ secretion in nasal secretions [230]. The IHC staining of nasal turbinates for IFN-γ revealed, however, a diffuse staining pattern that could not be localized to cells located in either the mucosal epithelium or submucosa (Figure 4.1d). Thus, it was not possible to directly link increased IFN-γ production following BHV-1 infection with the recruitment of a large number of non-conventional T cells. Future studies will be necessary to develop methods to isolate this unique T cell population from tissue following viral infection for the analysis of IFN-γ secretion or alternatively perform *in situ* analysis of IFN-γ transcript within individual cells co-expressing CD335 and CD3. It would also be interesting to determine if IFN-α is a potent activator of IFN-γ secretion by bovine non-conventional T cells since peak production of both types of IFN occur on day 5 post-BHV-1 infection [230].

Innate and acquired immune responses to BHV-1 infection have been an area of research interest for over 40 years [249], and a variety of cell-mediated cytotoxic mechanisms have been implicated as important for the control and clearance of a primary BHV-1 infection [71, 82, 192]. IFN-γ secretion has also been frequently used as an indicator of either NK cell or T-cell activation by BHV-1 proteins but these studies were

frequently performed with lymphocytes isolated from blood [113]. This is the first report that identifies effector lymphocyte populations recruited to mucosal surface of the URT following a primary BHV-1 infection. In this study, we combined IHC and IF analyses to demonstrate that $CD8^+$ non-conventional T cells were the primary lymphocyte population recruited early during a primary BHV-1 infection. Significant ($P < 0.05$) recruitment of this non-conventional T cell population to the lamina propria was, however, limited to day 5 pi (Figure 2). A previous study in mice demonstrated that NK cell recruitment to lymph nodes following poxvirus infection was dependent on IFN-γ production [228]. This observation may explain the coincidence of peak non-conventional T cell recruitment to nasal turbinates with maximum levels of IFN-γ in nasal secretions [230]. An alternative explanation may be that data showing recruitment of non-conventional T cells was biased by sampling nasal turbinate tissues at a fixed site. BHV-1 infection in the URT of naïve calves occurs within discrete foci of mucosal epithelium [230]. When non-conventional T-cells are first recruited from blood, they may be abundant throughout the lamina propria but the apparent decline in number of lymphocytes throughout the lamina propria on days 7 and 10 pi (Figure 4.2) may reflect further recruitment and localization of non-conventional T- cells to foci of viral infection. IHC studies analyzing lymphocyte subpopulations recruited specifically to foci of BHV-1 infection in the URT may better define the kinetics of effector lymphocyte populations recruited during viral infection.

Chemotactic migration of non-conventional T-cell subsets has been examined in mice and humans but the chemokines involved in non-conventional T-cell recruitment in

cattle is not known. Murine iNKT cells express CCR7, CXCR3, CXCR6, CCR4 and CCR6 chemokine receptors [194], of which CCR4 was critical for localization to the lung [195]. CXCR6, CCR1, and CCR6 are expressed on the surface of human NKT cells [196]. These studies indicate that non-conventional T cells express multiple chemokine receptors and individual receptors may be involved in tissue specific recruitment. Bovine $CD335^{+}CD2^{+}$ NK cells have been shown to express several chemokine receptors including CCR1, CCR8, CXCR6 and CX3CR1, while $CD335^{+}CD2^{-}$ NK cells expressed CCDR2, CCR5, CCR6, CCR7, CXCR3, CXCR4 and CXCR5 [199]. Thus, bovine NK cells also have the capacity to respond to abroad range of chemokines. The expression of 11 known bovine chemokine genes was analyzed in nasal turbinate tissue collected following BHV-1 infection and CCL4, CCL5, CXCL9 were significantly ($P < 0.05$) up-regulated (Figure 4.5). Transcript abundance for all three chemokine genes was greatest on day 7 pi and then remained at a similar level on day 10 pi. Significant chemokine gene expression on day 7 pi coincided with the highest level of BHV-1 replication and shedding in nasal secretions [230]. If these chemokines are involved in non-conventional T cell recruitment to viral infected mucosal epithelial cells then the temporal pattern of chemokine gene expression may explain the apparent decline in these T-cell subsets' abundance in the lamina propria on days 7 and 10 pi. It is also interesting that maximum CXCL9 gene expression occurred on day 7 pi, which is 2 days after peak IFN-γ production in the URT [230]. Increased expression of CXCL9, also known as Monokine induced by interferon gamma (MIG; Table 4.2), is consistent with the strong IFN-γ response observed following BHV-1 infection [230]. If CXCL9 is a chemokine involved in non-conventional T cell recruitment then the IFN-γ response induced during BHV-1

infection may play an indirect role in recruitment of this specific effector cell population. Further clarification of which chemokines are involved in the specific recruitment of non-conventional T cells will require an analysis of chemokine receptor expression by these cells.

4.6. Conclusion

The present study demonstrates that $CD335^{+}CD8^{+}$ non-conventional T-cells are the predominant lymphocyte population recruited to the lamina propria of nasal turbinates within 5 days after a primary BHV-1 infection. Dual-colour IF studies confirmed that over 75% of both $CD335^{+}$ cells and $CD3^{+}$ cells present in the lamina propria on day 5 pi could be classified as non-conventional T-cells. Although bovine $CD335^{+}CD8^{+}$ cells are a minor lymphoid population in blood [157], over 30 $CD335^{+}CD8^{+}$ T-cells/0.196 mm^2 were present in bovine nasal turbinate tissue on day 5 pi. This represents a highly specific recruitment of non-conventional T-cells and suggests that $CD335^{+}CD8^{+}$ T-cells may be an important effector population for the control or clearance of a herpesvirus infection. The recruitment of these non-conventional T-cells was associated with increased expression of three different chemokines, indicating that this tissue-specific recruitment may involve multiple chemoattractant signals. Finally, the T cell receptor repertoire of these cells needs to be determined before classifying these cells as either innate or adaptive T cells.

Acknowledgements

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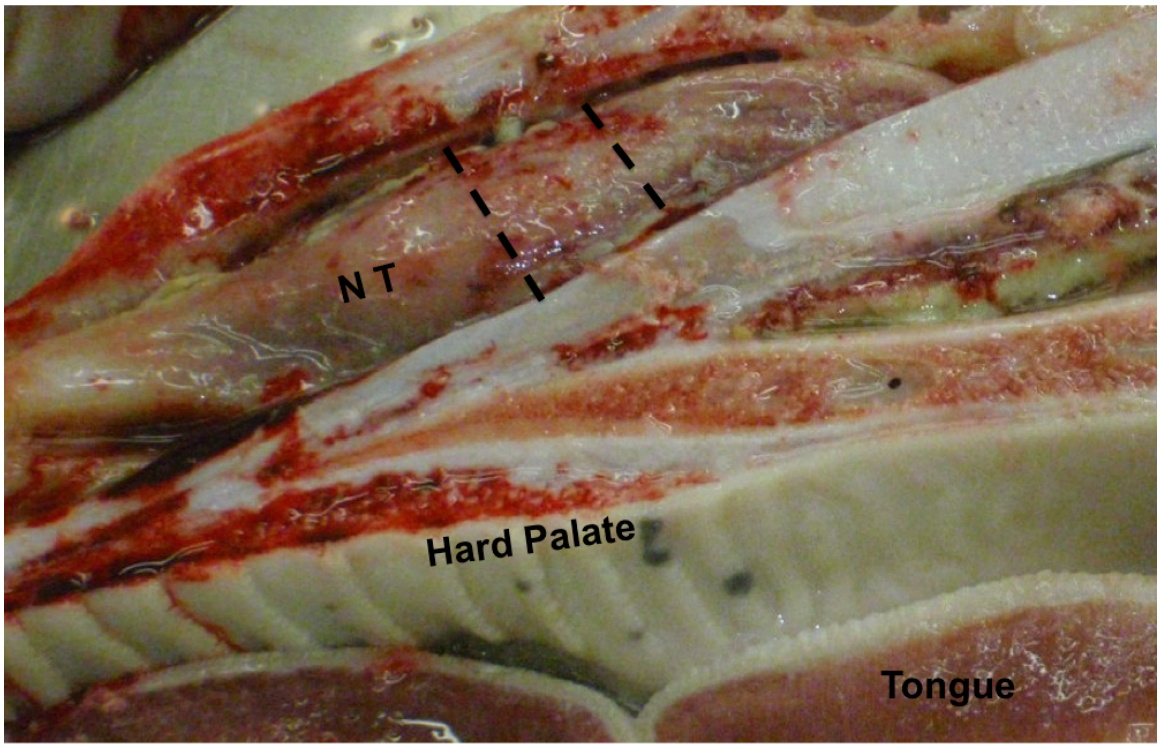
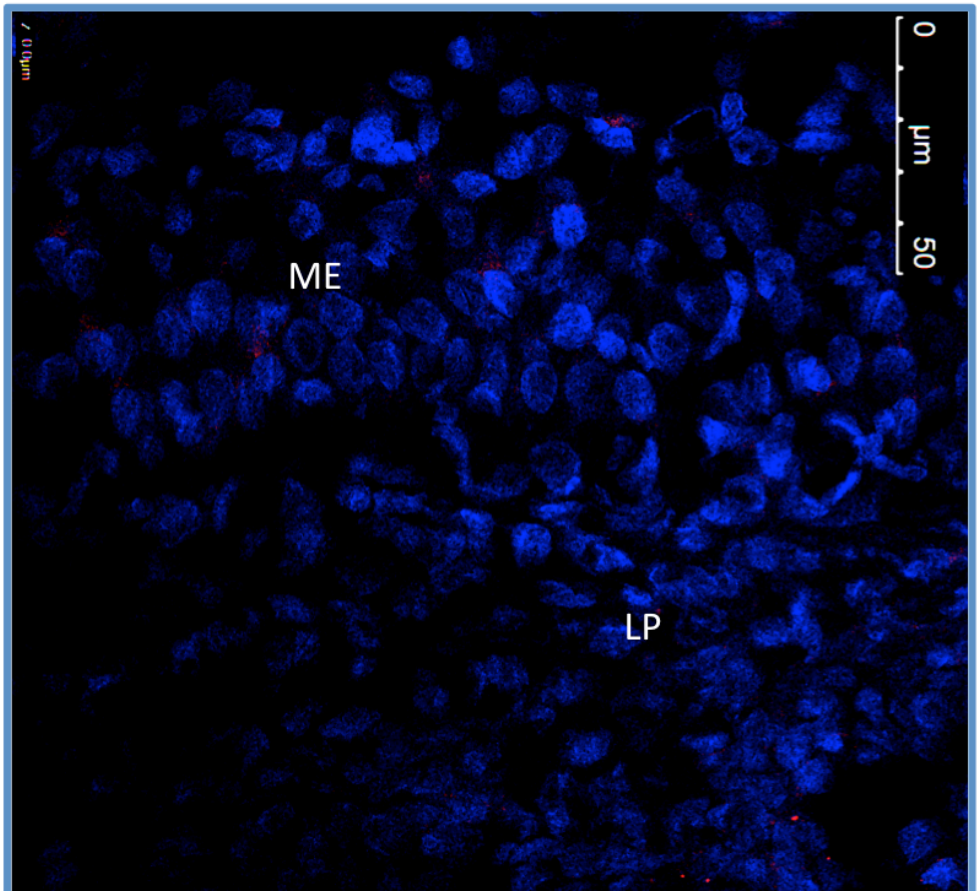
4.7. Supplemental Figures



Supplemental Figure 4A. Nasal turbinates in the upper respiratory tract of a 6 month old calf. A midsagittal cross-section of the head shows nasal turbinates located posterior to the external nares and photograph was taken on day 5 post-BHV-1 infection. Nasal turbinate samples were collected at the location indicated by the dashed lines on Days 0 (pre-infection) Day 3, 5, 7 and 10 post-infection. Tissue samples were collected from 6 calves at each time point. N T = Nasal turbinates



Supplemental Figure 4B. IgG1 Isotype Control staining of nasal turbinate tissue section on day 5 post primary BHV-1 infection. Nasal turbinate tissue was collected on day 5 pi. Tissues sections were stained with an equivalent concentration of IgG1 isotype control directly conjugated to Alexa Fluor® 594 (Red fluorescence) and was used as a specificity control when dual-staining for CD335 versus CD3 and CD335 versus CD8 (Figure 4.4). DAPI was used as a counterstain to identify cell nuclei. ME = Mucosal Epithelium, LP = Lamina Propria. Images were captured at 63X magnification.

CHAPTER 5. GENERAL DISCUSSION

Over 60 years of BHV-1 research has provided a wealth of information regarding the structure and biology of this virus. The BHV-1 genome has been sequenced and numerous studies have characterized the biology and function of individual viral proteins and their potential use as vaccine candidates [250]. Several generations of BHV-1 vaccines ranging from killed to modified-live and parenteral to mucosal vaccines have been formulated since the first BHV-1 vaccine was available in 1957 [251]. However, there is still limited information regarding the host immune defense mechanisms that play a critical role in the control and clearance of a primary BHV-1 infection.

Previous investigations documented high IFN-α and -γ production during primary BHV-1 infections, which suggested that IFNs may play an important role in the control of BHV-1 infection [67]. However, these studies also reported that high interferon levels coincided with shedding of over 10^6pfu/ml in nasal secretion [67]. Our animal studies confirmed that BHV-1 is a potent inducer of both type I and II interferons but *in vitro* studies demonstrated that BHV-1 replication was limited by less than 50% following the induction of antiviral effector mechanisms by both recombinant bovine IFN-α and -γ. These observations raised further questions regarding the role of IFNs in limiting BHV-1 infection.

I extended the analysis of Type I IFNs to include both the expression of IFN-β *in vivo* and the antiviral activity of IFN-β *in vitro*. These analyses confirmed that IFN-β expression is increased early during a primary BHV-1 infection and IFN-β has a greater capacity than either IFN-α or –γ to directly inhibit BHV-1 replication. Thus, IFN-β may be important in controlling the magnitude of BHV-1 replication during a primary respiratory infection. However, during a BHV-1 infection *in vivo*, the early induction of IFN-β was not associated with a rapid decline in virus replication and virus shedding continued to increase in the face of significant up-regulation of IFN-β gene expression in nasal turbinates and IFN-β secretion in the nasal cavity. The question remains, however, whether the multifaceted IFN response plays an important role in the indirect activation of either innate or adaptive defences against BHV-1 infection.

It is interesting to note that the current transcriptional and IHC studies indicate that both type I and II IFNs were expressed in nasal turbinates prior to BHV-1 infection. This observation contradicts the common belief that IFNs are induced only after a viral infection. One possible explanation for this phenomenon may be that IFNs are constitutively expressed at a relatively low level in healthy calves, as has been reported for swine [252]. Alternatively, other environmental factors, such as the respiratory virome, may act as an inducer of IFN production since animals were not housed in an isolation unit prior to BHV-1 challenge. The presence of varying stimuli within individual animals would be consistent with the highly variable level of type I and II IFN gene expression observed among animals prior to BHV-1 infections (Figure 3.3).

Background IFN activity may also explain why a relatively high dose of BHV-1 was used in the current animal studies to ensure that all animals became infected, showed clinical diseases and shed virus in nasal secretions (Figure 3.1).

Although IFN gene expression and IFN secretion increased significantly ($P < 0.05$) within three days after the infection (Figure 3.3) virus replication and shedding persisted for 10 days pi. Recombinant bovine IFNs have been reported to induce many of the clinical symptoms associated with BHV-1 infection, including fever, loss of appetite, diarrhea, and weight loss [55]. Therefore, it is not apparent that the multifaceted IFN is beneficial to the host. Concurrent production of IFN-α, -β and $-\gamma$ following BHV-1 infection also appears redundant when comparing the anti-viral effector genes induced by both type I and II IFNs (Figure 3.6). Although the potential benefit of a redundant IFN-response has not been studies in cattle, Zanotti et al have shown that porcine IFN-aFN-in-b subtypes vary in their capacity to induce antiviral, inflammatory and immune modulation effects and this redundancy contributes to host protection [253].

My current studies indicate that despite gene expression and secretion of multiple IFN types and sustained induction of antiviral effector genes in viral infected tissues, there was limited control of BHV-1 replication and shedding. The importance of individual IFNs during viral infections has been studied using a variety of animal models or natural gene defects that disrupt IFN genes or IFN receptors [254, 255]. These tools are not available in cattle at this time so I can only speculate to what extent IFNs play a critical role in controlling a primary BHV-1 infection. It may be logical to assume that in the

complete absence of either type I or type II IFN, the level of BHV-1 infection and shedding would be much greater than observed in the current study.

An alternate explanation for abundant IFN-γ secretion during the peak of BHV-1 replication [230] may be that IFN-γ production reflects the recruitment of activated NK-cells or cytotoxic T lymphocytes to the site of viral infection. One way to test this hypothesis was to analyze the phenotype of lymphocytes recruited to the URT during BHV-1 infection. IHC studies revealed that both $CD8^+$ and $CD335^+$ cells were recruited to BHV-1 infected nasal turbinates. The kinetics of cell recruitment over the 10 days of BHV-1 infection varied between tissues, such that peak recruitment of $CD3^+$, $CD8^+$ and $CD335^+$ cells occurred in nasal turbinates on day 5 pi. In contrast, no significant recruitment of these lymphocytes was detected in the trachea following BHV-1 infection (APPENDIX A). These observations suggest that lymphocyte recruitment following BHV-1 infection may be tissue-specific even though BHV-1 infection was confirmed in both tissues [230]. Furthermore, the recruitment of $CD3^+$ and $CD335^+$ cells in the URT coincided with peak IFN-γ levels in nasal secretions.

The phenotype analysis of lymphocytes present in nasal turbinates following BHV-1 infection revealed that peak IFN-γ levels coincided with peak recruitment of a non-conventional T-cell population (Figure 3.3 and Figure 4.4). We were unable to directly link increased IFN-γ production to non-conventional T cells recruitment on day 5 pi due to diffuse staining of IFN-γ. Future studies could possibly use *in situ* analysis of IFN transcripts to localize IFN gene expression to individual cells as has been done in other

studies [256]. Another alternative approach to determine if non-conventional T cells are the primary source of IFN-γ secreted in nasal secretions may be to develop tissue digestion methods to isolate single cell suspensions from the lamina propria and epithelium of nasal turbinates and trachea following BHV-1 infection. If sufficient viable lymphocyte numbers could be recovered from digested tissues then multi-color immunofluorescence could be used to co-stain for lymphocyte surface markers, intracellular IFN-γ, and other lymphocyte activation markers. These analyses would provide further evidence regarding the functional state of this unique T cell subpopulation following their recruitment to a mucosal site of viral infection.

The phenotype of lymphocytes recruited following BHV-1 infection was further clarified by dual-staining to analyze the expression of the CD8 cytotoxic lymphocyte marker on both $CD335^+$ and $CD3^+$ lymphocytes. This IF analysis revealed that the majority of lymphocytes recruited to nasal turbinates following BHV-1 infection were non-conventional $CD3^+$ T-cells that expressed both CD335 and CD8. It is currently not known whether these non-conventional T cells represent an innate or adaptive immune response. Further characterization of the T-cell repertoire of this recruited population is required before this distinction can be made. A limited TcR repertoire has been observed for iNKT cells [257]. iNKT cells express a TCR with an invariant chain which limits their antigen recognition repertoire and iNKT cells recognize lipid ligands presented in the context of the non-MHC surface molecule, CD1d. MAIT cells also express a semi-variant TCR which limits their T-cell repertoire relative to conventional T-cells. These cells recognize antigens in the context of the non-classical-MHC molecule, MR1 [163]

and bind vitamin B metabolite ligands [242]. While MAIT cells have been characterized in humans [240] and mice [163], there currently is no evidence for an equivalent population in cattle. Further analysis of TcR expression by bovine non-conventional T-cells is required to determine if these cells are analogous to either NKT [257] or MAIT cells [241] and should be classified as part of the innate or adaptive immune response to BHV-1 infection.

Although my IHC studies established a temporal association between recruitment of non-conventional T-cells and subsequent clearance of BHV-1 infection, further studies are required to determine whether $CD335^+$ T cells can function as effector cells able to eliminate BHV-1 infected cells. Based on the cytotoxic response mediated by bovine CD335+CD3+ cells during a parasitic infection, it is likely that these cells have the capacity to lyse BHV-1 infected cells [157]. Further IHC studies may be useful in demonstrating that the $CD335^+$ T cells migrate from the lamina propria and localize at foci of BHV-1 infected mucosal epithelial cells. Furthermore, $CD335^+$ T cells isolated from either blood or nasal turbinates, following BHV-1 infection, could be tested in *in vitro* assays to determine whether these non-conventional T cells display either MHC-restricted or non-MHC restricted cytotoxicity with BHV-1 infected cells. Bovine non-conventional T-cells have been shown to be activated through both CD335 and CD3 and have the potential to mediate both MHC-restricted and non-MHC-restricted cytotoxicity [157]. These studies may provide further evidence that non-conventional T cells are an important effector population for the control and clearance of a primary BHV-1 respiratory infection. IF results indicate, however, that not all $CD3^+$ lymphocytes present

in nasal turbinates during BHV-1 are $CD335^+$ (Figure 4.4i). Therefore, further analysis may be warranted to determine whether other T-cell subsets, such as γδT-cells, may also contribute to the control and clearance of a primary BHV-1 infection.

Differences in lymphocyte recruitment to nasal turbinates and trachea may reflect differences in the kinetics or magnitude of viral infection in these tissues. These differences in viral infection may then result in differences in the expression of tissue-specific adhesion molecules or chemokines required for cell recruitment. We observed a different combination of chemokine genes were significantly ($P < 0.05$) up-regulated in nasal turbinates and trachea following BHV-1 infection (APPENDIX B). CXCL-8, CCL2, CCL3, and CCL4 genes were upregulated in trachea. In contrast. CCL4, CCL5 and CXCL9 gene expression was significantly ($P < 0.05$) increased in nasal turbinates. Further information regarding the specific chemokines involved in the specific recruitment of non-conventional T cells is necessary before it can be concluded that differential chemokine production played a role in determining the differential cell recruitment observed in these two tissues.

Morphometric analyses revealed a recruitment pattern for $CD335^+$ and $CD8^+$ lymphocytes that was very brief with a significant ($P < 0.05$) increase in these cells in the lamina propria of nasal turbinates only on Day 5 pi (Figure 4.2). This temporal regulation of lymphocyte recruitment may reflect chemokine and adhesion molecule expression in this tissue but this conclusion is not consistent with the observed pattern of chemokine gene expression in nasal turbinates (Figure 4.5). Alternatively, morphometric

data may reflect the limitations associated with sampling tissues at a fixed site (10-12 mm into the external nares) throughout the course of viral infection and analyzing lymphocyte numbers in the lamina propria compartment. The lamina propria of nasal turbinates is highly vascular and likely to function as an important site for lymphocyte recruitment from blood. Further lymphocyte migration to foci of BHV-1 infected mucosal epithelial cells (Figure 3.2) would, however, create a dynamic balance between recruitment from blood and localization at sites of viral infection. Morphometric analyses of lymphocyte numbers in the lamina propria was predicated on the random distribution of lymphocytes. A more accurate representation of lymphocyte recruitment may have been achieved by selecting only those tissue sections that included both the lamina propria and a foci of BHV-1 infection. This may have provided a more complete analysis of both lymphocyte recruitment and localization to the site of BHV-1 infection.

Non-conventional T-cells have been characterized in bovine blood [157], porcine lungs [159] and humans [161]. The current study is the first demonstration that non-conventional T-cells can be recruited to a mucosal site following a viral infection in the URT. To further understand recruitment of this unique effector cell population we analyzed chemokine gene expression following BHV-1 infection. The expression of CCL4, CCL5 and CXCL9 genes were significantly ($P < 0.0332$) up-regulated in nasal turbinates following BHV-1 infection. This observation suggested that multiple chemokines may be involved in the specific recruitment of non-conventional T-cells. However, maximum chemokine expression did not coincide with the observed peak in non-conventional T-cell recruitment to the lamina propria. As discussed previously, the failure to correlate chemokine gene expression with lymphocyte recruitment may reflect

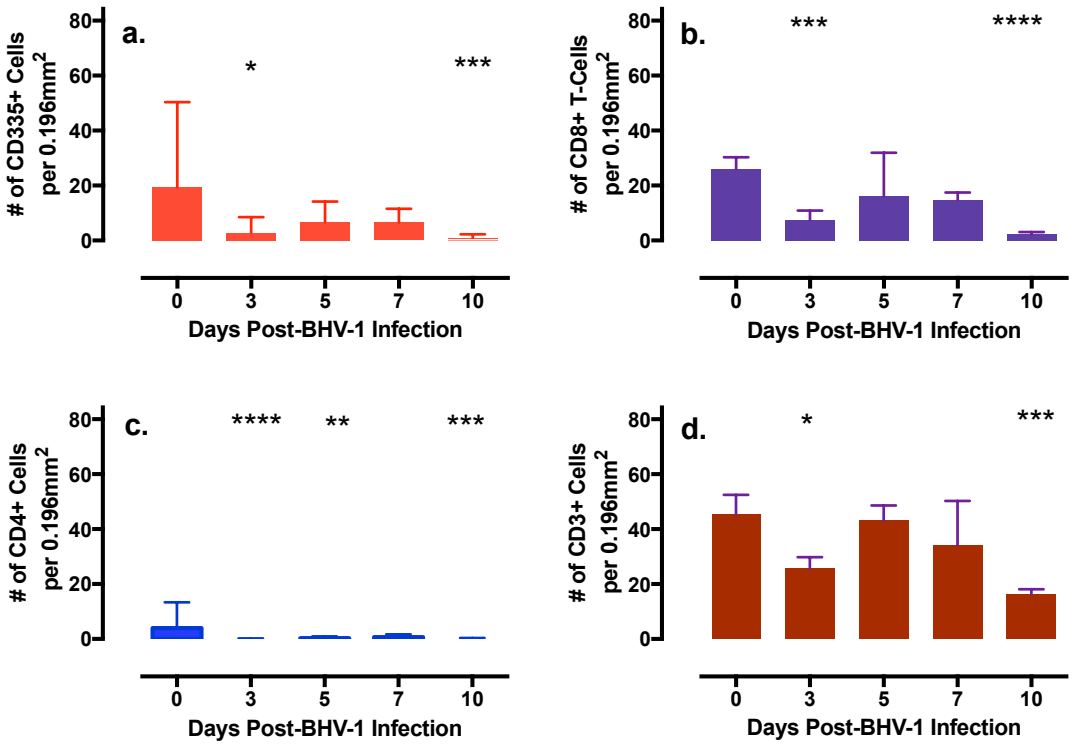
limitations of the current morphometric analysis methodology. In a recent study, *Mycobacterium bovis*-infected DCs selectively recruited $NKp46^{+}CD2^{-}$ cells following transcriptional upregulation of the CCL3, CCL4, CCL5, CCL20 and CXCL8 chemokine genes [199]. An alternative explanation for the lack of correlation between chemokine gene expression and non-conventional T cell recruitment may be that chemokine genes, other than those analyzed in this study, may be involved in recruitment of this unique T cell subpopulation. Chemotaxis studies with recombinant chemokine proteins may further clarify the capacity of non-conventional T cells to respond to the specific chemokines expressed in BHV-1 infected tissues. Alternatively, an analysis of chemokine receptor gene expression by non-conventional T cells purified from bovine blood may provide insight into which chemokine genes should be analyzed following BHV-1 infection.

In conclusion, my *in vivo* findings demonstrated that IFNs have limited capacity to control a primary BHV-1 infection despite early induction of a multifaceted type I and II IFN response and ISG expression. In contrast, recruitment of $CD335^{+}CD8^{+}$ T-cells within 5 days pi may provide an important host response contributing to the control of a primary BHV-1 infection. These non-conventional T cells were the major effector population recruited to nasal turbinates during a BHV-1 respiratory infection but it remains to be confirmed that $CD335^{+}CD8^{+}$T-cells have the capacity to lyse BHV-1 infected epithelial cells. Future studies are also needed to further characterize the TcR repertoire of $CD335^{+}CD8^{+}$T-cells before it can be concluded whether they constitute part of the innate or adaptive immune response to BHV-1 infection. If $CD335^{+}CD8^{+}$T-cells

are innate immune cells, then early recruitment of non-conventional T-cells following a primary viral infection may enable the host to recognize viral infected cells through NKp46. Alternatively, if these non-conventional T cells are part of the adaptive immune response then they may confer immune memory following a primary BHV-1 infection. If these cells are found to be adaptive and cytotoxic, then the specific induction of this unique T cell subpopulation may provide a surrogate measure for evaluating future BHV-1 vaccines that induce an improved cell-mediated immune response. It would then be necessary to investigate $CD335^{+}CD8^{+}$T-cell recognition of individual BHV-1 proteins and determine whether BHV-1 vaccination could increase the frequency of this relatively rare T-cell subpopulation [157].

APPENDICES

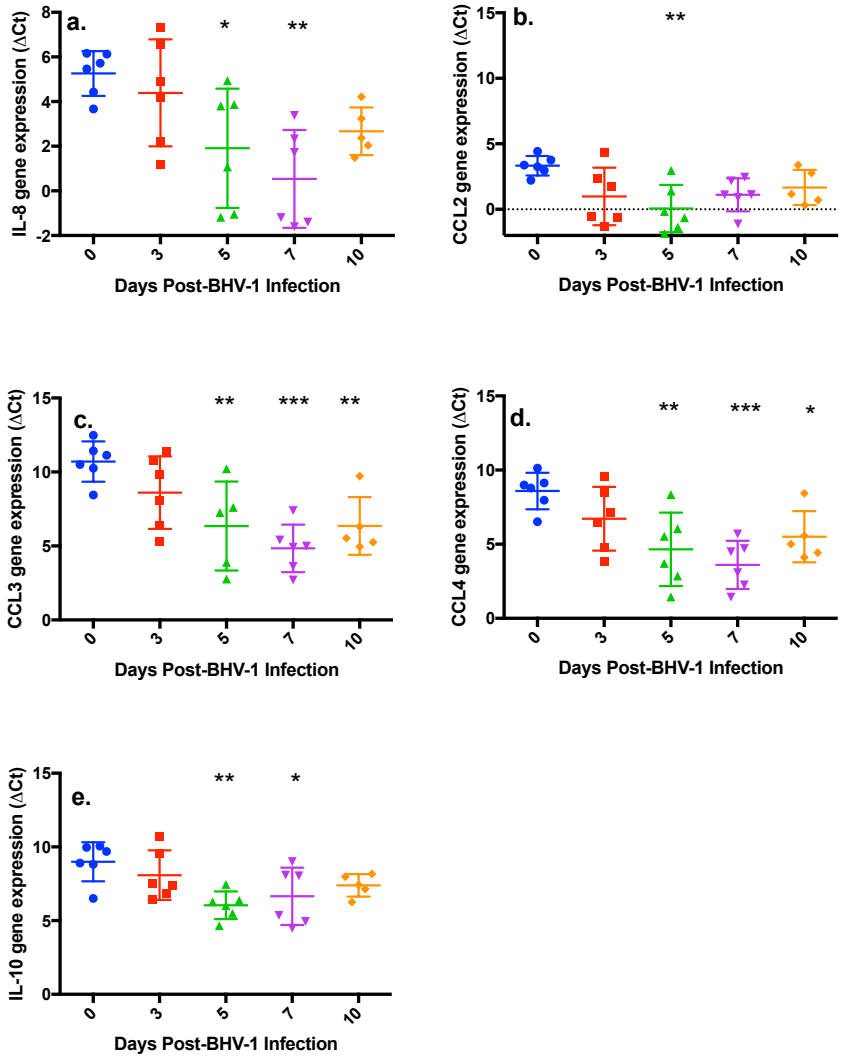
5.1 APPENDIX A: Lymphocyte recruitment to Bovine Trachea post-BHV-1 infection



APPENDIX A Figure 1. Frequency of lymphoid subpopulations present in trachea following a primary BHV-1 infection. Immunohistochemical staining was performed with monoclonal antibodies to identify CD335+ (**a**) CD8+ (**b**) CD4+ (**c**) and CD3+ cells (**d**). Morphometric analyses were used to quantify the frequency of each cell type located within the tracheal intraepithelial and submucosal compartments. Data presented are the mean and standard error of the mean for values from tissues sections analyzed from 6 animals/time point following BHV-1 infection. One-way ANOVA was used to compare values relative to pre-infection (Day 0) levels and significant changes in frequency of leukocytes relative to Day 0 are indicated as $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), $P \leq 0.0001$ (****).

APPENDICES

5.2 APPENDIX B: Chemokine expression in bovine trachea post-BHV-1 infection



APPENDIX B Figure 1. Expression of chemokines in tracheal tissue following primary BHV-1 infection. Tracheal tissue samples were collected (n = 6 animals/time point) prior to and following BHV-1 infection and RNA extracted for qRT-PCR analysis abundance of IL-8(a), CCL2 (b), CCL3 (c) and CCL4(d) and IL-10 (e) gene transcript abundance. Gene expression was calculated as the change in threshold cycle (ΔCq) relative to β-actin and data presented are values for individual animals. The horizontal bar represents the mean value for animals sampled at each time sampled. A Mann-Whitney test was used to compare values relative to pre-infection (Day 0) levels. Significant changes in gene expression relative to Day 0 are indicated: $P < 0.0332$ (*), $P \leq 0.0021$ (**), $P \leq 0.0002$ (***), $P \leq 0.0001$ (****).

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