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INFLAMMATORY RESPONSE IN THE BOVINE VIRAL DIARRHEA AND ITS ALTERATION IN SECONDARY INFECTIONS

RESPUESTA INFLAMATORIA EN LA DIARREA VÍRICA BOVINA Y SU ALTERACIÓN ANTE INFECCIONES SECUNDARIAS

MARÍA DE LOS ÁNGELES RISALDE MOYA

Departamento de Anatomía y Anatomía Patológica Comparadas

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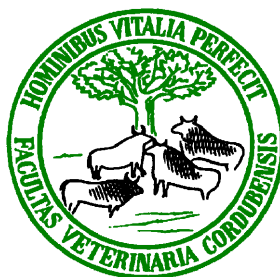
AUTOR: *María de los Ángeles Risalde Moya*

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Trabajo presentado por la Licenciada en Veterinaria
Dña. María de los Ángeles Risalde Moya para optar al
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TÍTULO DE LA TESIS:

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INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

José Carlos Gómez Villamandos, Catedrático del Departamento de Anatomía y Anatomía Patológica Comparadas de la Universidad de Córdoba, y Pedro José Sánchez Cordón, investigador contratado de la Universidad de Córdoba en el marco del programa Ramón y Cajal del Ministerio de Educación y Ciencia, informan que:

Este trabajo se ha realizado dentro de los proyectos de investigación AGL 2006-01536, titulado "Estudio de la respuesta inmune en terneros persistentemente infectados con el virus de la Diarrea Vírica Bovina e inoculados con Herpesvirus Bovino tipo 1", y AGR-4671, titulado "Respuesta inmune en la diarrea vírica bovina. Papel de las células dendríticas y células estromales de las estructuras linfoides frente a virus patógenos y vacunales" (Proyecto de excelencia).

El objetivo de esta tesis fue contribuir al estudio de las estrategias de evasión del sistema inmune inducidas por el virus de la Diarrea Vírica Bovina, así como analizar los mecanismos inmunológicos que se desencadenan ante una infección respiratoria secundaria, estableciendo las alteraciones que pudiesen inducir una respuesta inmune inadecuada y una mayor susceptibilidad a la infección.

Los resultados obtenidos han permitido realizar cinco artículos que serán enviados a revistas científicas indexadas, dos de los cuales ya han sido publicados en Veterinary Journal y *Veterinary Inmmunology and Immunopathology,* además de ser expuestos en diferentes congresos nacionales e internacionales.

Por todo ello, se autoriza la presentación de esta tesis doctoral.

Córdoba, 7 de diciembre de 2011

Fdo.: José Carlos Gómez Villamandos   Fdo.: Pedro José Sánchez Cordón

A mi familia.

A Israel.

"Defiende tu derecho a pensar, porque incluso pensar de manera errónea es mejor que no pensar"

Hipatia de Alejandría

Contents

Abbreviations

ºC	Celsius grades
ABC	avidin-biotin-peroxidase complex
Abs	antibodies
APCs	antigen-presenting cells
APPs	acute phase proteins
APR	acute phase response
BHV-1	bovine herpesvirus-1
BHV1 group	calves inoculated only with BHV-1
BRDC	Bovine Respiratory Disease Complex
BRSV	bovine respiratory syncytial virus
BVD	Bovine Viral Diarrhea
BVDV	bovine viral diarrhea virus
BVDV/BHV1 group	calves inoculated with BVDV and BHV-1
COX-2	cyclooxygenase-2
CP	cytopathogenic
CSFV	Classical Swine Fever Virus
Ct	cycle threshold
DCs	dendritic cells
DNA	deoxyribonucleic acid

dpi	days post-inoculation
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
gp	glycoprotein
HE	haematoxylin and eosin
Hp	haptoglobin
hpi	hours post-inoculation
IFN	interferon
IHC	immunohistochemistry
IIBs	intranuclear inclusion bodies
IL	interleukin
IMΦs	Interstitial macrophages
iNOS	inducible nitric oxide synthase
KCs	Küpffer cells
LPS	lipopolysaccharide
mAbs	monoclonal antibodies
MD	Mucosal Disease
MDBK	Madin Darby Bovine Kidney cells
MHC	major histocompatibility complex
min	minute
m-MΦs	monocytes/macrophages
MΦs	macrophages
NCP	non-cytopathogenic
NO	nitric oxide
NOS	nitric oxide synthases
O_2^-	superoxide anion
OD	optical density
OPD	O-phenylenediamine dihydrochloride

ORF	open reading frame
pAbs	polyclonal antibodies
PAMs	pulmonary alveolar macrophages
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PI	persistently infected animals
PI-3	parainfluenza-3
PIMs	pulmonary intravascular macrophages
RNA	ribonucleic acid
rpm	revolutions per minute
Rt	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SAA	serum amyloid A
Tc	T-cytotoxic
$TCID_{50}$	tissue culture infective dose 50%
TEM	transmission electron microscopy
Th	T helper
TNF	tumor necrosis factor
Tris	tris (hydroxymethyl) aminomethane
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling method
UI	un-infected animals
UTR	untranslated regions
VN	virus neutralization

Background

Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle, generating considerable economic losses for the livestock industry. This pestivirus is the causative agent of the bovine viral diarrhea (BVD), disease described for the first time by Olafson et al. in EEUU (1946) as an enteric bovine disease with high morbidity and low mortality that seemed to have viral etiology (Olafson et al., 1946). Then, another process with a sporadic presentation in cattle, extremely high mortality and produced by the same agent was reported in 1953, being named as mucosal disease (MD) (Ramsey, 1953; Thomson and Savan, 1963). Nowadays, BVD is known as a contagious disease that induces severe reproductive, respiratory and gastrointestinal pathologies mainly in bovine, although the BVDV can also infect ovine, caprine, wild ruminants and porcine (Tremblay, 1996; Ames, 2005).

BVD is widely distributed and presents high prevalence worldwide, seeing increased the number of BVDV-seropositive cases with the presence of immunotolerant animals in the herds. Nevertheless, the prevalence of BVDV infection is variable depending on diverse circumstances, such as density and size of the herds, housing system and management of the animals (Greiser-Wilke et al., 2003).

Since 1946, BVDV has shown to be responsible for a multifactorial disease with probably the most complicated pathogenesis of the bovine malignances. During the past 50 years, BVDV research has made many advances leading to the development of diagnostic tests, the production of vaccines and the design of successful control strategies for this virus. Likewise, the discovery of persistent infection was a key factor to understand BVDV maintenance in the host and to develop rational eradication strategies (Deregt, 2005).

1. Taxonomy, morphology and structure of BVDV

BVDV is a pestivirus classified within *Flaviviridae* family that displays high homology with other pestiviruses, such as classical swine fever virus (CSFV) and border disease virus of the sheep (Bolin and Grooms, 2004; Fauquet et al., 2005). Flaviviruses are a closely related group of small enveloped viruses with a single-stranded, positive sense ribonucleic acid (RNA) genome of approximately 12.5 kb in length (Ridpath and Bolin, 1997; Grummer et al., 2001). This RNA strand comprises an open reading frame (ORF) flanked on the 3' and 5' ends by untranslated regions (UTR), showing the 5' UTR extreme as the most conserved region in pestiviruses (Ridpath and Bolin, 1997). The ORF 5' end encodes the structural proteins of the virus (C, Erns, E1, E2), while the nonstructural proteins (Npro, NS23) are encoded at the 3' end (Thiel et al., 1996). The lipid envelope derives from the membrane of infected cells with a range from 40 to 60 nm in diameter and is found surrounding an icosahedral nucleocapsid of 25-37 nm (Lindenbach and Rice, 2001; Ridpath, 2005).

2. Genotypes and biotypes of BVDV

RNA viruses are characterized by their plasticity and ability to generate a selection of variants with different antigenic properties (Corapi et al., 1990; Ridpath, 1996), which helps BVDV to evade recognition by neutralizing antibodies (Abs) and escape the host immune response (Donis, 1995). BVDV has been classified into two different genotypes, BVDV-1 and BVDV-2, based on genetic differences (Ridpath et al., 1994; Heinz et al., 2000; Fulton et al., 2003a).

BVDV-1 includes the most common isolates on herds. This genotype causes processes with unapparent symptoms, characterized by a slight increase in body temperature and the presence of moderate lesions restricted to the digestive tract and organs of the lymphoid system, causing also abortions in pregnant cows and other reproductive disorders (Pellerin et al., 1994; Ridpath et al., 1994).

BVDV-2 isolates are associated with acute severe processes, presenting an intense lymphopenia, thrombocytopenia and body temperatures exceeding 40.6ºC (Carman et al., 1998; Liebler-Tenorio et al., 2002, 2003b), sometimes characterized by producing an acute bleeding disease, called hemorrhagic syndrome (Corapi et al., 1990; Stoffregen et al., 2000).

Independently of the genotype to which it belongs, BVDV is divided in two biotypes, cytopathogenic (CP) and non-cytopathogenic (NCP), depending on their lytic activity on cultured epithelial cells (Ridpath et al., 1994; Heinz et al., 2000; Fulton et al., 2003a). CP biotypes provoke a cytopathic effect which leads to cytoplasmic vacuolization and cell death, while NCP biotypes replicate in these cells without causing morphological changes (Bolin and Grooms, 2004; Ridpath, 2005). The biochemical hallmark of CP strains is the production of high levels of NS3 as a free protein after the early phase of infection. However, NCP BVDV variants produce small amounts of NS3 and

largely NS23 in the early phase of infection (Donis and Duvobi, 1987; Lackner et al., 2004). In this regard, there are evidences that NCP biotype of BVDV may originate CP strains, either by proteolytic cleavage of the NS23 protein (Lackner et al., 2004, 2005), gene duplication of the altered NS3 protein (Meyers et al., 1992), genetic deletion of the NS2 protein (Tautz et al., 1994) or mutation (Kümmerer et al., 2000).

Cytopathology *in vitro* does not correlate with virulence *in vivo* (Bezek et al., 1994). Indeed, the most severe clinical form of acute BVDV infection and the establishment of persistent infections are associated with NCP virus (Ridpath et al., 1994; Evermann and Ridpath, 2002; Fulton et al., 2002), which is the most common biotype in nature (Bolin and Grooms, 2004; Ridpath, 2005).

3. Clinical forms

As it has been reported, the biology of BVDV is very complex, depending on different factors such as genotype and biotype that causes the infection, the immune status and age of animals, as well as the gestational period of the cows. This leads to a broad spectrum of clinical manifestations and lesions that can be classified according to the type of BVDV infection in: acute infection, congenital infection and MD (*Figure 1*).

3.1. Acute infection

The infection of immunocompetent animals with BVDV can originate some clinical variants: subclinical infection, acute BVDV infection, severe acute BVDV infection and hemorrhagic BVDV infection (Grooms et al., 2002).

A significant percentage (70-90%) of BVDV infections results in *subclinical infections* (Rickey, 1996; Bolin and Grooms, 2004), showing the

animals only a slight increase in body temperature, decreased white blood cell count and immunosuppression (Wilhelmsen et al., 1990; Brock, 1995; Rickey, 1996; Bolin and Grooms, 2004; Pedrera et al., 2009b). This immunosuppression favors the emergence of opportunistic infectious agents (Potgieter, 1995), highlighting those that cause bovine respiratory disease (Brodersen and Kelling, 1998; Hamers et al., 2000). In these processes, clinical signs will depend on the nature of secondary infection, so that almost never are recognized as processes induced by BVDV (Brownlie, 1990; Rickey, 1996; Bolin and Grooms, 2004).

Acute infection with characteristic clinical symptoms is described as *BVD,* observing only moderate clinical manifestations as pyrexia, anorexia, lethargy, salivation, oculo-nasal discharge, cough and mild diarrhea (Müller-Doblies et al., 2004). Occasionally, erosions and ulcerations of the oral and gastrointestinal mucosa may be noticed (Wilhelmsen et al., 1990; Marshall et al., 1996; Spagnuolo-Weaver et al., 1997; Lambot et al., 1998). This form of disease is usually produced by BVDV-1 strains and some low virulence BVDV-2 strains, and although it is possible to obtain isolates from both strains, NCP biotypes are more frequent. These processes, that have a high morbidity and very low or no mortality, affect more frequently to calves with 6-24 months old (Wilhelmsen et al., 1990; Cherry et al., 1998).

There is an *acute severe form of BVD* characterized by high fever (39.7 to 41ºC), agalactia, watery diarrhea and respiratory disorders. These animals show a minimum reduction of 50% in the circulating lymphocytes and a marked thrombocytopenia together with pneumonic lesions, ulcerations in the oral mucosa and depletion of lymphoid organs (David et al., 1994; Rickey, 1996; Carman et al., 1998; Archambault et al., 2000). This clinical form is caused by NCP strains of BVDV-2 with high virulence, presenting elevated morbidity and mortality within 48 hours after the onset and affecting animals

of all ages (Wilhelmsen et al., 1990; Ridpath and Bolin, 1998; Archambault et al., 2000; Stoffregen et al., 2000; Jones and Weber, 2001; Liebler-Tenorio et al., 2002).

Hemorrhagic syndrome is a grave clinical form evolved from the acute severe form, where the animals show pyrexia, bloody diarrhea, conjunctiva and mucous congestion, petechial hemorrhages and ecchymoses in mucous membranes (Corapi et al., 1990; Stoffregen et al., 2000; Evermann and Barrington, 2005). In addition, this syndrome is characterized by a marked thrombocytopenia, leukopenia and neutropenia (Rebhun et al., 1989; Bolin and Grooms, 2004). Among the most characteristic lesions highlights an important depletion of lymphoid organs, increased lymphocytic apoptosis, vacuolization of epithelial cells and vasculitis in various organs (Ellis et al., 1998; Stoffregen et al., 2000; Liebler-Tenorio et al., 2003b). This clinical form is caused by NCP BVDV-2 highly virulent strains and has a mortality rate near to 25% (Pellerin et al., 1994; Ridpath et al., 1994; Bolin and Grooms, 2004).

3.2. Congenital infection

BVDV can also produce reproductive disorders, as it can be removed in the semen of infected animals, giving rise to venereal infections (Schlafer et al., 1990; Kirkland et al., 1991) that cause a decline in male fertility and reduced conception rates (Paton et al., 1990). In the case of females, all reproductive organs are permissive to BVDV, being the ovary the most affected organ, altering its function and preventing normal follicular dynamics which produces a temporary infertility (Fray et al., 2000; McGowan et al., 2003).

Adult immunocompetent pregnant cows can clear the virus and become immune, but during this period a transplacental infection involving any of

both genotypes may occur, although in turn only the NCP biotype causes the fetal infection (Wittum et al., 2001; Harding et al., 2002). Fetuses infected with NCP BVDV present differences in the severity of lesions depending on the stage of gestation at which the infection takes place (Goyal, 2005).

Thus, when the infection occurs before 60 days of gestation, it may lead to *fetal death* with mummifications or abortions from 10 days to 3 months after the virus entrance (Done et al., 1980; Evermann and Barrington, 2005).

The fetus infected between 50-120 days of gestation, which is prior to the development of fetal lymphoid tissues and to a functional acquired immune response, is unable to recognize the virus as foreign, which results in the acquisition of immunotolerance to the infecting BVDV strain and persistent infection (Stokstad and Loken, 2002; Goyal, 2005). After birth, these *persistently infected (PI)* animals appear clinically normal but they are viremic and shed virus in all excretions and secretions continuously, becoming the main reservoir of virus within the herd (Odeon et al., 1999; Brock, 2003; Bolin and Grooms, 2004; Confer et al., 2005) and being at risk of suffering MD. Some PI calves have reduced fertility and show immunosuppression that predispose them to secondary infections (Muñoz-Zanzi et al., 2003; Confer et al., 2005), occurring their death during the first year of life (Bock et al., 1997; Brackenbury et al., 2003).

Between 100-150 days of gestation, coinciding with the onset of fetal immunocompetence and organogenesis, *congenital malformations* appear and abortions are less frequent. Thus, the most characteristic lesions are thymic hypoplasia and pulmonary necrosis, alopecia, hypotrichosis, arthrogryposis, growth retardation and other skeletal and eye abnormalities (Baker, 1995; Bielefeldt Ohmann, 1995; Kahrs, 2001), presenting also severe malformations of the nervous system, such as microcephaly, hydrocephaly and cerebellar hypoplasia (Bielefeldt Ohmann, 1995).

When infection occurs in late stages of the gestation, the immune system is sufficiently developed and may start an immune response against the infecting virus. Therefore, these calves generally born immunocompetent and without problems, although *seropositves to BVDV* (Goyal, 2005).

3.3. Mucosal disease (MD)

MD is an sporadic and fatal disease produced by BVDV that only affects PI animals when exist a mutation of NCP strains into CP or a super-infection shortly after birth by a CP biotype antigenically homologous to the NCP biotype that caused the immunotolerance (Thiel et al., 1996; Sentsui et al., 2001; Confer et al., 2005; Smirnova et al., 2008).

Animals that suffer from this disease present bloody diarrhea, mucocutaneous erosions and death within 2-3 weeks from the onset of clinical signs. Microscopically, calves have fibrinous enteritis, erosions, ulcerations and hemorrhages in the mucous membranes of the oral cavity, esophagus, pre-stomachs, abomasum and intestine. Moreover, these animals show depletion of lymphoid tissues, especially those associated with mucous membranes, which induces a state of immunosuppression (Liebler et al., 1995; Wilhelmsen et al., 1991; Bolin and Grooms, 2004).
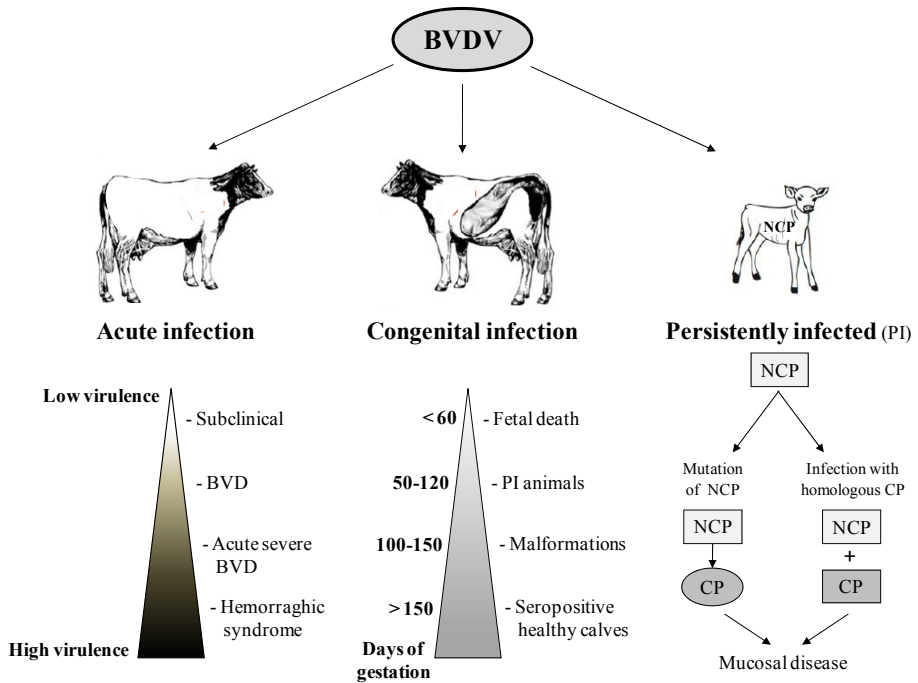
Figure 1. Schematic summary of different clinical manifestations described in cattle following BVDV infections.

4. Pathogenesis

After BVDV infection, there is a complex interaction between the etiologic agent and the infected host defined as "pathogenesis". This process evolves from the entry of virus to the development of disease and immune response in the host. The pathogenic mechanisms of BVDV have not yet been clarified, existing discrepancies about whether the direct action of the virus may or may not be the responsible for lesions appeared in different locations.

4.1. Distribution and target cells of BVDV

4.1.1. Acute infections

Regardless the virulence of the strain, the main route of postnatal infection with BVDV is the oronasal, being the nasal mucosa and tonsils the primary organs of virus replication. The virus spreads from the nasal cavity to regional lymph nodes through lymphatic and blood vessels and then to systemic distribution in a free form or associated with lymphocytes and monocyte-macrophages (m-MΦs) (Brodersen and Kelling, 1998; Bruschke et al., 1998b; Kelling et al., 2002). BVDV displays a special tropism for the mucosa-associated lymphoid tissue (tonsils and intestine) and lymphoid organs as lymph nodes, thymus and spleen. In these locations the presence of BVDV antigen is associated with a marked lymphoid depletion. Moreover, the antigen-presenting cells (APCs) such as dendritic cells (DCs) and m-MΦs, and the lymphocytes (T and B) appear as the main virus target cells (Bruschke et al., 1998b; Teichmann et al., 2000; Liebler-Tenorio et al., 2003a,b, 2004; Kelling et al., 2007; Pedrera et al., 2009b; Raya et al., 2011).

In studies performed after inoculation with strains of low virulence, viral antigen was only detectable in lymphoid tissues, not observing the presence of virus in the bone marrow during the infection (Wilhelmsen et al., 1990; Liebler-Tenorio et al., 2003a,b, 2004) (*Table 1*). However, in experimental infections of colostrum-deprived calves with BVDV strains of low virulence, the viral antigen can be also detected in the intestinal mucosa, liver, upper and lower respiratory tract (Liebler-Tenorio et al., 2003a,b, 2004; Da Silva et al., 2007; Pedrera et al., 2009b).

In inoculations with highly virulent strains, it was observed that the quantity and spread of viral antigen in tissues exceeded that produced by low virulence strains. The presence of antigen in processes caused by these strains is not only restricted to the follicles of lymphoid tissues, but it also extends to

other organs as skin, digestive and respiratory tracts, endocrine tissues, bone marrow and interstitium or vascular walls (Bruschke et al., 1998b; Ellis et al., 1998; Odeon et al., 1999; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2003b) (*Table 1*). Furthermore, other cells undergoing BVDV infection to a lesser extent are endothelial cells, neutrophils, epithelial cells, keratinocytes, megakaryocytes and platelets (Liebler et al., 1995; Marshall et al., 1996; Liebler-Tenorio et al., 2002, 2003b). Thus, the infection of epithelium in the upper digestive tract may cause erosive to ulcerative lesions (Marshall et al., 1996; Ellis et al., 1998; Odeon et al., 1999; Stoffregen et al., 2000). Bone marrow infection is related with the development of a marked thrombocytopenia characteristic of the infection with these strains (Spagnuolo et al., 1997; Ellis et al., 1998; Archambault et al., 2000; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2003b).

4.1.2. Congenital infections

Both BVDV biotypes infect the ovaries in experimental inoculations leading to a decreased conception rate (Bielanski et al., 1998; Grooms et al., 1998; McGowan et al., 2003). In the first stage of gestation, BVDV infects the placenta and numerous tissues of the fetuses, causing severe damage that is considered as an important initiator for abortion (Baszler et al., 1995; Fredriksen et al., 1999a). Nevertheless, if the fetus is not immediately expulsed, it can give rise to mummifications (Liebler-Tenorio, 2005).

PI animals present a wide distribution of BVDV in all their tissues, having more tropism for epithelial, lymphoid and central nervous system cells, although there are not morphological lesions associated with the presence of the virus (Fredriksen et al., 1999b; Njaa et al., 2000; Shin and Acland, 2001; Liebler-Tenorio et al., 2004).

Teratogenic effects of BVDV appear when the immune competence begins to develop, the ability to mount an inflammatory response sets in, and organogenesis is not completed. Such alterations affect mainly to brain and eyes because the organogenesis of these tissues occurs in the final stages of gestation (Liess et al., 1987; Liebler-Tenorio, 2005).

Table 1.

Distribution of BVDV strains in acute infections

Organs	Low virulence	Highly virulence
Lymphoid tissues		
Tonsils	+	+
Thymus	+	+
Spleen	+	+
Lymph nodes	+	+
Peyer's patches	+	+
Bone marrow	-	+
Digestive tract		
Oral mucosa	-	+
Esophagus	-	+
Intestines	-	+
Respiratory tract		
Nasal mucosa	-	+
Lung	-	+
Heart	-	+
Skin	-	+
Liver	-	+
Pancreas	-	+
Kidney	-	+
Adrenal	-	+
Thyroid	-	+
Pituitary	-	+
Ovary	-	+
Testis	-	+
Nervous system	-	-

*(-) absent; (+) presence

4.1.3. Mucosal disease

Like in PI animals, the NCP BVDV strains can be present in numerous organs and tissues of calves with MD, but are not associated with tissue lesions. When intranasal infection with CP BVDV occurs, primary replication of the virus is observed in epithelium of the tonsil. Following BVDV spread to the regional lymph nodes, it can be detected in Peyer's patches, lymphoid follicles of mucosa-associated to intestinal and respiratory tracts, and to a lesser extent, in peripheral lymph nodes, thymus, and spleen. Therefore, lesions occurring in MD are associated with the presence of CP BVDV antigen (Liebler, 1991, 1995; Liebler-Tenorio, 2005).

4.2. Immune response and immunosuppression

The vertebrate immune system posses sophisticated mechanisms to counter the multitude of pathogens that establish an infection and cause disease. However, at the same time, several pathogens evolve continuously developing complicated strategies to suppress or evade the host immune mechanisms. BVDV is not an exception to this phenomenon, being able to produce disease on its own and, which is perhaps more important, inducing a state of immunosuppression that predispose calves to infections by other micro-organisms.

As it has been seen before, BVDV has a special tropism for cells of the immune system, inducing cell death as an extreme event of the infection, or more subtle effects on cytokines and co-stimulatory molecules produced by immune or non-immune cells that could affect to both innate and adaptive immune response (*see Table 2*).

4.2.1. Innate immune response

Phagocytosis is a critical innate defense mechanism which implies the intracellular killing of pathogens and the secretion of pro-inflammatory cytokines. This internalization and killing of pathogens is an essential pre-requisite for microbial antigen presentation and induction of a specific adaptive immune response against pathogens (Henneke and Golenbock, 2004).

Specialized APCs, such as MΦs and DCs, are considered key components of innate immune system, developing the pathogens several strategies to combat these phagocytes (Coombes et al., 2004). Particularly, MΦs are of great importance in the defense of the body against viral infections, so that the valuation of their functions permits to determine the ability of the individual resistance or susceptibility to infection (Laskin et al., 2001).

Infection of MΦs with BVDV produces a decrease in chemotactic and phagocytic capacity by altering cellular metabolism (Ketelsen et al., 1979), an impaired microbicidal activity by decreasing superoxide anion (O_2^-) production and increasing nitric oxide (NO) synthesis in response to lipopolysaccharide (LPS) (Adler et al., 1994, 1996; Potgieter, 1995), and a stimulation of prostaglandin E2 (PGE2) synthesis (Welsh and Adair, 1995; Van Reeth and Adair, 1997). Furthermore, various authors have demonstrated that BVDV also induces an impaired production of cytokines by MΦs both *in vitro* and *in vivo*, which could affect to the generation of a subsequent immune response (Adler et al., 1996; Yamane et al., 2005; Lee et al., 2008; Pedrera et al., 2009a; Raya et al., 2011).

Proinflammatory cytokines as interleukin (IL)-1 and tumor necrosis factor-α (TNFα) are of great importance in the innate immune response, being secreted by MΦs in the inflammatory response or due to the existence of tissue

damage (Biron and Sen, 2001). These cytokines, together with type I IFN, mediate in the acute phase response (APR), an important defense mechanism that is activated before the specific immunity against disturbances in homeostasis by infections, tissue injury or immune disorders (Heinrich et al., 1990; Dinarello, 2006). This nonspecific mechanism include, among others, changes in the synthesis of certain plasma proteins denominated acute phase proteins (APPs) that can be considered as "positive'' or ''negative'', depending on the increase or decrease of their concentration in serum. Haptoglobin (Hp) and serum amyloid A (SAA), the main positive APPs in cattle, are used as indicators of disease severity (Eckersall, 2000; Petersen et al., 2004) (*Figure 2*).

About proinflammatory cytokines, *in vitro* BVDV infection of bovine MΦs does not provide consistent results; whereas some authors demonstrate a decrease in the production of TNFα with both CP and NCP BVDV strains (Adler et al., 1996; Lee et al., 2008), others maintain that CP strains induce the production of TNFα, which contributes to apoptosis of infected cells (Yamane et al., 2005). In this regard, acute experimental infection with a NCP strain also provided variable results depending on viral target organs (Pedrera et al., 2009b; Raya et al., 2011). The pro-inflammatory capacity of TNFα is limited by the lack of IL-1α, since these two cytokines act synergistically (Le and Vilcek, 1987; Van Reeth et al., 1999). Thus, BVDV-associated inhibition of IL-1 has been reported both *in vitro* and *in vivo*, regardless the biotype of the strain used (Jensen and Schultz, 1991; Adler et al., 1996; Yamane et al., 2005; Lee et al., 2008; Pedrera et al., 2009a; Raya et al., 2011).

On the other hand, the secretion of these proinflammatory cytokines in acute BVDV processes with NCP strains induced a progressive and late increase of disease indicators as Hp, SAA and fibrinogen in serum (Gånheim et al., 2003; Müller-Doblies et al., 2004).
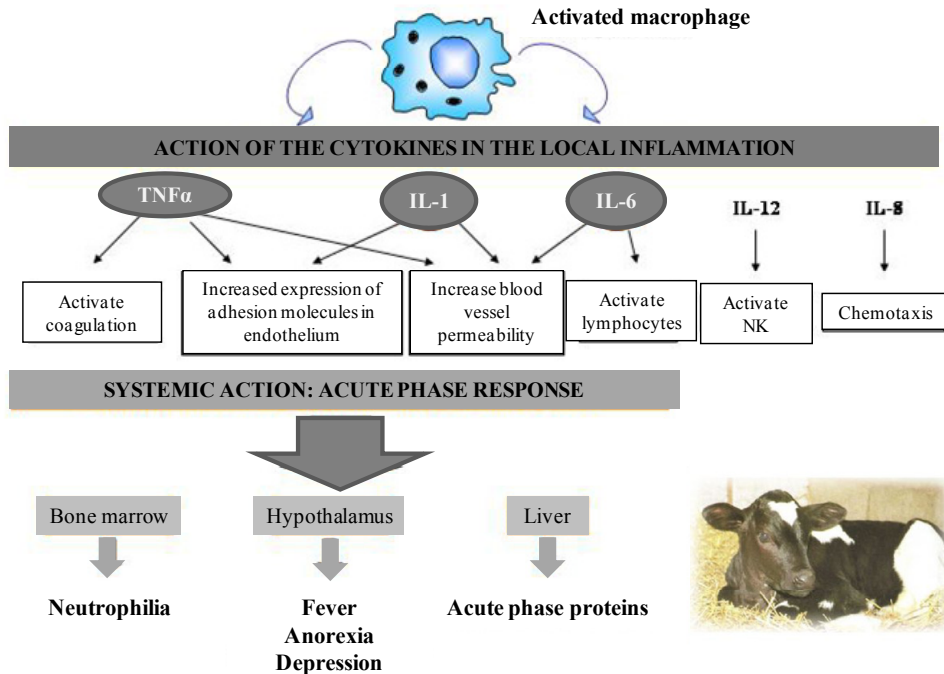
Figure 2. Diagram of inflammatory response associated to proinflammatory cytokines action.

Other cytokines as interferons (IFNs) were discovered due to their ability to protect cells from viral infection, reducing virus replication and dissemination. In acute processes, infection with CP BVDV strains produces a rapid and powerful local early response with the release of type I IFN (α/β) by m-MΦs or DCs, activating to the effector cells of innate immune response and limiting virus replication at mucosal level (Brackenbury et al., 2003). By contrast, NCP BVDV strains do not stimulate an early local cytokine response and the virus is conveyed to local lymph nodes where interacts with DCs that produce large amounts of IFNα, increasing their activation and limiting viral replication. This generates an effective primary immune response (Charleston et al., 2002; Glew et al., 2003) which, however, does not prevent the spread of

the virus (Palucka and Banchereau, 2002; Brackenbury et al., 2003), indicating the presence of other factors involved in the immunosuppression induced by BVDV (Charleston et al., 2002; Müller-Doblies et al., 2004).

The infection with both NCP and CP BVDV early in gestation gives rise to an elevated type I IFN production only in fetuses infected with CP BVDV. Thus, the evasion of innate immunity by inhibition of type I IFN following NCP BVDV infection favors the establishment of an immunotolerance state that allows virus persistence in the fetus (Charleston et al., 2001a, 2002). Since a functional fetal adaptive immune response does not occur at this time, virus replication will not be limited and this will spread throughout the body, not developing in PI calves an acquired immune response to BVDV attributed to tolerance of CD4+ cells (Collen et al., 2000; Schweizer and Peterhans, 2001; Peterhans et al., 2003; Smirnova et al., 2008).

4.2.2. Adaptive immune response

Adaptive immunity is an antigen-specific response with immunologic memory regulated by T and B lymphocytes, and the soluble factors produced by them – cytokines and Abs, respectively –. The immune response, whether it is cell or humoral mediated, begins with antigen recognition, processing and presentation (Goldsby et al., 2003; Tizard, 2008).

Cell-mediated immune response

This type of adaptive immune response, represented by T cells, recognizes peptide epitopes presented by the major histocompatibility complex (MHC) molecules on self-cells such as virus-infected cells. T cells are functionally divided into T-helper (Th) cells that act as inducers of the immune response through the release of different cytokines and T-cytotoxic

(Tc) cells for exerting a predominantly cytotoxic function, both generally expressing specific cell surface molecules CD4 and CD8, respectively.

Antigen recognition and its presentation by MHC molecules class I to CD8+ lymphocytes and class II to CD4+ lymphocytes are crucial for their proliferation and the successful induction of an immune response to any pathogen (Yewdell and Hill, 2002; Hewitt, 2003; Janeway and Travers, 2005). Many viruses, as BVDV, have developed strategies for interfere in these processes, compromising the capacity of infected APCs and affecting the proliferative response of lymphocytes, as means of immunosuppression.

DCs are more resistant than monocytes to *in vitro* infection with NCP strains; while DCs are not affected in their ability of viral antigen presentation to T cells, monocytes infected with NCP strains show an altered presentation function that reduce the proliferation of CD4+ T cells (Glew et al., 2003). Some authors report a decrease in the expression of MHC class II by monocytes infected with NCP (Archambault et al., 2000; Chase et al., 2004) and CP strains (Chase et al., 2004). In addition, it has been observed a decrease of MHC class I in monocytes infected by NCP and an increase in the case of CP (Archambault et al., 2000; Glew et al., 2003; Chase et al., 2004). Accordingly, T cell proliferative responses appear more lately in NCP BVDV infections than in CP (Collen and Morrison, 2000; Brackenbury et al., 2003).

In PI animals, infected monocytes do not seem to have affected his ability to present antigens, being able to stimulate responses of CD4+ and CD8+ lymphocytes (Archambault et al., 2000; Glew and Howard, 2001).

Th1/Th2 paradigm, postulated by Mosmann et al. (1986) from studies on cytokines produced by T lymphocytes in a murine model, is less well defined in ruminants (Estes and Brown, 2002). Th1 cytokines (IFNγ and IL-2) support MΦs activation, generation of cytotoxic T cells, induction of apoptosis

and production of opsonizing Abs, thereby enhancing resistance against viral infections (Biron and Sen, 2001; Samuel, 2001). These chemical mediators are produced by Natural killer (NK) cells, lymphocytes CD8+ and CD4+ Th1 in response to IL-12 (Hunter, 2005; Tizard, 2008). Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) stimulate the production of IgE by B cells that causes mast cell degranulation and activation of eosinophils, contributing to immune reactions in allergy and parasitic infections (Murphy and Reiner, 2002). IL-4 promotes the development of helper and cytotoxic T cells and the differentiation of immunoglobulins-producing plasma cells from B cells (Tizard, 2008). This cytokine is produced in response to antigen activation by CD4+ Th2 cells and some CD8+, NK1+ and γδT cells (Marcenaro et al., 2005). IL-10 is a regulatory cytokine with anti-inflammatory effects produced by Th cells in humans and cattle (Brown et al., 1994, 1998), inhibiting the activities initiated by proinflammatory cytokines (Biron and Sen, 2001; Pestka et al., 2004).

Studies on the type of immune response induced by BVDV do not provide consistent results. While some authors have demonstrated the establishment of a Th1 response (Howard et al., 1992; Charleston et al., 2002), others maintain that occurs a Th2 type immune response associated with a state of immunosuppression which might interfere with protective Th1 responses against other pathogens (Rhodes et al., 1999). Thus, experimental acute infections with NCP strains show an impaired IFNγ response against *Mycobacterium bovis* and bovine herpesvirus-1 (BHV-1) that would cause the inhibition of cellular immunity, diminishing host´s ability to contain these pathogens at the site of entry (Charleston et al., 2001b).

Humoral immune response

B lymphocytes are genetically programmed to recognize a particular antigen, multiply and differentiate giving rise to plasma cells that produce

large amounts of immunoglobulins. Humoral immunity may be caused by passive immunity for ingesting colostrum Abs or by an active immune response after exposure to antigen (Tizard, 2008).

High levels of maternal Abs can block B cell-mediated response to vaccination with BVDV (Ellis et al., 2001). However, the vaccination appears to be effective, protecting the animal against acute infections through a T cell-mediated and memory B cells response (Endsley et al., 2003; Ridpath et al., 2003).

Neutralizing Abs at the portal of entry are perhaps the most effective component of anti-viral immunity, since they can neutralize the virus and prevent their entry into the host. However, once the virus enters into the cell, cell-mediated immune response is critical for the defense against most viral infections. So, the disappearance of BVDV in acute infection cannot be attributed to the presence of specific Abs, which have a moderate and delayed response, not being detected until 2-4 weeks post-infection (Wilhelmsen et al., 1990; Archambault et al., 2000; Müller-Doblies et al., 2004).

4.2.3. Apoptosis

Apoptosis, or programmed cell death, can prevent the replication and spread of the viral infection, so many viruses have developed strategies to prevent this phenomenon. However, apoptosis might also facilitate virus dissemination and several viruses have developed potential mechanisms to activate the apoptotic pathway (Schweizer and Peterhans, 1999; Everett and McFadden, 1999).

During the BVD, there is a decrease in the number of lymphocytes of 50% in infections with strains of low virulence and 90% with high virulence strains (Ellis et al., 1998; Archambault et al., 2000; Ridpath et al., 2000; Stoffregen et al., 2000; Liebler-Tenorio et al., 2003a,b, 2004). Among T

lymphocytes subpopulations, the number of CD8+ is more reduced than the CD4+, with little affectation of the Tγδ circulating cells (Ellis et al., 1998; Brodersen and Kelling, 1999; Archambault et al., 2000). This lymphopenia correlates with infection and lesions in lymphoid tissues, since severe lymphoid depletion due to apoptosis is prominent in lymphoid follicles of Peyer's patches and, to a lesser extent, in thymic cortex (Marshall et al., 1996; Ellis et al., 1998; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2004; Pedrera et al., 2009a,b; Raya et al., 2011). It has not yet been clarified whether these lesions are induced directly by the virus or the immune response also contributes to their development. In this regard, different studies have attributed lymphoid depletion to the direct action of BVDV on lymphocytes (Wilhelmsen et al., 1990; Marshall et al., 1996; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2003a). However, other studies report that this apoptosis occurs subsequent to the elimination of viral antigen (Bolin and Ridpath, 1992; Marshall et al., 1996; Bruschke et al., 1998a; Liebler-Tenorio et al., 2003a, 2004), suggesting that cell death process would be mediated by CD4+ and CD8+ T lymphocytes (Hahn et al., 1995; Ellis and Yong, 1997; Liebler-Tenorio et al., 2003a).

It has also been indicated the existence of an indirect mechanism by which infected m-MΦs could play an important role in apoptosis of T lymphocytes through the release of pro-apoptotic cytokines (Zheng et al., 1995; Stennicke et al., 1998; Pedrera et al., 2009a), being the principal cause of lymphocyte apoptosis caused by other pestiviruses, such as CSFV (Gómez-Villamandos et al., 2001; Sánchez-Cordón et al., 2002, 2003, 2005). Thus, apoptosis of MΦs and epithelial cells induced by the release of type I IFN from MΦs infected with CP BVDV strains would contribute to the severe lesions observed in MD (Adler et al., 1997; Lambot et al., 1998; Perler et al., 2000). Other studies *in vitro* suggest the possibility that only the highly

virulent NCP strains could induce the production by MΦs of these pro-apoptotic factors (Chase et al., 2004), a fact that rules out the direct implication of virus replication in the lesions affecting lymphoid tissues (Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2003b).

There are two major regulatory pathways of apoptosis: the extrinsic pathway, which can be induced by members of the TNF family of cytokine receptors, associated with the cleavage and activation of caspase-8 (Rasper et al., 1998; Stennicke et al., 1998); and the intrinsic or mitochondria-dependent pathway, governed by Bcl-2 family proteins (Cory et al., 2003; Miller and Fox, 2004), which promotes the cleavage and activation of the caspase-9 (Li et al., 1997; Slee et al., 1999). Both pathways induce apoptosis via activation of effector caspases such as caspase-3, which, once activated, leads to irreversible cell death (Huppertz et al., 1999; Pedrera et al., 2009a).

The activation of initiator caspase-8 (extrinsic pathway) seems to play a major role in lymphocyte apoptosis within Peyer's patches during infection with NCP BVDV strains (Zheng et al., 1995; Stennicke et al., 1998; Pedrera et al., 2011). It has also been reported that the inactivation of caspase-9 accompanied by a moderate expression of the anti-apoptotic Bcl-2 protein dictates the resistance of MΦs to some apoptotic stimuli and favors the replication of BVDV within them (Levine et al., 1993; Reed, 2000; Pedrera et al., 2011). NCP BVDV replication undermines the biosynthetic functions of MΦs and impairs their immunological role as effector cells against virus infection (Pedrera et al., 2009a). Furthermore, other studies *in vitro* show that NCP strains are able to induce an apoptosis-inhibiting effect at the mitochondrial level in cell culture (Grummer et al., 2002b). This mechanism may be linked to the induction of *bcl-2* over-expression and the lack of increase in effector caspase-3, which would presumably favor the establishment of persistent infections (Bendfeldt et al., 2003).

CP strains of BVDV induce apoptosis in cell cultures (Lambot et al., 1998; Ridpath et al., 2006), although this phenomenon is not necessary for their replication (Schweizer and Peterhans, 1999). Studies realized on the mechanisms involved in the phenomenon of apoptosis indicate the activation of the intrinsic pathway (Grummer et al., 1998, 2002b), not ruling out the extrinsic (St-Louis et al., 2005), and pointing to the great presence of virus in cells infected with CP strains as the key factor for the induction of the intrinsic pathway (Vassilev and Donis, 2000).

Table 2.

Different mechanisms utilized by BVDV to induce a state of immunosuppression

BVDV immune evasion strategies	
Innate immunity	Decreased phagocytic activity
	Decreased chemotactic activity
	Decreased O_2^- synthesis
	Increased NO synthesis
	Stimulation of PGE2 synthesis
	Impaired TNFα response
	Inhibition of IL-1 synthesis
	Low type I IFN response to NCP strains
	Delayed increase of positive APPs
Adaptive immunity	Decreased expression of MHC I in m-MΦs by NCP strains
	Decreased expression of MHC II in m-MΦs
	Reduced proliferation of CD4+ T cells
	Impaired IFNγ response
	Delayed neutralizing antibodies response
Apoptosis	Lymphopenia
	Severe lymphoid depletion in lymphoid tissues
	Direct infection of lymphocytes
	Release of pro-apoptotic factors by infected MΦs
	Activation of extrinsic pathway by NCP strains
	Activation of intrinsic pathway by CP strains

4.3. Role of BVDV in the Bovine Respiratory Disease Complex (BRDC)

BRDC is characterized by a primary active viral infection with bovine respiratory viruses as BVDV, BHV-1, bovine respiratory syncytial virus (BRSV) and parainfluenza-3 (PI-3) that favors secondary bacterial infections. Bacterial pathogens implicated in this cattle pneumonia include *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni, Arcanobacterium pyogenes* and *Mycoplasma bovis* (Shahriar et al., 2002; Hodgson et al., 2005; Srikumaran et al., 2008; Fulton et al., 2009) (*Figure 3*). Several immune evasion strategies have been developed by these respiratory pathogens that help themselves and also the other agents to establish the infection, resulting in an exacerbation of the disease.

BVDV is considered as the main predisposing factor for the occurrence of this process through an alteration of the immune response, thus favoring the colonization by other pathogens (Potgieter, 1997; Srikumaran et al., 2008). In this regard, there is evidence that combined infections with BVDV have a potentiating effect on several pathogens, increasing in a more severe form the respiratory disease compared to calves infected only with rota- and coronavirus (Kelling et al., 2002; Niskanen et al., 2002), BHV-1 (Potgieter et al., 1984a; Castrucci et al., 1992), BRSV (Brodersen and Kelling, 1998) and *Mannheimia haemolytica* (Potgieter et al., 1984b, 1985; Gånheim et al., 2003).
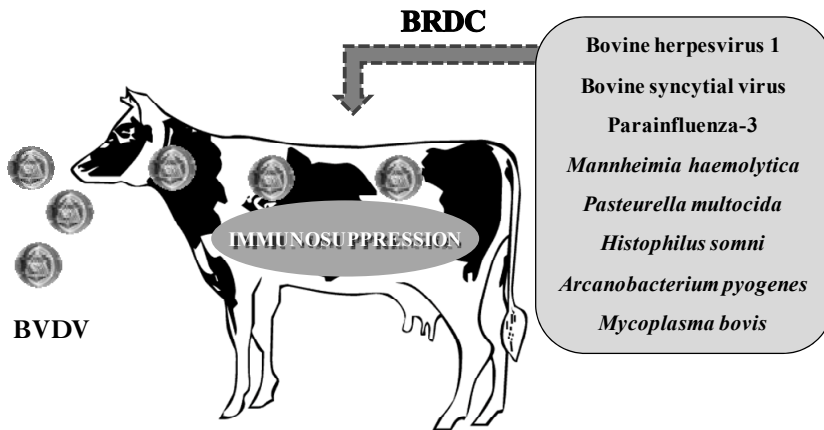
Figure 3. Scheme depicting the outcome of acute BVDV infection, where the animal is predisposed to suffer from BRDC upon infection with secondary viral and bacterial pathogens.

In these processes, the alteration of pulmonary MΦs activity by respiratory viruses enhances calves susceptibility against secondary infections, since these cells play an important role in nonspecific primary defense of the lung (Crystal, 1999; Zhang et al., 2000; Laskin et al., 2001), through their phagocytic, microbicidal and secretory functions (Nicod, 1999). Thus, it has been reported that BVDV infection of pulmonary alveolar MΦs can lead to a decrease in the expression of Fc receptor and complement C3 required for their phagocytic activity, reducing its antimicrobial activity and releasing chemotactic factors (Welsh et al., 1995; Adler et al., 1996; Liu et al., 1999; Glew et al., 2003; Peterhans et al., 2003).

The challenge for the animal in the BRDC is to initiate an innate immune response in order to defeat the virulence mechanisms utilized by the pathogens without eliciting extensive inflammation that can compromise lung function (Hodgson et al., 2005; Czuprynski, 2009). Indeed, inflammatory cytokines seem to play a central role in the lung injury produced during BRDC

since high levels of these mediators have been found in the airways of cattle infected with viruses or other respiratory pathogens (Rontved et al., 2000; Malazdrewich et al., 2001; Avraamides et al., 2007; Rivera-Rivas, 2009).

5. BHV-1 pathogenesis

BHV-1 is a member of the α-herpesvirinae subfamily classified in three subtypes, BHV-1.1, BHV-1.2a and BHV-1.2b, based on their antigenic and genomic differences (Fauquet et al., 2005; Muylkens et al., 2007). Subtype 1 is the causative agent of infectious bovine rhinotracheitis and the most frequently isolated in respiratory tract diseases or abortion cases (Oirschot, 1995; D'Arce et al., 2002).

The incubation period of BHV-1 is 2-6 days, appearing after the clinical symptoms. These signs include high fever, anorexia, coughing, excessive salivation, nasal discharge, conjunctivitis with lacrimal discharge, inflamed nares and sometimes dyspnoea. In the absence of bacterial pneumonia, recovery typically occurs 4-5 days after the onset of clinical symptoms.

Acute BHV-1 infection is initiated on mucosal surfaces and leads to high levels of virus in ocular, oral and nasal secretions, shedding virus until 7-10 days post-infection (Jones, 1998, 2003). BHV-1 has a special tropism for respiratory epithelial cells developing necrotic lesions in the nasal cavity, trachea, tonsils and bronchi that reduce the mucosal clearance due to mucous secretion and ciliary activities (Ohmann et al., 1991; Tikoo et al., 1995; Jubb and Kennedy, 2007). BHV-1 spreads into the infected animal using the local and systemic dissemination by viraemia and eventually neuroinvasion through the free enveloped particles or directly from cell-to-cell. BHV-1 neuroinvasion usually establishes a latent infection in ganglionic neurons (Schang and Jones, 1997; Inman et al., 2002; Muylkens et al., 2007), as well as in non-neural sites

as tonsils and lymph nodes (Mweene et al., 1996; Winkler et al., 2000; Perez et al., 2005).

During early stages after BHV-1 infection, IFN type I promotes leukocyte migration and increases NK cell activity stimulating cytolytic activities against virus-infected cells (Jensen and Schultz, 1990; Srikumaran et al., 2008).

BHV-1 can infect many different cell types in cattle, but the number of cells infected is small, and productive infection does not occur in most cells. Although the infected cells survive, their activity is affected (Jones and Chowdhruty, 2008). Thus, BHV-1 has evolved strategies to down-regulate expression of MHC class I molecules as a means to escape CD8+ lymphocytes recognition, key cells in the defense against cell-to-cell spread (Yewdell and Hill, 2002; Hewitt, 2003; Van Drunen Littel-van den Hurk, 2007). CD4+ T-cell function is also impaired during acute disease because BHV-1 infection of these cells carries out their apoptosis in peripheral blood and lymph nodes (Winkler et al., 1999). In addition to the destruction of infected cells, T-lymphocytes release a number of lymphokines such as IFNγ that modulate specific and non-specific immune response against BHV-1 (Jones and Chowdhruty, 2008).

Cattle infected with BHV-1 present a transitory immunosuppression that gives rise to secondary infections involved in the BRDC (Muylkens et al., 2007). In this regard, BHV-1 infections can diminish the activities of alveolar MΦs and polymorphonuclear neutrophils (Warren et al., 1996; Leite et al., 2004), as well as alter the profile of cytokines leading to inflammation and pneumonia (Ohmann et al., 1991; Muylkens et al., 2007).

Objectives

The main objective of this work was to contribute to the study of the immune-evasion strategies of BVDV and the different mechanisms by which primary BVDV infections enhance the susceptibility to secondary infections of the respiratory tract. For that, we have proposed the following partial aims:

1. To examine the response of cytokines in hepatic MΦs and their relationship with the APR during subclinical BVD: **Chapter 1**.

2. To evaluate and compare the clinical symptoms, severity of the pathological changes and antigen distribution between calves pre-infected with BVDV and challenged with other respiratory pathogen, such as BHV-1, versus others infected only with BHV-1: **Chapter 2**.

3. To estimate the effects of the pre-infection with BVDV in the pattern of cytokines and APPs implicated in the immune response against a secondary agent: **Chapter 3**.

4. To study the histopathological and ultrastructural changes in the lung of calves pre-infected with BVDV and challenged later with BHV-1, evaluating the role of MΦs and other immunocompetent cells in the development of pathological lesions in this organ: **Chapter 4**.

Materials and methods

1. Experimental designs

Based on the proposed objectives, we designed two experimental models: the first consisted of a single BVDV infection of colostrum-deprived calves with the aim of examine the immune response of hepatic MΦs and their relationship with the APR during subclinical BVD (**Experimental model 1**). Once valued the alterations in these cell populations and their influence on systemic response, our objective was to study the susceptibility of BVDV-infected calves against secondary infections. For that, it has been proposed a second experimental model that included a primary BVDV infection followed by a challenge with BHV-1.1 in order to reproduce the clinical and pathological symptoms associated with BRDC (**Experimental model 2**).

1.1. Experimental model 1

Ten colostrum-deprived male Friesian calves, aged 8-12 weeks, were used in this study. All calves were free of BVDV antigen and Abs by enzyme-linked immunosorbent assay (ELISA). Eight calves were each inoculated intranasally with 10 ml of NCP BVDV-1 strain 7443 (courtesy of the Institute für Virologie, TIHO, Hannover, Germany) containing 10^5 tissue culture infective dose 50% ($TCID_{50}$)/ml (Dean et al., 2003). This time point was

defined as day 0. Two animals used as uninfected (UI) controls received 10 ml of tissue culture fluid free of virus.

Animals were sedated with xylazine (Rompun® 2% solution; Bayer Healthcare, Kiel, Germany) and euthanased by overdosing with thiopental-sodium (Thiovet®; Vet Limited, Leyland, Lancashire, UK) in batches of two at 3, 6, 9 and 14 days post-inoculation (dpi). The two control animals were euthanased at the end of the experiment (*Figure 1*), which was approved by the University of Cordoba Ethics Committee (approval number 74/2006).
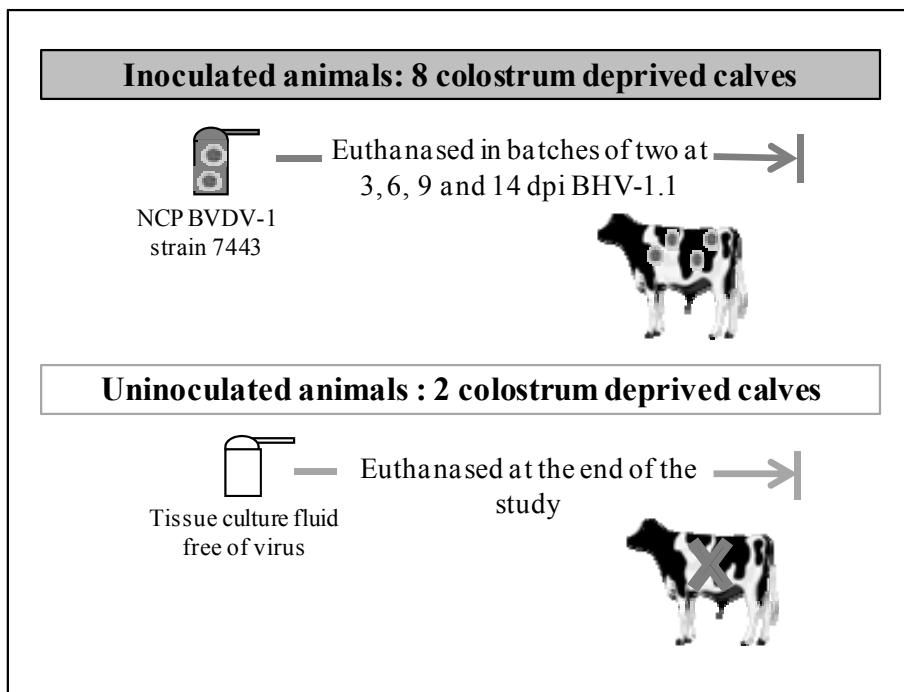
Figure 1. Schematic summary of experimental model 1.

EDTA blood and serum samples were collected from all calves before inoculation (day 0) to obtain baseline values. Samples from calves inoculated with BVDV were collected at 1, 2, 3, 5, 6, 8, 12, 13 and 14 dpi, and stored at -80ºC. At postmortem examination, samples of liver were fixed in 10% buffered formalin (pH 7.2), routinely processed and embedded in paraffin wax for histopathological and immunohistochemical studies.

1.2. Experimental model 2

Thirty male Friesian calves (8-9 months old) were obtained from a herd free of tuberculosis, brucellosis and bovine leucosis virus. The animals were tested to confirm their BVDV and BHV-1 antigen and antibody free status by ELISA. The calves were housed in the Animal Experimental Centre of Cordoba University (Spain), separated in three groups and inoculated as follows (*Figure 2*):

- BVDV/BHV1 group: 14 calves were inoculated in each nostril with 5 ml of a suspension of NCP BVDV-1 strain 7443 with a titration of 10^5 $TCID_{50}$/ml. Twelve days later, when the calves did not show clinical signs and viraemia against BVDV, 12 of them were challenged with 1 ml per nostril of BHV-1 subtype 1 (BHV-1.1) strain Iowa containing 10^7 $TCID_{50}$/ml (courtesy of Laboratorios Hipra). Animals were sedated with xylazine (Rompun 2% solution; Bayer Healthcare, Kiel, Germany) and euthanased by overdosing with thiopental-sodium (Thiovet; Vet Limited, Leyland, Lancashire, UK) in batches of two at 1, 2, 4, 7 and 14 dpi with BHV-1.1. The other 2 animals inoculated with BVDV-1 and BHV-1.1-free, were killed before BHV-1.1 inoculation (0 dpi) and used as BVDV infection controls.

- BHV1 group: 12 calves were only infected intranasally with 2 ml of BHV-1.1 and euthanased in batches of two at 1, 2, 4, 7 and 14 dpi.

- Negative control group: 4 calves received 2 ml of tissue culture fluid viruses-free and were killed at the end of the study (14 dpi BHV-1.1).

The entire experimental procedure was carried out in accordance with the Code of Practice for Housing and Care of Animals used in Scientific Procedures, approved by the European Economic Community in 1986 (86/609/EEC amended by the directive 2003/65/EC).
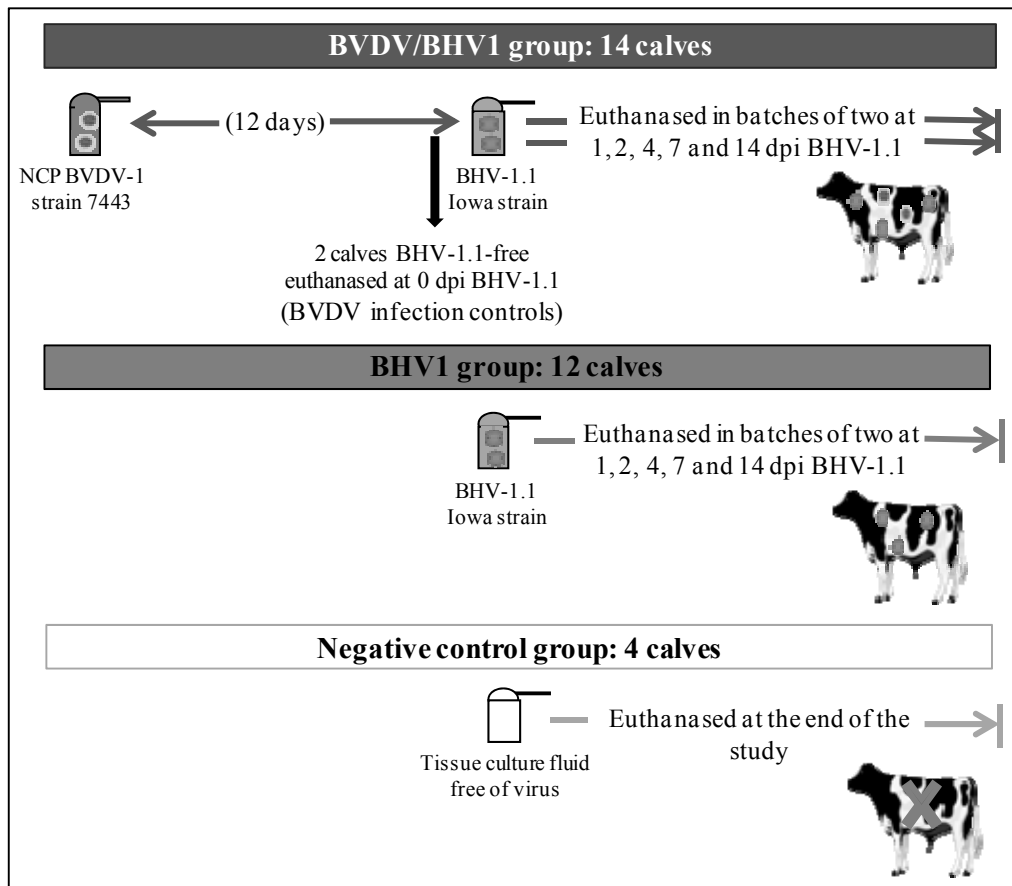
Figure 2. Schematic summary of experimental model 2.

Body temperature was recorded and clinical signs were assessed daily during the adjustment period and throughout the study. The clinical evaluation was carried out by a numerical score based on the sum of symptoms like depression, lacrimation, nasal discharge, cough, dyspnoea, nasal lesions and diarrhea valuated individually from 0 to 3, depending on the severity and specificity of the clinical sign.

EDTA blood obtained from coccygeal vein and nasal swabs samples were collected at 0, 1, 2, 4, 5, 7, 9 and 14 dpi and frozen at -80ºC until assayed. Blood samples without additive were taken at 0, 3, 6, 9, 12, 15, 18 and 21 hours post-inoculation (hpi), 1, 2, 4, 5, 7, 9 and 14 dpi. Blood was centrifuged at 4000 rpm for 10 min and the serum was separated and frozen in aliquots at -80ºC until assayed.

All euthanased calves were subjected to necropsy examination. Samples were collected from lymphoid tissues (retropharyngeal, tracheobronchial, mesenteric and ileocecal lymph nodes, pharingeal and lingual tonsils, thymus, spleen and bone marrow), respiratory tract (nasal mucosa, trachea, cranial and caudal lobes of the lung), digestive tract (liver, esophagus, duodenum, jejunum, distal ileum and ileocecal valve) and nervous system (anterior and posterior cerebrum, cerebellum, trigeminal ganglia, medulla oblongata and spinal cord). The collected tissue samples were immediately frozen at -80ºC for virological study; likewise, they were fixed in 10% buffered formalin solution for histopathological and immunohistochemical studies as well as in 2.5% glutaraldehyde in 0.1 M PBS for ultrastructural analysis.

2. Methods and techniques

2.1. Virological examination by Polymerase Chain Reaction (PCR)

BVDV RNA was extracted from EDTA blood and tissue samples using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) and the RNeasy Lipid tissue kit (Qiagen), respectively, according to the manufacturer's instructions. A one step Real-Time Reverse Transcription plus Polymerase Chain Reaction (RT-PCR) of the RNA was performed using the primers and Taqman probes (at the same concentration) based on conserved regions of the 5´-UTR of BVDV-1 described by Letellier and Kerkhofs (2003) and the Real Time Ready RNA Virus Master (Roche, Mannheim, Germany) following the manufacturer's instructions. The reactions were carried out in a LightCycler 1.5. Any sample that had a cycle threshold value less than or equal to 45 was considered as positive. The positive control was the NCP BVDV-1 strain 7443 at 10^5 TCID$_{50}$/ml.

BHV-1 deoxyribonucleic acid (DNA) was extracted from EDTA blood and nasal swabs samples using Genomic DNA Purification kit (Macherey-Nagel, Germany), and from tissue samples using Genomic DNA from tissue kit (Macherey-Nagel, Germany), according to the manufacturer's protocol. The real-time PCR analysis of the extracted DNA template was performed as describes the OIE Terrestrial Manual (OIE, 2010). Data were analyzed on an Applied 7300 detector (Applied Biosystems, USA). Any sample that had a cycle threshold value less than or equal to 45 was considered as positive. The positive control was the BHV-1.1 strain Iowa at $10^{8.3}$ TCID$_{50}$/ml.

2.2. Cytokines study

Serum cytokine levels were measured in duplicate by a sandwich ELISA that specifically detects soluble cytokine proteins. IL-1β, TNFα, IFNγ, IL-12, IL-4 and IL-10 protocols make use of commercially available monoclonal antibodies (mAbs) pairs (Serotec). Briefly, microplates (Nunc Maxisorb, Roskilde, Denmark) were coated with highly purified anti-cytokine Abs at 1 μg/ml in PBS (pH 7.5), except for the bovine IL-1β (2 μg/ml), and incubated at 4ºC overnight. After a blocking step with PBS, 2% Tween-20 and 3% BSA for 1 h at room temperature in agitation, the plates were washed 3 times with PBS/Tween-20 and incubated with the serum diluted 1:50 for 1 h at room temperature. Then, the plates were washed 3 times and incubated with secondary biotinylated Abs at 1 μg/ml, except IL-1β (2 μg/ml). This was followed by another washing step and addition of streptavidin–peroxidase (Sigma-Aldrich Química, Spain, 1:1000) for 45 min at room temperature. After a final wash, the chromogenic substrate (OPD) was added and absorbance values were measured spectrophotometrically using an ELISA-plate reader (Bio-Rad Laboratories, Spain) at 450 nm. Standard curves to calculate cytokine concentrations (ng/ml or pg/ml) were generated using recombinant bovine IL-1β, TNFα, IFNγ and IL-4 (Serotec). Because of the lack of commercial recombinant bovine IL-10 and IL-12, the results of these cytokines were presented as OD values.

2.3. APPs analysis

APPs were analyzed in duplicate in serum samples. Hp concentration was measured with a commercial spectrophotometrical assay based on the peroxidase activity of Hp-haemoglobin complex (Phase Haptoglobin assay, Tridelta). A commercial solid-phase sandwich ELISA was used to determine SAA concentrations (Phase Serum Amyloid A assay, Tridelta). Fibrinogen

serum concentration was measured with Biuret method (Total protein Kit, Spinreact SA), calculating the difference between total plasma protein and serum protein. Serum albumin was determined by the bromocresol green method (Albumin Kit, Spinreact SA). All these analysis were performed following the manufacturer's instructions.

2.4. Serum antibodies detection

The specific detection of BVDV Abs was tested in commercially available competitive ELISA, Ingezim BVD Compac (Ingenasa, Madrid, Spain), following the manufacturer's protocol.

Virus neutralization was applied for the evaluation of Ab response against BHV-1.1 Iowa strain, previously used for secondary infection. Briefly, the test protocol (OIE, 2010) was performed in 96-well microtitre plates with a 24 h virus/serum incubation period and Madin Darby Bovine Kidney cells (MDBK ATCC CCL-22). The plates were incubated for 4 days and the Ab titres were expressed as the reciprocal of the highest dilution that completely neutralized the virus effects in 50% of the wells. Any neutralization at an initial dilution titre of 1 or above was considered as positive.

2.5. Pathological and immunohistochemical studies

Formalin-fixed samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax by routine techniques for light microscopy. Samples were sectioned (3 µm) and stained by different methods as haematoxylin-eosin (HE) and the Fraser Lendrum technique, or processed for their immunohistochemical study using the avidin-biotin-peroxidase complex (ABC) method.

The type and location of histopathological lesions were graded as absent (-), mild (+), moderate (++) and severe (+++). For graphical representation and

statistical analysis of these findings, they were graded by a numerical score based on the sum of lesions valuated from 0 to 3, depending on their severity and specificity.

The ABC method for immunohistochemistry (IHC) was performed on serial sections of formalin-fixed samples, which were dewaxed and rehydrated as described previously Pedrera et al. (2009a,b). Briefly, endogenous peroxidase activity was exhausted by incubation with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The samples were subjected to different methods for antigen retrieval (*Table 1*). After pre-treatment, sections were rinsed three times in PBS pH 7.2 for 10 min and then covered with 1% normal horse serum (Pierce-Endogen, Woburn, USA) in 0.05M Tris buffer (pH 7.6) for 30 min at room temperature. After this blocking stage, sections were incubated with primary mAbs at 4ºC overnight. After primary incubation, the slides were washed in PBS (three times for 5 min each) and then incubated with biotinylated horse anti-mouse IgG secondary Ab (Pierce-Endogen), diluted in 0.05M Tris buffer containing 1% normal horse serum, for 30 min at room temperature. After three further 5 min washes in PBS, samples were incubated with the ABC complex (Vectastain® ABC Elite Kit, Vector Laboratories, CA, USA) for 1 h at room temperature. All tissue sections were finally rinsed in PBS and incubated with chromogen solution (NovaRED® Substrate Kit, Vector Laboratories). Slides were counterstained with Mayer's haematoxylin.

Details of the primary mAbs and polyclonal antibodies (pAbs) are summarized in *Table 1*.

Table 1. Detailed list of primary antibodies used in the immunohistochemical study.

Specificity	Antigen or cell detected	mAb/pAb	Dilution	Pre-treatment	Commercial origin
15c5	BVDV gp48	mAb	1:75	Proteinase K [a]	Dr Dubovi
F2	BHV-1 gC	mAb	1:1000	TC-microwave [b]	VMRD
Anti-human macrophage (MAC387)	Monocytes and macrophages	mAb	1:100	Protease [c]	Serotec
Anti-human CD79acy	B lymphocytes	mAb	1:25	TC-microwave [d]	DakoCytomation
Anti-human CD3	T lymphocytes	pAb	1:100	TC-microwave [b]	DakoCytomation
Anti-bovine CD4	Lymphocytes CD4+	mAb	1:10	TC-37°C [e]	Serotec
Anti-bovine CD8	Lymphocytes CD8+	mAb	1:25	TC-37°C [e]	Serotec
Anti-bovine WC1	Lymphocytes γδ	mAb	1:100	TC-37°C [e]	Serotec
Anti-bovine TNFα	TNFα	pAb	1:25	TC-microwave [b]	Serotec
Anti-human IL-1α	IL-1α	pAb	1:100	Tween-20 [f]	Endogen
Anti-bovine IFNγ	IFNγ	mAb	1:10	TC-microwave [g]	Serotec
Anti-murine iNOS/NOS Type II	iNOS/NOS Type II	pAb	1:100	TC-autoclave [h]	BD Transduction Lab.
Anti-murine COX-2	COX-2	pAb	1:75	TC-autoclave [h]	Cayman Chemical
Anti-Human Von Willebrand Factor	Von Willebrand Factor	pAb	1:800	Protease [c]	DakoCytomation

[a] Incubation with proteinase K (19,54 mg/ml, Roche) to 25 µg/ml in TRIS-HCl for 8 min at 37°C.
[b] Incubation with 0.1 M citric acid (pH 6), microwave for 5 min at sub-boiling temperature.
[c] Incubation with 0.1% protease type XIV (Sigma–Aldrich Chemie) in 0.01 M PBS, pH 7.2, for 10 min at room temperature.
[d] Incubation with 0.1 M citric acid (pH 6), microwave for 20 min at sub-boiling temperature.
[e] Incubation with 0.1 M citric acid (pH 3.2) for 30 min at 37°C in oven.
[f] Incubation with 0.1% Tween 20 (Merck) in 0.01 M PBS, p7.2, for 10 min at room temperature.
[g] Incubation with 0.1 M citric acid (pH 3.2), microwave for 5 min at sub-boiling temperature.
[h] Incubation with 0.1 M citric acid (pH 6) and autoclaved for 30 min at 121°C, 1 atm.

For gp48 detection by IHC, positive control tissues were from calves persistently infected with BVDV (kindly provided by Dr Dubovi, Cornell University, NY, USA) and for gC detection, positive controls were from abortion foetus samples positive to BHV-1.1 (courtesy of Moredum Research Institute, Scotland, UK), whilst negative control tissues were from specific pathogen-free calves not exposed to these viruses.

Tissues samples from cattle, in which reactivity for primary Abs against cytokines and cellular markers used in this study had been demonstrated, were used as positive controls in IHC (Pedrera et al., 2009a,b). Tissue sections for which the specific primary Abs were replaced by rabbit or mouse non-immune sera (DakoCytomation, Glostrup, Denmark) were used as negative controls.

Cell counting

To evaluate the number of immunolabelled cells and to correlate the results obtained using different Abs, two paraffin-wax blocks from liver and lung of each animal were selected. On tissue sections from these blocks, cell counts were carried out in 25-50 fields of 0.2 mm^2 chosen randomly. The results were given as the number of positive cells per 0.2 mm^2. Identification of different kind of immunolabelled cells was based on morphological features, location and size of the cells.

In the liver, circulating monocytes were located inside blood vessels and hepatic sinusoids and were round with a lobed nucleus and moderate amounts of cytoplasm. Küpffer cells (KCs) adherent to endothelial cells of sinusoids exhibited stellate morphology and had an indented nucleus with abundant cytoplasm. Interstitial MΦs (IMΦs) located among hepatocyte laminae, as well as in the interlobular connective tissue and in portal areas, exhibited rounded or elongated morphology, an indented nucleus and abundant cytoplasm.

In the lung, the different pulmonary MΦs exhibited rounded or elongated morphology, an indented nucleus and abundant cytoplasm. IMΦs, pulmonary intravascular MΦs (PIMs) and pulmonary alveolar MΦs (PAMs) are localized in distinct anatomical compartments of the lung, including connective tissue, adhered to endothelium in the pulmonary capillaries and air spaces, respectively. PIMs and IMΦs were grouped together and described as 'septal MΦs'.

A semiquantitative estimation of platelet aggregations Factor-VIII-positive were performed in 50 areas of 0.2 mm^2 chosen randomly. Results were given as presence of positive clusters of platelets per area as follows: absent (-), mild (+), moderate (++) and abundant (+++). For graphical representation and statistical analysis, they were scored from absent to severe (0 to 3).

Antigens distribution was valuated as absent (-), scarce (+), moderate (++), and intense (+++). The identification of target cells for both viruses was based on morphologic features, location and size of the cells.

2.6. Detection of apoptosis

Presence of apoptosis in formalin fixed samples was studied through the DNA fragmentation by a TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling staining) detection method (In situ Cell Death Detection kit, POD; Roche Diagnostics), according to the manufacturer's instructions. Internal positive control consisted on distal ileum sections with apoptotic lymphocytes from calves experimentally inoculated with BVDV-1 (Pedrera et al., 2009a). Negative controls were also included in each series of sections assayed.

2.7. Ultrastructural study

For transmission electron microscopy (TEM), glutaraldehyde-fixed samples were post-fixed in 2% osmium tetroxide, dehydrated in acetone and embedded in Epon 812® (Fluka Chemie AG, Buchs, Switzerland). Sections (50 nm) were counterstained with uranyl acetate and lead citrate, and examined with a Philips CM-10 transmission electron microscope.

2.8. Statistical analysis

Data were assessed to calculate mean ± standard error values and were analyzed with the SAS System for Windows, version 9.1 (SAS Institute, Cary, North Carolina, USA). Duncan's Multiple Range Test ($p<0.05$) was used to analyze significant differences of the values in the same group at various time points (*) and non-paired Student's *t*-test ($p<0.05$) was used between both inoculated groups at the same time point (**).

Chapter 1.

Hepatic immune response in calves during acute subclinical infection
with bovine viral diarrhea virus type 1

Hepatic immune response in calves during acute subclinical infection with bovine viral diarrhea virus type 1

Abstract

Eight colostrum-deprived calves aged 8-12 weeks were inoculated intranasally with a NCP strain of BVDV-1 and the effects on the hepatic immune response were studied. Two calves were sacrificed at each of 3, 6, 9 and 14 dpi and two UI animals were used as negative controls. BVDV was detected in hepatic MΦs and monocytes from 3 to 14 dpi and in KCs from 6 to 14 dpi. Increases in the numbers of MAC387+ KCs and monocytes, but not IMΦs, differentiated by morphological features, were evident in the liver following inoculation with BVDV. There was a substantial increase in the number of monocytes positive for TNFα, but only small increases in the numbers of TNF-α+ KCs and IMΦs and IL-6+ monocytes, KCs and IMΦs. There was an increase in the number of interstitial CD3+ T lymphocytes in the liver, but no substantial changes in the numbers of circulating CD3+ T lymphocytes, interstitial or circulating CD4+ or CD8+ T lymphocytes, or CD79αcy+ B lymphocytes. Serum Hp and SAA increased transiently at 12 dpi. Upregulation of some pro-inflammatory cytokines by hepatic MΦs is evident in subclinical acute BVDV-1 infection in calves.

Introduction

BVDV has been classified into two genotypes (genotypes 1 and 2) and NCP and CP biotypes (Ridpath et al., 1994; Fulton et al., 2003a). NCP strains, including NCP genotype 1 strains, produce mild disease in seronegative and immunocompetent infected cattle, characterized by a short febrile period, transient leucopenia and lymphoid depletion (Wilhelmsen et al., 1990; Hamers et al., 2000; Walz et al., 2001). BVDV exhibits tropism for lymphoid tissues, with m-MΦs, DCs and lymphocyte populations being the main target cells (Bruschke et al., 1998b; Liebler-Tenorio et al., 2003b).

To date, the liver has not been considered to be an important organ in the pathogenesis of BVDV. However, this organ plays a crucial role in the inflammatory response to infectious and toxic agents, as well as in the synthesis of APPs. Hepatic MΦs are important in the pathogenesis of liver disease, especially in viral hepatitis (Sánchez-Cordón et al., 2008). KCs, a resident population of hepatic phagocytes derived from circulating monocytes and located within the sinusoidal vascular space, represent the first line of defense against viruses entering the liver through the portal circulation (Gómez-Villamandos et al., 1995; Cabillic et al., 2006).

During viral infection, KCs exhibit phagocytic and biosynthetic changes characterized by the release of pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6 (Bilzer et al., 2006). These cytokines play an important role in the regulation of the immune response and stimulate the synthesis of APPs by hepatocytes (Heinrich et al., 1990; Gabay and Kushner, 1999). These proteins can be classified as 'positive' or 'negative' according to the magnitude of their increase or decrease in serum concentrations during the APR. The APR is an early non-specific mechanism against local or general disturbances in homeostasis attributable to several stimuli, such as infection, inflammation,

stress or tissue injury (*Figure 1*). Hp and SAA are considered to be the main positive APPs in cattle, while albumin is considered to be a negative APP (Eckersall, 2000; Petersen et al., 2004).

APPs can be used as non-specific markers of clinical and subclinical disease in cattle. The expression of APPs during BVDV infections in cattle has been described by Gånheim et al. (2003) and Müller-Doblies et al. (2004). However, production of cytokines by the hepatic MΦs population and their relationship with serum APPs during BVDV infection has not been investigated yet.
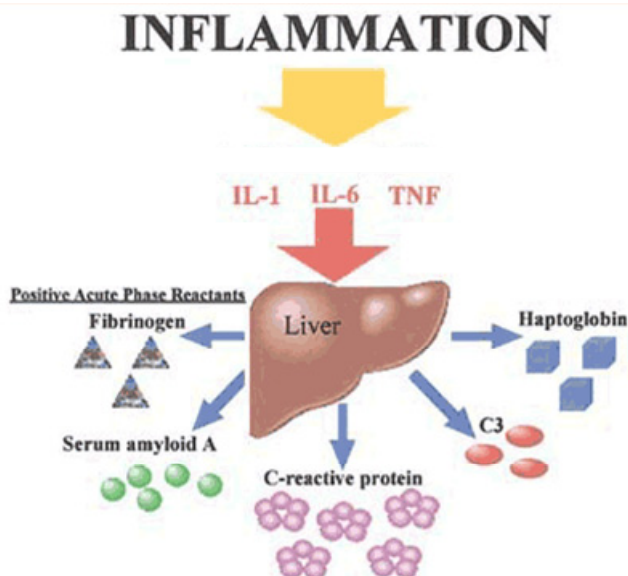
Figure 1. Stimulation and synthesis of positive acute-phase reactants during inflammation. Inflammation caused by infection or tissue damage stimulates the circulating inflammation-associated cytokines, including IL-1, IL-6, and TNFα. These cytokines stimulate hepatocytes to increase the synthesis and release of positive APPs.

With this objective, eight colostrum-deprived calves aged 8-12 weeks were inoculated intranasally with the NCP strain 7443 of bovine BVDV-1 and the effects on the hepatic immune response were studied. Two calves were sacrificed at each of 3, 6, 9 and 14 dpi and two UI animals were used as negative controls. The presence of BVDV was detected in liver by RT-PCR and IHC. The hepatic immune response was analyzed through immunohistochemical study (MAC387, TNFα, IL-1α, IL-6, CD3, CD4, CD8, CD79αcy and IFNγ). Serum APP concentrations (Hp, SAA and albumin) were determined by ELISA and colorimetric methods *(See Materials and methods section, page 57. Experimental model 1).*

Results

Clinical signs, haematological and serological findings, virus detection in blood by antigen ELISA and conventional RT-PCR after experimental infection with BVDV have been described by Pedrera et al. (2009b). No macroscopic lesions were found in the livers of inoculated calves.

Detection of bovine viral diarrhea virus

BVDV was detected in the livers of inoculated calves by RT-PCR from 3 dpi onwards. Small numbers of monocytes and some IMΦs were positive for BVDV antigen (gp48) by IHC from 3 to 14 dpi, whereas small numbers of gp48-positive KCs were observed from 6 to 14 dpi (*Figures 2 and 3*).
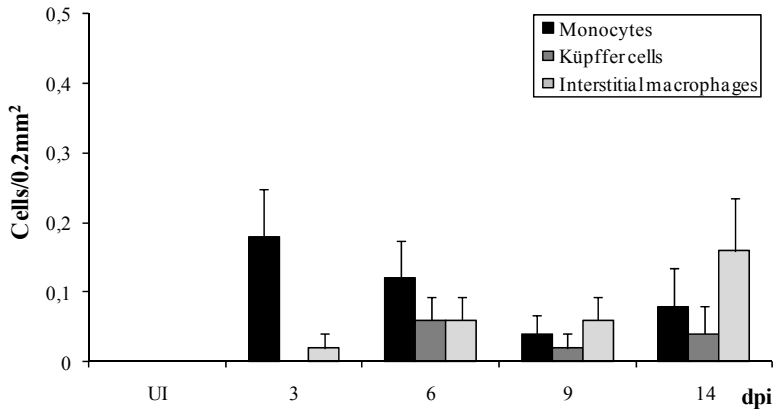
Figure 2. Numbers of hepatic MΦs (mean ± standard error) positive for BVDV antigen (gp48) in the livers of UI animals and animals inoculated with BVDV.
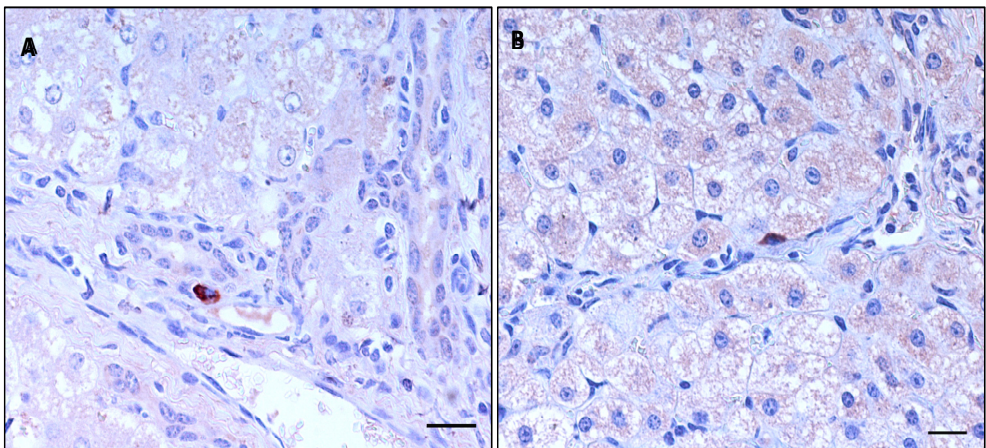
Figure 3. IHC for BVDV antigen in calves inoculated with BVDV. (A) IMΦ positive for BVDV gp48 at 3 dpi. Bar = 30 µm. (B) KC positive for BVDV gp48 at 6 dpi. Bar = 30 µm.

Histopathology and immunohistochemistry

On histopathological examination, moderate oedema, with dilatation of lymphatic vessels, was evident in portal areas at 3 dpi (*Figure 4A*). There were increased numbers of mononuclear cells (*Figure 4B*) composed mainly of MAC387+ MΦs (*Figure 4C*), CD3+ T lymphocytes (*Figure 4D*) and a few interspersed CD79αcy+ B lymphocytes. From 9 dpi onwards, oedema was observed in fewer portal areas, while cellular infiltrates were reduced in size.
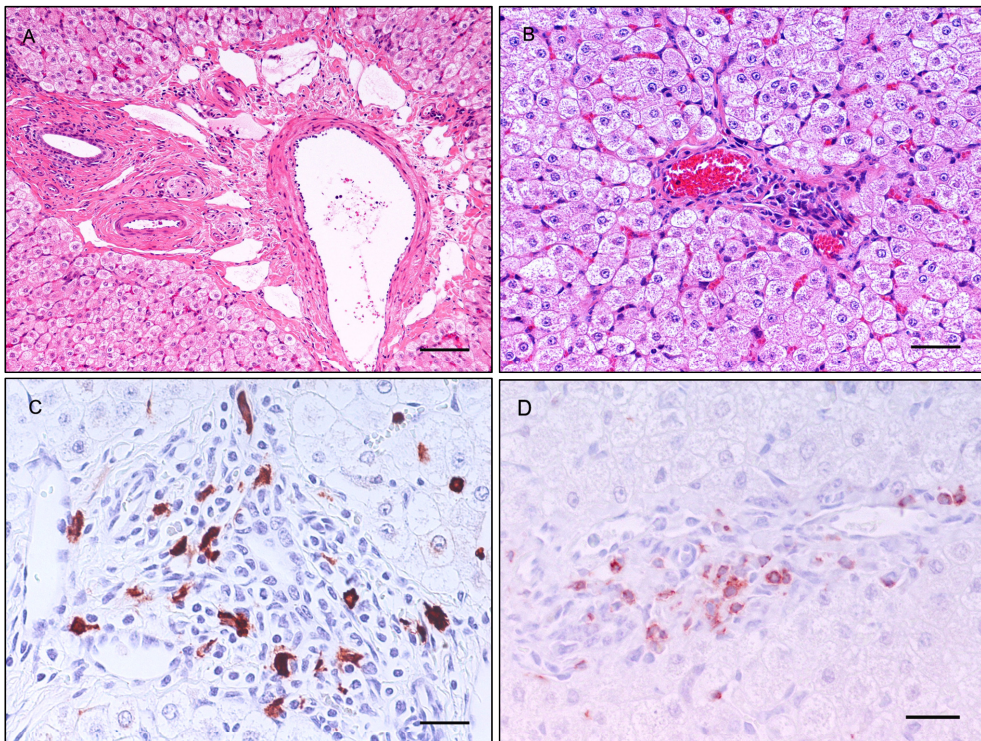
Figure 4. Histopathology (HE) and IHC at 3 dpi in the livers of calves infected with BVDV. (A) Periportal oedema and dilatation of lymphatic vessels. HE. Bar = 130 μm. (B) Mononuclear cells in the periportal area. HE. Bar = 65 μm. (C) Mononuclear cells, mainly MΦs, immunolabelled against MAC387 in the periportal area. IHC. Bar = 40 μm. (D) CD3+ T lymphocytes in the cluster of mononuclear cells in the periportal area. IHC. Bar = 30 μm.

Monocytes, KCs and IMΦs were MAC387+ and were differentiated on the basis of location and histomorphology, whereas lymphocytes were negative for MAC387. Numbers of monocytes increased from 3 to 14 dpi in calves inoculated with BVDV compared to UI calves, while numbers of KCs increased from 3 to 6 dpi and there were few changes in the numbers of IMΦs (*Figure 6*).

Following inoculation with BVDV, there was a substantial increase in the numbers of monocytes positive for TNFα, but only small increases in the numbers of TNFα+ KCs and IMΦs (*Figures 5 and 6*). Monocytes exhibited positive staining for IL-1α following inoculation with BVDV, but KCs and IMΦs were negative. There were mild increases in numbers of IL-6+ monocytes, KCs and IMΦs at 9 dpi (*Figure 6*).
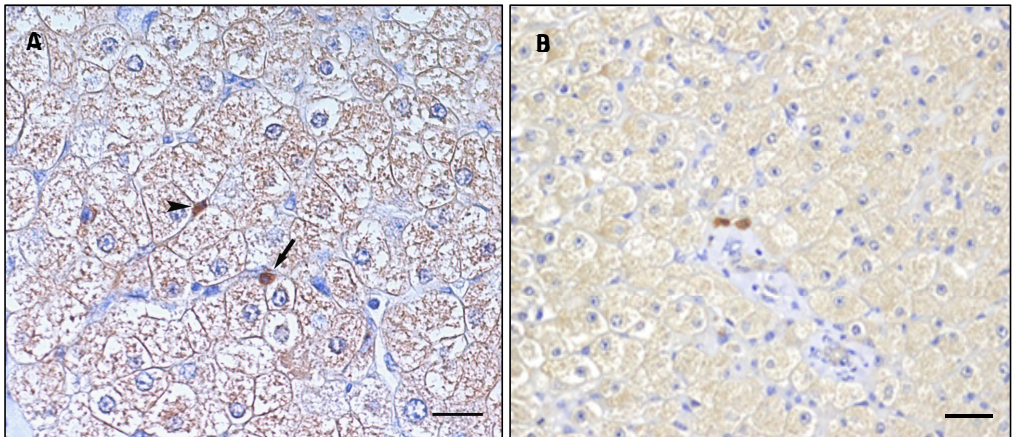
Figure 5. (A) Monocyte (arrow) and KC (arrowhead) positive for TNFα at 9 dpi. Bar = 30 µm. (B) IMΦs positive for TNFα at 9 dpi. Bar = 40 µm.
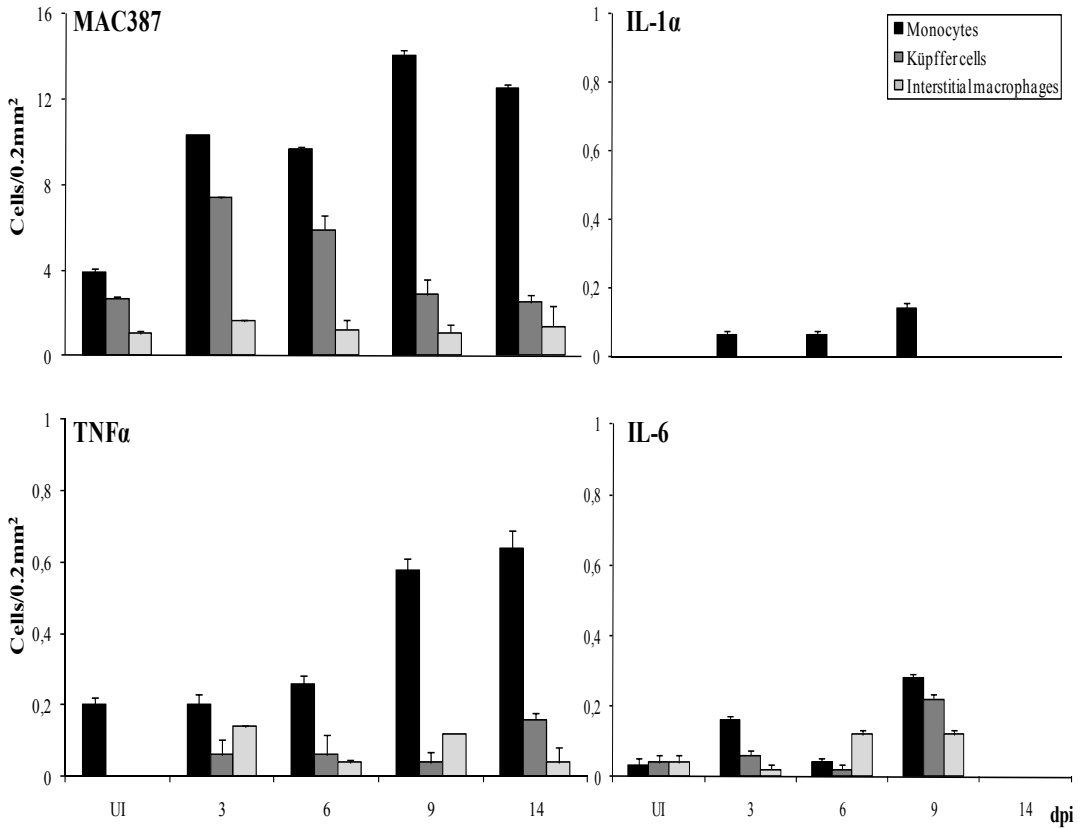
Figure 6. Numbers of monocytes, KCs and IMΦs (mean ± standard error) positive for MAC387, TNFα, IL-1α and IL-6 by IHC in the liver of UI animals and animals inoculated with BVDV.

There was an increase in the numbers of interstitial CD3+ T lymphocytes from 3 to 9 dpi, mainly in periportal areas, but little change in the numbers of circulating CD3+ T lymphocytes in the liver. Numbers of circulating γδ+ T lymphocytes increased from 6 to 9 dpi, but no changes were observed in the numbers of interstitial or circulating CD4+ or CD8+ T lymphocytes, interstitial γδ+ T lymphocytes or interstitial or circulating CD79αcy+ B lymphocytes. There were no consistent changes in the numbers of IFNγ+ lymphocytes (*Figure 7*).
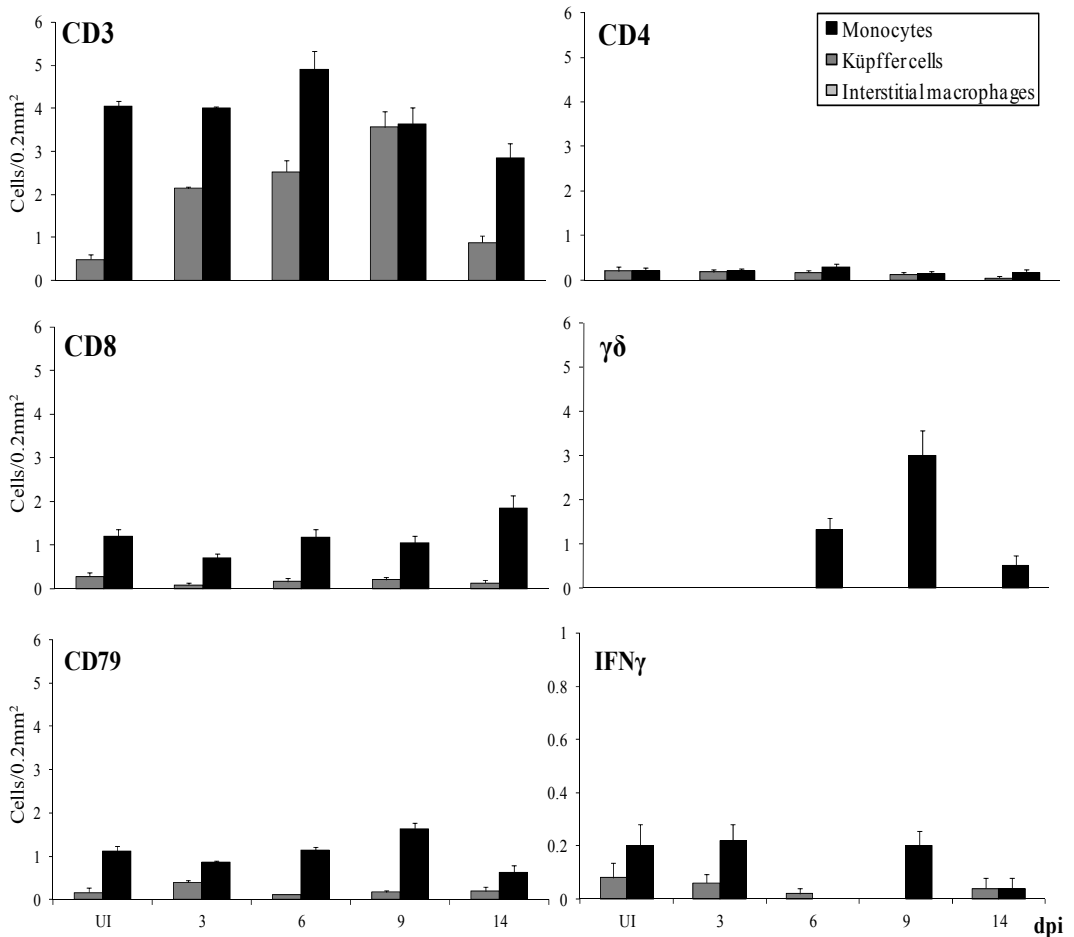
Figure 7. Numbers (mean ± standard error) of interstitial and circulating CD3+ T lymphocytes, CD4+ T lymphocytes, CD8+ T lymphocytes, γδ+ T lymphocytes, CD79αcy+ B lymphocytes and interferon-γ+ lymphocytes in the livers of calves inoculated with BVDV and UI animals.

Acute phase proteins

Calves inoculated with BVDV had mild but variable increases in serum Hp and progressive increases in SAA from 3 to 9 dpi. From a mean pre-inoculation value of 0.09 g/l, the concentration of Hp peaked at 0.16 g/l at 12 dpi, before returning to normal levels. SAA increased from a mean pre-

inoculation concentration of 15.8 µg/ml to 60.3 µg/ml at 12 dpi, and then decreased to 4.2 µg/ml by 14 dpi. There were few changes in the concentrations of albumin during the experiment (*Figure 8*).
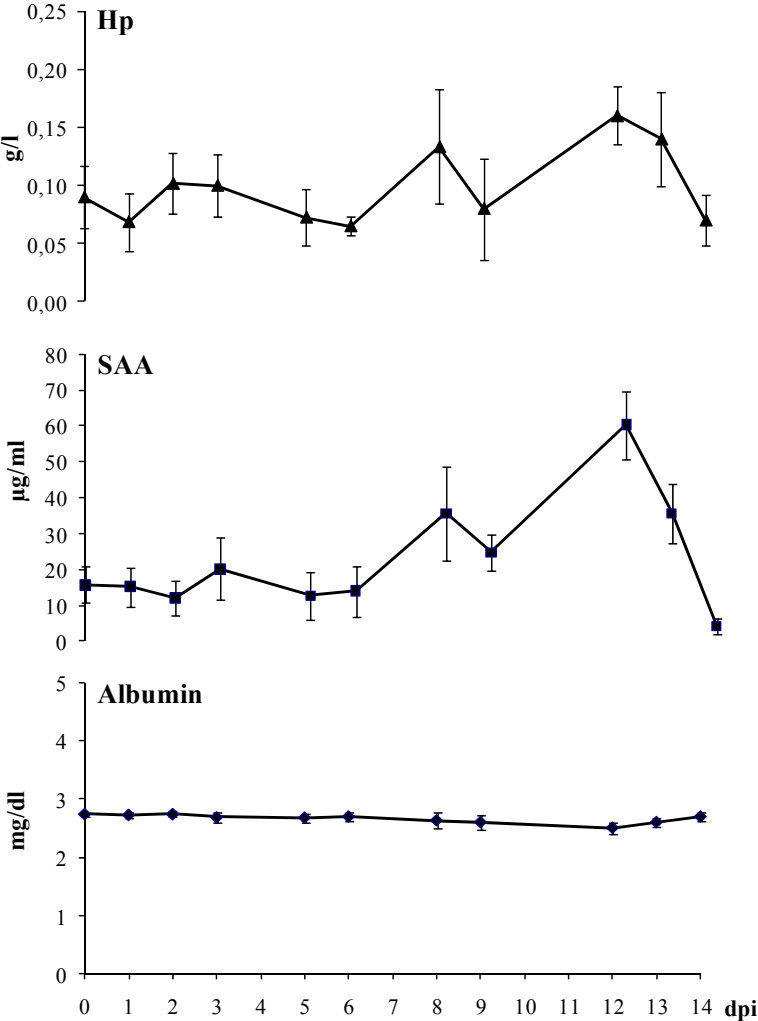
Figure 8. Serum concentrations (mean ± standard error) of Hp, SAA and albumin in calves before and after inoculation with BVDV.

Discussion

Inoculation of calves with NCP BVDV-1 strain 7443 results in subclinical infection, with lesions of moderate severity confined mainly to the digestive and lymphoid systems, with limited expression of cytokines at these sites (Da Silva et al., 2007; Pedrera et al., 2009a; Raya et al., 2011). In the present study, histopathological examination of the livers of calves inoculated with BVDV strain 7443 revealed vascular changes in portal areas, along with clusters of mononuclear cells composed mainly of MAC387+ MΦs and CD3+ T lymphocytes. There were increases in the numbers of KCs, monocytes and interstitial CD3+ T lymphocytes in the liver. A substantial increase in the number of monocytes positive for TNFα was observed, while the numbers of TNFα+ KCs and IMΦs were moderately increased.

The presence of some IMΦs positive for BVDV by IHC from 3 dpi was accompanied by a local increase in T lymphocytes and MΦs in portal areas. This increase in numbers of mononuclear cells in periportal areas could be due to immune complex deposition in the basement membranes of vessel walls, infection of endothelial cells or release of chemical mediators (Hewicker et al., 1987; Liebler-Tenorio et al., 2003b). The first two mechanisms are considered to be unlikely, since there was no evidence of vasculitis or endothelial cell damage or infection.

Instead, the release of chemotactic mediators may account for increases in lymphocytes and MΦs in portal areas in BVDV infected calves (Iwai et al., 2003; Fainboim et al., 2007). A moderate increase in immunoreactivity for TNFα was observed in IMΦs, coinciding with detection of BVDV gp48 in these cells, but there were few changes in expression of IL-1. The increase in numbers of TNFα+ IMΦs corresponded with an increase in the numbers of T lymphocytes in portal areas. However, since there were no changes in the

numbers of IFNγ+ cells following infection with BVDV, findings were not consistent with a TNFα-modulated Th1 response. Inhibition of antiviral function associated with IFNγ has been reported in previous BVDV studies (Rhodes et al., 1999; Schweizer and Peterhans, 2001; Lee et al., 2008).

KCs are able to produce chemical mediators with pro-inflammatory properties and contribute to the cell-mediated immune response by presenting antigens to T lymphocytes (Bilzer et al., 2006; Kolios et al., 2006). In the present study, small increases in the numbers of TNFα+ and IL-6+ KCs were observed in the livers of calves inoculated with BVDV. However, the pro-inflammatory capacity of TNFα would be limited by the low number of IL-1α-producing cells, since these two cytokines act synergistically (Le and Vilcek, 1987; Van Reeth et al., 1999). Impaired production of cytokines during BVDV infection has been reported *in vitro* (Adler et al., 1996; Yamane et al., 2005; Lee et al., 2008) and *in vivo* in the small intestine, the main target organ (Pedrera et al., 2009a).

Increased serum concentrations of SAA, a type 1 APP, are induced predominantly by IL-1 and TNFα acting synergistically with IL-6, whereas increased serum concentrations of Hp, a type 2 APP, are induced predominantly by IL-6 (Petersen et al., 2004). Changes in serum concentrations of some APPs, such as Hp, occur more slowly, whereas others, such as SAA, exhibit a faster response; the combined use of both APPs is recommended in cattle to distinguish between acute and chronic inflammatory processes (Horadagoda et al., 1999).

In our study, there was a progressive and therefore late increase in serum concentrations of SAA during subclinical infection with BVDV strain NCP 7443, but few changes in serum concentrations of Hp, which only peaked at 12 dpi. Similar findings have been reported in other studies using BVDV NCP strains (Gånheim et al., 2003; Müller-Doblies et al., 2004) and

may reflect the lack of activation of KCs early in the course of BVDV infection (Heinrich et al., 1990; Knolle et al., 1995; Gabay and Kushner, 1999). The relationship between elevated levels of pro-inflammatory cytokines and the onset of an intense early APR has been demonstrated in infections caused by other pestiviruses, such as classical swine fever virus, in which pigs exhibit severe clinical signs accompanied by high serum pro-inflammatory cytokine concentrations and secretory activation of KCs (Núñez et al., 2005; Sánchez-Cordón et al., 2007).

Conclusions

Increases in the numbers of KCs, monocytes and interstitial CD3+ T lymphocytes are evident in the liver during subclinical infection of calves with BVDV-1 NCP strain 7443. These changes are accompanied by increased immunohistochemical expression of TNFα in hepatic MΦs and increased serum concentrations of SAA and Hp. These findings indicate that there is upregulation of some pro-inflammatory cytokines in hepatic MΦs during subclinical BVDV infection in calves.

Chapter 2.

Comparative study between healthy and subclinical BVD-infected calves challenged with BHV-1: lesions and viral antigen distribution

Comparative study between healthy and subclinical BVD-infected calves challenged with BHV-1: lesions and viral antigen distribution

Abstract

As it is known BVDV is one of the pathogens involved in the BRDC, being able to produce disease on its own and, which is perhaps more important, it also can predispose calves to infections by other micro-organisms. The aim of this work was to carry out a detailed and sequential study in tissues of the effects of BVDV pre-infection in calves challenged with another respiratory viral pathogen, such as BHV-1. For it, we studied lesions and viral antigen distribution by IHC in healthy calves and calves with subclinical BVD, both experimentally inoculated with BHV-1. The results obtained indicate that compared with their healthy counterparts, calves with subclinical BVD displayed the earlier development of more severe inflammatory processes, leading to a worsening of tissue lesions – limited to lymphoid tissues, respiratory and digestive tracts– and more spread of BHV-1. This data suggest that BVDV facilitates the establishment of BHV-1, thereby potentiating its pathogenic action and increasing host susceptibility to other infections. Furthermore, the presence of BHV-1 favors the persistence of BVDV in target organs, which was

detected during very late stages of the disease, revealing a synergic effect of both agents.

Introduction

Virus and bacteria co-infections in cattle are considered key factors in the aetiology of respiratory disease (Fulton et al., 2002; Shahriar et al., 2002). As it is known BVDV is one of the pathogens involved in the BRDC, being able to produce disease on its own and, which is perhaps more important, it also can predispose calves to infections by other micro-organisms (Potgieter, 1997; Peterhans et al., 2003; Risalde et al., 2011b-*Chapter 3*-). Experimental inoculation with BVDV of low virulence demonstrates that even infections that have a subclinical course and would go unnoticed under field conditions can cause a marked, although transient, immunosuppression (Liebler-Tenorio et al., 2003a; Pedrera et al., 2009b). This explains why combined infections with BVDV have a potentiating effect on several pathogens, increasing the severity of rota- and coronavirus, (Kelling et al., 2002; Niskanen et al., 2002), BHV-1 and BRSV infections (Castrucci et al., 1992; Brodersen and Kelling, 1998). Furthermore, sequential inoculation of calves with BVDV and *Mannheimia haemolytica* also increased the severity of lung lesions (Gånheim et al., 2003; Fulton et al., 2009).

Based on antigenic and genetic differences, cattle pestivirus isolates can be classified into two genotypes, BVDV-1 and BVDV-2, which are divided in NCP and CP biotypes, depending on their effect on cell cultures (Ridpath et al., 1994; Fulton et al., 2003a). BVDV displays a special tropism for the mucosa-associated lymphoid tissue (tonsils and intestine) and lymphoid organs, appearing m-MΦs, DCs and lymphocytes as the main virus target cells (Bruschke et al., 1998b; Liebler-Tenorio et al., 2003b).

BHV-1 is an important cattle pathogen with worldwide distribution that causes significant economical losses in the bovine industry. This virus is an α-herpesvirinae classified in three subtypes: BHV-1.1, BHV-1.2a and BHV-1.2b, based on antigenic and genomic analysis. BHV-1.1 isolates are the causative agents of infectious bovine rhinotracheitis and are found in the respiratory tract as well as in aborted fetuses (Fauquet et al., 2005; Muylkens et al., 2007). Moreover, this pathogen can establish latent or persistent infections in ganglionic neurons and in non-neural sites as tonsils and lymph nodes (Mweene et al., 1996; Winkler et al., 2000; Perez et al., 2005).

To the authors' knowledge, a detailed and sequential study in tissues of the effects of BVDV pre-infection in calves experimentally inoculated with a respiratory viral pathogen has not been performed yet. So, the purposes of this study were: (1) to evaluate the clinical symptoms of calves co-infected with BVDV and BHV-1 and compare them with those of calves infected only with BHV-1.1; (2) to characterize and evaluate the severity of the pathological changes occurring in both inoculated groups; and (3) to determine whether BHV-1.1 antigen distribution is affected by BVDV pre-infection.

For it, fourteen calves were inoculated intranasally with the NCP strain 7443 of BVDV-1 and twelve days later, when the calves did not show clinical signs and viraemia against BVDV, twelve of them were challenged with BHV-1.1 Iowa strain (BVDV/BHV1 group). The other 2 animals inoculated with BVDV-1 and BHV-1.1-free, were killed before BHV-1.1 inoculation (0 dpi) and used as BVDV infection controls. Twelve calves were only inoculated with the BHV-1.1 (BHV1 group). The infected calves were sacrificed in batches of two at 1, 2, 4, 7 and 14 dpi. Four UI animals were used as controls and sacrificed at the end of the study (negative control group).

Clinical examinations were performed daily. Blood and serum samples were collected at 0, 3, 6, 9, 12, 15, 18 and 21 hpi, 1, 2, 4, 5, 7, 9, 12 and 14 dpi. The presence of BVDV and BHV-1.1 in blood was assessed by PCR. At post-mortem examination, samples collected from lymphoid tissues, respiratory tract, digestive tract and nervous system were fixed and routinely processed for histopathological and ultrastructural examination. Identification of BVDV and BHV-1.1 surface glycoproteins, gp48 and gC respectively, was carried out through immunohistochemical study in formalin fixed samples *(See Materials and methods section, page 57. Experimental model 2).*

Results

Assessment of clinical symptoms and post-mortem lesions

The calves in the control group remained clinically unaffected throughout the study. In both inoculated groups, the calves had elevated body temperature and their general appearance was affected in varying degrees. Calves of the BHV1 group had a slightly affected appearance, showing depression, lacrimation and serous nasal discharge, mainly between 7 and 9 dpi ($p < 0.0001$). These calves had a significant elevation of the rectal temperature at 5 and 11 dpi (39.6 ∘C; $p < 0.0001$). Animals of the BVDV/BHV1 group displayed more significant severe clinical symptoms, including cough, mucopurulent nasal discharge, dyspnoea, open-mouth breathing, nasal lesions and recurrent diarrhea between 4 and 11 dpi ($p < 0.0001$). Moreover, the animals of this group showed more significant increase of the rectal temperature at 4 and 5 dpi BHV-1.1 (>40 ∘C; $p < 0.0001$) (*Figure 1*).
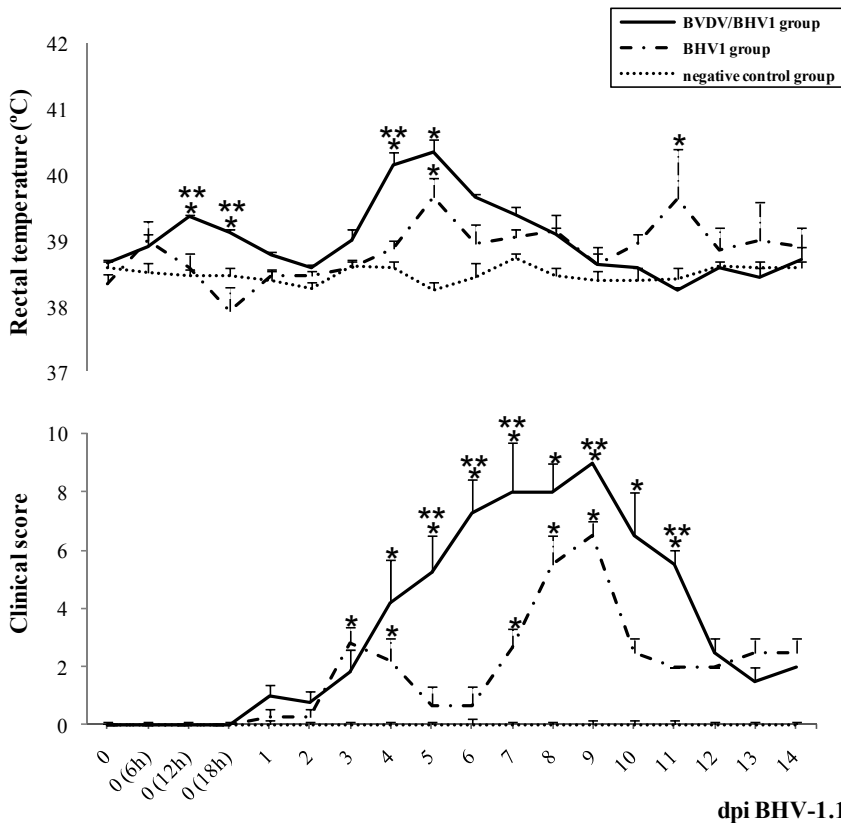
Figure 1. Means of the rectal temperature (ºC) and clinical score values of calves inoculated with BHV-1.1 versus co-infected with BVDV and BHV-1.1., and UI control animals (0, pre-inoculation values; h, h post-inoculation with BHV-1.1; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Pathological study

There were no remarkable gross or histological lesions in calves of the negative control group. In both inoculated groups, the histological lesions were limited to the digestive tract, respiratory and lymphoid tissues. Digestive lesions were observed in BVDV-inoculated animals which displayed severe depleted lymphoid follicles in the Peyer's patches of the ileum and ileocecal valve throughout the study. In both inoculated groups, the main lesions in the respiratory tract and lymphoid tissues were inflammatory alterations

characterized by mononuclear infiltrate composed of MΦs, lymphocytes and plasmatic cells associated with vascular changes. The most severe inflammatory changes were observed in the BVDV/BHV1 group between 4 and 7 dpi (2.03 and 1.83 score values, respectively; $p<0.0001$), presenting significant differences in magnitude with respect to BHV1 group, except at 7 dpi, due to a peak in the single infection (1.4 score value) (*Figure 2*).
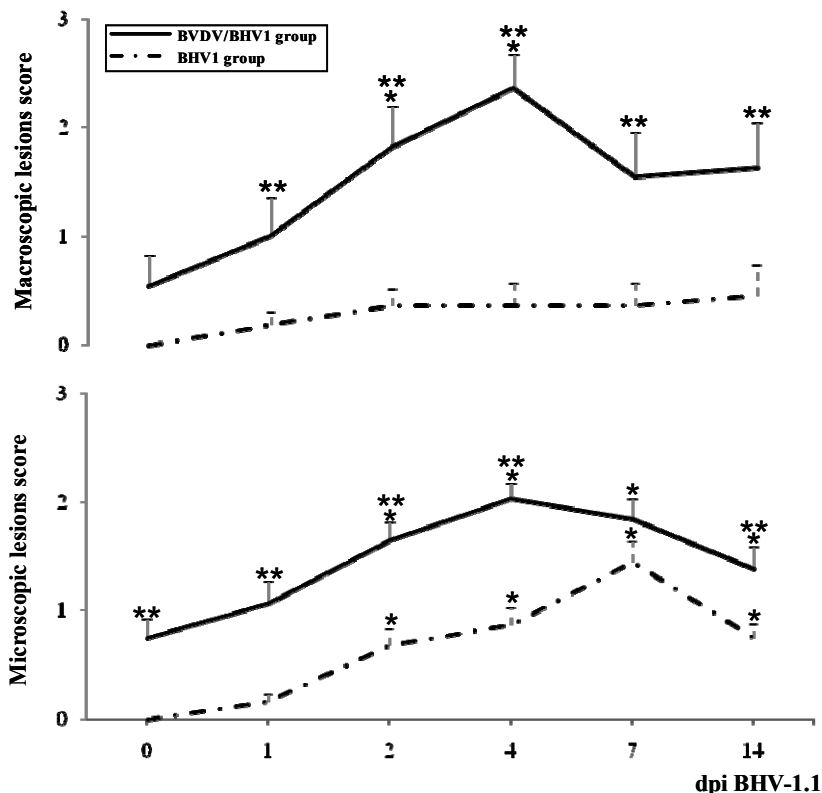
Figure 2. Means of the gross and microscopic lesions score values of calves inoculated with BHV-1.1 versus BVDV/BHV1 group calves. (0, BHV-1.1 UI animals; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Type, location and evolution of these lesions in both inoculated groups are summarized in *Tables 1* and *2*.

Lymphoid tissues

Both infected groups had large lymph nodes with petechial haemorrhages that were more evident over the course of the study, being the lesions more intense in retropharyngeal lymph nodes. Additionally, BVDV/BHV1 group showed petechial haemorrhages in lingual tonsil, congestive splenomegaly and atrophic bone marrow.

Microscopically, the tonsils displayed hyperaemia, petechial haemorrhages within lymphoid follicles and dense accumulations of leukocytes in epithelium with occasional ulceration, mainly in the co-infected calves. From 2 dpi onwards, these animals showed progressive depleted lymphoid follicles together with the presence of pyknosis, cellular fragmentation and MΦs showing phagocyted cell debris (tingible bodies MΦs), characteristic of apoptosis TUNEL-positive (*Figure 3A*). Moreover, between 4 and 7 dpi, we observed focal necrosis in epithelium, tonsil crypts and lymphoid follicles, together with an inflammatory response of infiltrated MΦs, lymphocytes and neutrophils. Two types of intranuclear inclusion bodies (IIBs) were seen in epithelial cells of the margins of necrotic lesions: slightly basophilic IIBs occupying the entire nucleus and small eosinophilic IIBs (*Figure 6C*).

The thymic lesions appeared before BHV-1.1 inoculation and were more evident in the BVDV/BHV1 group. Thymic cortex of co-infected calves was reduced in size due to the marked depletion of lymphocytes associated with apoptotic images TUNEL-positive, increased visible stellate cells and proliferation of adjacent interlobular stroma (*Figure 3B*).
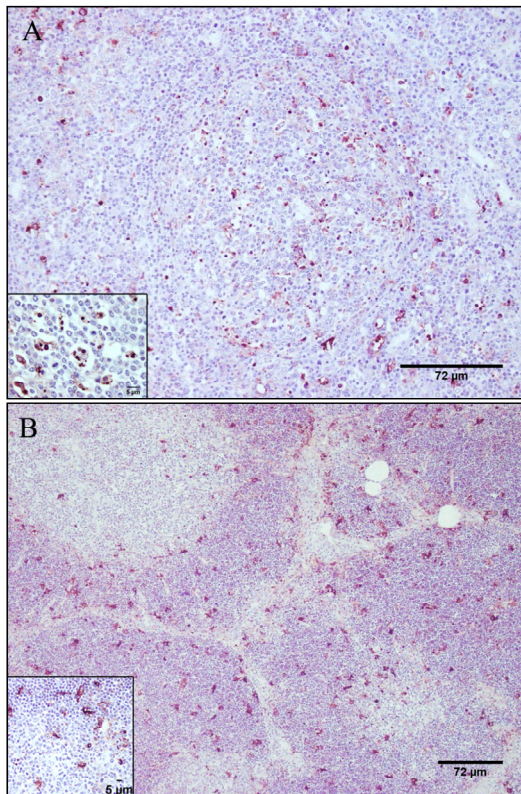
Figure 3. (A) Lymphoid follicles of the pharyngeal tonsil with free and phagocyted apoptotic bodies TUNEL-positives in BVDV/BHV1 group at 14 dpi. (B) Pyknotic lymphocyte-like cells and apoptotic bodies TUNEL-positives in the thymic cortex of co-infected animals at 7 dpi.

Microscopic examination confirmed the congestive splenomegaly, characterized by dilated splenic sinuses with packed red cells and widely separated germinal centres. From 2 dpi, both inoculated groups displayed a mild to moderate depopulation of follicular cells and apoptosis within germinal centres. Although the vascular changes were more intense in the animals of the BVDV/BHV1 group, there were no apparent differences between both groups in the rest of lesions described.

In the bone marrow of BVDV-infected animals, there was a severe hypoplasia caused by the lack of myeloid progenitor cells (*Figure 4A*).

Hypoplasia signs in the bone marrow were almost inexistent in the BHV1 group (*Figure 4B*).

Vascular alterations as hyperaemia and petechial haemorrhages within lymphoid follicles were seen in lymph nodes of all inoculated calves. After BHV-1.1 inoculation, the lymph nodes showed an intense hyperplasia that was more evident over the course of the disease, mainly in the BVDV/BHV1 group. Thus, in early stages, there was a follicular and paracortical hyperplasia due to the increased number and size of the follicles, together with the intense scattering of lymphocytes. Sinus hyperplasia was produced by a migration of lymphocytes and MΦs occluding the lumen (sinus catarrh) (*Figure 4C,D*). Retropharyngeal lymph nodes displayed the most important alterations.

Respiratory tract

The gross respiratory lesions described were almost unapparent in the BHV1 group and mild in the co-infected group, consisting in congestion of the nasal mucosa after BHV-1.1 inoculation and occasional mucosal tracheitis in later stages of the study. The ulcerated lesion of the nares appeared only in BVDV/BHV1 group.

Histologically, the epithelial lesions were described as necrotic ulcerations, accompanied by connective tissue proliferation and mononuclear infiltrate in the dermis. Eosinophilic IIBs appeared in epithelial cells of the margins of necrotic lesions.

The upper respiratory tract of infected calves showed hyperaemia in lamina propria, besides diffuse and periglandular infiltrate, composed of MΦs, lymphocytes and plasmatic cells. Some epithelial syncytia with basophilic IIBs were seen in nasal mucosa of the BVDV/BHV1 group (*Figure 4E*).

Inflammatory changes were also observed microscopically in the lung after BHV-1.1 infection. In both inoculated groups, pulmonary parenchyma

was affected by interstitial pneumonia with alveolar septal thickening produced by interstitial aggregates of lymphocytes, MΦs and, to a lesser extent, neutrophils. This alteration appeared earlier and in a more severe form in the BVDV/BHV1 group, associated with occasional alveolar oedema and haemorrhages (*Figure 4E,F*). Moreover, in these animals were observed sporadic epithelial syncytia in pulmonary alveoli between 2 and 7 dpi.

Digestive tract

Gross lesions in the alimentary tract were observed in BVDV-infected calves. Mucosa of the small intestine showed congestion and oedema from the start of the experiment. These changes increased after BHV-1.1 infection and were accompanied by an intense inflammation of the biliary vesicle and petechial haemorrhages in colon.

Microscopically, the ileum showed hyperaemia, sporadic haemorrhages in interfollicular areas and mononuclear infiltrate in lamina propria throughout the study. Ileal submucosa displayed intense dilation of lymphatic vessels with migration of lymphocytes, accompanied by depleted lymphoid follicles in the Peyer's patches. This lesion was characterized by the reduction in follicle size due to severe lymphoid depletion, as well as infiltrated MΦs and enlarged stellate cells. Moreover, we observed pyknosis, cellular fragmentation and tingible body MΦs TUNEL-positives in the interfollicular lymphoid tissue.

The most characteristic lesion found in the ileocecal valve was a proliferation of Lieberkühn crypts resulting in crypthyperplasia. Affected crypts were dilated and filled with mucus, epithelial debris and inflammatory cells. Some of these crypts appear herniated into the submucosal space previously occupied by involuted lymphoid follicles.

Microscopic examination of the liver in the infected animals showed acute inflammation signs after BHV-1.1 infection. Vascular changes as

hyperaemia and sporadic oedema in the portal triads and dilation of lymphatic vessels were more intense between 2 and 7 dpi. Evidence of mononuclear infiltrate in the portal tracts and occasionally in the hepatic parenchyma, together with an increased number of leukocytes in sinusoids (sinusoidal leukocytosis) was seen from 2 dpi in both inoculated groups.

Nervous system

There were no apparent gross or histological lesions in the sections of nervous tissue analyzed in this study.

Table 1

Macroscopic lesions observed at postmortem examination.

	UI	BVDV control	1 dpi		2 dpi		4 dpi		7 dpi		14 dpi	
			BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1
LYMPHOID TISSUES												
Enlarged lymph nodes	-	+	+	+	+	++	++	+++	++	+++	+++	+++
Petechial haemorrhages in lymph nodes	-	+	+	++	+	++	+	+	-	+	-	-
Petechial haemorrhages in lingual tonsil	-	-	-	+	-	++	-	++	-	-	-	-
Congestive splenomegaly	-	-	-	+++	-	++	-	++	+	++	+	+++
Atrofic bone marrow	-	+++	-	+++	+	+++	-	+++	-	+++	-	+++
RESPIRATORY TRACT												
Congestive nasal mucosa	-	-	-	-	+	+++	+	+++	-	-	-	-
Ulcerated nares	-	-	-	-	-	-	-	-	-	+++	-	++
Mucosal tracheitis	-	-	-	-	-	-	-	+++	-	-	+	++
DIGESTIVE TRACT	-											
Congestive and oedematous small intestinal mucosa	-	+	-	+	-	+++	-	+++	-	++	-	++
Congestive and oedematous biliary vesicle mucosa	-	-	-	-	-	+++	-	+++	-	-	-	-
Petechial haemorrhages in colon	-	-	-	-	-	-	-	+++	-	+++	-	+++

(-) no gross lesion; (+) mild; (++) moderate; (+++) severe.

Means of the gross lesions valuation (n = 2 per time point) of calves inoculated with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1. (UI, uninoculated animals of the negative control group; BVDV control, calves inoculated with BVDV-1 and BHV-1.1-free).

Table 2. Type and location of histopathological findings after BHV-1.1 inoculation.

	UI	BVDV control	1 dpi BHV1	1 dpi BVDV/BHV1	2 dpi BHV1	2 dpi BVDV/BHV1	4 dpi BHV1	4 dpi BVDV/BHV1	7 dpi BHV1	7 dpi BVDV/BHV1	14 dpi BHV1	14 dpi BVDV/BHV1
LYMPHOID TISSUES												
Tonsils												
Hyperaemia	-	-	-	-	-	+	++	+	+++	+	++	+
Petechial haemorrhages	-	-	-	-	-	+++	-	++	-	-	-	+
Apoptosis in lymphoid follicles	-	-	-	-	-	+	+	++	+	++	-	+++
Focal necrosis	-	-	-	-	-	-	-	+++	-	+++	-	-
Thymus												
Hyperaemia	-	+	+	++	+	-	+	-	++	-	+	-
Petechial haemorrhages	-	+	-	+	+	+	++	++	-	-	-	-
Lymphoid depletion	-	++	-	++	-	++	-	+++	-	+++	+	++
Apoptosis	-	+	-	+	-	++	-	++	+	+++	+	+
Spleen												
Hyperaemia	-	-	-	+++	-	++	-	++	+	++	+	+++
Lymphoid depletion	-	-	-	-	+	++	+	+	++	+	++	-
Apoptosis	-	-	-	-	+	-	+	+	+	++	++	++
Bone marrow												
Hypoplasia	-	+++	+	+++	+	+++	+	+++	+	+++	+	+++
Lymph nodes												
Hyperaemia	-	-	-	-	-	++	+	++	+	++	-	+
Petechial haemorrhages	-	++	+	++	+	++	-	++	-	+	+	+
Apoptosis in lymphoid follicles	-	+	-	+	-	+	+	+	+	++	+	+++
Hyperplasia	-	-	+	++	+	++	++	+++	++	+++	++	+++
RESPIRATORY TRACT												
Upper respiratory tract												
Hyperaemia	-	-	-	-	++	++	+	++	-	-	-	-
Mononuclear infiltrate	-	-	+	++	+	++	++	++	++	++	++	++
Lung												
Hyperaemia	-	+	-	+	++	++	++	++	+	++	+	++
Haemorrhages	-	-	-	-	-	++	-	++	-	+	-	+
Interstitial pneumonia	-	-	-	+	+	++	++	+++	+	+++	+	+
DIGESTIVE TRACT												
Ileum												
Hyperaemia	-	+	-	++	-	++	-	++	-	++	-	+
Haemorrhages	-	-	-	-	-	-	-	+	-	++	-	+
Lymphoid depletion	-	+++	-	+++	+	+++	+	+++	+	+++	+	+++
Lymphatic vessels dilation	-	+++	-	+++	-	++	-	++	-	+++	-	+++
Mononuclear infiltrate	-	++	-	++	-	++	-	++	-	+++	-	+
Ileocecal valve												
Hyperaemia	-	+	-	++	-	+++	-	+++	-	++	-	-
Proliferation Lieberkühn's crypts	-	+	-	+	-	+++	-	+++	-	++	-	-
Liver												
Hyperaemia	-	-	-	-	++	-	+++	++	+++	-	+	-
Periportal mononuclear infiltrate	-	-	-	-	++	++	+	++	+	++	+	++
Sinusoidal leucocytosis	-	-	-	-	+++	+	++	++	++	++	+	++

(-) no gross lesion; (+) mild; (++) moderate; (+++) severe.
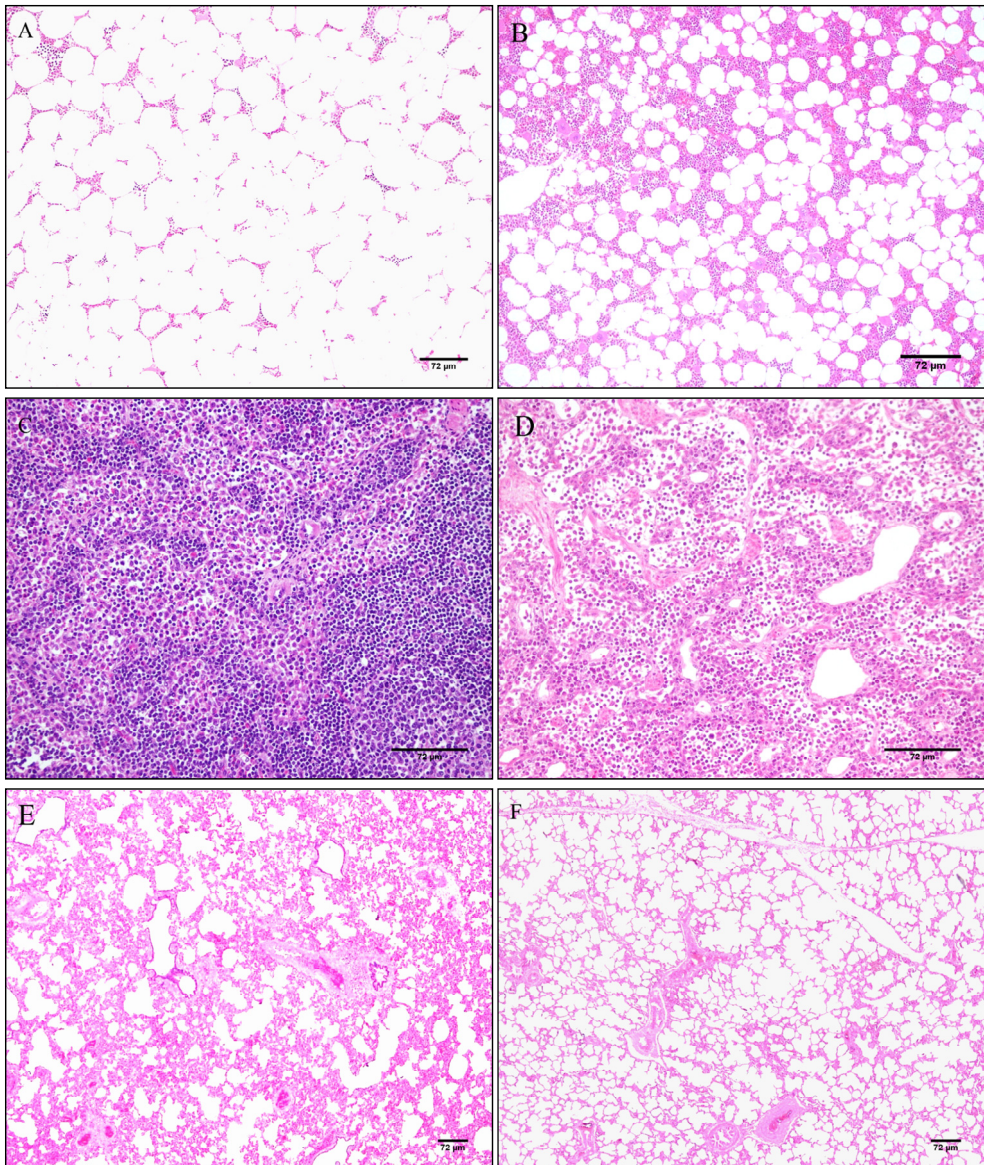
Figure 4. HE. Severe hypoplasia of the bone marrow caused by the lack of myeloid progenitor cells in BVDV/BHV1 group at 4 dpi (A), compared with an almost inexistent lesion in the BHV1 group (B). Sinus hyperplasia in retropharyngeal lymph node of the BVDV/BHV1 group produced by lymphocytes and MΦs occluding the lumen (sinus catarrh) together with an intense scattering of lymphocytes at 4 dpi (C) versus a mild lesion observed in the single infection (D). Lung of the BVDV/BHV1 group showing interstitial pneumonia with alveolar septal thickening at 7 dpi (E), compared with minor changes in the BHV1 group (F).

Virological examination

Polymerase Chain Reaction (PCR)

There was no evidence of any viral infection from blood samples in the animals of the negative control group. BVDV was not detected by RT-PCR in the blood of calves of the BVDV/BHV1 group at the moment of BHV-1.1 inoculation (12 dpi BVDV). However, BVDV was detected in the blood of these calves between 1 and 5 dpi, showing only significant values at 1 and 2 dpi BHV-1.1 (p=0.046). The presence of BHV-1.1 was confirmed in both inoculated groups by PCR of nasal swabs samples from 1 dpi onwards, detecting only viraemia in the BVDV/BHV1 group from 4 dpi onwards and peaking at 7 dpi BHV-1.1 ($10^{1.94}$ $TCID_{50}$/ml) (*Figure 5*).
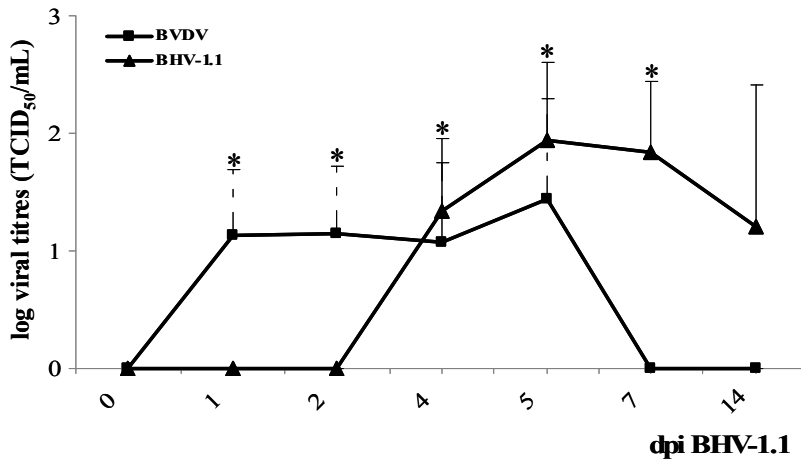
Figure 5. Mean of viruses titres in the blood of BVDV/BHV1 group (0, BHV-1.1 pre-inoculation values; dpi, days post-infection with BHV-1.1; *p<0.05 significant differences in the group at various time points).

Immunohistochemistry

In positive control samples, gp48 and gC labelling appeared as evenly distributed dark red granules or a diffuse cytoplasmic staining. There was no labelling of any tissue when BHV-1 or BVDV-specific Abs were replaced by murine non-immune serum.

Neither BVDV nor BHV-1 antigens were detected in tissue sections from negative control calves. The distribution of both viral antigens, principally associated to lymphoid tissues and intestinal mucosa of inoculated animals, is represented in *Table 3*.

BVDV antigen was only detectable in the BVDV/BHV1 group, being present in lymphoid tissues, specifically in the thymus and ileocecal lymph node, as well as mucosa-associated lymphoid tissue of the ileum and ileocecal valve. Gp48 was not seen in the respiratory tract, central nervous system, upper digestive tract, liver, spleen and bone marrow of any of the BVDV-infected animals.

In the thymus of the BVDV inoculated animals, viral gp48 was detected throughout the experiment, being scarce its presence at 14 dpi BHV-1.1. Positive staining was associated with reticular epithelial cells, MΦs and occasional lymphocytes of the thymic cortex as well as with fibroblasts of the adjacent stroma (*Figure 6A*).

In the ileocecal lymph nodes, a great presence of BVDV antigen was observed until 4 dpi BHV-1.1. The widest viral distribution was seen in the medullary sinuses where the antigen was confined to the cytoplasm of MΦs, stellate-like cells and some lymphocytes. In one calf, viral gp48 was detected in the tunica media of muscular arteries of the retropharyngeal lymph node, associated with lymphocytic arteritis.

BVDV antigen was present in great amounts within depleted lymphoid follicles and interfollicular areas in ileal Peyer's patches until 4 dpi. In this

period, MΦs and stellate cells as well as some lymphocytes, fibroblasts and apoptotic bodies were positive to virus (*Figure 6B*). Thereafter, we observed a gradual decrease of the labelled cells towards the end of the study, where viral gp48 was only detected in MΦs.

BHV-1 antigen distribution was mainly confined to ulcerations in the nares, and both pharingeal and lingual tonsils of BVDV/BHV1 group at 4 and 7 dpi. Viral gC was observed in epithelial and lymphoid necrotic areas, as well as in cell debris within tonsil crypts. The target cells of the BHV-1.1 were epithelial cells and, to a lesser extent, lymphocytes and MΦs (*Figure 6D*).

Transmission electron microscopy

Ultrastructural study revealed BHV-1-like particles and virus-like replication sites in epithelial cells of the tonsil and nasal necrotic lesions, pulmonary alveolar MΦs and necrotic cells in alveoli of the BVDV/BHV1 group calves. Intranuclear viral particles measured 105 to 115 nm in diameter, were hexagonal in shape and showed variable electron-density in the cores. Other intracellular changes indicative of viral infection were margination of chromatin, intranuclear aggregation of small particles and intranuclear filamentous structures with concentric distribution. Moreover, enveloped (200 to 250 nm in diameter) and non-enveloped virions were found in the cytoplasm of infected cells (*Figure 6F*).

Table 3. Organ and tissue distribution of BVDV and BHV1 antigens.

	UI	BVDV control	1 dpi BHV1	1 dpi BVDV/BHV1	2 dpi BHV1	2 dpi BVDV/BHV1	4 dpi BHV1	4 dpi BVDV/BHV1	7 dpi BHV1	7 dpi BVDV/BHV1	14 dpi BHV1	14 dpi BVDV/BHV1
BVDV (gp48) DETECTION												
Lymphoid tissues												
Pharyngeal tonsil	-	-	-	-	-	-	-	+	-	-	-	-
Lingual tonsil	-	-	-	-	-	-	-	-	-	-	-	-
Thymus	-	+++	-	+++	-	++	-	++	-	++	-	+
Spleen	-	-	-	-	-	-	-	-	-	-	-	-
Bone marrow	-	-	-	-	-	-	-	-	-	-	-	-
Retropharyngeal lymph node	-	-	-	-	-	-	-	-	-	+	-	-
Tracheobronchial lymph node	-	-	-	-	-	-	-	-	-	-	-	-
Mesenteric lymph node	-	-	-	-	-	-	-	-	-	-	-	-
Ileocecal lymph node	-	+++	-	+++	-	+++	-	++	-	-	-	-
Digestive tract												
Liver	-	-	-	-	-	-	-	-	-	-	-	-
Esophagus	-	-	-	-	-	-	-	-	-	-	-	-
Duodenum	-	-	-	-	-	-	-	-	-	-	-	-
Jejunum	-	-	-	-	-	-	-	-	-	-	-	-
Ileum	-	+++	-	+++	-	++	-	++	-	+	-	+
Ileocecal valve	-	+	-	+	-	-	-	-	-	-	-	-
BHV1 (gC) DETECTION												
Lymphoid tissues												
Pharyngeal tonsil	-	-	-	-	-	-	+	+++	-	+	-	-
Lingual tonsil	-	-	-	-	-	-	-	+	-	+++	-	-
Thymus	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-	-	-	-	-
Bone marrow	-	-	-	-	-	-	-	-	-	-	-	-
Retropharyngeal lymph node	-	-	-	-	-	-	-	-	-	-	-	-
Tracheobronchial lymph node	-	-	-	-	-	-	-	-	-	-	-	-
Mesenteric lymph node	-	-	-	-	-	-	-	-	-	-	-	-
Ileocecal lymph node	-	-	-	-	-	-	-	-	-	-	-	-

(-) absent; (+) scarce; (++) moderate; (+++) intense.

Means of the antigen distribution assessment in tissues (n = 2 per time point) of calves inoculated with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1. (UI, uninoculated animals of the negative control group; BVDV control, calves inoculated with BVDV-1 and BHV-1.1-free).
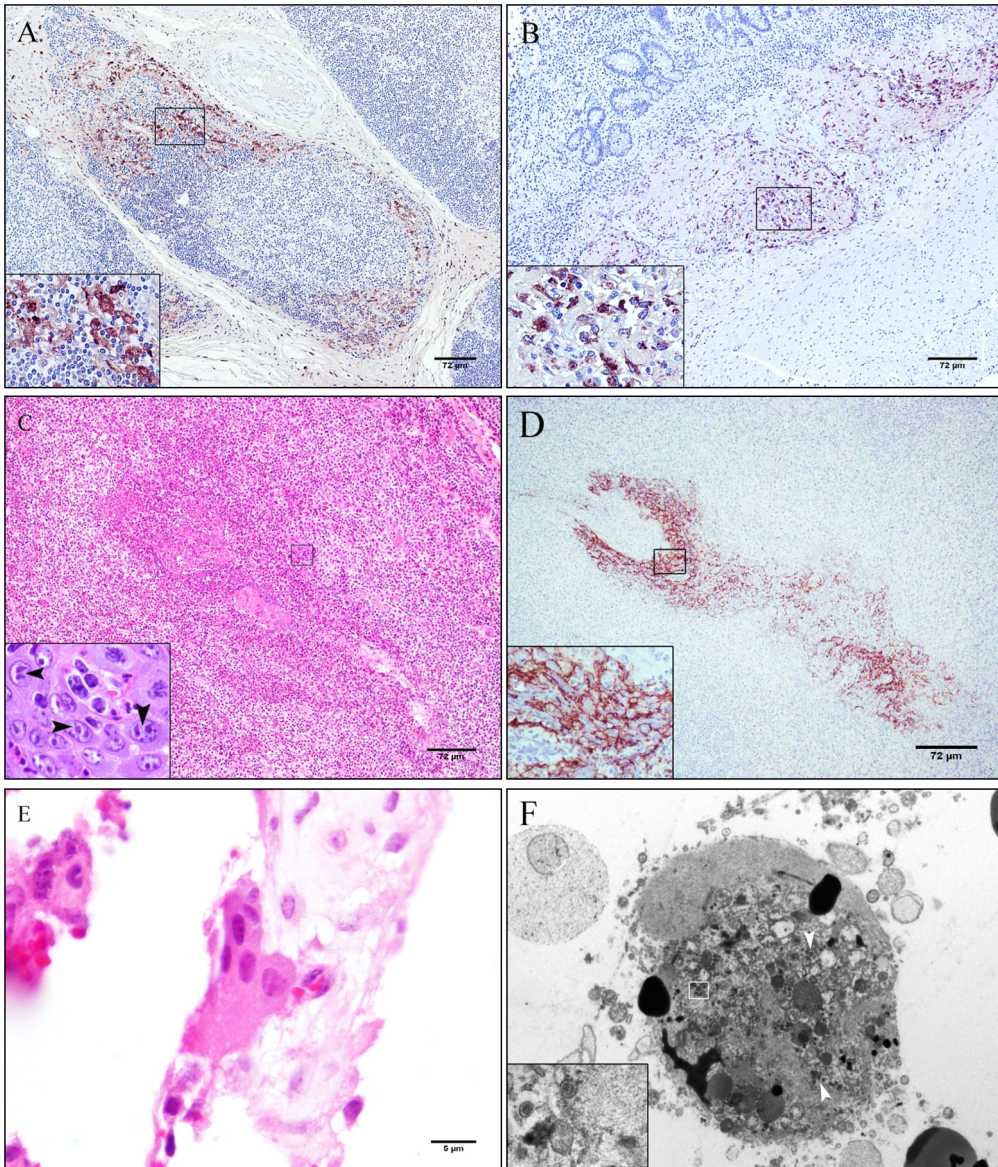
Figure 6. (A) IHC. Multifocal depletion of lymphocytes in the thymic cortex associated with distribution of viral gp48 in MΦs and cells with dendritic morphology of the BVDV/BHV1 group at 1 dpi. (B) IHC. Stellate cells, MΦs and fibroblasts expressing BVDV antigen within the medulla of depleted Peyer's patches follicles in BVDV/BHV1 group at 2 dpi. (C) HE. Pharyngeal tonsil of a co-infected animal: focal necrosis in a tonsil crypt with infiltrated MΦs, lymphocytes and neutrophils at 4 dpi. Detail of eosinophilic IIBs observed in epithelial cells of the necrotic margins (arrowheads). (D) IHC. BHV-1 gC associated with the necrotic lesion of the pharyngeal tonsil crypt. (E) HE. Epithelial syncytia with slightly basophilic IIBs occupying the entire nucleus in nasal mucosa of BVDV/BHV1 group at 2 dpi. (F). TEM. Necrotic alveolar cell in a co-infected animal showing BHV-1-like particles at 4 dpi (arrowheads).

Discussion

In the present work, a descriptive study of lesions and viral antigen distribution was carried out in healthy calves and calves with subclinical BVD, both inoculated with BHV-1.1. Compared with their healthy counterparts, calves with subclinical BVD displayed a worsening of tissue lesions –particularly inflammatory lesions– and more dissemination of BHV-1.1. Moreover, BVDV was detected in this group using immunohistochemical techniques during very late stages of the disease, a finding not reported in studies of calves inoculated with BVDV alone (Liebler-Tenorio et al., 2003a, 2004; Pedrera et al., 2009b; Raya et al., 2011).

The greater intensity of the clinical manifestations and lesions observed in the BVDV/BHV1 group may have been due to: 1) more dissemination of the agent, with direct action on target cells; 2) the triggering of a more intense host inflammatory response; or 3) a combination of the two mechanisms (Beutler, 2004; Muylkens et al., 2007; Tizard, 2008), as occurs in this study.

In the BHV1 group, only a small amount of viral antigen was found in tonsils at 4 dpi. By contrast, in the BVDV/BHV1 group, abundant presence of viral antigen together with IIBs were observed in tonsils at 4 and 7 dpi, associated with necrotic lesions due to the cytopathic effect of BHV-1 (Schuh et al., 1992; Winkler et al., 2000; Perez et al., 2005). These differences highlight a widespread dissemination of the secondary agent in calves inoculated with BVDV, which might be linked to an inadequate local cell-mediated response and the consequent failure to stop BHV-1 at its entry site, thus favoring systemic spread (Potgieter et al., 1984). It has been suggested that changes in resident CD8+ T lymphocytes, with cytotoxic capacity against infected cells (Tizard, 2008), may contribute to a delayed local immune

response to BHV-1, and to inefficient elimination of the pathogenic agent (Schuh et al., 1992).

Moreover, in the BVDV/BHV1 group, BVDV was detected in association with MΦs in thymus and Peyer's patches throughout the experiment (26 dpi BVDV), a finding not reported in single infection studies, in which this virus starts to clear from 12-14 dpi (Liebler-Tenorio et al., 2003a, 2004; Pedrera et al., 2009b; Raya et al., 2011). Thus, just as BVDV facilitates the dissemination of BHV-1, the presence of BHV-1 favors the persistence of BVDV in target organs and the reappearance of digestive-tract changes attributed to BVDV (Potgieter et al., 1984; Castrucci et al., 1992; Risalde et al., 2011b-*Chapter 3*-), thereby potentiating its pathogenic action and increasing host susceptibility to other infections (Hodgson et al., 2005).

With regard to inflammatory lesions, calves of the BHV1 group displayed only moderate changes as hyperaemia and mononuclear cell infiltrate in the upper airways, lung and, especially, liver; similar cell recruitment is reported in other studies of BHV-1 infection (Babiuk et al., 1996; Leite et al., 2002b, 2005; Rivera-Rivas et al., 2009). By contrast, in the BVDV/BHV1 group these inflammatory changes occurred earlier and were more severe, particularly at 4 dpi, remaining these differences until the end of the experiment. Calves in this group also displayed marked activation of lymph nodes from 1 dpi, particularly retropharyngeal nodes, which underwent greater antigen stimulation because of their proximity to the inoculation route.

Massive recruitment of mononuclear cells in the lungs of BVDV/BHV1 group calves prompted interstitial pneumonia, with a marked thickening of the lung parenchyma between 4 and 7 dpi, coinciding with the onset of respiratory symptoms (Risalde et al., 2011b-*Chapter 3*-). This finding may be attributable to the direct action of both viruses, detected by PCR in the lung (data not shown), showing that pre-infection with BVDV favors the development of

more severe respiratory symptoms in response to secondary infections (Castrucci et al., 1992; Fulton et al., 2002; Confer et al., 2005). Despite the identification of both agents using molecular techniques, the occasional detection of syncytial cells in alveoli, associated with BHV-1 replication (Jubb and Kennedy, 2007), and the detection of BHV-1 virions in alveolar MΦs by TEM, at no stage was viral antigen detected in lungs using IHC techniques, though this had been reported by other authors (Babiuk et al., 1996; Narita et al., 2000; Shahriar et al., 2002; Fulton et al., 2003b, 2009; Liebler-Tenorio et al., 2004).

Calves of the BVDV/BHV1 group displayed other lesions not directly related to the inflammatory response and prior to infection with BHV-1. These included numerous TUNEL-positive apoptotic bodies phagocyted by MΦs in B-dependent areas of lymph nodes, thymus and ileal Peyer's patches, together with marked lymphoid depletion associated with BVDV. These lesions have been reported in other *in vivo* studies of this virus (Liebler-Tenorio et al., 2003a, 2004; Pedrera et al., 2009b; Raya et al., 2011), being more intense in our study after BHV-1 inoculation, which can induce apoptosis both *in vivo* and *in vitro* (Devireddy and Jones, 1999; Winkler et al., 1999; Perez et al., 2005; Geiser et al., 2008). These results suggest that the concomitance of the two agents has a synergic effect, thus potentiating the immunosuppressive action of the BVDV.

Another lesion observed in the BVDV/BHV1 group prior to infection with BHV-1 was an intense bone marrow hypoplasia, which persisted until the end of the study. To our knowledge, this lack of hematopoietic nests has not been reported hitherto in infections with low-virulence BVDV-1 or BVDV-2 strains, although it has been observed in inoculations with more virulent BVDV-2 strains (Wood et al., 2004; Keller et al., 2006). Immunohistochemical analysis detected no BVDV antigen in bone marrow,

suggesting that the changes observed may have been prompted by indirect virus action due to altered cytokine expression by stromal cells, leading to a modification of the hematopoietic microenvironment, a finding reported with other pestiviruses (Gómez-Villamandos et al., 2003). Further research is therefore required into the mechanisms triggered by BVDV to alter the environment regulating these cells.

Conclusions

The results obtained indicate that BVDV does not prevent the development of a cell-mediated response, but in fact favors the earlier development of more severe inflammatory processes following BHV-1 infection, leading to a worsening of clinical signs and lesions limited to lymphoid tissues, airways and the digestive tract. Moreover, BVDV facilitates the establishment and dissemination of BHV-1, thereby potentiating its pathogenic effect and predisposing the host to secondary infections. At the same time, the presence of BHV-1 favors the persistence of BVDV in target organs.

Chapter 3.

Response of pro-inflammatory and anti-inflammatory cytokines in calves with subclinical bovine viral diarrhea inoculated with bovine herpesvirus-1

Response of pro-inflammatory and anti-inflammatory cytokines in calves with subclinical bovine viral diarrhea challenged with bovine herpesvirus-1

Abstract

The aim of this work was to investigate the susceptibility of calves infected with BVDV against secondary infections. For this purpose, the profile of cytokines implicated in the immune response of calves experimentally infected with a NCP strain of BVDV-1 and challenged with BHV-1.1 was evaluated in comparison with healthy animals challenged only with BHV-1.1. The immune response was measured by serum concentrations of cytokines (IL-1β, TNFα, IFNγ, IL-12, IL-4 and IL-10), APPs (Hp, SAA and fibrinogen), and BVDV and BHV-1.1 specific Abs. BVDV-infected calves displayed a great secretion of TNFα and reduced production of IL-10 following BHV-1 infection, leading to an exacerbation of the inflammatory response and to the development of more intense clinical symptoms and lesions than those observed in healthy animals BHV-1-inoculated. A Th1 immune response, based on IFNγ production and on the absence of significant changes in IL-4 production, was observed in both groups of BHV-1-infected calves. However, whereas the animals inoculated only with BHV-1 presented an IFNγ response from the start of the study and high expression of IL-12, the BVDV-infected calves showed a delay in the IFNγ production and low levels of IL-12. This

alteration in the kinetics and magnitude of these cytokines, involved in cytotoxic mechanisms responsible for limiting the spread of secondary pathogens, facilitated the dissemination of BHV-1.1 in BVDV-infected calves.

Introduction

BVDV is an endemic ruminant pestivirus in populations worldwide. Two genotypes, BVDV-1 and BVDV-2, are recognized as distinct species within this genus, being classified in CP and NCP based on their activity on cultured epithelial cells (Ridpath et al., 1994; Heinz et al., 2000; Fulton et al., 2003a). Although acute BVDV infections are often asymptomatic or produce only mild clinical symptoms, there is evidence that they induce lymphopenia and a range of effects on the immune response which allow the appearance of secondary infections (Peterhans et al., 2003; Liebler-Tenorio et al., 2004; Pedrera et al., 2009b). BVDV infects a wide variety of cell types but has a predilection for cells of the immune system as m-MΦs, DCs and lymphocyte populations (Glew et al., 2003; Liebler-Tenorio et al., 2003b). The consequences of infection include the death of these cells populations as an extreme event, or more subtle effects on cytokine expression and synthesis of co-stimulatory molecules (Brackenbury et al., 2003; Glew et al., 2003; Peterhans et al., 2003). Thus, the changes in the profile of cytokines, produced by immune or non-immune cells, could affect to both innate and specific immunity (Nobiron et al., 2001).

Pro-inflammatory cytokines as IL-1 and TNFα are of great importance in the innate immune response (Biron and Sen, 2001), causing leukocyte chemoattraction, phagocyte stimulation and enhancement of downstream cytokine and chemokine production (Krishnadasan et al., 2003; Pfeffer, 2003). Moreover, these cytokines mediate in the APR through the regulation of the

profile of "positive" or "negative" APPs, depending on the increase or decrease of their concentration in serum. Hp and SAA, the main positive APPs in cattle, are used as indicators of disease severity (Eckersall, 2000; Petersen et al., 2004). In this regard, various authors have demonstrated that BVDV induces an impaired production of these chemical mediators both *in vitro* and *in vivo*, which could affect to the generation of a subsequent immune response (Adler et al., 1996; Yamane et al., 2005; Lee et al., 2008; Pedrera et al., 2009a; Risalde et al., 2011a-*Chapter 1*-).

Th1/Th2 paradigm, postulated by Mosmann et al. (1986) from studies on cytokines produced by T lymphocytes in a murine model, is less well defined in ruminants (Estes and Brown, 2002). The functions of Th1 cytokine IFNγ include the stimulation of immunoglobulin production and specific cytotoxicity of T cells, induction of apoptosis and activation of resting tissue MΦs, thereby enhancing resistance against viral infections (Biron and Sen, 2001; Samuel, 2001). This chemical mediator is produced by NK cells, lymphocytes CD8+ and CD4+ Th1 cells in response to IL-12 (Hunter, 2005; Tizard, 2008). On the other hand, the Th2 cytokine IL-4 promotes the development of helper and cytotoxic T cells and the differentiation of immunoglobulins-producing plasma cells from B cells (Tizard, 2008). This cytokine is produced in response to antigen activation by CD4+ Th2 cells and some CD8+, NK1+ and γδT cells (Marcenaro et al., 2005) (*Figure 1*). IL-10 is a regulatory cytokine with anti-inflammatory effects, inhibiting the activities initiated by pro-inflammatory cytokines (Biron and Sen, 2001; Pestka et al., 2004). This mediator is produced by all subtypes of Th cells in humans and cattle (Brown et al., 1994, 1998).

Previous studies on the immune response to BVDV do not provide consistent results. Whereas some authors demonstrate the establishment of a Th1 response (Charleston et al., 2002; Lee et al., 2008), others maintain that

occurs a Th2 type immune response which might interfere with protective Th1 responses against other pathogens (Rhodes et al., 1999). However, to our knowledge, no studies have been made on pro-inflammatory and Th1/Th2 cytokines during co-infections with BVDV and secondary agents in calves.

BHV-1 is an α-herpesvirinae subfamily member that, together with other bacterial and viral pathogens as BVDV, is implicated in the BRDC (Hodgson et al., 2005; Muylkens et al., 2007; Srikumaran et al., 2007). Innate immune responses against BHV-1 include the antiviral action of IFN, alternative complement pathway and local infiltration of lymphoid cells, MΦs, neutrophils or NK cells (Babiuk et al., 1996). In the specific cellular immunity, T helper lymphocytes mediate the lysis of BHV-1 infected cells by activating MΦs and NK cells through IFNγ and IL-2 secretion, and by recruiting and promoting the proliferation of specific cytotoxic T lymphocytes (Babiuk et al., 1996; Van Drunen Littel-van den Hurk, 2007). The specific humoral immunity also participates in BHV-1 infection clearance by mediating the antibody-dependent cell cytotoxicity and by neutralizing cell-free virus particles (Muylkens et al., 2007).

In the present study, we have evaluated the effects of the pre-infection with BVDV in the pattern of cytokines implicated in the immune response of calves BHV-1-challenged with the purpose of investigate the susceptibility of these animals against secondary infections.
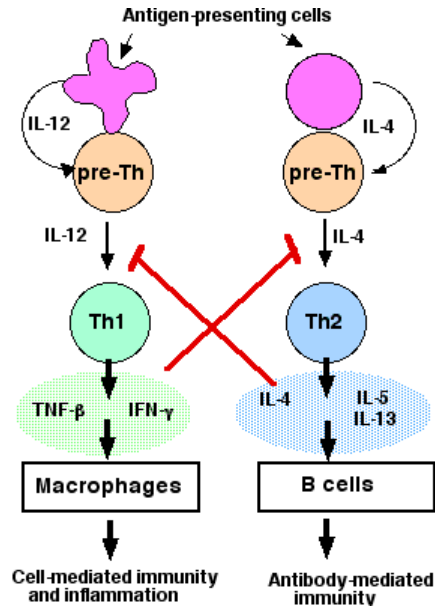
Figure 1. Schematic representation of cytokines influencing the development of Th1 and Th2 responses. APCs present their antigens to T cells, which recognize them through their T-cell receptors. Intracellular organisms as bacteria or viruses are recognized inducing the secretion of IL-12 and differentiation of T cells into the Th1 lineage that produces IFNγ and develops such cell-mediated immunity. When APCs recognize larger pathogens, the end result is differentiation of Th2 effector cells regulated by T-cell-produced IL-4 that start an antibody-mediated immunity.

Thus, fourteen Friesian calves were inoculated intranasally with the NCP strain 7443 of BVDV-1 and twelve days later, twelve of them were challenged with BHV-1.1 Iowa strain (BVDV/BHV1 group). Twelve calves were only inoculated with the BHV-1.1 (BHV1 group). Four UI animals were used as negative controls (negative control group). Blood and serum samples from calves inoculated with BVDV were collected at 0, 3, 6, 9, 12, 15, 18 and 21 hpi, 1, 2, 4, 5, 7, 9, 12 and 14 dpi.

The systemic inflammatory and immune response was analyzed through the study of serum concentrations of cytokines (IL-1β, TNFα, IFNγ, IL-12, IL-4 and IL-10) measured by ELISA, and serum APPs levels (Hp, SAA and

fibrinogen) determined by ELISA and colorimetric methods. BVDV and BHV-1.1 specific Abs were measured by ELISA and VN, respectively. *(See Materials and methods section, page 57. Experimental model 2).*

Results

Cytokines study

The different analyzed cytokines in the negative control group maintained low and constant levels without changes in this study. The cytokine results of both inoculated groups are depicted in *Figures 2* and *3*.

Pro-inflammatory cytokines

The pro-inflammatory cytokines, IL-1β and TNFα, presented differences in magnitude and kinetics between single and dual infections, mainly from 1 dpi onwards. While the animals of the BHV1 group did not show changes on IL-1β serum concentration after BHV-1.1 inoculation, the calves of the BVDV/BHV1 group displayed a significant decrease of this cytokine (p=0.03), maintaining low levels until the end of the study (*Figure 2*). TNFα concentration in the BHV1 group only increased in the later stages, at 9 dpi (59 ng/ml; p<0.001), whereas BVDV/BHV1 group showed an earlier and longer response subsequent to BHV-1.1 inoculation. These animals presented a significant increase of this chemical mediator from 1 dpi onwards (p<0.001), peaking at 4 dpi with values 5 times above the baseline level (*Figure 2*).
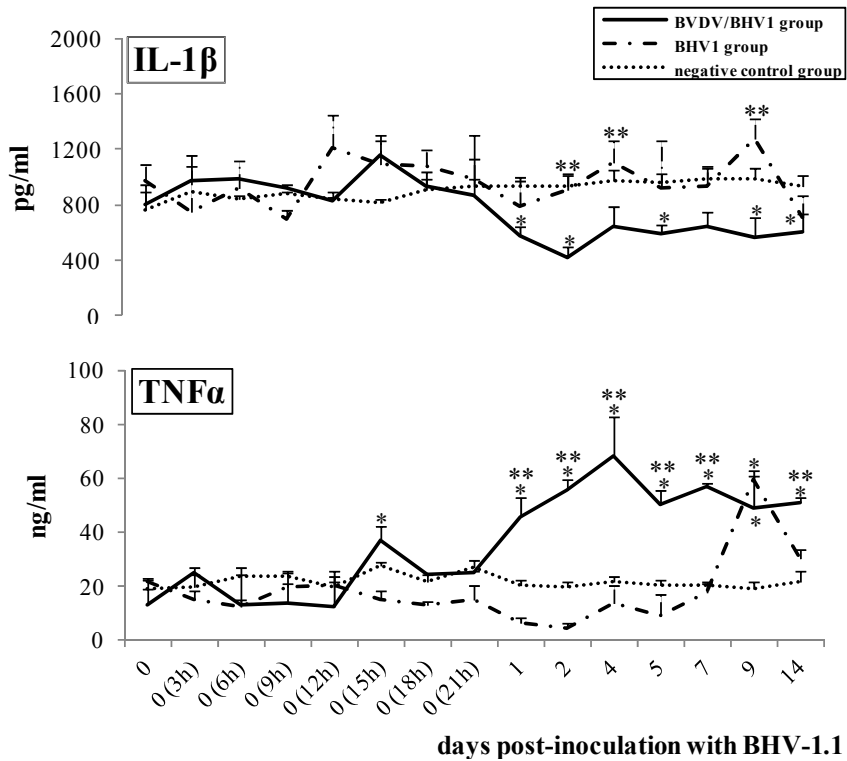
Figure 2. Serum concentrations of the pro-inflammatory cytokines (IL-1β and TNFα) in calves co-infected experimentally with BVDV and BHV-1.1, calves inoculated only with BHV-1.1 and uninfected control calves. (0, BHV-1.1 pre-inoculation values; h, hour post-inoculation with BHV-1.1; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Th1/Th2 cytokines

The concentration of Th1 cytokines, IFNγ and IL-12, were elevated in both inoculated groups, although presented differences in magnitude. IFNγ concentration in the BHV1 group displayed an early increase that was maintained with oscillations throughout the study, showing significant values at 5 dpi (4214.62 pg/ml; p<0.0001). However, in the BVDV/BHV1 group, IFNγ serum concentration remained constant from the start of the experiment, peaking only between 4 and 5 dpi (p<0.0001). There was a similar pattern of IL-12 serum concentration, because the BHV1 group presented a significant increase between 4 and 9 dpi, while the IL-12 levels remained low during the course of the study in the BVDV/BHV1 group, except for an increase at 4 dpi (*Figure 3*).

Th2 cytokine (IL-4) serum concentration did not present changes in any of the studied groups, maintaining low and constant levels throughout the study (*Figure 3*).

Anti-inflammatory cytokine (IL-10) concentration displayed a significant increase of twice the baseline level (from 0.18 to 0.45 of OD, approximately) associated with the peak of TNFα in both infected groups, although this increase was longer in BHV1 group (between 7 and 9 dpi) (*Figure 3*).
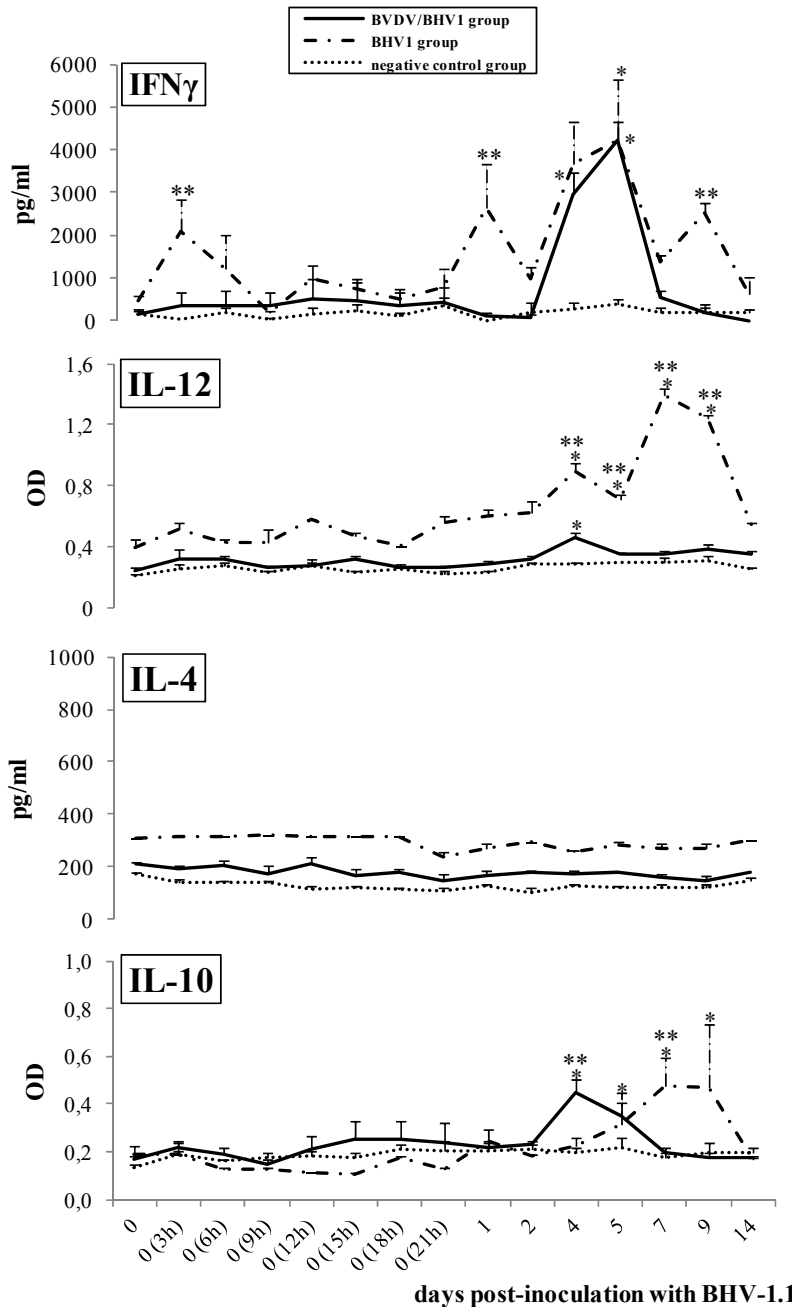
Figure 3. Th1 (IFNγ and IL-12), Th2 (IL-4) and (IL-10) cytokines in calves co-infected experimentally with BVDV and BHV-1.1, calves inoculated only with BHV-1.1 and uninfected control calves. (0, BHV-1.1 pre-inoculation values; h, hour post-inoculation with BHV-1.1; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

APPs analysis

APPs serum concentration of the negative control group remained low and without changes throughout the experiment.

The serum levels of Hp displayed no significant changes in both inoculated groups in this study. However, SAA protein showed a significant increase in the animals of the BVDV/BHV1 group at 5 and 7 dpi, whereas the calves of the BHV1 group did not present changes in this study. Fibrinogen concentrations followed a similar pattern than SAA protein, showing only the animals of the BVDV/BHV1 group a significant increase at 7 and 9 dpi. Albumin, a negative APP, presented non-significant changes in any group during the experiment (*Figure 4*).

Serum antibodies detection

All animals were negative for BVDV or BHV-1 specific Abs at the start of the study and the UI animals of the control group remained seronegative until the end of the experiment. Appearances of BVDV-specific serum Abs were only detected in the calves of the BVDV/BHV1 group from 4 dpi BHV-1.1 (16 dpi BVDV). On the other hand, in both inoculated groups were found neutralizing Abs against BHV-1.1 at 14 dpi, being greater the magnitude in the BVDV/BHV1 group (data not shown).

Figure 5 depicts a schematic summary of the time-ordered manifestation of clinical signs, virological and immunological parameters addressed in the animals of both BHV1 and BVDV/BHV1 groups.
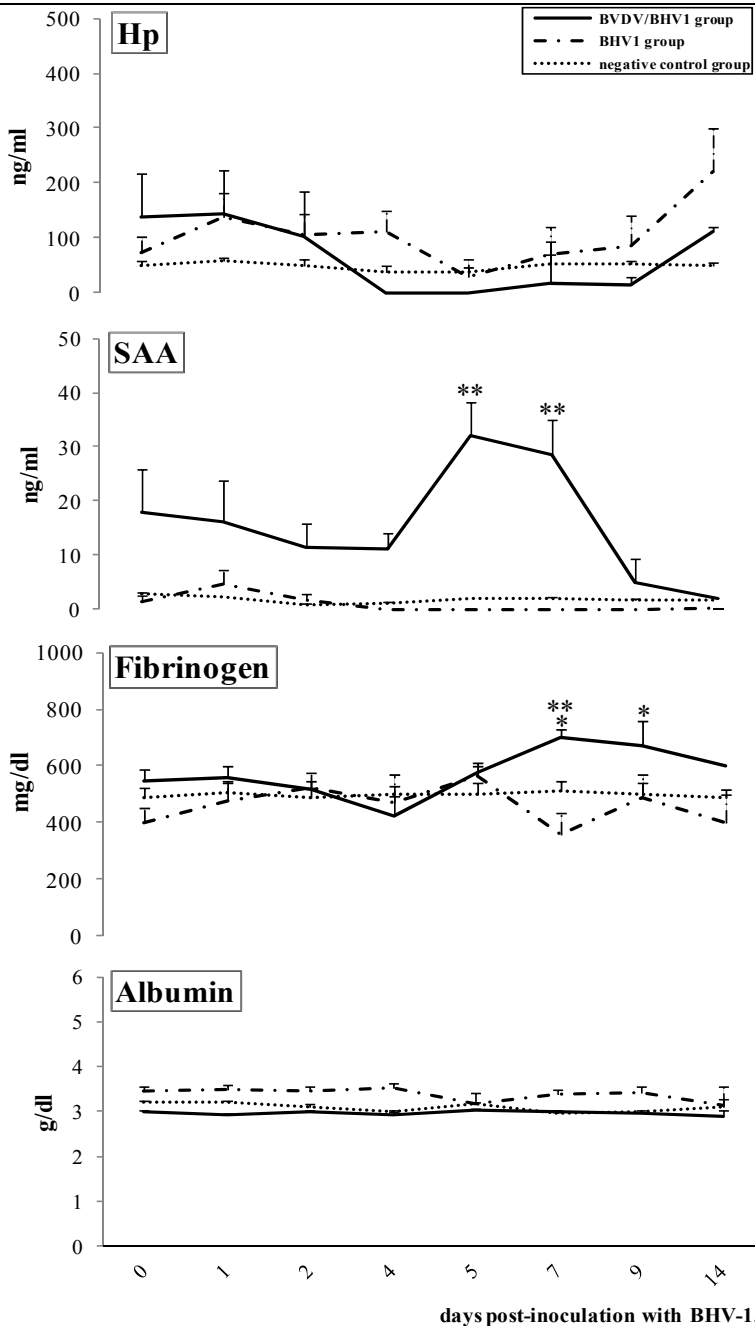
Figure 4. Serum concentrations of Hp, SAA, fibrinogen and albumin in calves co-infected experimentally with BVDV and BHV-1.1, calves inoculated only with BHV-1.1 and uninfected control calves. (0, BHV-1.1 pre-inoculation values; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).
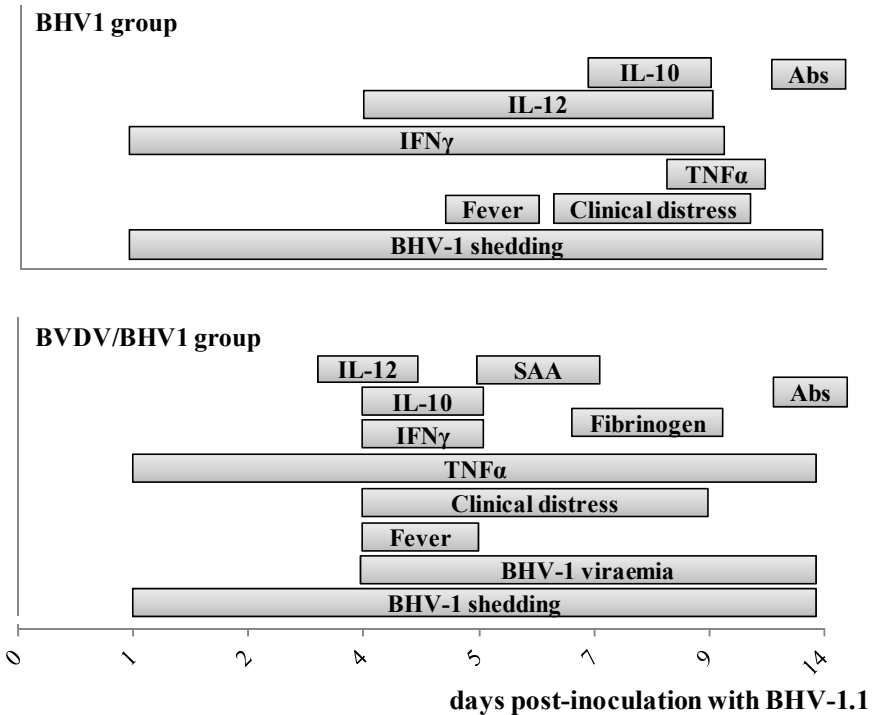
Figure 5. Schematic summary of clinical signs, virological and immunological events occurring in calves infected with BHV-1.1 versus calves co-infected with BVDV and BHV-1.1. The length of the bars represents the duration of the parameters studied over the time course of the experiment.

Discussion

Clinical symptoms and lesions in the BVDV/BHV1 group were more varied and intense than in the BHV1 group; inflammatory processes, in particular, were exacerbated and more widely distributed. This marked inflammatory response in the BVDV/BHV1 group was confirmed by analysis of APPs, which showed an increase in SAA protein and fibrinogen, associated with greater secretion of pro-inflammatory cytokines (TNFα) and reduced production of anti-inflammatory cytokines (IL-10) (Petersen et al., 2004;

Sánchez-Cordón et al., 2007; Risalde et al., 2011a-*Chapter 1*-). The appearance of viraemia, clinical signs, fever and more intense inflammatory lesions also coincided with a peak in TNFα levels. However, IL-1β synthesis rather than increasing actually declined, avoiding the appearance of a synergic pro-inflammatory effect involving TNFα and IL-1β (Pedrera et al., 2009a; Risalde et al., 2011a-*Chapter 1*-). This BVDV-associated inhibition of IL-1 has also been reported *in vitro* coinciding with a lack of TNFα response (Adler et al., 1996; Yamane et al., 2005; Lee et al., 2008), in contrast with our results where TNFα was the main inflammatory mediator. The enhanced inflammatory response observed in the BVDV/BHV1 group highlights the fact that prior inoculation of BVDV does not inhibit the development of a non-specific response to the secondary agent (Gånheim et al., 2003). The results also suggest that the mild inflammatory signs observed in healthy BHV-1-inoculated calves were associated with a transitory increase in TNFα during the final phase of the experiment.

Differences in pro-inflammatory cytokine profiles were accompanied by differences in the synthesis of IL-10, a cytokine with anti-inflammatory activity that plays a major role in regulating the immune response (Grutz, 2005). Thus, in the BHV1 group, IL-10 synthesis coincides with that of the pro-inflammatory cytokines studied, modulating their effect, whereas in the BVDV/BHV1 group the lesions observed suggest that the transitory increase in IL-10 synthesis failed to counteract the action of TNFα.

Coinciding with similar findings reported by other authors, both groups of BHV-1-inoculated calves developed a Th1 immune response based on IFNγ production and on the absence of significant changes in IL-4 production over the course of the experiment (Takashima et al., 2002; Abril et al., 2004). However, while the BVDV does not appear to impair the development of a Th1 immune response, differences between inoculated groups were apparent

with regard to the kinetics and magnitude of IFNγ and IL-12 production, two cytokines that interfere with the spread of BHV-1 by mediating the lysis of infected cells. In the BHV1 group, IFNγ levels rose from the start of the experiment; this increase, together with intense expression of IL-12, could prevent the distribution of BHV-1 from primary replication sites into the blood (Babiuk et al., 1996; Hodgson et al., 2005). In the BVDV/BHV1 group, by contrast, IFNγ did not increase until 4 and 5 dpi, coinciding with the detection of BHV-1 in blood and with mild IL-12 production, suggesting an inhibition of the cytotoxic effect on infected cells at the start of the process, associated with the action of BVDV (Lee et al., 2008; Risalde et al., 2011a-*Chapter 1-*). Moreover, these changes may well be linked to the increase of TNFα in the BVDV/BHV1 group, and to the ability of this cytokine to induce integrins expression on leukocytes, contributing to their recruitment at the inflammation site (Ley et al., 2007; Rivera-Rivas et al., 2009) and favoring viral spread by increasing the number of target cells. The results obtained suggest that the protective cell-mediated immune response against BHV-1 was ineffective in the BVDV/BHV1 group, and failed to prevent the persistence and dissemination of the pathogen throughout the experiment.

BHV-1-specific humoral response normally starts at around 7-10 dpi (Murphy and Reiner, 2002; OIE, 2010), a fact that was not observed in any group of our study until 14 dpi, attributing this delay to the virulence of the studied strains, as well as to the age and immune status of the calves. These results indicate that the specific humoral response to BHV-1 was not impaired by pre-infection with BVDV; indeed, calves in the BVDV/BHV1 group displayed higher Ab titres, with no decline in the Ab response to BVDV, which was detected from 16 dpi of BVDV, as reported by other authors (Wilhelmsen et al., 1990; Archambault et al., 2000; Müller-Doblies et al., 2004). Similarly, healthy animals vaccinated against BVDV and later against

BHV-1 produce higher Ab titres than those vaccinated only against BHV-1 (Alvarez et al., 2007). Because of this, our data suggest that the greater stimulation of the immune system in the BVDV/BHV1 group might favor the development of the humoral response to the secondary agent.

Conclusions

The results showed that BVDV-infected calves displayed changes in pro- and anti-inflammatory cytokine profiles following BHV-1 infection, leading to an exacerbation of the inflammatory response and to the development of more intense clinical symptoms and lesions than those observed in healthy animals as a response to a secondary infection. Alterations were also observed in cytokines involved in the cytotoxic mechanisms responsible for limiting the spread of pathogens giving rise to secondary infections, such as BHV-1, thus facilitating the dissemination of this virus. By contrast, prior inoculation of BVDV did not modify the development of a BHV-1-specific humoral response in comparison with that of healthy animals.

Chapter 4.

Pathogenic mechanisms of vascular and inflammatory lesions in the lung of BVDV-infected calves challenged with BHV-1

Pathogenic mechanisms of vascular and inflammatory lesions in the lung of BVDV-infected calves challenged with BHV-1

Abstract

Resistance to respiratory disease in cattle requires host defense mechanisms that protect against pathogens which have evolved sophisticated strategies to evade them, including an altered function of pulmonary MΦs or the induction of inflammatory responses that cause lung injury and sepsis. The aim of this study was to clarify the mechanisms responsible for histopathological and ultrastructural changes occurring in the lung of calves infected with BVDV and challenged later with BHV-1, evaluating the role of MΦs and other immunocompetent cells in the development of pathological lesions in this organ. For this purpose, pulmonary lesions and the local immune response were compared between co-infected calves and healthy animals inoculated only with BHV-1 through immunohistochemical studies (Factor-VIII, MAC387, TNFα, IL-1α, iNOS, COX-2, CD3, CD79αcy, CD4, CD8 and IFNγ). Both groups of calves presented mononuclear aggregates of IMΦs and T lymphocytes in the pulmonary parenchyma, along with important vascular alterations produced by fibrin microthrombi and platelet aggregations within the blood vessels. These findings were earlier and more severe in the co-infected group, indicating that the concomitance of BVDV and BHV-1 in

lung disrupts the pulmonary homeostasis by facilitating the establishment of an inflammatory and procoagulant environment modulated by inflammatory mediators released by pulmonary MΦs. In this regard, the co-infected calves, despite presenting a greater number of IMΦs than single-infected group, showed a significant decrease in iNOS expression coinciding with the presence of more coagulation lesions. Moreover, animals pre-inoculated with BVDV displayed an alteration in the response of pro-inflammatory cytokines (TNFα and IL-1), which play a key role in activating the immune response, as well as in the local cell-mediated response marked essentially by an inhibition in the CD8+ and CD4+ Th1 response to the secondary respiratory pathogen.

Introduction

The BRDC is an important problem for the cattle industry, often resulting in severe economic losses (Barrett, 1998; Caldow and Nettleton, 2000). This fatal bovine respiratory infection is a multi-factorial disease associated with a primary viral infection followed by a secondary bacterial infection, although frequently, is characterized by concurrent infections of several pathogens. The etiologic agents related to feedlot pneumonias include bovine viral diarrhea virus 1 and 2 (BVDV-1 and BVDV-2), BHV-1, bovine PI-3, BRSV, bovine adenovirus A-D, bovine coronavirus, *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma spp.* (Griffin, 1997; Fulton et al., 2000, 2009; Hodgson et al., 2005). Resistance to respiratory disease in cattle requires host defense mechanisms that protect against viral and bacterial pathogens which have also evolved sophisticated strategies to evade the host immune responses, including among others an altered pulmonary MΦs function or the induction of profound inflammatory responses that cause lung injury and sepsis (Hodgson et al., 2005).

The inflammatory process is a protective response that occurs in response to trauma, infection or tissue injury (Zedler and Faist, 2006; Mariathasan and Monack, 2007). Increased blood supply, enhanced vascular permeability and migration of immune cells occur at damaged sites. In this process, MΦs play a central role in managing different immunopathological phenomena through the secretion of inflammatory mediators such as NO, prostaglandins, and the pro-inflammatory cytokines TNFα and IL-1 (Seibert et al., 1994; Esposito and Cuzzocrea, 2007; Tizard, 2008).

NO is a potent vasodilator, acting to maintain vascular tone and function within the vessel wall, generated from L-arginine by the action of nitric oxide synthase (NOS) enzymes. There are three main isoforms of NOS with distinct functions and patterns of expression: endothelial NOS, neuronal NOS and inducible NOS (iNOS) highly expressed in MΦs (Cooney et al., 2006; Esposito and Cuzzocrea, 2007).

Prostaglandins are other important inflammatory mediators implicated in the vascular homeostasis since in low levels can prevent or reverse aggregation of platelets and induce vasodilatation (Seibert et al., 1994; Yoon et al., 2009). These mediators are produced from arachidonic acid metabolites by the catalysis of cyclooxygenase-2 (COX-2) (Wehbrink et al., 2008).

Pro-inflammatory cytokines induce integrin expression and redistribution of leukocytes, which contribute to their recruitment and activation to the site of inflammation (Leite et al., 2002a,b), as well as to increase vascular permeability and tissue injury (Ohmann et al., 1991; Peper and Van Campen, 1995). There is evidence that inflammatory cytokines are the main orchestrators of the inflammatory cascade in BRDC, detecting high levels of TNFα and IL-1 in airways of cattle infected with respiratory pathogens (Rontved et al., 2000; Malazdrewich et al., 2001; Muylkens et al., 2007; Rivera-Rivas et al., 2009).

BVDV is a pestivirus that although is not a primary agent in the pathogenesis of BRDC, it can suppress the host immune system and increase the risk of secondary infections, thus enhancing pulmonary colonization by other pathogens as BHV-1 (Castrucci et al., 1992; Cusack et al., 2003; Ridpath et al., 2003). The mechanisms of the immunosuppressive action of BVDV are object of debate, including changes related to decreased lymphocyte proliferation (Brown et al., 1991; Brodersen and Kelling, 1999), severe lymphoid depletion in lymphoid tissues (Liebler-Teneorio et al., 2004; Pedrera et al., 2009b; Raya et al., 2011), decreased chemotaxis and phagocytic activity (Ketelsen et al., 1979), increased production of PGE2 (Welsh and Adair, 1995; Van Reeth and Adair, 1997), increased NO synthesis in response to lipopolysaccharide (Adler et al., 1994, 1996) and impaired production of pro-inflammatory cytokines (Yamane et al., 2005; Lee et al., 2008; Pedrera et al., 2009a; Raya et al., 2011; Risalde et al., 2011a-*Chapter 1*-).

Recently, we have undertaken a series of studies to examine *in vivo* the specific mechanisms by which a primary BVDV infection favors the dissemination of BHV-1, observing an impairment of the systemic immune response based on a delay in the IFNγ production and low levels of IL-12. Furthermore, BVDV pre-infected calves displayed a great TNFα secretion in serum and reduced production of IL-10 following BHV-1 infection, leading to an exacerbation of the inflammatory response and to the development of intense clinical symptoms and lesions (Risalde et al., 2011b-*Chapter 3*-).

These inflammatory lesions have also been observed in the lung of BVD-infected calves challenged with BHV-1, which pulmonary parenchyma was affected by interstitial pneumonia produced by aggregates of mononuclear cells (Risalde et al., 2011c-*Chapter 2*-). Among the immune modulatory mechanisms that might play an important role in this viral synergy, it have been suggested a compromised local cell-mediated immunity, the main barrier

to contain BHV-1 infection in the respiratory tract of BVDV-infected calves (Potgieter et al., 1995), as well as an alteration in the antimicrobial activity of pulmonary MΦs (Welsh et al., 1995; Glew et al., 2003; Peterhans et al., 2003), key cells in the nonspecific primary defense of the lung (Zhang et al., 2000; Laskin et al., 2001). However, experimental studies of these immune modulatory processes in the lung have not been performed yet.

Therefore, the aim of this study was to clarify the mechanisms responsible for histopathological and ultrastructural changes occurring in the lung of calves pre-infected with BVDV and challenged later with BHV-1. For this purpose, we characterized the interstitial aggregates observed in the pulmonary parenchyma, in addition to analyze the role of MΦs in the appearance of inflammatory lesions and the possible alteration of the local cell-mediated immunity induced by BVDV. This work will contribute to gain a better understanding about how this virus predispose to secondary airborne infections.

For it, fourteen calves were inoculated intranasally with the NCP strain 7443 of BVDV-1 and twelve days later, when the calves did not show clinical signs and viraemia against BVDV, twelve of them were challenged with BHV-1.1 Iowa strain (BVDV/BHV1 group). The other 2 animals inoculated with BVDV-1 and BHV-1.1-free, were killed before BHV-1.1 inoculation (0 dpi) and used as BVDV infection controls. On the other hand, twelve animals were only inoculated with BHV-1.1 (BHV1 group). The infected calves were sacrificed in batches of two at 1, 2, 4, 7 and 14 dpi. Four UI animals were used as controls and sacrificed at end of the study (negative control group).

Blood samples were collected at 0, 3, 6, 9, 12, 15, 18 and 21 hpi, 1, 2, 4, 5, 7, 9, 12 and 14 dpi. Samples collected from lung were fixed and routinely processed for histopathological and ultrastructural examination. The presence of BVDV and BHV-1 in lung was assessed by PCR, IHC and TEM.

Pulmonary lesions and the local immune response were analyzed through immunohistochemical studies (Factor-VIII, MAC387, TNFα, IL-1α, iNOS, COX-2, CD3, CD79αcy, CD4, CD8 and IFNγ) (*See Materials and methods section, page 57. Experimental model 2*).

Results

Respiratory signs and hematological findings

The main respiratory sings were detailed in *Chapter 2* (Risalde et al., 2011b). Briefly, the calves pre-infected with BVDV showed more intense respiratory signs as cough, mucopurulent nasal discharge, dyspnoea and open-mouth breathing, while the calves of BHV1 group only presented a moderate serous nasal discharge.

Platelet numbers were within the clinically normal range in all calves throughout the study. In 2 animals of both inoculated groups, an important decrease of platelet numbers to 57% approximately was observed at 12 hpi.

Detection of BVDV and BHV-1 in the lung

Virological study of the lung by IHC and ultrastructural analyses have been described in *Chapter 2*. In brief, there was no evidence of any viral infection in tissue samples from animals of the negative control group. Neither BVDV nor BHV-1 antigens were detectable by IHC in both inoculated groups. However, the subcellular study revealed the existence of BHV-1-like particles and virus-like replication sites in PAMs and necrotic cells in alveoli of the BVDV/BHV1 group calves (Risalde et al., 2011c).

The presence of viral agents in lung of both inoculated groups was confirmed using molecular techniques. In the BVDV/BHV1 group, BVDV was detected by RT-PCR between 0 and 7 dpi (12-19 dpi BVDV), and BHV-1

was detected by PCR from 2 to 14 dpi. However, in the BHV1 group, BHV-1 was only detected between 4 and 7 dpi, whereas BVDV was not detected at any time of the experiment.

Respiratory lesions

The main morphological changes observed in the respiratory tract were described previously in *Chapter 2* (Risalde et al., 2011c). Briefly, there were no remarkable lesions in the calves of the negative control group. The pulmonary parenchyma of inoculated calves was affected by interstitial pneumonia with alveolar septal thickening produced by interstitial aggregates of mononuclear cells. The appearance of this alteration was earlier in the co-infected calves and was associated with occasional alveolar oedema and haemorrhages (*Table 1*).

Table 1. Type and score of histopathological findings in lung after BHV-1.1 inoculation

	UI	BVDV control	1 dpi		2 dpi		4 dpi		7 dpi		14 dpi	
			BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1	BHV1	BVDV/ BHV1
Hyperaemia	-	+	-	+	++	++	++	++	+	++	+	++
Alveolar oedema	-	-	-	++	-	+++	+	++	+	-	-	-
Haemorrhages	-	-	-	-	-	++	-	++	-	+	-	+
Interstitial mononuclear aggregates	-	-	-	+	+	++	++	+++	+	+++	+	+
Alveolar septal thickening	-	-	-	+	+	++	++	+++	+	+++	+	+

(-) no histopathological lesion; (+) mild; (++) moderate; (+++) severe.

Means of the pulmonary lesions valuation (n = 2 per time point) of calves inoculated with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1. (UI, uninoculated calves of the negative control group; BVDV control, calves inoculated with BVDV-1 and BHV-1.1-free; BVDV/BHV1, calves infected with BVDV and BHV-1; BHV1, calves infected with BHV-1).

The most intense histological changes were also observed in the BVDV/BHV1 group between 2 and 4 dpi (P<0.0001), being these changes significant with respect to BHV1 group, except at 4 dpi due to a peak in the single infection and at 14 dpi when both groups presented a recovery of the pathological signs (*Figure 1*).
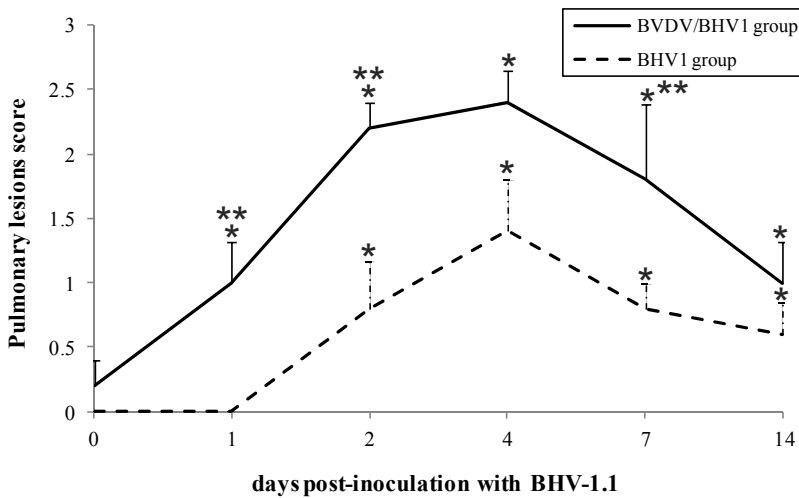

Figure 1. Means±standard errors (n = 2 per time point) of microscopic lesions score values in lung of calves inoculated with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1. (0, BHV-1.1 UI animals; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Fraser Lendrum technique revealed fibrin microthrombi in some pulmonary venules and capillaries of inoculated calves, being more severe in co-infected calves, mainly from 4 dpi (*Figure 2A*). These changes were confirmed by the ultrastructural study together with the presence of fibrin in alveoli associated with PAMs in both inoculated groups at early stages (*Figure 2B*). Moreover, these animals displayed an intense hyperaemia as well as interstitial and alveolar oedema during this period.
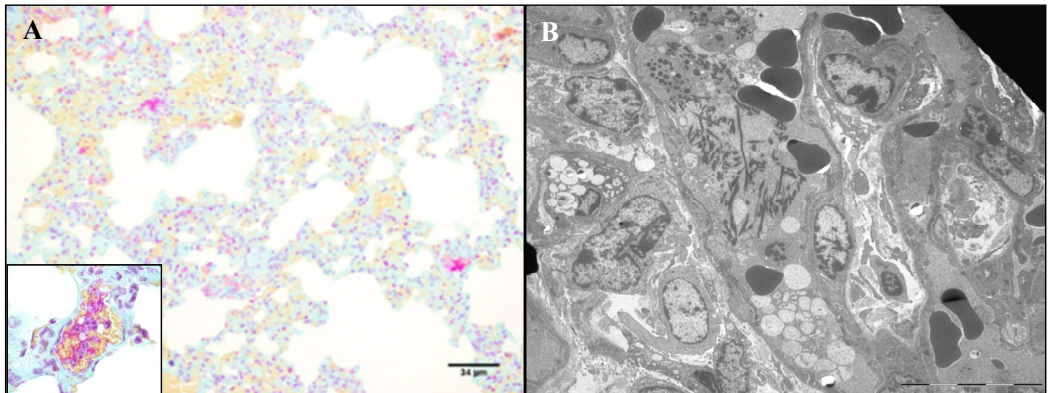
Figure 2. Fibrin microthrombi in a pulmonary venule of a co-infected animal observed by Fraser Lendrum technique (A) and TEM (B) at 4 dpi.

These findings were associated with a great quantity of platelets, which detection was performed by IHC using anti-Factor-VIII Ab. Thus, in the negative control group, this Ab prompted positive granular immunostaining among sheathed capillary cells, free in the interstitium and occasionally in MΦs cytoplasm. However, in the inoculated groups, clusters of immunostained granular material were observed in blood vessels. Moreover, numerous IMΦs and periarterial MΦs, together with some PAMs, were swollen and displayed an intense positive granular and cytoplasmic reaction in both inoculated groups between 4 and 7 dpi (*Figure 3A,B*). In the BVDV/BHV1 group, clusters of platelets were observed from 1 dpi, being more evident throughout the study associated with MΦs engulfing platelets that peaked at 4 dpi (3 score values; $p<0.0001$) (*Figure 4*). Subsequently, the positive reaction in MΦs and vascular lumina diminished considerably without recovering thereafter (*Figure 3C*). In the BHV1 group, these findings were observed from 2 dpi with similar values to the co-infected group (1.5 score values; $p<0.0001$), decreasing the number of platelet clusters until the end of the study (*Figure 3D* and *4*).
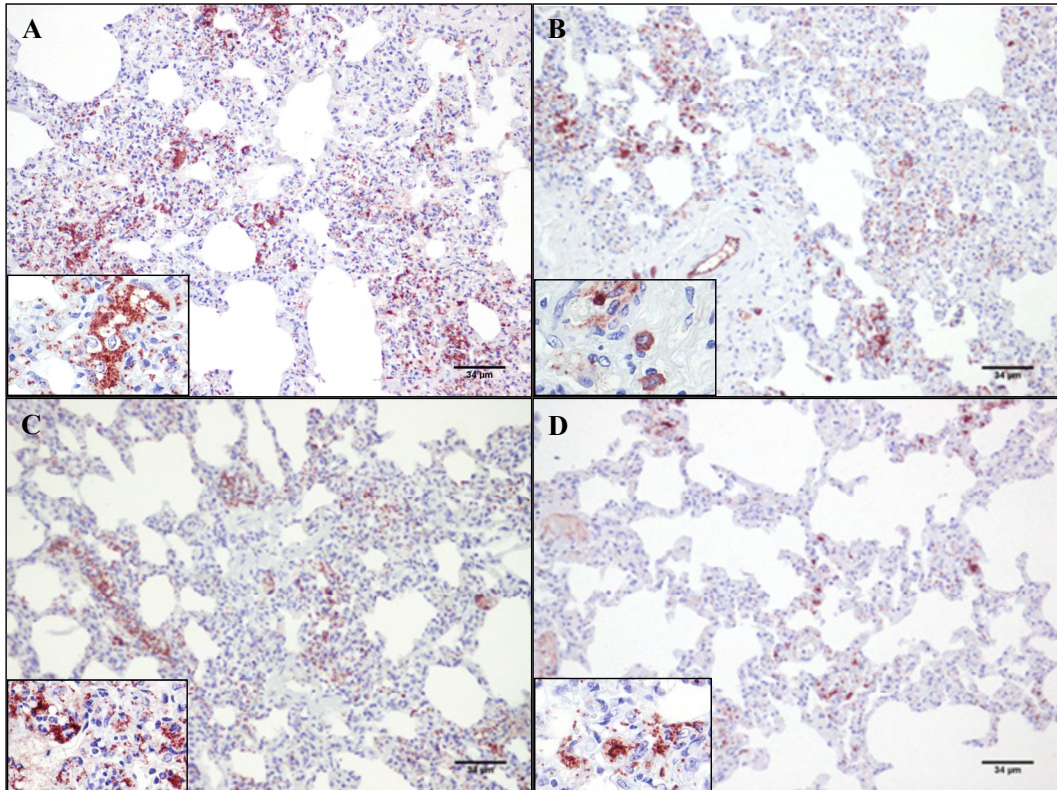
Figure 3. IHC. (A) Lung of the BVDV/BHV1 group showing great quantity of Factor VIII-positive clusters of platelets in some pulmonary venules and capillaries at 4 dpi, compared with minor changes in the BHV1 group (B), where were observed IMΦs and periarterial MΦs engulfing positive granular material. (C) Presence of less quantity of platelet clusters in the BVDV/BHV1 group at 14 dpi versus an almost inexistent lesion in the BHV1 group (D).

Ultrastructural study confirmed that there was an increase in the number of activated platelets forming multiple aggregations within the blood vessels, which vascular lumina usually appeared completely occluded. The changes indicative of this activation were an enlarged and deformed shape of the cells, a partial or total decrease in granule numbers and dilation of the open canalicular system. The most common form of platelet aggregation was the appearance of mosaic-like clusters comprising fully degranulated platelets with fusion of cytoplasmic membranes. Occasionally, these membranes

completely disappeared, giving rise to a finely granular structure with low electron density, containing vestiges of platelet organelles and surrounded by a membrane layer. In the BVDV/BHV1 group, platelet aggregation occurred sooner and was more intense, increasing between 4 and 7 dpi (*Figure 5A*) and not showing a totally recovery at the end of the study (*Figure 5C*). In the BHV1 group this lesion was moderate, presenting retrieval signs from 7 dpi (*Figure 5B,D*).

Subcellular changes indicative of a slight secretory activation in IMΦs as enlargement, proliferation and dilation of rough endoplasmic reticulum and Golgi complex cisternae were mainly observed in the BHV1 group at 4 dpi (*Figure 5E*). Moreover, from 4 dpi, some PIMs and PAMs of both inoculated groups appeared enlarged and rounded, with loss of filopodia, increased number of lysosomes and varying amounts of cell debris in their cytoplasm, characteristic signs of phagocytic activation (*Figure 5F*).
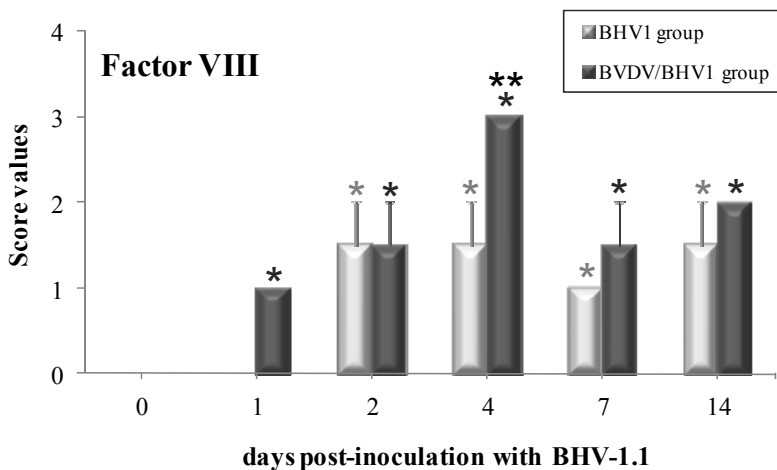
Figure 4. Means±standard errors (n = 2 per time point) of score values of Factor VIII-positive clusters of platelets in lung of calves inoculated with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1. (0, BHV-1.1 UI animals; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).
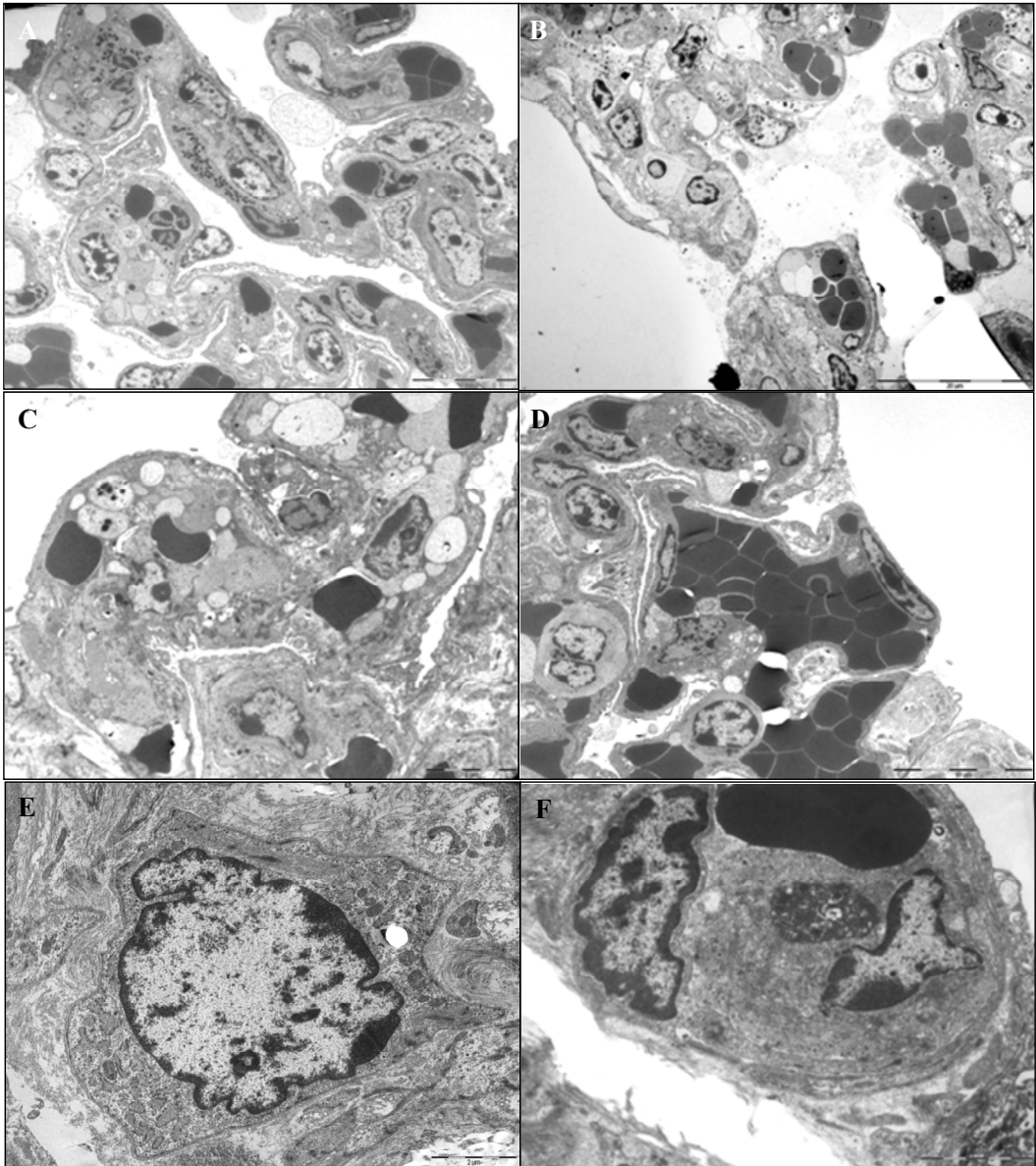
Figure 5. TEM. Multiple aggregations of platelets within the blood vessels with some vascular lumina completely occluded in the lung of the BVDV/BHV1 group at 4 dpi (A), versus a mild lesion observed in the BHV1 group (B). Pulmonary parenchyma showing recovery signs of this lesion in the co-infected group at 14 dpi (C), compared with a totally recuperation in the single infection (D). IMΦ in the BHV1 group with signs of secretory activation as enlargement, proliferation and dilation of rough endoplasmic reticulum at 4 dpi (E). PIM enlarged and rounded engulfing cell debris in the BVDV/BHV1 group at 4 dpi (F).

Changes in pulmonary immunocompetent cells

It was observed by immunohistochemical examination that mononuclear aggregates present in the pulmonary parenchyma were composed of IMΦs and T lymphocytes (CD3+), which were mostly TCD4+, TCD8+ and Tγδ; a few interspersed CD79αcy-positive B cells were also observed.

Immunolabeling of pulmonary MΦs showed no significant numerical changes of the PIMs in any of the inoculated groups, except for an increase in the BVDV/BHV1 group at 4 dpi; however, the number of PAMs was significantly higher in the co-infected calves at the start of the study, showing a decrease from 4 dpi. IMΦs displayed similar kinetics in single and dual infections after BHV-1.1 inoculation, although presented differences in the magnitude of their response. Thus, the BVDV/BHV1 group showed a higher number of these cells during the study, peaking at 4 dpi (p<0.0001) (*Figure 6*).
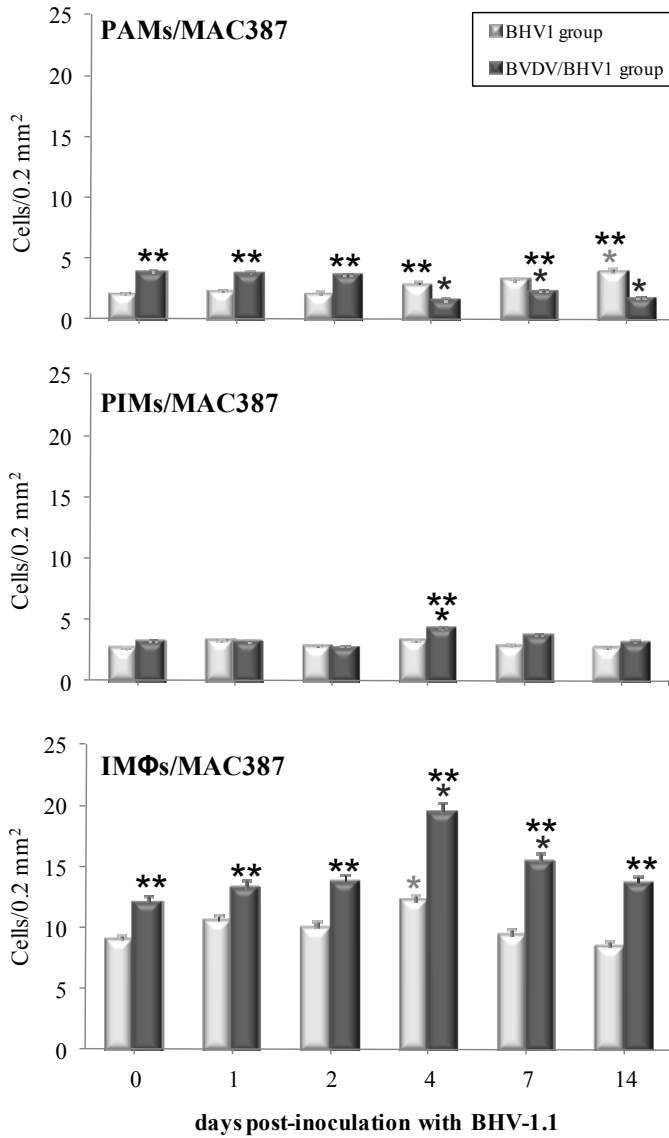
Figure 6. Means±standard errors (n = 2 per time point) of PAMs, PIMs and IMΦs positive for MAC387 by IHC in the lung of calves co-infected experimentally with BVDV and BHV-1.1 compared with calves inoculated only with BHV-1.1. (0, BHV-1.1 UI animals; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Secretory activity of MΦs observed ultrastructurally was confirmed by IHC, which enabled the detection of MΦs-secreted inflammatory mediators. For their evaluation, PIMs and IMΦs were grouped together and described as 'septal MΦs'.

TNFα and IL-1α-producing cells, identified as septal MΦs and PAMs, were detected immunohistochemically in the lungs of control and infected animals. These proinflammatory cytokines presented differences in magnitude and kinetics between single and dual infections. TNFα-positive septal MΦs were associated with sites of inflammation in the BVDV/BHV1 group (*Figure 7A*), showing only a slight peak at 2 dpi ($p<0.008$), whereas BHV1 group presented a longer response of this chemical mediator in peribronchial areas (from 4 dpi; $p<0.0001$) (*Figure 7B*). On the other hand, IL-1α-reactive septal MΦs were significantly different between both infected groups before BHV-1 inoculation (0 dpi BHV-1). The calves of the BVDV/BHV1 group maintained lower numbers of IL-1α-positive septal MΦs throughout the study (*Figure 7C*), showing a delayed response to BHV-1 inoculation (from 7 dpi onwards). By contrast, the calves of the BHV1 group displayed an early increase of this cytokine associated with peribronchial areas (at 2 dpi; $p<0.007$) (*Figure 7D*). The number of PAMs positive for studied cytokines was low in both inoculated groups, presenting only a slight response at end of the study, with the exception of an IL-1α peak in the single infected group between 1 and 2 dpi (*Figure 9*).
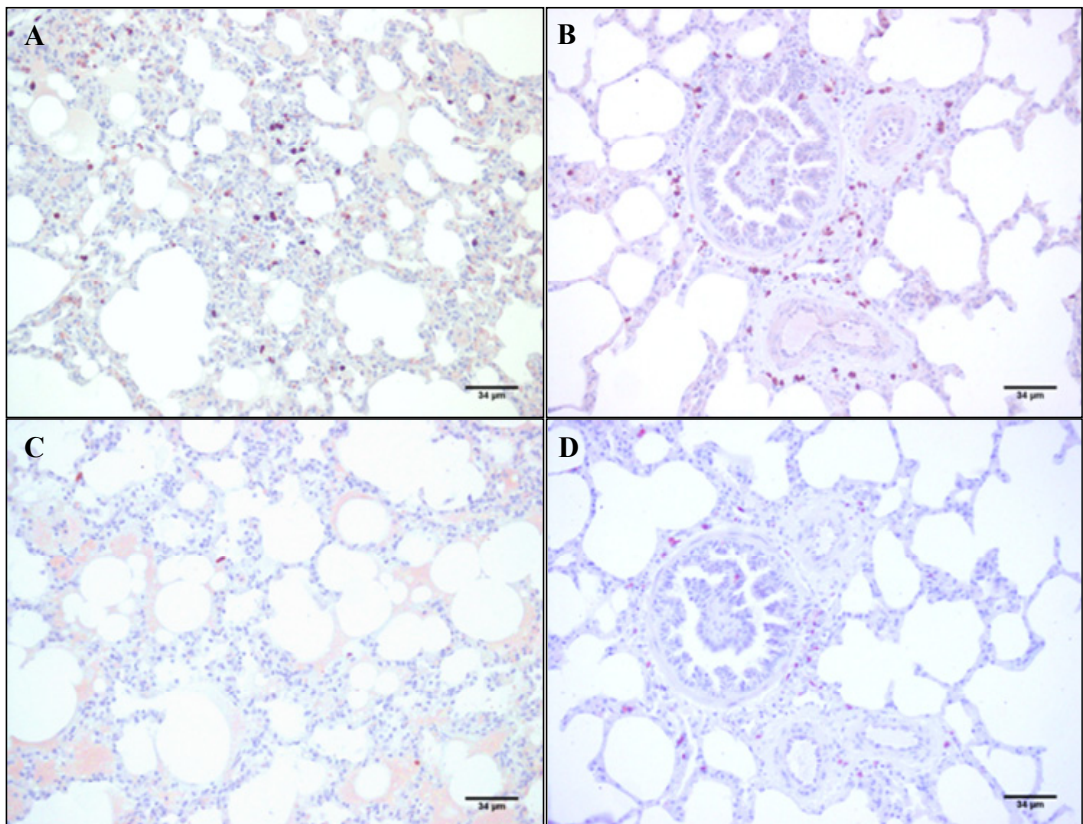
Figure 7. IHC. Septal MΦs and PAMs positive for TNFα (A) and IL-1α (C) associated with sites of inflammation in the pulmonary parenchyma of the BVDV/BHV1 group at 2 dpi. Higher number of IMΦs reactive to TNFα (B) and IL-1α (D) in peribronchial areas of the lung in the BHV1 group at 2 dpi.

Numerous groups of immunolabeled septal MΦs presenting strongly iNOS-positive cytoplasmic granules, mainly IMΦs, were observed in the pulmonary parenchyma of animals infected only with BVDV (*Figure 8A*). After BHV-1 inoculation, in the calves of the BVDV/BHV1 group, septal MΦs expressing iNOS decreased significantly until the end of the experiment (p<0.0001), showing values of approximately twice below the baseline level from 4 dpi (*Figure 8B*). In healthy calves, septal MΦs presented a similar response after BHV-1 inoculation; although in these animals the decrease of this mediator was observed later than in the BVDV/BHV1 group. On the other

hand, the number of iNOS-positive PAMs was higher in the BVDV/BHV1 group and did not suffer changes throughout the study, while the calves of BHV1 group showed a significant decrease from 7 dpi (*Figure 9*).

Intracellular localization of COX-2 was perinuclear and cytoplasmic in the MΦs, being its expression scarce in the lung during the study (*Figure 8C*), except for septal MΦs of the BVDV/BHV1 group at 4 dpi (p<0.0001) (*Figure 8*). This intense expression occurred at sites of inflammation and injury, mainly oedemas, and was correlated with the degree of pulmonary inflammation (*Figure 8D*).
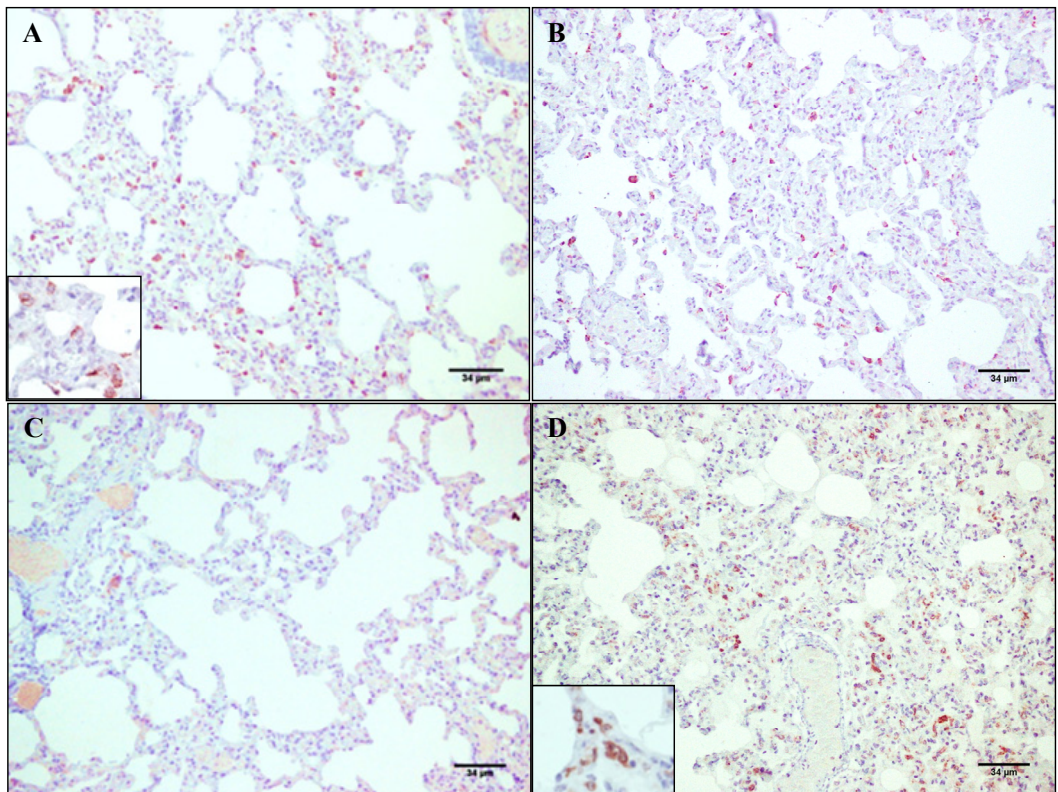
Figure 8. IHC. (A) Numerous immunolabeled septal MΦs iNOS-positive in the lung of calves inoculated only with BVDV (0 dpi of BVDV/BHV1 group), versus a minor number of these cells at 4 dpi BHV-1 (B). Pulmonary parenchyma of calves inoculated only with BVDV (0 dpi of BVDV/BHV1 group) showing an absence of MΦs positive for COX-2 Ab (C), compared with numerous immunolabeled MΦs COX-2-positive in sites of inflammation at 4 dpi (D).
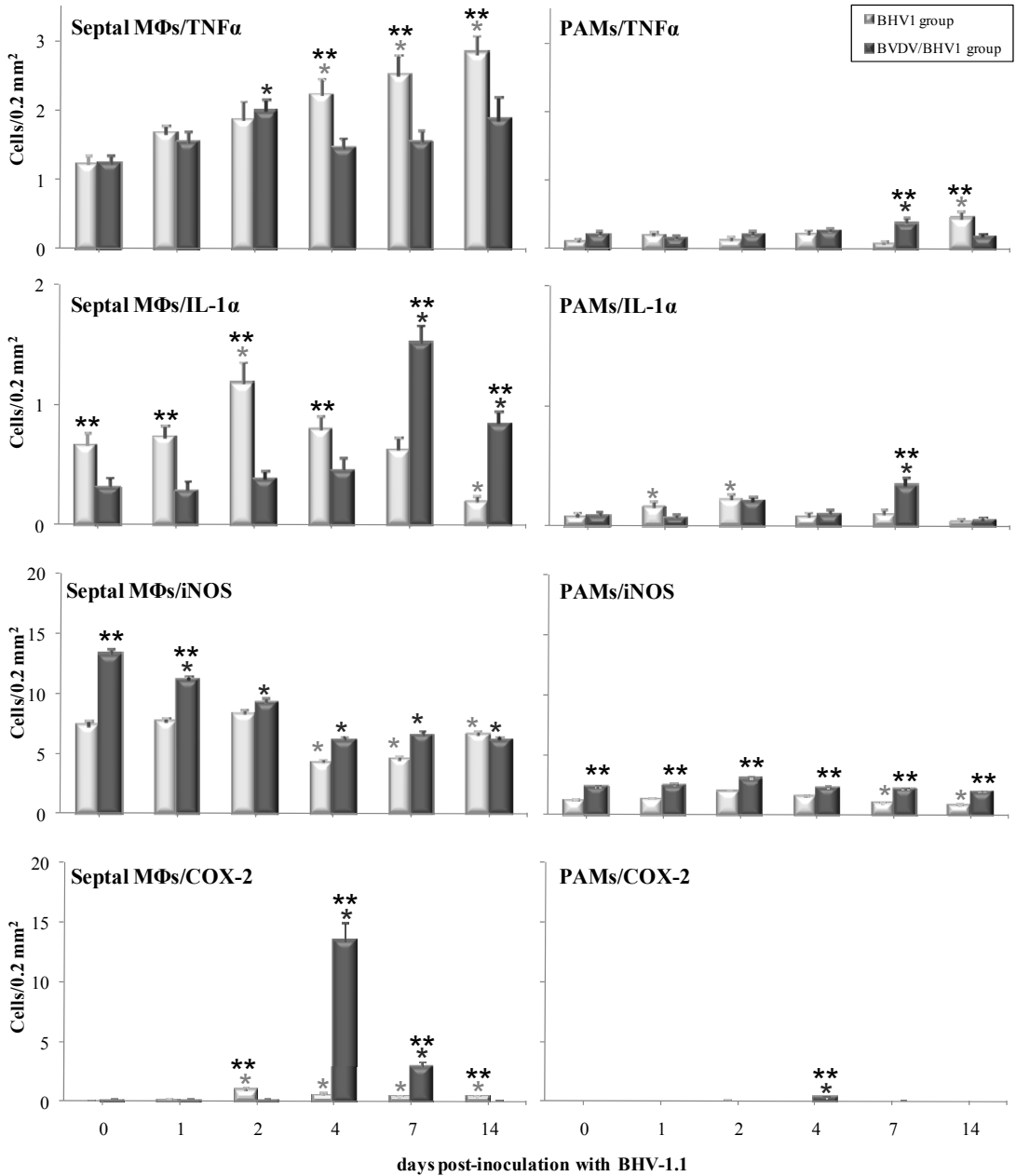
Figure 9. Number of septal MΦs and PAMs (mean ± standard error) positive for TNFα, IL-1α, iNOS and COX-2 by IHC in the lung of calves inoculated with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1. (0, BHV-1.1 UI animals; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Interstitial CD3+ T lymphocytes showed a similar behavior to BHV-1.1 infection in both inoculated groups, presenting an increase in their number between 2 and 7 dpi. However, the magnitude of this response was significantly higher in the BVDV/BHV1 group (p<0.0001), except at 4 dpi due to a peak in the single infection (21.85 cells/0.2 mm^2) (*Figure 10*).

In both inoculated groups, the number of B lymphocytes rose after BHV-1.1 inoculation. This response was earlier and more intense in the BHV1 group, showing a peak at 14 dpi with values 4 times above the baseline level (from 3.3 to 13.85 cells/0.2 mm^2, approximately; p<0.0001) (*Figure 10*).
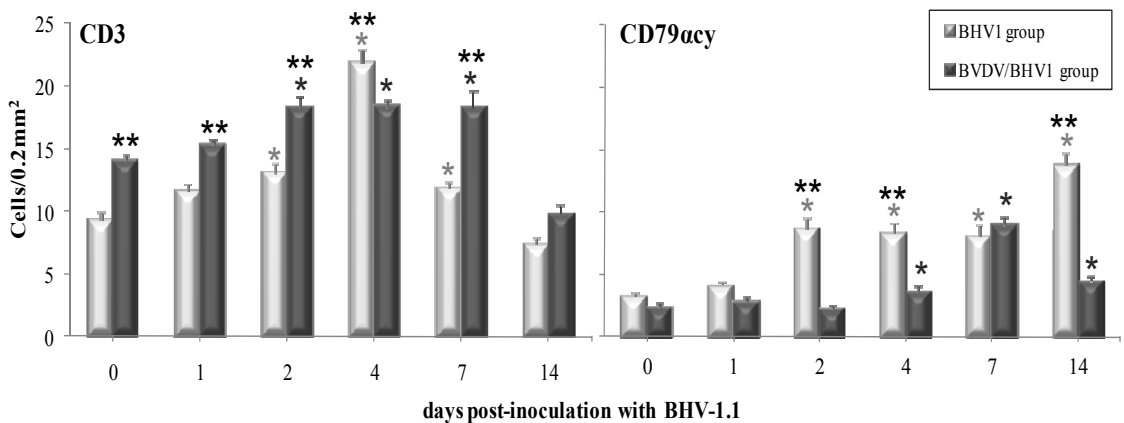
Figure 10. Means±standard errors (n = 2 per time point) of interstitial CD3+ T lymphocytes and CD79αcy+ B lymphocytes in the lung of calves co-infected experimentally with BVDV and BHV-1.1 compared with calves inoculated only with BHV-1.1. (0, BHV-1.1 UI animals; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Immunolabeling of T lymphocyte subpopulations revealed that CD4+, CD8+ and γδ+ lymphocytes also presented a greater number in BVDV pre-infected calves with respect to BHV1 group from the start of the study. Following inoculation with BHV-1.1, these cell populations suffered a progressive decrease from 4 dpi (p<0.0001) in the co-infected calves, reducing

twice their baseline level at 14 dpi. In the BHV1 group, γδ+ lymphocytes presented a similar behavior, while CD8+ and CD4+ T lymphocytes showed a significant increase at 2 and 4 dpi, respectively. IFNγ, an important antiviral cytokine produced by lymphocytes, presented a significant increase in the single infection at 4 dpi, displaying also a peak in later stages (7 dpi) of the dual infection (*Figure 11*).
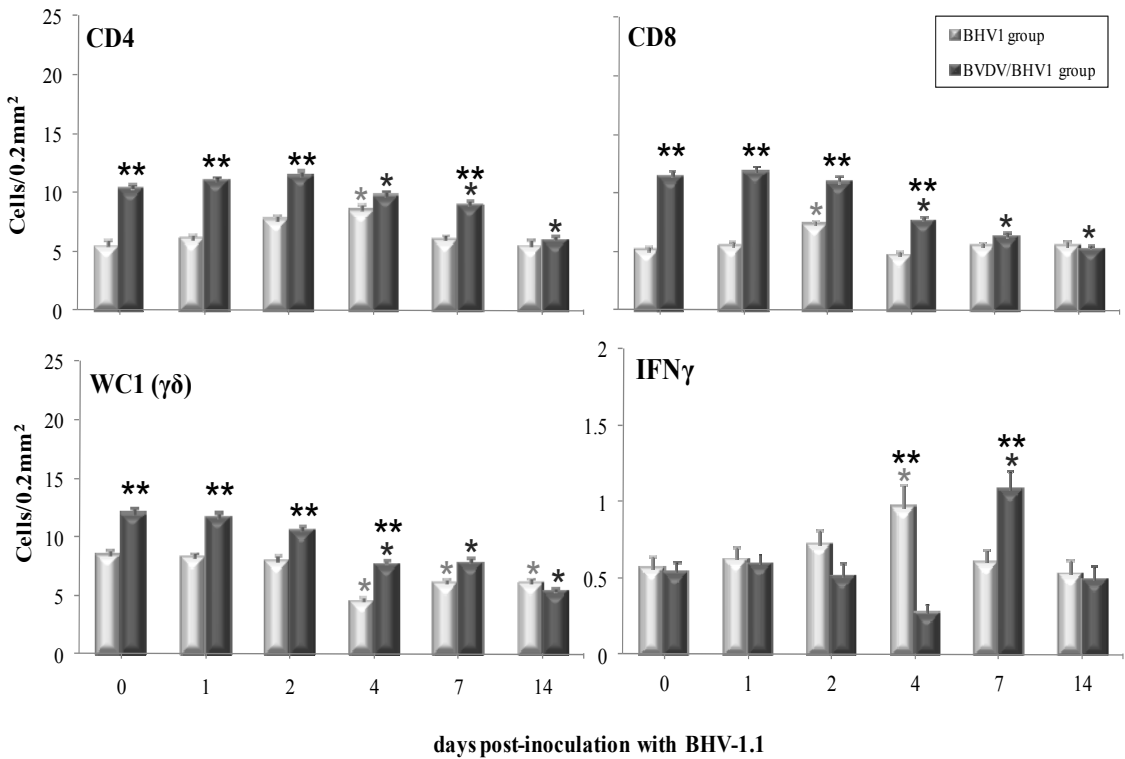
Figure 11. Number (mean ± standard error) of CD4+ T lymphocytes, CD8+ T lymphocytes, γδ+ T lymphocytes and interferon-γ+ lymphocytes in the lung of calves inoculated with BHV-1.1 versus calves inoculated with BVDV and BHV-1.1. (0, BHV-1.1 UI animals; *p<0.05 significant differences in the same group at various time points; **p<0.05 significant differences between inoculated groups at the same time point).

Discussion

The objective of this study was to evaluate and characterize the mononuclear cell infiltrates observed in the lung parenchyma of healthy calves and calves with subclinical BVD experimentally inoculated with BHV-1.1, and to clarify the role of these immunocompetent cells in the local response to the secondary pathogen.

The results showed that following BHV-1 inoculation, both groups of calves displayed a mononuclear cell infiltrate comprising mainly IMΦs and T lymphocytes. This infiltrate occurred earlier, and was more severe, in calves with subclinical BVD. The most intense lesions were observed in the BVDV/BHV1 group at 4 dpi, coinciding with the most severe respiratory symptoms (Risalde et al., 2011b-*Chapter 3*-).

Inoculation with BHV-1 also prompted major vascular alterations in the lungs of both groups, marked by intense intravascular coagulation in small and medium-sized blood vessels from an early stage of the disease. Three mechanisms, jointly known as Virchow's triad, can induce thrombosis (Chung and Lip, 2003): 1) endothelial wall injury; 2) abnormalities of blood constituents (platelets, coagulation and fibrinolytic pathways); and 3) abnormalities of blood flow.

With regard to endothelial damage, infection by human herpes simplex virus – belonging to the same subfamily as BHV-1 (Fauquet et al., 2005) – has been shown to cause endothelial injury, favoring the exposure of subendothelial tissue and the release of procoagulant mediators (Key et al., 1990; Russell et al., 1999). Here, however, neither histopathological nor ultrastructural examination disclosed any morphological evidence of endothelial damage in either of the inoculated groups.

Analysis of the cell components involved in coagulation pathways revealed fibrin deposits and intense platelet aggregation in the pulmonary

microvasculature of both groups; these findings were particularly marked in the BVDV/BHV1 group at 4 dpi. According to this, in the course of certain acute viral infections, platelets may be activated *in vivo*, leading to their degranulation, aggregation and withdrawal from circulation (Boudreaux et al., 1990a,b; Bautista et al., 2002). The procoagulant activity of BVDV and BHV-1 has been reported *in vitro* (Olchowy et al., 1997), being increased in the co-infected calves of our experimental study due to the concomitance of both agents in lung between 2 and 7 dpi; however, at no stage was there any evidence of direct interaction between these viruses and platelets. This would suggest that platelet activation may be enhanced by indirect mechanisms including the expression of inflammatory mediators released by MΦs, which are known to play a major role in the maintenance of tissue homeostasis (Gordon and Taylor, 2005; Tizard, 2008).

The study of pro-inflammatory cytokines revealed alterations in the kinetics and magnitude of TNFα and IL-1 expression; both these mediators can prompt changes in coagulation by increasing the number of endothelial adhesion molecules or increasing vascular permeability (Tolcher et al., 1995; Tizard, 2008). Thus, coinciding with the onset of platelet aggregation in the lungs (2 dpi), calves in the BVH-1 group displayed an increase in IL-1 synthesis by septal MΦs; this, together with the subsequent action of TNFα, would favor maintenance of the procoagulant setting. By contrast, calves in the BVDV/BHV1 group, whilst exhibiting a higher number of IMΦs – the main producers of these cytokines – displayed inhibited IL-1 expression until 7 dpi, along with a minimal TNFα response; these results indicate that the synergic action of both mediators can be ruled out as a potential mechanism for inducing platelet aggregation in co-infected group.

Calves inoculated only with NCP BVDV (0 dpi for the BVDV/BHV1 group) displayed greater expression of iNOS by septal MΦs than healthy

calves (Adler et al., 1994, 1996; Potgieter, 1995). However, following BHV-1 inoculation, calves in the BVDV/BHV1 group exhibited an early decline in iNOS (1 dpi), an inflammatory mediator that limits the extent and duration of pathogen-induced platelet activation (Moore et al., 2011). This finding, together with the moderate response of TNFα, may have favored the appearance of platelet aggregates in the early stages of the disease, and intense aggregation coinciding with the greatest decrease in iNOS levels (4 dpi).

The intense platelet aggregation observed in lung microvasculature of the BVDV/BHV1 group at 4 dpi, together with the increase in number and size of PIMs as a result of phagocytic and secretory activation would indirectly prompt a slowdown in blood flow and a subsequent response by the COX-2 enzyme aimed at reversing that process (Yoon et al., 2009). However, in view of the damage observed at later stages, this action was presumably unable to counter the procoagulant events associated with the drop in iNOS expression, these additionally being enhanced by the delayed action of IL-1 in co-infected animals. Slowed blood flow, together with cytokine release, may lead to increased vascular permeability and extravasation of leukocytes into the pulmonary parenchyma (Tolcher et al., 1995; Feldmann et al., 1996; Law et al., 2007; Tizard, 2008).

With regard to the other immunocompetent cells analyzed in interstitial aggregates, a difference was observed between groups in the behavior of CD8+ and CD4+ T lymphocytes in response to the secondary agent. After BHV-1 inoculation and according to the systemic findings (Molina et al., 2011), healthy calves showed at pulmonary level an early increase of CD8+ T lymphocytes, cytotoxic cells with a major role in preventing the spread of the pathogenic agent (Tizard, 2008). There was also an increase in the number of CD4+ T lymphocytes at 4 dpi, coinciding with the detection of BHV-1 and with a marked IFNγ response in the lung; this

cytokine is known to have a major antiviral function (Biron and Sen, 2001; Samuel, 2001). Taken in conjunction, these results suggest that calves in the BHV-1 group develop an adequate adaptive immune response to BHV-1 at lung level, contributing to elimination of the virus from 7 dpi. However, BVDV pre-infected calves – though displaying greater CD8+ and CD4+ T lymphocyte numbers at the time of BHV-1 inoculation – failed to respond to the presence of this pathogen in the lung (from 2 dpi); indeed, significant lymphocyte depletion was observed from 4 dpi. This inhibition in the proliferative response of CD8+ and CD4+ T lymphocytes, also reported in other infections with NCP strains of BVDV (Howard et al., 1992), coupled with alterations in IFNγ production (Lee et al., 2008; Risalde et al., 2011a,b-*Chapters 1 and 3*-), might impair the development of an adaptive immune response to the secondary viral agent, thus favoring its persistence in the lung, where it was detected until the end of the study (14 dpi).

A marked decrease in the number of Tγδ+ lymphocytes was observed in both inoculated groups from 4 dpi; these cells play a key role in the early stages of inflammation, by stimulating the rapid flow of lymphocytes and monocytes to the site of infection (Tizard, 2008). Thereby, this inter-group similarity suggests that pre-infection with BVDV does not affect the response of these cells to BHV-1 in the lung, being more important their role in protecting mucosal surfaces (Haas et al., 1993; Bruschke et al., 1998b; Pollock and Welsh, 2002).

On the other hand, the increase in B lymphocyte numbers was more marked, and occurred earlier, in calves inoculated with BHV-1 alone. The attenuated B-lymphocyte response observed in the BVDV/BHV1 group may be linked to marked lymphocyte depletion in BVDV target lymphoid organs, lesion already described in these calves (Risalde et al., 2011c-*Chapter 2*-) and

in other *in vivo* infections with BVDV (Liebler-Tenorio et al., 2003a, 2004; Pedrera et al., 2009a,b; Raya et al., 2011).

Conclusions

The results of this study indicate that the concomitance of BVDV and BHV-1 in lung enhance a synergic action of their pathogenic mechanisms, disrupting the maintenance of pulmonary homeostasis by facilitating the establishment of an inflammatory and procoagulant environment, characteristic of the BRDC, which appears to be modulated by inflammatory mediators released by pulmonary MΦs. Moreover, animals pre-inoculated with BVDV - despite suffering a transient infection - exhibit an alteration in the response of pro-inflammatory cytokines which play a key role in activating the immune response, as well as an impaired local cell-mediated response to the secondary respiratory pathogen, marked essentially by an inhibition in the response of CD8+ and CD4+ Th1 lymphocytes.

Conclusions

1. BVDV infection of colostrum-deprived calves induces a transitory and late APR characterized by the production of SAA and Hp in serum, as well as moderate inflammatory changes in liver regulated by TNFα secreted by hepatic MΦs: **Chapter 1**.

2. The primary BVDV infection followed by a challenge with BHV-1 give rise to the earlier development of more intense clinical symptoms and inflammatory lesions –limited to lymphoid tissues, respiratory and digestive tracts– and more spread of BHV-1 than in the single infection with this virus. In addition, the presence of BHV-1 favors the persistence of BVDV in target organs, facilitating a synergic effect of both agents: **Chapter 2**.

3. BVDV-infected calves present at systemic level an inhibition of IL-1 production and greater secretion of TNFα than healthy animals after BHV-1 inoculation, leading to an exacerbation of the inflammatory response confirmed by an increase of APPs. These calves also display an alteration in the Th1 immune response against the

secondary agent based on a delay in the IFNγ production and low levels of IL-12, thus favoring BHV-1 dissemination: **Chapter 3**.

4. BVDV pre-infection facilitates the establishment of an inflammatory and procoagulant environment in the lung, increasing the vascular changes induced by BHV-1 inoculation, which are modulated by inflammatory mediators released by pulmonary MΦs. Furthermore, BVDV pre-infection gives rise to an impaired local cell-mediated response due to an inhibition in the CD8+ and CD4+ Th1 response to the secondary respiratory pathogen: **Chapter 4**.

Summary

BVDV is an important pathogen of cattle, generating considerable economic losses for the livestock industry. This agent has a special tropism for cells of the immune system, inducing cell death as an extreme event of the infection, or more subtle effects on cytokines and co-stimulatory molecules produced by immune or non-immune cells that could affect to both innate and adaptive immune response. Thus, BVDV can produce disease on its own and, which is perhaps more important, induces a state of immunosuppression that predispose calves to infections by other micro-organisms. Consequently, the main objective of this work was to contribute to the study of the immune-evasion strategies of BVDV and the different mechanisms by which primary BVDV infections enhance the susceptibility to secondary infections.

For this purpose, we designed a first experimental model that consisted of a single BVDV infection of colostrum-deprived calves with the aim of examine the immune response of hepatic MΦs and their relationship with the APR during subclinical BVD. Thus, eight colostrum-deprived calves aged 8-12 weeks were inoculated intranasally with the NCP BVDV-1 strain 7443. Two calves were sacrificed at each of 3, 6, 9 and 14 dpi and two UI animals were used as negative controls. The presence of BVDV was detected in liver by RT-PCR and IHC. The hepatic immune response was analyzed through

immunohistochemical study using different monoclonal and polyclonal antibodies to detect BVDV (15c5), MΦs (MAC387), B-lymphocytes (CD79αcy), T-lymphocytes (CD3, CD4, CD8, WC1), and cytokines (TNFα, IL-1α, IL-6 and IFNγ). Serum APP concentrations (Hp, SAA and albumin) were determined by ELISA and colorimetric methods.

Inoculation of these calves results in subclinical infection with inflammatory lesions in the liver, such as vascular changes in portal areas, along with clusters of mononuclear cells composed mainly of MAC387+ MΦs and CD3+ T lymphocytes. BVDV was detected in hepatic MΦs and monocytes from 3 to 14 dpi and in KCs from 6 to 14 dpi.

There were increases in the numbers of KCs, monocytes and interstitial CD3+ T lymphocytes in the liver following inoculation with BVDV. These changes are accompanied by increased immunohistochemical expression of TNFα in hepatic MΦs and transiently increased serum concentrations of SAA and Hp at 12 dpi, indicating that there is upregulation of this pro-inflammatory cytokine in hepatic MΦs during subclinical BVDV infection in calves.

Once valued the alterations in these cell populations and their influence on systemic response, our objective was to study the susceptibility of BVDV-infected calves against secondary infections. For that, it was proposed a second experimental model that included a primary BVDV infection followed by a challenge with BHV-1, in order to reproduce the clinical and pathological symptoms associated with BRDC. Thus, fourteen calves were inoculated intranasally with the NCP strain 7443 of BVDV-1 and twelve days later, when the calves did not show clinical signs and viraemia against BVDV, twelve of them were challenged with BHV-1.1 Iowa strain (BVDV/BHV1 group). The other 2 animals inoculated with BVDV-1 and BHV-1.1-free, were killed before BHV-1.1 inoculation (0 dpi) and used as BVDV infection controls.

Twelve calves were only inoculated with the BHV-1.1 (BHV1 group). The infected calves were sacrificed in batches of two at 1, 2, 4, 7 and 14 dpi. Four UI animals were used as controls and sacrificed at end of the study (negative control group).

Clinical examinations were performed daily. Blood and serum samples were collected at 0, 3, 6, 9, 12, 15, 18 and 21 hpi, 1, 2, 4, 5, 7, 9, 12 and 14 dpi. The presence of BVDV and BHV-1 in blood was assessed by PCR. BVDV and BHV-1 specific Abs were measured by ELISA and VN, respectively. The systemic inflammatory and immune response was analyzed through the study of serum concentrations of cytokines (IL-1β, TNFα, IFNγ, IL-12, IL-4 and IL-10) measured by ELISA, and serum APPs levels (Hp, SAA and fibrinogen) determined by ELISA and colorimetric methods.

At post-mortem examination, samples collected from lymphoid tissues, respiratory tract, digestive tract and nervous system were fixed and routinely processed for histopathological and ultrastructural examination. Identification of BVDV and BHV-1.1 surface glycoproteins, gp48 and gC respectively, was carried out through immunohistochemical study in formalin fixed samples. Apoptosis in tissue samples was examined by TUNEL detection method.

The calves of BHV1 group displayed subclinical infection, while the calves of BVDV/BHV1 group presented fever, mucopurulent oculonasal discharge, respiratory distress and recurring diarrhea. These results indicate that BVDV pre-infection induces more severity of the symptoms prompted by BHV-1, and that inoculation of this secondary agent favors the reappearance of digestive alterations attributed to BVDV.

BVDV was not detected in the blood of calves of the BVDV/BHV1 group at the moment of BHV-1.1 inoculation; by contrast, it was detected between 1 and 5 dpi with BHV-1. The presence of BHV-1 was confirmed in nasal swabs samples of both inoculated groups from 1 dpi onwards. Viraemia

was not detected in the BHV1 group at any stage, the only finding being small amounts of viral antigen in the tonsils at 4 dpi. In the BVDV/BHV1 group, by contrast, viraemia was detected from 4 dpi onwards, coinciding with the appearance of fever and clinical symptoms characteristic of BHV-1 infection, together with abundant viral antigen and IIBs in tonsils, associated with necrotic lesions due to the cytopathic effect of BHV-1.

BVDV antigen was only detectable in tissues of the BVDV/BHV1 group, being present in lymphoid tissues, specifically in the thymus and ileocecal lymph node, as well as mucosa-associated lymphoid tissue of the ileum and ileocecal valve during very late stages of the disease, a finding not reported in studies of calves inoculated with BVDV alone. Moreover, these animals displayed lesions prior to BHV-1 infection, including apoptosis-associated lymphoid depletion in B-dependent areas and thymus. These changes were more intense following BHV-1 inoculation, which can also induce apoptosis. Thus, it seems that the presence of BHV-1 favors the persistence of BVDV in target organs and that the synergic effect of both agents would enhance the immunosuppressive action of BVDV.

Both groups of calves displayed inflammatory alterations in the respiratory tract and lymphoid tissues following BHV-1 inoculation, characterized by mononuclear infiltrates composed of MΦs, lymphocytes and plasmatic cells associated with vascular changes, which were earlier and more severe in the BVDV/BHV1 group. This marked inflammatory response in the co-infected calves was confirmed by analysis of APPs, which showed an increase in SAA protein and fibrinogen, associated with greater secretion of TNFα and reduced production of IL-10. However, IL-1β synthesis rather than increasing actually declined, avoiding the appearance of a synergic proinflammatory effect.

After BHV-1 infection, both groups of calves developed a Th1 immune response based on IFNγ production and on the absence of IL-4. However, differences between groups were apparent with regard to the kinetics and magnitude of IFNγ and IL-12 production. In the BHV1 group, IFNγ levels rose from the start of the study; this increase, together with an intense expression of IL-12, could prevent the distribution of BHV-1 from primary replication sites into the blood. In the BVDV/BHV1 group, by contrast, IFNγ did not increase until 4 and 5 dpi, coinciding with the detection of BHV-1 in blood and with mild IL-12 production, suggesting an inhibition of the cytotoxic effect on infected cells at the start of the process.

On the other hand, prior inoculation of BVDV did not modify the development of a BHV-1-specific humoral response in comparison with that of healthy animals, detecting neutralizing Abs against BHV-1.1 in both inoculated groups at 14 dpi.

BVDV is considered as the main predisposing factor for the occurrence of BRDC through an alteration of the immune response, thus favoring the lung colonization by other pathogens. There is evidence that combined infections with BVDV have a potentiating effect on several pathogens, increasing in a more severe form the respiratory disease compared with calves single-infected. Therefore, the last objective was to clarify the mechanisms responsible for histopathological and ultrastructural changes occurring in the lung of calves pre-infected with BVDV and challenged later with BHV-1, evaluating the role of MΦs and other immunocompetent cells in the development of pathological lesions in this organ. For this purpose, pulmonary lesions and local immune response were compared between both inoculated groups through immunohistochemical studies (Factor-VIII, MAC387, TNFα, IL-1α, iNOS, COX-2, CD3, CD79αcy, CD4, CD8 and IFNγ).

In the BVDV/BHV1 group, BVDV was detected by molecular techniques between 0 and 7 dpi (12-19 dpi BVDV), and BHV-1 was detected from 2 to 14 dpi. However, in the BHV1 group, this virus was only detected between 4 and 7 dpi.

The pulmonary parenchyma of inoculated calves was affected by an interstitial pneumonia produced by mononuclear aggregates of IMΦs and T lymphocytes, which were mostly TCD4+, TCD8+ and Tγδ; a few interspersed B cells were also observed. Moreover, both groups presented important vascular alterations as fibrin microthrombi and platelet aggregations within the blood vessels. These changes were earlier and more severe in the co-infected calves, where were associated with occasional alveolar oedema and haemorrhages.

Study of the pro-inflammatory cytokines revealed that BVH-1 group displayed an increase in IL-1 synthesis by septal MΦs, that together with the subsequent action of TNFα, would favored the onset and maintenance of the procoagulant environment. By contrast, BVDV/BHV1 group displayed an alteration in the response of these cytokines, presenting a delayed IL-1 expression, along with a minimal TNFα response, ruling out a synergic action of both mediators as a potential mechanism for inducing platelet aggregation in these calves. In this regard, BVDV pre-infected calves showed after BHV-1 inoculation a higher decline in iNOS expression, which acts limiting the action of pathogen-induced platelet activation; in fact, the greatest decrease of this enzyme coincided with the most severe coagulation lesions (4 dpi). This intense platelet aggregation, together with an increase in number and size of PIMs by their phagocytic and secretory activation, would indirectly prompt a slowdown in blood flow and a subsequent response by the COX-2 enzyme, which action was unable to counter the procoagulant events. These results indicate that the concomitance of BVDV and BHV-1 in lung produces a

greater disruption of pulmonary homeostasis by facilitating the establishment of an inflammatory and procoagulant environment modulated by inflammatory mediators released by pulmonary MΦs.

With regard to the other immunocompetent cells studied in interstitial aggregates, calves infected with BHV-1 alone showed a greater number of B lymphocytes than the co-infected calves, which attenuated response may be linked to B cells depletion in BVDV target lymphoid organs.

T lymphocytes response was characterized by differences in the CD8+ and CD4+ lymphocytes, not affecting the BVDV pre-infection to Tγδ+ lymphocytes response against BHV-1 in the lung. Thus, the calves of the BHV1 group developed an adequate adaptive immune response to BHV-1 in lung, based on an increase of CD8+ and CD4+ T lymphocytes, as well as a marked IFNγ response, which contributed to virus elimination from 7 dpi. However, BVDV pre-infected calves, despite displaying greater numbers of these cells before BHV-1 inoculation, exhibited an impaired local cell-mediated response to the presence of BHV-1, marked by an inhibition in the response of CD8+ and CD4+ Th1 lymphocytes.

Resumen

El virus de la diarrea vírica bovina (VDVB) es un importante patógeno del ganado vacuno que genera considerables pérdidas económicas en la industria ganadera. Este agente tiene un especial tropismo por células del sistema inmune, induciendo la muerte celular como efecto extremo de la infección, o alteraciones más sutiles sobre las citoquinas y otras moléculas co-estimuladoras producidas por células inmunes o no inmunes que podrían afectar tanto a la respuesta inmune innata como a la adaptativa. Por lo tanto, VDVB puede producir enfermedad por sí mismo y, lo que es más importante, inducir un estado de inmunosupresión que predispone a la infección por otros microorganismos. El objetivo principal de este trabajo fue contribuir al estudio de las estrategias de evasión del VDVB ante el sistema inmune y de los diferentes mecanismos por los que las infecciones primarias con este virus aumentan la susceptibilidad a infecciones respiratorias secundarias.

Con este fin, diseñamos un primer modelo experimental que consistió en la infección de terneros no encalostrados con el VDVB con el objetivo de estudiar la respuesta inmune local de los MΦs hepáticos ante esta infección y su relación con la respuesta de fase aguda (RFA). Para ello, ocho terneros no encalostrados de 8-12 semanas fueron inoculados intranasalmente con la cepa

7443 del VDVB-1 NCP y sacrificados en grupos de dos a los 3, 6, 9 y 14 dpi. Además, 2 terneros no inoculados fueron utilizados como controles negativos de la infección y sacrificados al final de la experiencia. La presencia del VDVB fue detectada en el hígado por RT-PCR e inmunohistoquímica (IHQ). Para analizar la respuesta inmune hepática se utilizó el estudio inmunohistoquímico mediante diferentes anticuerpos monoclonales y policlonales para detectar VDVB (15c5), MΦs (MAC387), linfocitos B (CD79αcy), linfocitos T (CD3, CD4, CD8, WC1) y citoquinas (TNF α, IL-1α, IL-6 e IFN). Las concentraciones séricas de diferentes proteínas de fase aguda (PFAs) (haptoglobina –Hp-, amiloide A del suero –AAS- y albúmina) se determinaron por ELISA y métodos colorimétricos.

La infección de estos terneros dio lugar a una enfermedad subclínica con lesiones inflamatorias en hígado, tales como cambios vasculares y agregados monucleares en áreas periportales, constituidos por MΦs MAC387+ y linfocitos T CD3+. El VDVB fue detectado en MΦs hepáticos y monocitos desde los 3 dpi y en las células de Kupffer (CKs) desde los 6 dpi.

Además, tras la inoculación con el VDVB se observó un aumento en el número de CKs, monocitos y linfocitos T intersticiales CD3+. Estos cambios estuvieron acompañados de un aumento en la expresión inmunohistoquímica de TNFα en MΦs hepáticos y una respuesta transitoria en las concentraciones séricas de AAS y Hp a los 12 dpi, lo que indica que existe una respuesta de esta citoquina pro-inflamatoria por parte de los MΦs hepáticos durante la infección subclínica del VDVB.

Una vez valoradas las alteraciones en estas poblaciones celulares y su influencia en la respuesta sistémica, nuestro objetivo fue estudiar la susceptibilidad de los terneros infectados con el VDVB ante infecciones

secundarias. De este modo, se propuso un segundo modelo experimental que incluía una infección primaria por el VDVB seguida de una infección secundaria con el herpesvirus bovino 1 (HVB-1), con el fin de reproducir los síntomas clínicos y patológicos asociados al complejo respiratorio bovino (CRB). Para ello, 14 terneros fueron inoculados intranasalmente con la cepa 7443 del VDVB-1 NCP y doce días después, cuando los terneros no mostraron signos clínicos de enfermedad ni viremia frente al VDVB, 12 de ellos fueron inoculados con la cepa Iowa del HVB-1.1 (grupo VDVB/HVB1). Los otros 2 animales, inoculados con el VDVB y libres del HVB-1.1, fueron sacrificados antes de la inoculación con el HVB-1.1 (0 dpi) y utilizados como controles para el grupo pre-infectado con el VDVB. Otro grupo de 12 terneros fueron inoculados solamente con el HVB-1.1 (grupo HVB1). Los terneros infectados fueron sacrificados en grupos de dos a los 1, 2, 4, 7 y 14 dpi. Por otro lado, 4 terneros no infectados fueron utilizados como controles negativos y sacrificados al final del estudio (grupo control negativo).

El examen clínico de los terneros fue realizado diariamente. Las muestras de sangre y el suero se recolectaron a las 0, 3, 6, 9, 12, 15, 18 y 21 hpi, 1, 2, 4, 5, 7, 9, 12 y 14 dpi. La presencia del VDVB y el HVB-1 en sangre se determinó mediante PCR. Los anticuerpos específicos frente al VDVB y HVB-1 se examinaron por ELISA y seroneutralización, respectivamente. La respuesta inflamatoria sistémica e inmune se analizó a través del estudio de las concentraciones séricas de citoquinas (IL-1β, TNFα, IFNγ, IL-12, IL-4 e IL-10) medidas por ELISA y de PFAs (Hp, AAS y fibrinógeno) determinadas por ELISA y métodos colorimétricos.

En el examen post-mortem, las muestras obtenidas de tejidos linfoides, tracto respiratorio, tracto digestivo y sistema nervioso fueron fijadas y procesadas rutinariamente para su estudio histopatológico y ultraestructural.

La identificación de las glicoproteínas de superficie del VDVB y HVB-1, gp48 y gC respectivamente, se llevó a cabo por IHQ de las muestras fijadas en formol. La determinación de apoptosis en tejidos fue realizada por el método de detección de TUNEL.

Los terneros del grupo HVB1 mostraron una infección subclínica, mientras que los del grupo VDVB/HVB1 presentaron fiebre, secreción oculonasal mucopurulenta, dificultad respiratoria y diarrea recurrente. Estos resultados indican que la pre-infección con el VDVB induce síntomas más severos asociados al HVB-1, y que la inoculación de este agente secundario favorece la reaparición de los signos digestivos atribuidos al VDVB.

El VDVB no fue detectado en la sangre de los terneros del grupo VDVB/HVB1 en el momento de la inoculación con el HVB-1, en cambio, se detectó entre los 1 y 5 dpi HVB-1. La presencia del HVB-1 fue confirmada en muestras de hisopos nasales de ambos grupos inoculados a partir del 1 dpi. La viremia no se detectó en el grupo HVB1 en ningún momento, siendo el único hallazgo una pequeña cantidad de antígeno vírico en las tonsilas a los 4 dpi detectada por IHQ. En el grupo VDVB/HVB1, por el contrario, la viremia se detectó a partir de los 4 dpi, coincidiendo con la aparición de fiebre y los síntomas clínicos característicos de la infección por el HVB-1, junto con una abundante cantidad de antígeno vírico y cuerpos de inclusión en las tonsilas, asociados con lesiones necróticas por el efecto citopático del HVB-1.

El antígeno del VDVB sólo se detectó en los tejidos del grupo VDVB/HVB1, estando presente en tejidos linfoides, especialmente en nódulos linfáticos ileocecales, timo y tejido linfoide asociado a la mucosa de la válvula ileocecal y del íleon en fases muy avanzadas de la enfermedad, hallazgo que no se encontró en otros estudios con inoculaciones simples del VDVB. Por otra parte, estos animales mostraron lesiones previas a la inoculación con el

HVB-1, incluyendo apoptosis asociada a la depleción linfocitaria en las zonas B dependientes y timo. Estos cambios fueron más intensos tras la inoculación con el HVB-1, el cual también puede inducir apoptosis. Por lo tanto, parece que la presencia de HVB-1 favorece la persistencia del VDVB en sus órganos diana y que el efecto sinérgico estos agentes potencia la acción inmunosupresora del VDVB.

Los dos grupos de animales muestran alteraciones inflamatorias en el tracto respiratorio y los órganos linfoides tras la inoculación con el HVB-1, caracterizadas por un infiltrado mononuclear compuesto de MΦs, linfocitos y células plasmáticas asociados con cambios vasculares anteriores y más graves en el grupo VDVB/HVB1. Esta marcada respuesta inflamatoria en los animales co-infectados fue confirmada por el estudio de PFAs, observándose un incremento de la proteína SAA y el fibrinógeno, asociado con una mayor secreción de TNFα y una reducción de la IL-10. Sin embargo, la síntesis de IL-1β disminuyó, evitando la instauración de un efecto pro-inflamatorio sinérgico entre estas citoquinas.

Tras la infección con el HVB-1, los dos grupos desarrollaron una respuesta inmune Th1 con la producción de IFNγ y la ausencia de IL-4. Sin embargo, las diferencias entre grupos fueron evidentes con respecto a la cinética y magnitud de respuesta del IFNγ y la IL-12. Así, en el grupo HVB1, los niveles de IFNγ fueron elevados desde el inicio del estudio; este fenómeno, junto con la intensa expresión de la IL-12, podría prevenir la diseminación del HVB-1 desde los sitios de replicación primaria a sangre. En el grupo VDVB/HVB1, por el contrario, el IFNγ no aumentó hasta los 4-5 dpi, coincidiendo con la detección de HVB-1 en sangre y con una ligera respuesta de la IL-12, lo que sugiere una inhibición de la acción citotóxica de estos mediadores frente a las células infectadas en el inicio del proceso.

Por otra parte, la pre-infección con el VDVB no alteró el desarrollo de una respuesta humoral específica frente al HVB-1, detectándose anticuerpos neutralizantes en ambos grupos inoculados a los 14 dpi.

El VDVB es considerado como el principal factor predisponente en la aparición del CRB, dando lugar a una alteración de la respuesta inmune del hospedador para favorecer la colonización pulmonar por otros patógenos respiratorios. Existen evidencias de que las infecciones combinadas con el VDVB tienen un efecto potenciador sobre otros patógenos, aumentando en una forma más severa la enfermedad respiratoria. Por lo tanto, el último objetivo consistió en aclarar los mecanismos responsables de los cambios histopatológicos y ultraestructurales que ocurren en el pulmón de terneros pre-infectados con el VDVB e inoculados más tarde con el HVB-1, así como evaluar el papel de los MΦs y otras células inmunocompetentes en el desarrollo de lesiones patológicas en este órgano. Para ello, las lesiones pulmonares y la respuesta inmune local fueron comparadas entre ambos grupos inoculados por IHQ mediante diferentes anticuerpos monoclonales y policlonales (Factor-VIII, MAC387, TNFα, IL-1α, iNOS, COX-2, CD3, CD79αcy, CD4, CD8 e IFNγ).

En el grupo VDVB/HVB1, el VDVB fue detectado mediante técnicas moleculares entre los 0 y los 7 dpi (12-19 dpi VDVB) y el HVB-1 a partir de los 2 dpi. Sin embargo, en el grupo HVB1, este virus sólo se detectó entre los 4 y los 7 dpi.

El parénquima pulmonar de los terneros inoculados se vio afectado por una neumonía intersticial producida por agregados mononucleares de MΦs intersticiales y linfocitos T, principalmente CD4+, CD8+ y Tγδ, además de algunas células B. Por otra parte, ambos grupos presentaron importantes

alteraciones vasculares como la presencia de microtrombos de fibrina y numerosos agregados plaquetarios en vasos sanguíneos del pulmón. Estos cambios se presentaron de forma más temprana y severa en los animales co-infectados, donde además se asociaron a la presencia ocasional de edema alveolar y hemorragias.

El estudio de las citoquinas pro-inflamatorias reveló que los terneros del grupo HVB1 mostraron un aumento en la síntesis de IL-1 por MΦs septales, lo que unido a la acción posterior del TNFα favoreció la aparición y el mantenimiento de un ambiente procoagulante. Por el contrario, los terneros del grupo VDVB/HVB1 mostraron una alteración en la respuesta de estas citoquinas, basada en una expresión retrasada de la IL-1 y una mínima respuesta del TNFα, descartando una acción sinérgica entre ambos mediadores como mecanismo potencial para inducir la agregación plaquetaria en estos terneros. En este sentido, los terneros pre-infectados con el VDVB mostraron tras la inoculación con el HVB-1 una mayor disminución en la expresión de iNOS por los MΦs septales, enzima que actúa limitando la acción de patógenos inductores de la activación plaquetaria, coincidiendo su mayor descenso con una mayor presencia de fenómenos de coagulación (4 dpi). Esta intensa agregación plaquetaria, junto con el aumento en número y tamaño de los MΦs intravasculares pulmonares debido a su activación fagocítica y secretora, provocaría indirectamente un enlentecimiento del flujo sanguíneo y una posterior respuesta de la enzima COX-2, cuya acción fue incapaz de contrarrestar los mecanismos procoagulantes. Estos resultados indican que la concomitancia del VDVB y del HVB-1 en pulmón produce una mayor alteración en la homeostasis pulmonar, facilitando la creación de un ambiente inflamatorio y procoagulante modulado por mediadores inflamatorios liberados por los MΦs pulmonares.

Con respecto a las otras células inmunocompetentes que formaron parte de los agregados intersticiales, los terneros infectados solamente con el HVB-1 mostraron un mayor número de linfocitos B que los terneros co-infectados, cuya respuesta pudo estar atenuada por la marcada depleción de estas células en los órganos linfoides diana del VDVB.

La respuesta de los linfocitos T se caracterizó por las diferencias entre los linfocitos T CD8+ y CD4+, no afectando la pre-infección con el VDVB a la respuesta frente al HVB-1. Así, los terneros del grupo HVB1 desarrollaron una adecuada respuesta inmune adaptativa ante el HVB-1 en pulmón, caracterizada por un aumento de los linfocitos T CD8+ y CD4+, así como una marcada respuesta del IFNγ, lo que contribuyó a la eliminación del virus a partir de los 7 dpi. Sin embargo, los terneros pre-infectados con el VDVB, pese a mostrar un mayor número de estas células antes de la inoculación con el HVB-1, presentaron una alteración de la respuesta inmune local celulomediada ante la presencia del HVB-1, marcada por una inhibición en la respuesta de los linfocitos T CD8+ y CD4+ Th1.

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Annex

PUBLICATIONS

Risalde MA, Gómez-Villamandos JC, Pedrera M, Molina V, Cerón JJ, Martínez-Subiela S, Sánchez-Cordón PJ (2011). Hepatic immune response in calves during acute subclinical infection with bovine viral diarrhea virus type 1. The Veterinary Journal 190: e110-e116.
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Risalde MA, Molina V, Sánchez-Cordón PJ, Pedrera M, Panadero R, Romero-Palomo F, Gómez-Villamandos JC (2011). Response of pro-inflammatory and anti-inflammatory cytokines in calves with subclinical bovine viral diarrhea challenged with bovine herpesvirus-1. Veterinary Immunology and Immunopathology 144(1-2): 135-143.
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Risalde MA, Molina V, Romero-Palomo F, Sánchez-Cordón PJ, Pedrera M, Gómez-Villamandos JC (2011). Rinotraqueitis infecciosa bovina: clínica, patogenia e inmunosupresión. Tierras (ganadería) (en prensa).
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CHAPTERS IN BOOKS

Risalde MA, Sánchez-Cordón PJ, Pedrera M, Molina V, Romero-Palomo F, Gómez-Villamandos JC (2011). Bovine viral diarrhea virus: biology, clinical forms and pathogenesis. In: Ruminants: Anatomy, Behavior and Diseases. Ed. Nova Science Publishers, Inc. Hauppauge, NY, USA. (in press).

COMMUNICATIONS AT MEETINGS

Risalde MA, Molina V, Pedrera M, Romero-Palomo F, Sánchez-Cordón PJ, Gómez-Villamandos JC (2011). Estudio de la implicación del virus de la Diarrea Vírica Bovina en la respuesta inflamatoria frente a infecciones secundarias. En: XXXVI Congreso de la Sociedad Española de Inmunología, Pamplona (España). Tipo de participación: Póster.

Risalde MA, Molina V, Sánchez-Cordón PJ, Pedrera M, Romero-Palomo F, Gómez-Villamandos JC (2010). Implication of the pulmonary macrophages in the innate immune response in a mixed infection (BVDV/BHV-1.1). In: 28th Meeting of the European Society of Veterinary Pathology and European College of Veterinary Pathologsits, Belgrade (Serbia). Type of participation: Poster.

Risalde MA, Molina V, Pedrera M, Sánchez-Cordón PJ, Romero-Palomo F, Gómez-Villamandos JC (2010). Respuesta inmunitaria en pulmón durante una infección experimental mixta con el vDVB y HVB-1.1. En: XXXV Congreso de la Sociedad Española de Inmunología, San Sebastián (España). Tipo de participación: Comunicación Oral.

Risalde MA, Molina V, Pedrera M, Sánchez-Cordón PJ, Moreno A, Gómez-Villamandos JC (2010). Estudio secuencial de los macrófagos pulmonares en la IBR y su implicación en la patogenia de la enfermedad. En: XXXV Congreso de la Sociedad Española de Inmunología, San Sebastián (España). Tipo de participación: Póster.

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