UNIVERSIDAD DE CÓRDOBA
FACULTAD DE VETERINARIA

TESIS DOCTORAL

SYSTEMIC AND LOCAL CELL-MEDIATED IMMUNE RESPONSE AGAINST BHV-1 IN HEALTHY AND BVDV-INFECTED CALVES

RESPUESTA INMUNE CELULAR SISTÉMICA Y LOCAL FRENTE AL HVB-1 EN TERNEROS SANOS Y TERNEROS CON EL vDVB

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Departamento de Anatomía y Anatomía Patológica Comparadas

Córdoba, Marzo 2013

TITULO: *RESPUESTA INMUNE CELULAR SISTÉMICA Y LOCAL FRENTE AL HVB-1 EN TERNER0OS SANOS Y TERNEROS CON vDVB.*

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TÍTULO DE LA TESIS:

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DOCTORANDO/A: VERÓNICA Mª MOLINA HERNÁNDEZ

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

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 immune en terneros persistentemente infectados con el virus de la Diarrhea Vírica Bovina e inoculados con Herpesvirus Bovino tipo 1", y AGR-4671, titulado "Respuesta immune 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 evasion del sistema immune inducidas por el Virus de la Diarrea Vírica Bovina, así como analizar los mecanismos inmunológicos que se desencadenan ante una infección secundaria, estableciendo las alteraciones que pudiesen inducir una respuesta immune inadecuada y una mayor susceptibilidad a la infección. Los resultados obtenidos han permitido realizar tres artículos enviados a revistas científicas indexadas, dos de los cuales ya han sido publicados en *Transboundary and Emerging Diseases y Research in Veterinary Science,* además de ser expuestos en diferentes congresos nacionales e internacionales.

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

Córdoba, 22 de Marzo de 2013

Firma del/de los director/es

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

A mi familia
A Pedro

A todos los que iluminan mi camino

"Casi todos los que desconfían de sus propias fuerzas ignoran el maravilloso poder de la atención prolongada"

De "Reglas y consejos sobre investigación científica: Los tónicos de la voluntad"

D. Santiago Ramón y Cajal

TABLE OF CONTENTS

ABBREVIATIONS

°C	Celsius grades
ABC	avidin-biotin-peroxidase complex
Abs	antibodies
Ag	antigen
APCs	antigen-presenting cells
APPs	acute phase proteins
APR	acute phase response
BALT	bronchus-associated lymphoid tissue
BDV	border disease virus
BHV-1	bovine herpesvirus-1
BHV1 group	calves inoculated with BHV-1
BRDC	bovine respiratory disease complex
BRSV	bovine respiratory syncytial virus
BS	Bouin's solution
BSA	bovine serum albumin
BVD	bovine viral diarrhea
BVDV	bovine viral diarrhea virus
BVDV/BHV1 group	calves inoculated with BVDV and BHV-1
CD	cluster of differentiation
cp	cytopathogenic
CSFV	classical swine fever virus
Ct	cycle threshold
DCs	dendritic cells
DNA	deoxyribonucleic acid
dpi	days post-inoculation
dpi BHV1	days post-inoculation with BHV-1.1

dpi BVDV	days post-inoculation with BVDV
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
gp or g	glycoprotein
h	hours
HE	haematoxylin and eosin stain
HEVs	high endothelial venules
Hp	haptoglobin
hpi	hours post-inoculation
hpi BHV1	hours post-inoculation with BHV-1.1
IBR	infectious bovine rhinotracheitis
i.e.	*id est*; means *that is, which is to say*
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
Kb	kilobase
l	liters
mAbs	monoclonal antibodies
MD	mucosal disease
MDBK	Madin Darby Bovine Kidney cells
mg	miligrams
MHC	major histocompatibility complex
min	minutes
ml	milliliters
mm^2	millimeters squared
MW	microwave
ncp	non-cytopathogenic
ng	nanograms

nm	nanometers
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
PE	phycoerythrin
PI	persistently infected animals
PI-3	parainfluenza-3
PMN	polymorphonuclear
RNA	ribonucleic acid
rpm	revolutions per minute
Rt	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SAA	serum amyloid A
SEM	standard error media
$TCID_{50}$	tissue culture infective dose 50%
TCR	T-cell receptor
TEM	transmission electron microscopy
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
Tris	tris (hydroxymethyl) aminomethane
UI	uninfected animals
UTR	untranslated regions
VN	virus neutralization
μm	micrometers

PREFACE

Aim and scope of the thesis

Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* of the *Flaviviridae* family that also include classical swine fever virus (CSFV) and border disease virus (BDV). BVDV is a major pathogen of cattle encompassing viruses of two recognized different species, BVDV-1 and BVDV-2 that are of varying virulence from avirulent to highly virulent. BVDV strains of either species exist as two biotypes, a non-cytopathic form (ncp) that is widely distributed and a cytopathic form (cp) that is associated predominantly with animals that develop mucosal disease (MD). The way BVDV interacts with the immune system is critical both for the pathogenesis of the BVDV disease complex and for the development of immunity.

Bovine herpesvirus-1 (BHV-1) is a member of the subfamily *Alphaherpesvirinae* belonging to the family *Herpesviridae*. One group of the viruses, classified as subtype 1 (BHV-1.1), is the causative agent of the infectious bovine rhinotracheitis (IBR) and causes severe respiratory tract disease, conjuntivitis and abortion. This subtype is considerer one of the most important of the respiratory viruses in cattle.

Bovine respiratory disease complex (BRDC) is a major cause of economic loss in the cattle industry. Even a mild case of bovine respiratory disease can set the stage for infection by other pathogens, weakening the immune system and making the animal more susceptible to secondary infections. Several infectious agents have been associated with BRDC, including BVDV and BHV-1, which are present in almost all herds. Frequently, severe respiratory tract disease in cattle is associated with concurrent infections of these pathogens. Although both viruses can colonize the respiratory tract on its own, their participation in the BRDC is mainly due to its enhancer role in mixed infections derived from its significant immunosuppressive effect.

Both BVDV and BHV-1 enter the susceptible host primarily by the oronasal route and initially replicate in the nasal mucosa followed by the dissemination of the virus to tonsils, and then spreads to the regional lymph nodes followed by a systemic viremia wich can be as free virus form or cell-associated virus, mainly to lymphocytes and monocytes-macrophages.

However, cell-immune responses to ncp BVDV-1 and BHV-1 infections in vivo have not been fully studied in detail. Unraveling the BVDV and BHV-1 interactions with host will be a clue to better understand the mechanisms involved in the viral pathogenesis.

In the thesis, the author described interactions of BVDV on systemic and local immune response, primarily focusing on cell-mediated immunity, and their enhanced effect in the susceptibility to secondary infections such as BHV-1.1. In the GENERAL INTRODUCTION, known the basics about background, overview and update on aspects of the pathogenesis of BVD and IBR were overviewed for the background of the thesis.

In CHAPTER 1, the author described blood parameters implicated in the cell-mediated immune response in BVDV infected calves.

With the purpose of deepen in the study the consequences of the immunosuppressive effect of BVDV and the enhancement of susceptibility against secondary infections, the author propose the CHAPTER 2, examinining the systemic changes of immunocompetent cells in apparently recovery calves of BVD, challenged with BHV-1.1.

In CHAPTER 3, the author described and discussed the role of organs such as nasal mucosa and palatine tonsil located in the entry of the oronasal route as important site of initial replication following BVDV and BHV-1.1 infections.

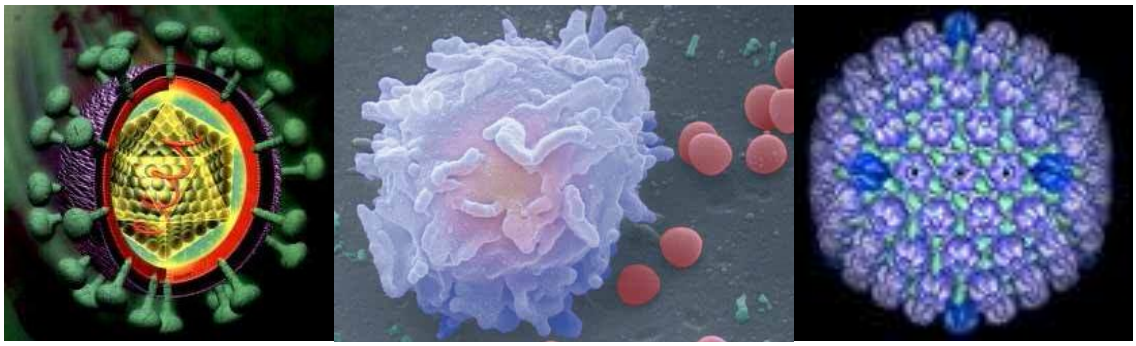
In GENERAL CONCLUSIONS, the author presents the major conclusions drawn from this thesis.

The realization of the present thesis has been possible thanks to following projects:

"Estudio de la respuesta inmune en terneros persistentemente infectados con el virus de la Diarrea Vírica Bovina e inoculados con Herpesvirus Bovino tipo 1" (AGL2006-01536) funded by the Spanish Ministry of Education and Science.

"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" (P09-AGR-4671) funded by the Andalusian Autonomous Regional Government-ERDF.

The author was supported by a pre-doctoral grant (BES-2007-16257) from the Spanish Ministry of Education and Science.



GENERAL INTRODUCTION

I. BOVINE VIRAL DIARRHEA VIRUS (BVDV)

1. History of Bovine Viral Diarrhea (BVD)

BVD is an endemic viral disease affecting both economical and welfare aspects of the cattle industry globally since many years ago. BVD was first described in the 1940s after an outbreak of severe diarrhea that affected cattle herds of the province of Saskatchewan, Canada and in the state of New York, USA. The disease was first termed “X disease” and subsequently “virus diarrhea” due to suspicions of a viral etiology (Childs, 1946; Olafson et al., 1946). The disease presented a high morbidity and low mortality. After the first report of this new transmissible disease of cattle, a viral etiology was confirmed (Baker et al., 1954). Also, a similar virus was subsequently associated with a new sporadically-occurring disease termed “mucosal disease” (MD). Contrary to the first descriptions of BVD, MD presented low morbidity and high mortality (Ramsey and Chivers, 1953). In 1957, researchers isolated and cultured a virus from a case of MD (Underdahl et al., 1957). The virus was cytopathic (cp) to the cultured cells, causing morphological changes such as vacuolation and cell death. In the same year, researchers propagated in culture cells a non-cytopathic (ncp) virus from cases of typical BVD of cattle (Lee and Gillespie, 1957). The relationship, if any, between these 2 isolates was unknown at the time. This led to the first classification of BVDV into cp and ncp biotypes. The discovery of different BVDV biotypes would initiate one of the most studied topics on BVDV infection, the pathogenesis of persistent infection (PI) and MD. Studies determined that cattle persistently infected with ncp BVDV developed MD after a superinfection with cp BVDV (Bolin et al., 1985b; Brownlie et al., 1987). Moreover, the study of BVDV in the last two decades has rapidly increased the knowledge of the epidemiology and molecular biology of BVDV. A newly found viral isolate associated with severe acute disease and high mortality rates in cattle was determined to be genetically different from the initial BVDV isolate (Pellerin et al., 1994; Carman

et al., 1998). The genetic dissimilarity between these isolates promoted the classification of BVDV in two genotypes (1 and 2) (Ridpath et al., 1994; Ridpath, 2005).

2. Classification, Morphology and Structure of BVDV

BVDV is a member of the Pestivirus genus within the family Flaviviridae (Figure 1). Members of the Flaviviridae include West Nile virus, dengue virus, yellow fever virus, and hepatitis C virus and are classified as a single family based on common genetic and structural characteristics. The other members of the Pestivirus genus are Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV) that possess the flavivirus features but also encode two unique proteins, Npro and Erns. In recent years, a new putative pestivirus species, tentatively called "HoBi-like," "BVDV-3," or "atypical pestiviruses," are related to BVDV at the genetic and antigenic levels (Calisher and Gould, 2003; Peterhans et al., 2010; Bauermann et al., 2013; Neill, 2013).

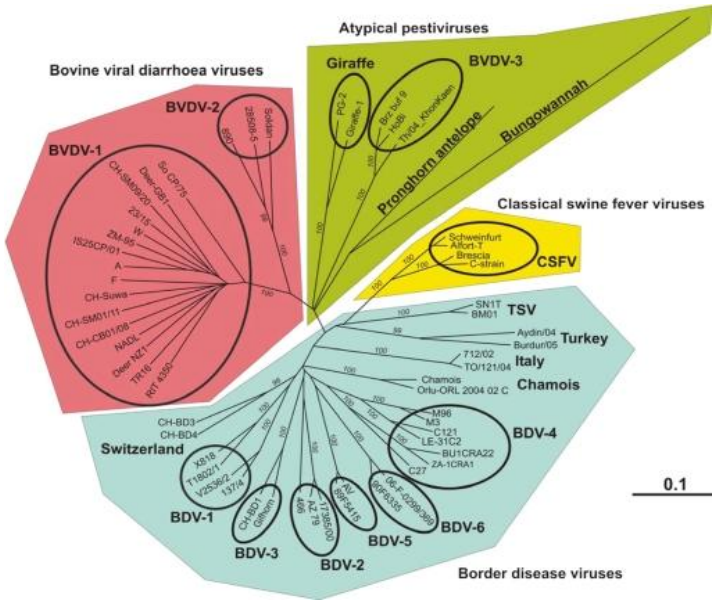


Figure 1. Phylogenetic analysis and classification of pestiviruses. *Source: Peterhans et al., 2010*

Although cattle are considered the natural host of BVDV, some isolates are capable of infecting heterologous species including pigs, sheep, goats, bison, deer and camelids; however, implications of transmission between these species are still unknown (Passler and Wlaz, 2010; Passler et al., 2010). The prevalence of seropositive animals is influenced by management conditions, vaccination status, and the presence of PI cattle (Houe et al., 1995; Houe, 1999); although, it is possible that the presence and identification of PI individuals within heterologous species different from cattle may be of critical importance in the epidemiology of BVDV (Passler and Walz, 2010; Henningson et al., 2013).

The genetic and antigenic differences between BVDV isolates lead to the recognition of two distinct BVDV species, BVDV-1 and BVDV-2 (Figure 1) that vary from avirulent to highly virulent; however, genetic mutations and constant antigenic variation are common within BVDV viruses (Ridpath et al., 1994; Hamers et al., 2001; Becher and Thiel, 2002; Bachofen et al., 2008; Peterhans et al., 2010; Strong et al., 2013). BVDV-1 viruses have a worldwide distribution, while BVDV-2 viruses are as yet largely restricted to the USA and Canada in high prevalence; in contrast to occur in European countries (Wolfmeyer et al., 1997; Vega et al., 2000; Tajima et al., 2001; Hurtado et al., 2003; Fulton et al., 2005b; Jackova et al., 2008; Brulisauer et al., 2010). BVDV strains of either species exist as two biotypes that are distinguished by their effect on cultured cells; ncp isolates infect permissive host cells without causing cell death, whereas cp isolates cause cytopathic effects and kill cells via apoptosis (Zhang et al., 1996; Hoff and Donis, 1997). Studies have shown that final protein and functions of both biotypes are different showing to have different tissue tropism (Ridpath, 2010). A third biotype of BVDV has been recently reported. This biotype consists of a subpopulation of ncp strains of BVDV capable of causing cytopathic effects in cultures of lymphocytes *in vitro* (Ridpath et al., 2006). The classification of BVDV by biotype is not related with virulence and pathogenicity of the virus *in vivo.* Cp, ncp and lymphocytopathic strains can be equally pathogenic and capable of causing severe disease. Ncp BVDV represent between 60 to 90% of the BVDV isolates from diagnostic laboratories, and it is believed that is the most common biotype in nature (Fulton et al., 2000b; Fulton et al., 2005b; Chase, 2013). It is usually involved in various manifestations of the disease where as the cp biotype is involved in causing MD through superinfection of the PI calf (Fulton et al., 2005a; Ridpath et al., 2006).

At molecular level, BVDV virion (Figure 2), although mainly spherical is pleomorphic in nature, has a diameter of about 40-60 nm and has a tightly adherent envelope. The genome (Figure 3) is made up of a single molecule of linear positive-sense single-stranded ribonucleic acid (RNA) molecule containing approximately 12.5 kb in one large open reading frame (ORF), flanked by 5'- and 3'-untranslated regions (UTR) (Collett et al., 1988a; Deng and Brock, 1992). The highest level of conservation within pestiviruses is observed in 5'-UTR fragment of genomic sequence (Ridpath and Bolin, 1995; 1997). Sequence variations in 5'-UTR are conserved among viruses within the same pestiviral genotype (Ridpath and Bolin, 1998). Sequence analysis of BVDV 5'-UTR can distinguish BVDV-1 and BVDV-2 genotype (Pellerin et al., 1994; Ridpath et al., 1994). Moreover, the UTR of positive-strand RNA viruses are thought to contain important signals for translation, transcription, replication, and probably also packaging of viral genomes (Topliff and Kelling, 1998; Becher et al., 2000). The genome encodes a single polyprotein that is proteolytically processed during virus replication to produce a variety of viral structural and non-structural proteins (Murphy et al., 1999).

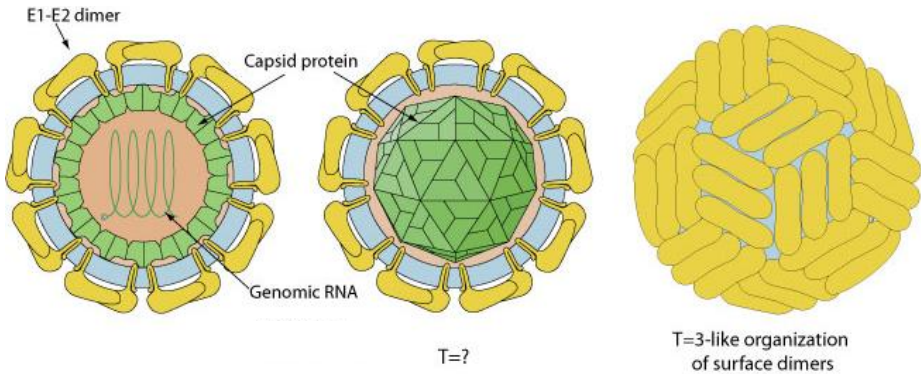


Figure 2. Virion of Pestivirus. Enveloped, spherical, about 50 nm in diameter. Mature virions contain three virus-encoded membrane proteins (Erns, E1 and E2) in addition to the capsid protein. *Source: ViralZone 2010. Swiss Institute of Bioinformatics. http://viralzone.expasy.org*

The ORF of BVDV can be divided into distinct regions that encode the individual viral proteins (Figure 3). The viral proteins are encoded in the order of NH2-Npro-capsid-Erns-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. With the exception of Npro, the first coding region

of the ORF encodes the structural proteins, or the proteins that are integral components of the viral particle. These include the capsid or core protein, Erns, E1 and E2 (Collett et al., 1988b; Meyers et al., 1991; Neill, 2013).

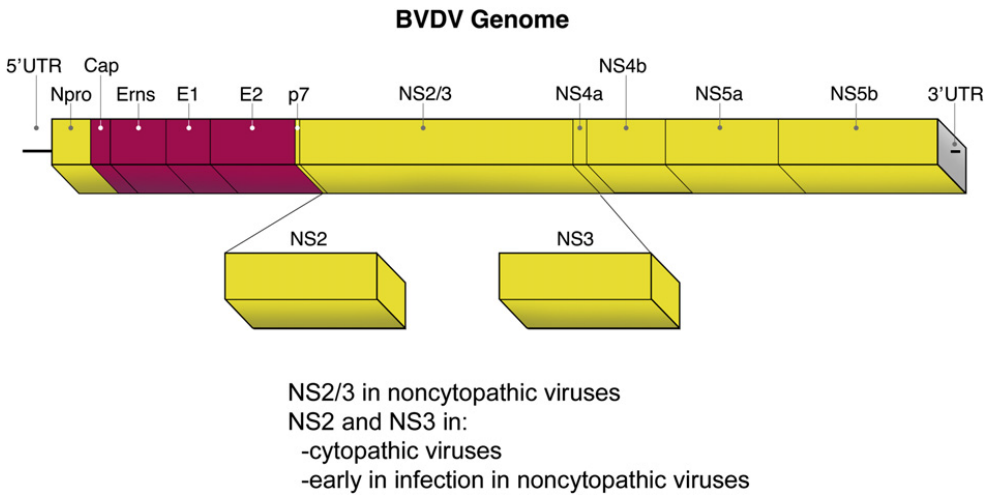


Figure 3. Diagrammatic representation of differences in non-cytopathic (ncp) and cytopathic (cp) BVDV genomes. *Source: Neill, 2013.*

The latter three proteins (gpErns, E1 and E2) are envelope glycoproteins and are displayed on the exterior of the virus particle and the fourth structural protein is the capsid protein (C) (Figure 2). The E1 and E2 proteins are inserted in the membrane via stretches of hydrophobic amino acid residues. The heterodimers of E1 and E2 are necessary for infectivity of the virus particle (Ronecker et al., 2008). The Erns protein is not inserted directly into the membrane, rather it has a unique C-terminal domain that interacts with the outer membrane providing a weak attachment, or it may be secreted into the extracellular matrix (Fetzer et al., 2005). The Erns exhibits ribonuclease activity, which seems to be essential in the viral life cycle (Schneider et al., 1993). A second form of Erns is secreted into the environment of the infected cell and can be detected in the serum and plasma of infected animals (Weiland et al., 1999; Kampa et al., 2007). Of the structural proteins, E2 is the major target for BVDV neutralising antibodies (Abs) (Donis et al., 1988; Deregt et al., 1998; Kalaycioglu et al., 2012) and probably involved in the initial binding of BVDV to the surface of permissive cells (Xue and Minocha, 1993). The highly

variability within E2 sequence pointed to be the most important source of antigenic variability between different BVDV strains (Toth et al., 1999).

Among the non-structural proteins, the first non-structural protein, Npro, is an autoprotease that cleaves itself. This protein is unique to the Pestiviruses. For sometime, the function of this protein was unclear. However, in vivo, Npro protein functions to block interferon production in virus infected cells and is not required for replication of the virus (Tratschin et al., 1998; Chen et al., 2007). The NS2-3 protein seems to be the most important because cp BVDV but not ncp BVDV cleaves NS2-3 to produce NS3 (Figure 3), which can be used as a marker for infection (Meyers and Thiel, 1996; Meyers et al., 1996; Kummerer and Meyers, 2000). Likewise, NS2-3 display high level of genetic conservation and formes the basis of several commercially available Ab and virus detection assays (Paton et al., 1991). A few cp strains have been isolated with genomic changes in the Npro, capsid and NS4b proteins as well (Qi et al., 1998; Nagai et al., 2003). The ncp biotype contain an unaltered genome and produce primarily an uncleaved NS2/3 and a small amount of NS2 and NS3 as required for replication (Lackner et al., 2004).

According to biophysical properties, the lipid envelope confers sensitivity to heat, detergents and organic solvents, making it relatively easy to inactivate. The virus has a density of 1.134 g/ml. The virus is not stable in the environment and is easily inactivated by heat, only surviving for 1 hour (h) at 56°C. At room temperature (Rt) it survives for up to 5 days but the virus can be stored for up to 16 months at –40°C. The virus survives best at pH range 5.7 to 9.3 with maximum viability at pH 7.4. Common disinfectants like phenols and chlorhexidine readily inactivate the virus (Murphy et al. 1999; Neill, 2013).

3. Clinical Manifestations of BVDV Infections in Cattle

Depending on the interactions between host, environmental and viral factors, infection of cattle associated with both BVDV-1 and BVDV-2 induces a wide variety of disease conditions that range from clinically asymptomatic to fatal disease (Baker, 1995); although BVDV-2 is generally associated with the more severe outcomes of acute BVDV infections (Pellerin et al., 1994; Carman et al., 1998). The clinical manifestations can divide mainly in three: acute BVD, congenital PI and MD, as is shown in Figure 4 (Baker, 1995; Ridpath et al., 2006; Peterhans et al., 2010).

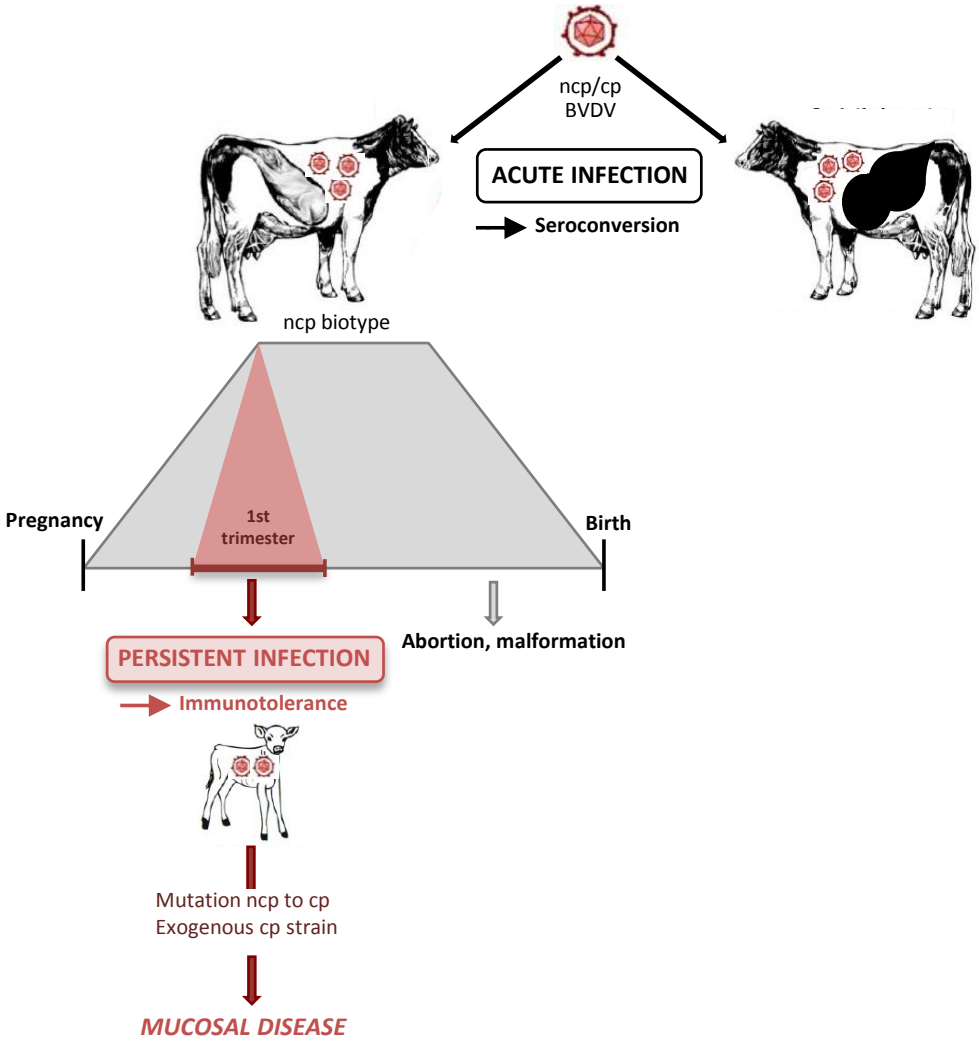


Figure 4. Schematic summary of clinical manifestations in BVDV infections. *Adapted from Peterhans et al., 2010.*

3.1. Acute BVD

Primary postnatal infection or infection in seronegative-immunocompetent cattle with BVDV is the cause of acute BVD. Seropositive cattle, dependent on the levels of Ab titers, are usually not susceptible (Liebler-Tenorio, 2005). Acute infections are transient and can be caused by both biotypes of BVDV, where the outcome is highly variable mostly due to virulence of the individual strain. Typically, ncp BVDV is isolated from cases of acute BVD. However, cp BVDV may

induce acute BVD, but is rare and seldom isolated unless MD has occurred in the herd to serve as a source of virus. Most BVDV strains are not virulent, so acute BVD often is a clinically inapparent to mild disease. Manifestations of acute BVD frequently are limited to slight elevation in body temperature around 6-9 day post-infection (dpi), diarrhea, depression, oculonasal discharge, anorexia, cough, decreased milk production, mucosal lesions, leukopenia from about 3 to 7 dpi and increased incidence of reproductive failure. However, the infection is often associated with respiratory and gastrointestinal symptoms (Traven et al., 1991; Moerman et al., 1994; Baker, 1995; Potgieter, 1997). Such symptoms can also be a result of secondary, or concurrent infections (Brodersen and Kelling, 1998; Elvander et al., 1998; Fulton et al., 2000a) since BVDV acts as an immunosuppressive agent by impairing immune functions mainly associated with the cellular response (Potgieter, 1995; Adler et al., 1996; Bruschke et al., 1998a; Fulton, 2013). There is usually very limited recovery of the virus from the blood and nasal secretions lasting about 3 to 7 dpi and in some cases may persist up to 15 dpi, but may be longer dependent on virus strain, stress, and other pathogens (Baker, 1990; Evermann and Barrington, 2005; Pedrera et al., 2011; Risalde et al., 2011b). This is followed by a rise in Abs that leads to a protective immunity. High serum levels of Abs to BVDV can be detected from 14-28 dpi onwards, which reach a maximum 10 to 12 weeks post infection and continues until up to three years after infection and immunity is assumed to last a lengthy period, possibly the animals life time (Fredriksen et al., 1999; Peterhans et al., 2010; Risalde et al., 2011b). Moreover, colostral immunity is considered to protect at least partially against disease during the first months of live, being the mean half-life of passively acquired BVDV Abs approximately 23 days, but low titers can remain detectable until more than 6 months of live (Howard et al., 1989; Bolin and Ridpath, 1995; Fulton et al., 2004).

Severe acute BVD can be caused under certain circumstances by some virulent ncp BVDV-2 strains and manifests high fever, depression, inappetence, diarrhea, leukopenia, thrombocytopenia, hemorrhaging, dehydration, and death (Corapi et al., 1989; Rebhun et al., 1989; Corapi et al., 1990; Bolin and Ridpath, 1992; Carman et al., 1998; Ellis et al., 1998; Walz et al., 2001). Likewise, outbreaks of severe acute BVDV infections have also been caused by strains identified as BVDV-1 (Hamers et al., 2000).

Hemorrhagic syndrome is another form of severe BVDV infection in cattle associated with ncp isolates of BVDV-2 with a mortality rate near to 25% (Ridpath et al., 1994; Bolin and Grooms, 2004). Clinical manifestations of hemorrhagic syndrome include severe thrombocytopenia, hemorrhage, bloody diarrhea, epistaxis, petechial hemorrhages, ecchymotic hemorrhages, bleeding from injection sites, and death (Corapi et al., 1989; Rebhun et al., 1989; Corapi et al., 1990; Bolin and Ridpath, 1992). Marked thrombocytopenia induced by BVDV infection is characterized by altered function of platelets and viral infection of megakaryocytes in the bone marrow (Walz et al., 2001).

3.2. Congenital persistent infection (PI)

Fetal infection is a key feature of BVDV transmission. Fetal infection has only been shown as a result of infection in seronegative animals. BVDV rarely infects the fetuses of seropositive or PI cows (Radostits and Littlejohns, 1988; Houe, 1999). Whereas cp BVDV infection seems to be cleared by the fetus, ncp BVDV infection of a seronegative cow results in abortions, congenital damage and in the birth of a PI animal (Brownlie et al., 1989; Brownlie, 1991). This difference between cp and ncp fetal infection is associated with a different interaction of the two biotypes with the innate immune response against viral infection (Peterhans et al., 2003). However, fetal exposure to BVDV with virus clearance and seroconversion is also a common outcome during the second and third trimesters of gestation when the fetus is immunocompetent, leads to the birth of clinically normal calves with pre-colostral BVDV Abs (Brownlie et al., 1989). Thus, the outcome of fetal infection depends on the stage of pregnancy (Tremblay, 1996; Grooms, 2009). Moreover, reproductive and ovary dysfunctions have also been associated with BVDV infections. Infertility could well be due to ovaritis at the time of infection or due to failure of fertilisation as a result of poor quality semen from PI bulls. Seronegative cows inseminated with infected semen generally fail to conceive until they develop an immune response to the virus (Grooms et al., 1998a,b,c; McGowan et al., 2003).

An important outcome of fetal infection occurs during the first trimester (42 and 125 days gestation), after development of fetal membranes, but before the immune system is functional. The fetus will not develop virus neutralising Abs and the virus will be recognised as self (Lambot et al., 1997). The fetus may be carried normally to term and be born with a PI (McClurkin et al., 1984; Fray et al., 2000). However, congenital defects and malformations may occur as a result of

BVDV infection between days 100-150 and are dependent on the critical age for organ development (Baker, 1987; Brownlie et al., 1987; Brownlie, 1990; Taylor et al., 1997a; Brownlie et al., 2000; Grooms, 2009). In all recorded field and experimental data there is no evidence for persistence with the cp biotype (Brownlie et al., 1989). From birth PI animals are persistently viremic and specifically immunotolerant to the infecting ncp strain and may respond with antibody production to either heterologous field strains or vaccine strains (Fulton et al., 2003; 2009). PI animals normally shed virus from 3-4 months of age following depletion of maternal Abs (Palfi et al., 1993; Fray et al., 2000), and shedding continues throughout the animal's life, creating an important transmission route (Brownlie et al., 1986; Houe et al., 1995; Fulton et al., 2009). PI animals have impaired immune functions and they tend to be more susceptible to other infections than calves born Ab negative (Potgieter, 1995; Munoz-Fanzi et al., 2003). It is therefore quite common that they die or are culled before they reach adult age (Barber et al., 1985; Taylor et al., 1997b; Houe, 1999). However, they may also be clinically healthy. PI cows that reach adult age can conceive (McClurkin et al., 1979). If so, the infection will be transmitted to the fetus, and thus, the offspring will always be PI (Baker, 1987). The extent/prevalence of these congenitally infected calves remains to be determined (Fulton, 2013).

The main route of BVDV transmission is via direct contact to a PI animal (Ezanno et al., 2007). Virus spread from an acutely infected animal seems to be possible (Moen et al., 2005), probably even for a considerable duration (Collins et al., 2009), but by far less efficient than spread from a PI (Niskanen et al., 2000; 2002a,b). Likewise, airborn transmission of BVDV has been demonstrated under experimental conditions (Mars et al., 1999), but the epidemiological relevance is less important than the direct contact (Niskanen and Lindberg, 2003).

3.3. Mucosal disease (MD)

MD had been described as a highly fatal disease of cattle with low morbidity and high mortality (Ramsey and Chivers, 1953; Fulton, 2013). Typically, cases exhibit fever, anorexia, massive mucosal erosions throughout the gastrointestinal canal and profuse diarrhoea leading to progressive wastage and death (Baker, 1995). The disease could be similar to a severe form of acute digestive tract disease, but is differentiated from the acute disease by two features: (1) in MD, both the cp and ncp are isolated from the affected animal or from necropsy tissues; and (2) the animal with MD will have evidence of PI status, i.e., positive skin test samples detected by

immunohistochemistry (IHC) or antigen (Ag) capture ELISA (Fulton, 2013). Also, apart from gastrointestinal symptoms, dermatological symptoms like erosive lesions on the skin and laminitis may develop (Peterhans et al., 2010). MD arises when a PI animal (usually between 6-18 months of age) gets superinfected with a cp virus antigenically homologous with the persistent ncp virus (Figure 4). Rarely calves as young as 4 months of age, or cattle older than two years are affected (Bolin et al., 1985b; Thiel et al., 1996; Grooms, 2009). MD can result of a mutation in the non-structural part of the genome, resulting in a change in biotype from ncp to cp (Tautz et al., 1994; Kummerer et al., 2000). Likewise, MD can also develop as a result of RNA recombination between the persisting ncp strain and an exogenous cp strain. This can occur, for example, if live BVDV vaccines containing cp strains are used (Becher et al., 2001).

4. Pathogenesis of Acute BVD

The pathogenesis of any disease reveals the balance between the ability of the host to resist pathogen invasion and the capacity of the pathogen to produce damage on the host. The BVDV disease complex is characterized by a wide variety of clinical signs that it demonstrates the complexity of this balance and consequently, the complication for description of pathogenicity (Brownlie, 1990).

4.1. Target cell and Spread of BVDV

The most important determinant for the outcome of acute BVDV infection in susceptible animals is the virulence of the individual BVDV strain (Liebler-Tenorio, 2005). Both low and high virulent BVDV strains enters the susceptible host primarily by the oro-nasal route (Figure 5) and initially replicate in the nasal mucosa followed by the dissemination of the virus to tonsils and then spreads to the regional lymph nodes followed by a systemic viremia wich can be as free virus form or cell-associated virus, mainly to lymphocytes and monocytes-macrophages (Brodersen and Kelling, 1998; Bruschke et al., 1998b; Baule et al., 2001; Kelling et al., 2002). After viremia that is evident 2 to 4 days after exposure, the virus can be found in a wide range of host tissues, especially in case of high virulent BVDV strains that show a more widespread distribution than low virulent strains. In both BVDV replication occurs mainly in epithelial cells of lymphoid tissues, being possible to detect infected cells in tonsils, lymph nodes and Peyer's

patches and then in spleen and thymus (Liebler-Tenorio et al., 2000; 2003a,b; Pedrera et al., 2009b; Raya et al., 2012).

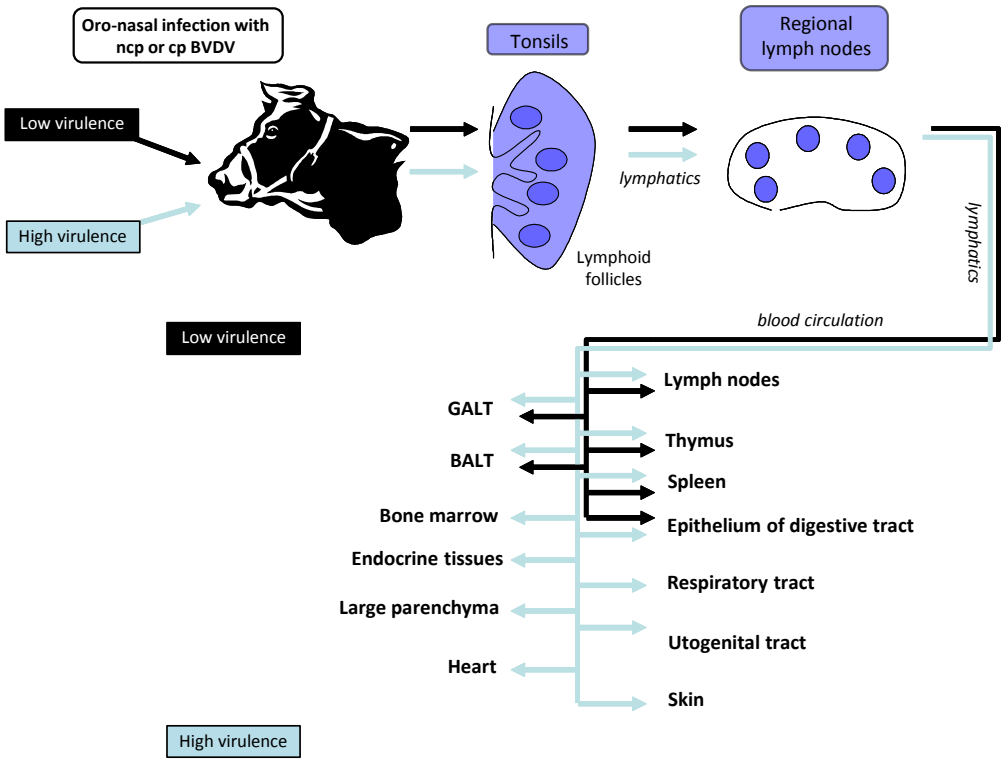


Figure 5. Spread of BVDV of low and high virulence in acute BVD. *Adapted from Liebler-Tenorio, 2005*.

Based on comparisons to related members of the *Flaviviridae,* the binding and entry of BVDV involves a series of steps, beginning with attachment or interaction of the virion with specific host cell receptors, followed by internalization and pH dependent fusion of the viral envelope and cell membrane. Envelope gp coded by E2 and Erns have been demonstrated to independently bind to cell surfaces (Hulst and Moormann, 1997; Iqbal et al., 2000). The ability of BVDV to infect a relatively diverse range of cell-types, as well as, the tissue and host species tropisms observed for BVDV have been associated with the E2 envelope gp (Liang et al., 2003). The gpE2 may additionally contribute to the ability of BVDV to escape the host immune response (Ridpath et al., 2003). After entry into the host cell is complete, viral RNA is released into the

host cell cytoplasm and RNA translation begins. All replication take place in the citoplasm of the cell and peak release of virions occurs between 12-24 h after infection occurrs. After replication in the cytoplasm, virus obtains the outer envelope from host membranes before being released by exocytosis (Ridpath, 2005).

Severe clinical signs are correlated with a higher level of viremia but specific virulence factors for BVDV have not yet been characterized (Walz et al., 2001). As virus Ag is not generally associated with lesions in non-lymphoid tissues, tissue damage might not only be a function of viral replication but also be attributed to a reaction of the host (Tajima et al., 1999; Liebler-Tenorio et al., 2002). The pathogenesis of tissue lesions is unsolved; the discrepancy between the presence of viral Ag and lesions as well as the delayed onset of lesions seen with low and highly virulent BVDV strains might indicate that immune-mediated reactions contribute to the development of lesions (Liebler-Tenorio, 2005).

After infection with *BVDV-1 and BVDV-2 strains of low virulence*, the calves did not develop much clinical signs of the disease (Table 1), except a mild pirexia for 1–2 days and mild leukopenia (Liebler-Tenorio et al., 2003a; Pedrera et al., 2009b; Risalde et al., 2011b; Risalde et al., 2013). Viral Ag was initially found in lymphoid organs being predominantly present within lymphoid follicles and the thymic cortex associated with lymphocytes, stromal cells and dendritic cells (DCs) (Liebler-Tenorio et al., 2003a; Raya et al., 2012; Risalde et al., 2013) (Figure 5). However, viral Ag was not detectable in the bone marrow (Wilhelmsen et al., 1990; Liebler-Tenorio et al., 2003a,b; Pedrera et al., 2009b; Risalde et al., 2013). Furthermore, in experimental infections of colostrum-deprived calves with BVDV strains of low virulence, the viral Ag 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; Risalde et al., 2011b; 2013).

In *highly virulent strains*, clinical signs (Table 1) are severe but are often nonspecific consisting of severe pyrexia, anorexia, depression, and frequently diarrhea. In experimental infections, severe progressive lymphopenia and moderate to severe thrombocytopenia are consistent findings. Moreover, some animals develop haemorrhages associated with severe acute BVDV infections (Corapi et al., 1990; Bolin and Ridpath, 1992; Marshall et al., 1996; Ellis et al., 1998; Odeon et al., 1999; Archambault et al., 2000; Hamers et al., 2000; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002). The amount of viral Ag in tissues rapidly exceeds that caused

by low virulence strains (Figure 5) and beyond of localization in follicles of lymphoid tissues, the virus extended to T-cell–dependent areas. Moreover, exist BVDV spreads to other organs as bone marrow, digestive and respiratory tract, endocrine tissues, skin and interstitium or vascular walls. Likewise, severe depletion of lymphoid organs and necrosis of epithelium in the digestive tract are also find (Marshall et al., 1996; Carman et al., 1998; Ellis et al., 1998; Odeon et al., 1999; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002).

Table 1. Clinical signs and distribution in acute BVD caused by strains of high and low virulence.

Virulence	Clinical signs and findings	Virus spread
Low virulence	No evident clinical signs	Mainly confined to lymphoid organs.
	Mild pyrexia	
	Mild leukopenia	Also detected in the intestinal mucosa
	Decreased milk production	
High virulence	Severe pyrexia	
	Anorexia	Predominantly in lymphoid tissues,
	Depression	mucosa of the gastrointestinal tract,
	Diarrhea	respiratory tract, bone marrow
	Occulo-nasal discharge	and endocrine organs.
	Respiratory distress	
	Haemorrhage	Eventually general spread to most organs
	Severe lymphopenia	
	Thrombocytopenia	

4.2. BVDV and Immunity

The interaction between BVDV and the host's immune system is a key feature in the BVDV disease complex. The way BVDV interacts with the innate and adaptive immune system is critical both for the pathogenesis of the BVDV and for the development of immunity.

Immune responses to acute BVDV infections take place in two steps. After initial infection, there is an immunosuppression that is usually followed by a protective immunity, so-called adaptive immunity, as the animal recovers from infection and the virus is cleared from the body.

4.2.1. Immunosuppression

One of the most dramatic and controversial outcomes of BVDV is transient immunosuppression. Many diseases have been shown to worsen when there is a concurrent BVDV infection (*see 4.3. BVDV and Concurrent Infections, in General introduction section, pp. 40-41*). BVDV has been shown to can infect many immune cells including T cells, B cells, monocytes-macrophages and DCs (Bolin et al., 1985a; Ellis et al., 1988; Sopp et al., 1994; Welsh et al., 1997; Walz et al., 2001; Glew et al., 2003). *In vitro*, the virus replicates in lymphocytes and macrophages, while *in vivo* there is a decrease of circulating B and T lymphocytes (Bolin et al., 1985a). Furthermore, the function of many of these cells is depressed. BVDV has been shown to alter function of Toll-like receptors (TLRs), expression of cytokines and costimulatory molecules (Franchini et al., 2006; Lee et al., 2008) resulting in an adverse affect on their ability to stimulate Th cells (Glew et al., 2003). Many neutrophil functions are affected and there is decreased migration and neutrophil-mediated Ab-dependent cell-mediated cytotoxicity (Atluru et al., 1990). Deppessed neutrophil function may last as long as weeks after infection with the virus (Brown et al., 1991). But there is also an associated depression of PMN cells function through a decrease in total numbers (Roth et al., 1981; Roth and Kaeberle, 1983). Lymphocyte and monocyte responses are impaired as well. Infecting antigen presenting cells (APCs) such as monocytes-macrophages, DCs and B cells (Table 2) has the potential to strongly affect the immune response since they have a key role in inducing and shaping the adaptive immune response. However, no impairment of APC function has however been demonstrated for BVDV infected DCs. Both cp and ncp strains of both genotypes have been described as having a negative impact on the innate and cell-mediated immune system. This depression of cell-mediated immunity is accompanied by a reduced humoral immune response (Lambot et al., 1997).

4.2.2. Innate Immunity

Macrophages, neutrophils, IFN production, and circulating lymphocytes are all negatively affected in acute infections (Potgieter, 1995). Early studies examining the innate immune system indicated that cattle experimentally infected with BVDV have impaired degranulation of PMNs (Roth et al., 1981). Calves intranasally infected with type 2 BVDV showed a significant drop in the number of circulating neutrophils, lymphocytes and monocytes between 3 and 5 dpi

(Archambault et al., 2000). Monocytes infected with ncp strains of BVDV had decreased ability to stimulate allogeneic and memory CD4+ T cell responses (Glew et al., 2003). Cp biotypes induced apoptosis in peripheral blood mononuclear cells. Apoptosis was also detected in bovine CD4+ and CD8+ T cells (Lambot et al., 1998).

Table 2. Summary table of APCs

	Macrophage	Dendritic Cell	B cell
MHC-II Expression	Low levels. Induced by Bacteria and/or Cytokines	Always Expressed.	Always Expressed. Inducible upon Activation
Antigen type and presentation by MHC	Extracellular Ags: presentation via MHC-II	Intracellular and Extracellular Ags: presentation via MHC-I and II	Extracellular Ag binds to specific Ig receptors: presentation via MHC-II
Co-Stimulation (B7 expression)	Low levels. Induced by Bacteria and/or Cytokines	Always expressed at high Levels	Low levels. Inducible upon Activation
Location	Lymphoid tissue Connective tissue Body Cavities	Lymphoid tissue Connective tissue Epithelium	Lymphoid tissues. Blood

IFN is an important component in the activation of the innate immune response. Ncp biotypes of BVDV failed to induce IFN type I in cultured bovine macrophages, whereas cp biotypes were capable of inducing the IFN response. The failure of ncp BVDV to induce IFN is speculated to be an important feature in the development of PI calves and immunotolerance (Peterhans et al., 2003; Peterhans and Schweizer, 2013). However, other studies confirmed that infection with ncp BVDV is associated with failure to induce type 1 IFN *in vitro* (Charleston et al., 2001) but also showed that alpha/beta and gamma IFNs are induced by infection with ncp BVDV *in vivo* (Charleston et al., 2001; 2002; Palomares et al., 2013).

4.2.3. Adaptive Immunity

CD4+, CD8+ and γδ T cells, together with B cells, form the adaptive immune response to viral infections. The hallmarks of adaptive immunity include Ag specificity and memory. These features allow T cells to elaborate responses wich specifically target the numerous viruses which may infect the host. The ability to stablish long-lived immunological memory provides a unique

mechanism to better protect the host during subsequent viral exposures. Due to their importance in controlling pathogens, cell-mediated immune response may be widely studied in viral infections.

The effect of BVDV infection on the adaptive immune system begins at the earliest stage of the innate immune response, which is the recognition of danger, Ag presentation and co-stimulatory signals (Chase, 2013).

4.2.3.1. Humoral immune response

B lymphocytes are genetically programmed to recognize a particular Ag, multiply and differentiate giving rise to plasma cells that produce inflammatory response in the BVD and its alteration in secondary infections large amounts of Igs. Humoral immunity may be caused by passive immunity for ingesting colostrum Abs or by an active immune response after exposure to Ag (Tizard, 2008; Risalde et al., 2012).

The humoral immune responses are the best defined of the immune responses to BVDV infection and it is felt that this is the primary mechanisms of protection. Studies have shown that maternal BVDV neutralizing Abs will provide protection against severe infections; however, calves with low titres of maternal Abs or that not receive colostrum may be at risk for acute BVDV infection. Interference by high titres of maternal Abs prevents the development of an Ab response following vaccination but may generate BVDV specific memory T and B cells (Endsley et al., 2003; Fulton et al., 2004).

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 (Potgieter, 1995; Risalde et al., 2012).

4.2.3.2. Cell-mediated immune response

Cell-mediated immune response plays a critical role in combating viral infections, including BVDV infections (Figure 6). They are comprised of T cell responses, which fundamentally differ from Ab (humoral) responses in the way they bring about infection control.

The involvement of local and the cell-mediated immunty, in clearing BVDV infection, is not clear. Few studies have been done measuring cell-mediated immunity. However, cell-mediated

immunity may be an important component of protection against pathogens. The first studies about lymphocyte depletion showed primarily CD4+ T cells involvement with CD8+ T cells little response to BVDV infections. This may indicate that there is a MHC-II restricted cytotoxicity T cell activity (Sheffy et al., 1962). However, γδ T lymphocytes also may have a role in the highly complex mechanism involved in cell-mediated immune responses due to have a wide range of functions, including cytokine production, immunomodulation and regulation of inflammation (Kaufmann et al., 1993; Welsh et al., 1997; Carding and Egan, 2000; Price et al., 2007), as well as, a cytotoxic activity in a non Ag-dependent stimulation form supporting a role in the innate immunity, in addition to their role in Ag-dependent responses distinctive of adaptive immunity (Endsley et al., 2004; Guzman et al., 2012).

As a virus infection becomes established in the host, a series of molecular and cellular signals are initiated which activate cell-mediated immune responses. These signals include the production of IFNs, other cytokines, and inflammatory mediators, in addition to the mobilization of local DCs (Figure 6) (Brackenbury et al., 2003; Glew et al., 2003; Peterhans et al., 2003; Al-Haddawi et al., 2007; Zajac and Harrington, 2008).

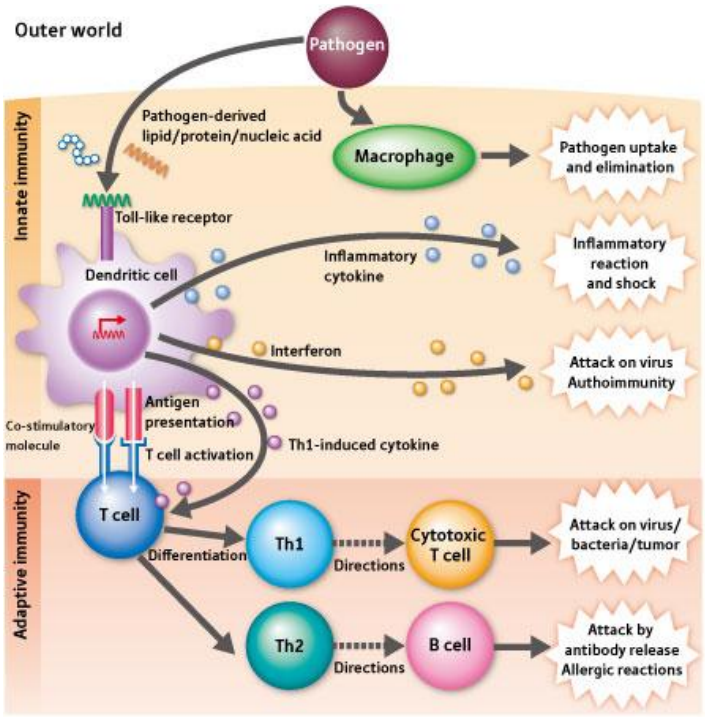


Figure 6. Interactions of molecular and cellular signals activating cell-mediated immune responses. S*ource: Riken Research Center web.*

DCs are thought to provide a critical cellular link for priming naive CD4+ and CD8+ T cells. The primary activation events, which induce cell-mediated immunity predominately, occur in secondary lymphoid organs including tonsils and regional lymph nodes. During the early stages of viral infections DCs residing at the initial sites of infection take up viral Ags, become activated, and migrate to regional lymph nodes (Figure 7). Within the lymph nodes these DCs encounter naive T cells which are circulating through these organs as part of their normal immunosurveillance protocol. Engagement of T-cell receptors (TCRs) on the naive T cells with viral–peptide MHC complexes presented by the DCs results in sequestration of the T cells and launches the antiviral T-cell response. The ensuing proliferation and differentiation of virus-specific T cells also occur in conjunction with inflammatory mediators such as INFs and other signals. Many parameters, including the duration and strength of antigenic stimulation, co-stimulatory interactions, the presence of cytokines, and the provision of CD4+ T cells help guide the developing response. These early events play a critical role in driving the generation of both the effector T cells as well as the subsequent establishment of the memory T cell pool.

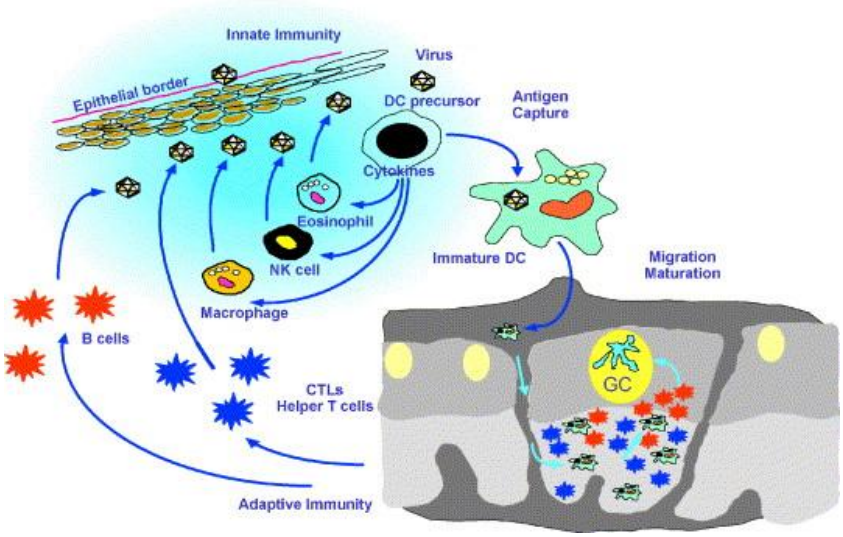


Figure 7. Interaction between virus and innate and adaptive immune system. S*ource: Brackenbury et al, 2003.*

In BVDV, a dichotomy of the host interaction with the two cp and ncp biotypes has been demonstred (Brownlie et al., 1989; Charleston et al., 2001; Brackenbury et al., 2003). Infection with cp BVDV at a mucosal or epitelial surface will cause cytokines such as IFN-α activating

effector cells of innate immune response such as DCs that will limit the replication of virus; while ncp BVDV infections will not stimulate an early cytokine response and DCs do not become highly activated and virus will disseminate throughout the cattle (Brackenbury et al., 2003).

4.2.4. Role of Cytokines in BVD

Understanding how immune responses are regulated in ruminants is critical for devising strategies to direct an immune response toward a disered effector function required for either preventing infection or eliminating disease.

Cytokines are critical for immunological control of virus infection. Cytokines and chemokines, produced largely by macrophages and T lymphocytes, play a pivotal role in antiviral immune responses. A wide range of mechanisms are involved, including alteration of the expression of MHC molecules (Wong et al., 1983; Bukowski and Welsh, 1985), adhesion molecules (Springer, 1994) and co-stimulatory molecules (Croft, 1994), and direct activation or inactivation of immune cells (Stout, 1993; Paul and Seder, 1994). These changes may lead to the activation of cell-mediated antiviral responses, and Ab-mediated virus clearance (Zinkernagel, 1993).

Pro-inflammatory cytokines characteristically are those cytokines that are rapidly induced and expressed early in a disease or injury process in an Ag-independent manner. Monocytes and macrophages are the cell types responsible for the majority of pro-inflammatory cytokine expression, but a broad spectrum of cell types secrete these proteins (Lippolis, 2008). The principal pro-inflammatory cytokines are tumor necrosis factor (TNF-α), interleukin-1 (IL-1) and IL-6. TNF-α and IL-1 mediate the inflammatory response at both the local and systemic levels (Dinarello, 1996). Locally, these cytokines induce vascular endothelial adhesion molecule expression, thereby promoting neutrophil transendothelial migration to the site of infection. Systemically, TNF-α and IL-1 are potent inducers of fever and the acute-phase response (APR). Induction of the APR results in increased hepatic synthesis of several acute phase proteins (APPs) such as haptoglobin (Hp), serum amyloid A (SAA), fibrinogen and albumin among others in cattle (Uthaisangsook et al., 2002; Petersen et al., 2004; Gruys et al., 2005; Eckersall and Bell, 2010).

The Th1/Th2 paradigm defined in murine (Mosmann and Sad, 1996) has provided a useful framework for understanding the observed bias in the immune responses that are often

dominated by either cell-mediated immunity and humoral responses. Both type 1 and type 2 immune responses have been reported in cattle, depending on the cytokines secreted by the two T helper cell subsets (Th1 and Th2) (Figure 8) and on the involvement of cytotoxic T cells and natural killer cells (Brown et al., 1993; 1994; Abbas et al., 1996; Mosmann and Sad, 1996). Specifically, the type 1 response (IFN-γ and IL-12) relates to cell-mediated immunity, contributing to inflammation process; whereas the type 2 response (IL-4) stimulates the production of neutralizing Abs associated with humoral responses in cattle. Bovine IL-12 is considered a key cytokine with a likely central role in the initiation and control of cell-mediated immunity by stimulating a rapid production of IFN-γ, an activator of neutrophils and macrophages (Trinchieri, 1997). In contrast, IL-4 is a central cytokine in the differentiation and maturation of B lymphocytes and the synthesis of immunoglobulins (Igs) (Mosmann et al., 1986). In addition, it is a potent cytokine in the suppression of the inflammatory activities of stimulated macrophages (Zhou et al., 1994). Since the initial response to the disease is inflammatory, it stands to reason that, in a successful immune response, the host must modulate the inflammation to prevent excessive inflammatory cytokine production and host damage. Finally, resolution of the inflammatory process is mediated by the upregulation of IL-10, which downregulates pro-inflammatory cytokine production (Lippolis, 2008). A balance between type 1 and type 2 bias seems to be necessary for efficient elimination of infectious agents (Collins et al., 1999; Waldvogel et al., 2000).

Cell-mediated immune response to BVDV also differs between biotypes. Ncp BVDV can induce a stronger, faster humoral Th2-biased response, in contrast to cp BVDV which mostly results in a Th1-biased (Lambot et al., 1997; Rhodes et al., 1999; Collen and Morrison, 2000). There is evidence for the presence of BVDV specific CD4+ and CD8+ memory T cells after acute infection (Glew and Howard, 2001). However, CD4+ cells seem to be more important than CD8+ cells (Collen and Morrison, 2000; Collen et al., 2002). However, the type of immune response against BVDV has yet to be clearly defined. In fact, Charleston et al. 2002 established that during acute infections with ncp BVDV infections, there are no evidences of a type 2-like response.

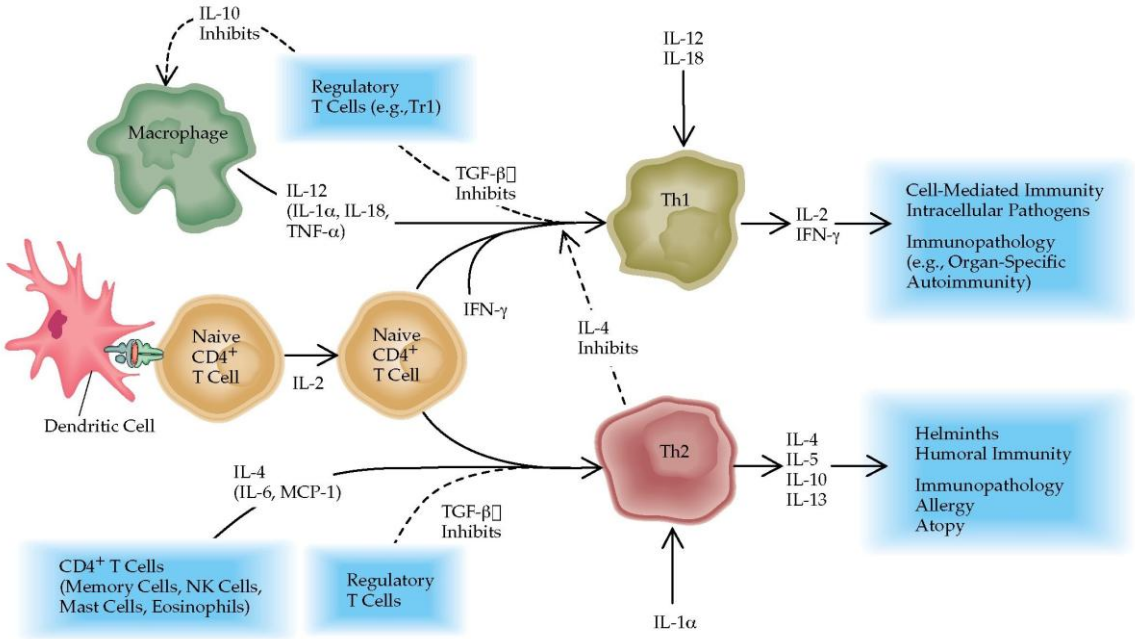


Figure 8. Cytokines regulating immune response.

4.3. BVDV and Concurrent Infections

BVDV might also play a role in the bovine respiratory disease (BRDC) (Potgieter, 1985; Baule et al., 2001) due to the fact of the result of immunosuppression in acute BVDV infections is an increased susceptibility to other microbial infections. Several reports suggest that BVDV is a synergistic agent in mixed infections of the respiratory tract (Potgieter et al., 1984a; Castrucci et al., 1992; Kelling et al., 2007; Fulton, 2009). Viral pathogens most frequently linked with BVDV in respiratory disease include bovine herpesvirus 1 (BHV-1), parainfluenza virus 3 (PI-3V), and bovine respiratory syncytial virus (BRSV) (Richer et al., 1988; Graham et al., 1998a; 1998b; Fulton et al., 2000a), and bacteria such as Mannheimia hemolytica and Mycoplasma bovis (Ridpath, 2010). BRDC viral pathogens have, in fact, even been show to have a synergistic effect on each other. Although, infection with BVDV has also been reported to enhance other infectious conditions such as enteritis caused by Salmonella spp., colibacillosis, metritis, and mastitis (Ames, 2005).

Compared with disease caused by a single pathogen, mixed infections of BVDV and BRSV, BVDV and BHV-1, or BVDV and *Mannheimia haemolytica* cause more severe disease, delayed

and reduced Ab response, greater dissemination of virus, prolonged viral shedding, and more severe lesions (Potgieter et al., 1984a; Potgieter et al., 1984b; Brodersen and Kelling, 1998; Elvander et al., 1998; Graham et al., 1998a). 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 (Risalde et al., 2012). Field and experimental data indicate the contribution BVDV makes to respiratory disease is to alter or impair local and systemic defense mechanisms. By doing this, BVDV allows other pathogens to propagate to higher numbers and to persist for an extended period, leading to severe disease.

II. BOVINE HERPESVIRUS TYPE 1 (BHV-1)

1. History, Classification and Clinical Manifestations

BHV-1 is an enveloped DNA virus that belongs to the *Herpesviridae* family and the *Alphaherpesvirinae* subfamily (Figure 9). It is the causative agent of respiratory and genital tract infections such as infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/infectious balanopostitis (IPV/IBP), and abortion. Thus, BHV-1 is an economically important pathogen of cattle, with worldwide distribution (Kahrs, 1977; Straub, 2001); however, some European countries have set up eradication programmes and are actually free (including Switzerland and the Scandinavian countries).

IBR was first reported from outbreaks among dairy cattle in North America in the 1950s as a disease characterized by sudden onset, pyrexia, abrupt cessation of milk flow, salivation, dyspnoea and severe inflammation of the upper respiratory tract including the trachea (Schroeder and Moys, 1954). The IBR virus was isolated in 1956 (Madin et al., 1956). In Europe, the genital form of the disease was probably already observed in the 19th century and described as “coïtal vesicular exanthema” (Bläschenausschlag). The viral nature of the etiological agent was established in 1928 (Reisinger and Reimann, 1928). The respiratory form (IBR) only started to spread in Europe in the second half of the 20th century after importation of dairy cattle from North America. Although IPV is usually not observed in respiratory outbreaks (and viceversa), occasionally the respiratory and the genital form occur simultaneously in herds (Kahrs and Smith, 1965).

According to antigenic and genomic characteristics, BHV-1 is subdivided into distinct but closely related subtypes, BHV-1.1 and BHV-1.2 and the latter further divided into 1.2a and 1.2b

(Metzler et al., 1986). Initially it was proposed that the two subtypes 1.1 and 1.2 may be cause distinct manifestations of disease, with BHV-1.1 causing IBR and BHV-1.2 being related to the genital form (Engels et al., 1992). The current understanding is that both subtypes are able to infect the respiratory and genital tract of cattle, however, it has been suggested that BHV-1.1 is better adapted to the respiratory tract and BHV-1.2 to the genital tract (Rijsewijk et al., 1999).

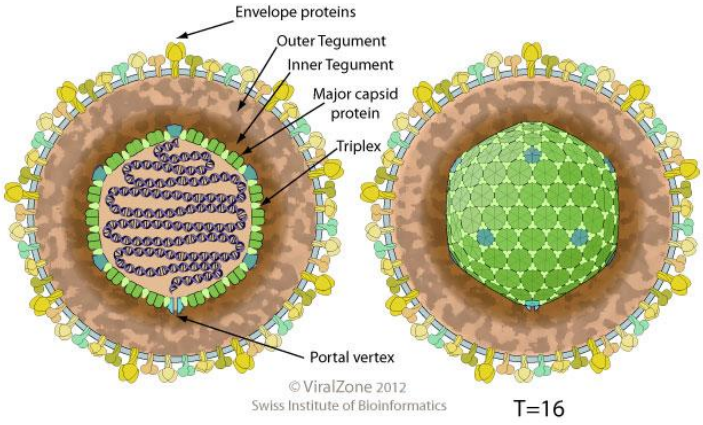


Figure 9. Virion of alphaherpesvirinae. Enveloped, spherical to pleomorphic, 150-200 nm in diameter, T=16 icosahedral symmetry . Capsid consists of 162 capsomers and is surrounded by an amorphous tegument. Glycoproteins complexes are embeded in the lipid envelope. *Source: ViralZone 2010. Swiss Institute of Bioinformatics. http://viralzone.expasy.org*

The cell-derived virus envelope contains virally encoded membrane proteins and a tegument protein. A capsid surrounds the genomic double-stranded linear DNA consisting of approximately 135 kb pairs (Figure 9). The complete sequence of the BHV-1 genome has been determined using a composite of the Cooper and Jura strains of BHV-1 (Schwyzer et al., 1997). The genome has the characteristics of a class D genome. It comprises two unique sequences, a unique long and a unique short sequence, of which the latter is flanked by inverted internal repeated and terminal repeated sequences. During DNA replication, both unique regions can flip- flop relative to the other unique region. All herpesviruses encode a large number of proteins involved in nucleic acid, DNA synthesis and protein processing (Roizman and Pellett, 2001). At least 33 of the BHV-1 encoded proteins are structural proteins (Misra et al., 1981). Of these,

thirteen are probably associated with the envelope (Liang et al., 1996) and ten of these have the potential to encode glycoptroteins (Schwyzer and Ackermann, 1996). These BHV-1 glycoproteins (g) have been characterized so far: gB, gC, gD, gE, gG, gI, gH, gK, gL, and gM (Robinson et al., 2008). The glycoproteins play an important role in the interaction with the virus host. Some glycoproteins like gC, gB and gD are major envelope glycoproteins and are indispensable for virus replication *in vitro*, they are referred to as essential. Others, the so-called non-essential glycoproteins like gE can be deleted without abolishing the virus replication *in vitro*. The currently used IBR marker vaccines are based on gE deletion mutants of BHV-1. The gC, gD, gE, gG, gI, UL49h and thymidine kinase genes are involved in viral virulence (Kit et al., 1985; 1986; Smith et al., 1994; van Engelenburg et al., 1994; Young and Smith, 1995; Van Oirschot et al., 1996; Liang et al., 1997; Kaashoek et al., 1998). There is no evidence either *in vitro* or *in vivo* that BHV-1 produces toxins.

BHV-1 causes disease in cattle, with the severity of disease relating to the subtype and strain of the virus. The clinical signs of acute disease are caused by the destruction of BHV-1-infected cells (Engels and Ackermann, 1996), binding of the virus triggers programmed host cell death (apoptosis) (Lovato et al., 2003). BHV-1 infection can cause respiratory, ocular, reproductive, central nervous system, enteric, neonatal and dermal disease in cattle (Biuk-Rudan et al., 1999; Kahrs, 2001), and can cause mastitis under experimental, but not field, conditions (Wellenberg et al, 2002). Infections with BHV-1 alone do not cause death in healthy mature cattle (Kahrs, 2001). Deaths from BHV-1 occur when viral infection is established throughout the blood and the body (viremia). Engels and Ackermann, 1996 described that BHV-1 infection does not cause viremia in healthy mature cattle. However, Risalde et al. 2011b demostrated BHV-1 viremia in mature cattle with subclinical BVD. Moreover, BHV-1 infections can cause fetal infection, viremia, death and abortion following genital infection of pregnant cows (Kahrs, 2001), and can cause fatal viremia in newborn calves in the absence of maternal Abs in milk (Mechor et al., 1987). Acute BHV-1 infection, whether or not there are clinical signs of disease, leads to latent infection. Latently infected cattle show no disease unless the latent infection is reactivated.

The respiratory disease IBR is manifested by sudden onset of high fever, reduced appetite, increased respiration rate, and dyspnoea. The animals have excess nasal and sometimes also occular discharge that is initially clear and later becomes mucopurulent (Kahrs, 2001).

Hyperemia and reddening of the muzzle and nasal turbinates inspired the term “red nose disease”. Necrotic lesions on the nasal mucosa are almost pathognomonic for IBR. Apart from the damage caused by the infection BHV-1 or the host’s immune reaction, BHV-1 can play an important role in the BRDC, by causing immunosuppression, facilitating superinfections with other viruses or bacteria (Babiuk et al., 1988; Winkler et al., 1999; Lovato et al., 2003). The reduction in milk yield, that can be observed in the corse of an acute infection is probably related to the rise in temperature. When pregnant cattle are affected, abortions may occur between the 5th and 8th month of pregnancy up to 100 days after infection. Cases of encephalitis with loos of coordination, hyperexcitation and depression have been reported in association with certain clinical forms of IBR, mainly in young calves. However, bovine encephalitis is more often caused by the closely related bovine herpesvirus 5 (BHV-5) (Rissi et al., 2008). The morbidity and the severity of the clinical signs vary according to the percentage of seropositive of exposed animals and the strain of virus. The mortality might reach 10% in some severe cases (Kapil and Basaraba, 1997).

2. Pathogenesis

Animals with IBR shed large amounts of virus with nasal and to a lesser extent occular discharge; virus is nasally shed for 10-14 days during acute respiratory infection (van Engelenburg et al., 1995). The virus is mainly transmitted by inhalation of droplets containing infectious virus mainly through contact with an infected animal. Airborn transmission has been demonstrated under experimental conditions (Mars et al., 1999). Virus entry happens via the mucous membranes of the upper respiratory tract or the conjunctival epithelium. Infection via the mucous membranes of the genital tract takes place during mating and results in IPV/IBP.

The entry of the virus occurs mainly by nasal route after contact with infected secretions or by inhalation and the virus spread in the animal by viremia and neuronal invasion (Lemaire et al., 1994). Conjunctivitis with an ocular discharge is frequently observed in the first phase of the disease. BHV-1 can only cause a productive infection in certain cell types, since only some cells produce the proteins required for virus entry and subsequent virus production. BHV-1 infects epithelial cells of the upper respiratory tract, vaginal and prepuce mucous membranes, and the tonsils and conjunctivae (Tikoo et al., 1995a), as well as CD4+ T cells (Lovato et al., 2003),

monocytes and macrophages (Nyaga and McKercher, 1979; Forman et al., 1982). Thus, the infection induces a necrosis of the epithelium of the muzzle, nose, nasopharynx, trachea and the first bronchi. In addition, BHV-1 impairs the macrophage, neutrophil and lymphocyte functions, favouring bacterial colonisation and virus infection of the respiratory tract. After infection the virus migrates to sites such as the trigeminal and sacral ganglia where it can become latent for life (Rock et al., 1992). Latency may also occur in tonsillar lymphoid cells and peripheral blood lymphocytes (Mweene et al., 1996). Infectious virus is not present during latent infection (Rock, 1994; Engels and Ackermann, 1996).

Virus attachment to the cell surface is mediated by gB and/or gC (Li et al., 1995) that bind to cell surface structures like heparan sulphate. This low affinity binding is followed by stable binding of gD to specific cellular receptors (Campadelli-Fiume et al., 2000). The subsequent virus penetration occurs via fusion of the viral envelope with the cell membrane. At least four glycoproteins are involved in this process: gD, gB, as well as gH and gL that form a heterodimer (Liang et al., 1995; Gerdts et al., 2000). The capacity of BHV-1 to spread directly from an infected to neighboring uninfected cells allows viral spread in the presence of neutralising Abs. BHV-1 glycoproteins gE and gG were shown to function independently from each other in cell-to-cell spread, because an additive effect on plaque formation was observed for a gE/gG double deletion mutant (Trapp et al., 2003). Recent studies suggest that gE is also required for anterograde transport of BHV-1 from neuronal cell bodies in the trigeminal ganglion to their nerve processes (Brum et al., 2009).

After entry into the host cell, BHV-1 is transported along microtubules to the nucleus for replication using host cell proteins. The virus becomes enveloped as it buds through the nuclear envelope and is then transported within intracellular vesicles to the cytoplasmic membrane and released from the cell (Hunter, 2001; Knipe et al., 2001). BHV-1 replication starts within 2 h of infection in cattle (Meurens et al., 2004), with cell surface Ag expression within 3-4 h after infection and viral release and spread starting at 8 h after infection (Babiuk et al., 1996). After the primary infection, the virus spreads within the host via three different routes: 1) by cell-to-cell spread, 2) via the blood stream, and 3) via the nervous system (Engels and Ackermann, 1996). BHV1 infections spread through a herd within a few weeks if the animals are seronegative. It is via the latter route that the virus reaches the cells of the trigeminal ganglion (respiratory infection) or the sacral ganglion (genital infection), where it establishes a life-long

latent infection (Pastoret et al., 1982). Latent carriers of the virus are clinically inapparent. Most of them are serologically positive for Abs against BHV-1, but there is evidence that some latent carriers can become seronegative (Hage et al., 1998). Moreover, administration of a live-attenuated vaccine in passively immunized calves resulted in BHV-1 seronegative latent carriers (Lemaire et al., 2000a). Following immunosuppression caused by various factors including transport, calving and treatment with glucocorticoids, the latent virus can be reactivated. Reactivation generally passes clinically unnoticed but is associated with re-excretion of BHV-1 and an anamnestic immune response.

BHV-1 infects cells of the upper respiratory tract, causing rhinitis, conjuntivitis and tracheitis. Respiratory tract infections with BHV-1 also may contribute to establishment of bronchopneumonia by impairing host defenses, such as by diminishing lung clearance mechanisms and by immunosuppression. If infection occurs in non-immune pregnant females, systemic infection, fetal infection and abortion are the likely consequences. Genital infections may result in development of IPV in females or IBP in males. Genital infections are characterized by formation of variable numbers of small nodules, vesicles, focal erosions or ulcers visible on inflammed mucosal membranes and occur transiently and resolve spontaneusly in 1 to 2 weeks.

Most BHV-1 vaccines reduce the severity of the disease, and also virus replication and transmission, but are not able to prevent infection and the establishment of latency (Ackermann and Engels, 2006).

3. BHV-1 and Immunity

The immunology of BHV-1 infection relates to both the host immune response to viral infection, and to mechanisms that the virus uses to overcome the host immune response. The immune response to BHV-1 infection is triggered when the virus begins to replicate (Babiuk et al., 1996). Adaptive cell-mediated and Ab-mediated immune response occur by 7 dpi (Engels and Ackermann, 1996; OIE, 2010). Ab is thought to be critical in preventing infection and viral spread, while cell-mediated immunity is involved in recovery from infection (Babiuk et al., 1996).

Humoral and cellular immunities protect against clinical signs. After natural infection with BHV-1, the first response of the immune system is the production of IFN alpha/beta. These cytokines can be detected within several hpi (Babiuk et al., 1996). They might contribute to the

early immunity after intranasal vaccination with a BHV-1 live vaccines (Makoschey and Keil, 2000). Only after a few dpi the host develops a humoral and a cell mediated immune response (Engels and Ackermann, 1996).

3.1. Immunosuppression

BHV-1 causes a broad immunosuppression in infected cattle, which often leads to secondary viral and bacterial infections (Winkler et al., 1999), contributing to BRDC. Immunosuppression is caused by impairment of macrophage, neutrophil and lymphocyte function (Tikoo et al., 1995a), and by decreased IL-2 receptor expression, decreased mitogenic stimulation of peripheral blood mononuclear cells and reduced numbers of circulating T lymphocytes (Winkler et al., 1999). Infection of monocytes and macrophages leads to impaired phagocytosis, Ab-dependent cellular cytotoxicity function and poor T cell stimulation (Forman et al., 1982). The effect of immunosuppression is partly mediated by the BHV-1 gG, a broad-spectrum chemokine-binding protein that blocks chemokine binding and activity (Bryant et al., 2003). BHV-1 also infects CD4+ T cells, inducing a loss of CD4 expression followed by apoptosis of these cells (Winkler et al., 1999; Lovato et al., 2003).

3.2. Humoral Immune Response

The Ab response includes neutralising Abs, and may either neutralize free virus or contribute to antiviral Ab-dependent cellular cytotoxicity (Tikoo et al., 1995a), which is enhanced by complement (Rouse et al., 1977). Abs directed against the glycoproteins involved in virus attachment or entry (gB, gC, gD, gE) are critical in preventing infection (Babiuk et al., 1996). This defence mechanism has a major impact to control virus infectivity and spread after secondary infections or in case of reactivation. After natural infection, Ab response against BHV-1 is longlasting (Kaashoek et al., 1996). Maternal Abs protect the calves against the clinical disease, but they do not prevent infection or the establishment of latency (Lemaire et al., 2000b). Moreover passive Abs have been shown to negatively affect efficacy of a live vaccine (Patel, 2005; Patel and Shilleto, 2005).

3.3. Cell-mediated Immune Response

Like other viruses, also BHV-1 applies mechanisms to evade the immune system. The most efficient ones are the establishment of latency and the direct cell-to-cell spread. During latency viral proteins are not expressed, and infection occurs at immunoprivileged sites that do not express MHC-I Ags (Tyler and Nathanson, 2001). Another mechanism of immune evasion that has been demonstrated is the inhibition of the proliferative response of peripheral blood mononuclear leukocytes to Ag (Hutchings et al., 1990a). Moreover, BHV-1 evades the host immune response by interfering with Ag processing and presentation and by infecting monocytes and macrophages (Nyaga and McKercher 1979; Forman et al., 1982). Alphaherpesviruses, including BHV-1, also have immunomodulatory activity mediated by herpesvirus proteins that mimic key molecules of the host immune system (Raftery et al., 2000).

Once the infection is established, Abs are of limited value in preventing direct cell-to-cell spread. Cell-mediated immunity is thought to be involved in recovery from infection (Babiuk et al., 1996). The cell-mediated immune response to BHV-1 infection includes macrophages, IL-2 and IFN-γ production, natural killer cells, gC and gD specific CD4+ T cells, and stimulation of cytotoxic T-lymphocyte activity (Hutchings et al., 1990b; Tikoo et al., 1995b). BHV-1 causes a general immunosuppression in infected cattle, which often leads to secondary viral and bacterial infections in the context of the BRDC (Bielefeldt Ohmann and Babiuk, 1985). BHV-1 has been shown to infect CD4+ T cells in cattle, leading to apoptosis and suppression of cell-mediated immunity (Winkler et al., 1999). IFN-γ has been shown to both protect against infection and to prevent viral spread in experimental infection in mice (Abril et al., 2004). Responses to BHV-1 infection are broad based and include both T helper 1 and T helper 2 responses (Babiuk et al., 1996), although, as with other intracellular pathogens, there is a skew towards a T helper 1-type response (Mena et al., 2002).

III. NASAL MUCOSA AND PALATINE TONSIL

1. Mucosa-Associated Lymphoid Tissue (MALT)

The mucous membranes are continuously exposed to various Ags, to which the body must either mount an immune response or maintain immunological tolerance (Hiller et al., 1998; Debertin et al., 2003). As they form a weak mechanical barrier (Kuper et al., 1992), mucosal surfaces are provided with a local immune system (mucosa-associated lymphoid tissue [MALT]) (Davis, 2001; Debertin et al., 2003). The MALT consists of organized, non-encapsulated lymphoid tissue with follicular structures and is found in the gastrointestinal, respiratory and urogenital tracts and the conjunctival surface of the eye (Hellings et al., 2000). One of the main functions of the MALT is to generate and disseminate Ag-sensitized B lymphocytes, which diferentiate into IgA producing plasma cells in the lamina propria of the various mucosal sites and the associated secretory tissues (e.g., salivary or lacrimal glands) (Hellings et al., 2000; Davis, 2001; Zuercher et al., 2002). MALT is the initial inductive site for mucosal immunity: Ags are sampled from mucosal surfaces and cognate naive B and T lymphocytes stimulated. MALT structures are the origin of the lymphocyte trafficking to mucosal effector sites (Brandtzaeg and Pabst, 2004; Liebler-Tenorio and Pabst, 2006).

Within the respiratory tract, MALT is represented by the nasal-associated lymphoid tissue (NALT), Waldeyer's ring of the pharyngeal mucosa, and the bronchus-associated lymphoid tissue (BALT) (Liebler-Tenorio and Pabst, 2006).

2. Composition of the Bovine Waldeyer's ring

The tonsils form a ring of lymphoid tissue in the pharyngeal wall called the Waldeyer's ring (von Waldeyer-Hartz, 1884; Perry and White, 1998; Hellings et al., 2000). Its location at the crossing of the digestive and respiratory tracts plays a key role in immunity as this is the site where vast amounts of foreign Ags enter the body during feeding and breathing (Cassidy et al., 1999; Brandtzaeg and Pabst, 2004; Liebler-Tenorio and Pabst, 2006). In the bovine, five distinct tonsils are described: the lingual tonsil, the palatine tonsil and the tonsil of the soft palate protect the oropharynx; and the pharyngeal and the tubal tonsils in the nasopharynx. The palatine tonsils and the pharyngeal tonsil account for the major part of the lymphoid tissue of Waldeyer's ring (Casteleyn et al., 2011).

The palatine tonsils are global masses of lymphoid tissue embedded in the submucosa of the lateral wall of the oropharynx. Anatomically, the large ovoid bovine palatine tonsil is bilobated with a central cavity called 'sinus tonsillaris' visible at the oropharynx surface that communicates with the oral cavity. Many tonsillar fossules open into this tonsillar sinus and lead numerous crypts that lie in the centres of tonsillar follicles. The crypt epithelium is stratified squamous and often is infiltrated by lymphoid cells (Figure 10). The lymphoid tissue surrounding the crypts is composed of many secondary lymphoid follicles with distinct light, dark and mantle zones and with interfollicular lymphoid tissue (Liebler-Tenorio and Pabst, 2006; Casteleyn et al., 2011).

3. Tonsillar Structures

The tonsils are secondary lymphoid organs containing aggregations of lymphoid cells, located in the lamina propria of the pharyngeal wall. The subepithelial lymphoid compartments of tonsils are formed by numerous secondary lymphoid follicles (B-cell areas), surrounded by interfollicular regions (T-cell areas).

Tonsils possess several unique characteristics: (1) unlike the spleen or the lymph nodes, they are not fully encapsulated; (2) like the spleen but unlike the lymph nodes, they do not possess afferent lymphatics; (3) like both the spleen and lymph nodes, they are lymphoreticular structures, but unlike them, tonsils are also lymphoepithelial organs; and (4) the tonsillar epithelium not only provides a protective surface cover but also invaginates and lines the

tonsillar crypts. Tonsillar crypts are narrow epithelial diverticula, which considerably increase the available surface area for direct antigenic stimulation (Perry and Whyte, 1998).

3.1. Tonsillar Epithelium

The pharyngeal surfaces are protected by epithelial layers that are avascular and only a very few nonepithelial cells are found here. Epithelium of the bovine palatine tonsil is characterized as stratified non-keratinized epithelium. The surface epithelia are underlined by a band of thick connective tissue containing many vessels, nerves and lymphatics (Palmer et al., 2009). By contrast, the epithelia lining the crypts are not uniform. They contain patches of reticulated, morphologically reshaped and rearranged strands of epithelial cells, which are infiltrated with nonepithelial cells (mainly lymphocytes), and are underlined with disrupted basement membrane but no thick connective tissue band (Figure 10) (Perry and White, 1998; Palmer et al., 2009). The degree of reticulation and lymphocytic infiltration varies and is more pronounced in the palatine tonsils and pharyngeal tonsil than in the lingual tonsil or tubal tonsils. It is this type of specialized 'lymphoepithelium' that is of functional importance and of renewed interest to immunologists (Perry and White, 1998).

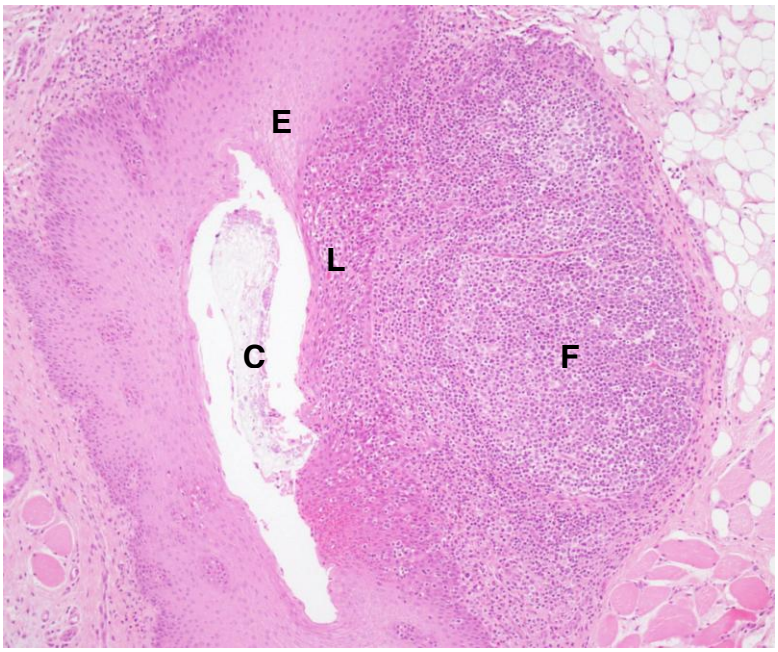


Figure 10. Histology of the palatine tonsils. (E) Tonsillar epithelium, (L) Lymphoepithelium, (F) Lymph follicle and (C) Tonsillar crypt. HE

3.2. Lymphoepithelium

Lymphoepithelium consist in epithelial cells, which are altered in shape and cellular contents but which represent a scaffold held together by desmosomes, infiltrating, motile nonepithelial cells and intraepithelial vasculature (Figure 10). Moreover, there are follicle-associated to epithelium, referring to an epithelium that can sample antigens and translocate them to the underlying lymphoid tissue where appropriate clones of T and B lymphocytes can be selected and amplified prior to their migration into the surrounding mucosa (Perry and Whyte, 1998).

Between the epithelial cells with their slender cytoplasmic processes, a vast continuum of intercellular spaces is formed, which is filled with mobile and motile 'free cells' such as T lymphocytes and B lymphocytes (producing IgG, IgA and IgM), macrophages, interdigitating DCs and Langerhans cells. The infiltrating nonepithelial cells are therefore considered a physiological characteristic of this epithelium, which postnatally are constantly exposed to airborne and alimentary Ags. Any inflammatory changes, with the additional presence of PMN leukocytes, are superimposed onto the normal pattern of reticulation (Scadding, 1990; Perry and Whyte, 1998). Studies on human palatine and pharyngeal tonsils showed that the tonsillar crypt epithelium functions as an additional lymphoid compartment by contributing to the production of immunocytes and to the protection of the mucosal surface; it also provides direct, transepithelial access for Ags (Brandtzaeg and Halstensen, 1992). To be efficient, the reticulated epithelium needs to contain not only the immigrant lymphoid cells but also sufficient epithelial cells, some of which can synthesize secretory components. Within the tonsillar lymphoepithelium, there is a network of intraepithelial blood vessels (Figure 11). In both the palatine tonsil and pharyngeal tonsil, capillaries are arranged in loops oriented perpendicularly to the crypt surface, and high endothelial venules (HEVs) are located in the lower border regions of many reticulated patches (Perry, 1994; Perry and Whyte, 1998). The rich intraepithelial blood flow provides for the metabolic needs of this site, as well as increasing the area for interactions between endothelial cells and leukocytes, and the transport of Igs and other substances across the vessel walls.

In the intestine and reticular epithelium in tonsils of some species, specialized epithelial cells with characteristic short microvilli and microfolds on their luminal surface are known as M cells. These are always in close contact with macrophages, DCs and lymphocytes. M cells allow

for selective sampling through endocytosis and transport of luminal Ags with exocytosis to immune cells within or below the mucosal layer (Palmer et al., 2009). In bovine tonsils, the presence of M cells is still in debate due to M cells have been identified in bovine nasopharyngeal tonsils (Schuh and Oliphant, 1992), but not bovine palatine tonsils (Palmer et al., 2009).

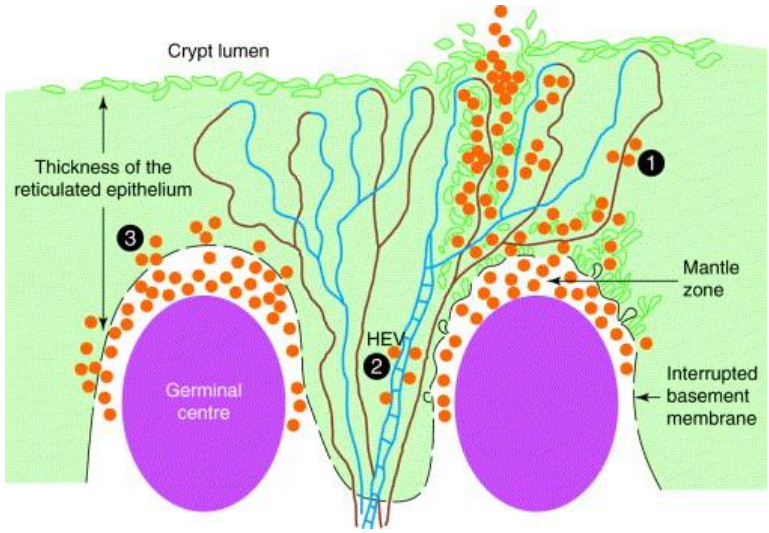


Figure 11. Routes of entry of nonepithelial cells into lymphoepithelium of palatine tonsil. Routes 1 and 2 involve direct homing through the walls of capillaries and HEVs; and route 3 involves entry from the subepithelial lymphoid comprtiments through the disrupted basement membrane. *Source: Perry and Whyte, 1998.*

4. Role of tonsils in infection

Tonsils are important for inducing immunity at mucosal sites (Liebler-Tenorio and Pabst, 2006). However, some pathogens have developed mechanisms to overcome tonsillar defences and may use them as the port of entry, replication and colonization (Horter et al., 2002). Bovine tonsils are sites for entry and replication of several pathogens and have been investigated in the context of several infectious diseases (Brandtzaeg and Halstensen, 1992). The tonsils are infected early in the course of BHV-1 infection. Viral Ag is present in the tonsillar epithelium and lymphoid tissue and causes necrosis/apoptosis of the tonsillar epithelium and lymphoid tissue in neonates, calves and adult cows (Narita et al., 1982; Schuh et al., 1992; Winkler et al., 1999, 2000). Tonsils are also important for diagnosing asymptomatic carriers of BHV1, since the virus becomes latent in the lymphoid tissue of the tonsil and can be reactivated by immunsuppression

causing renewed shedding (Winkler et al., 2000; Perez et al., 2005). In BVDV infection, the tonsils are the initial site of infection in acute postnatal infection and replicates to very high titres in the tonsils (Liebler-Tenorio et al., 1997; Bruschke et al., 1998b; Liebler-Tenorio et al., 2002).

OBJECTIVES

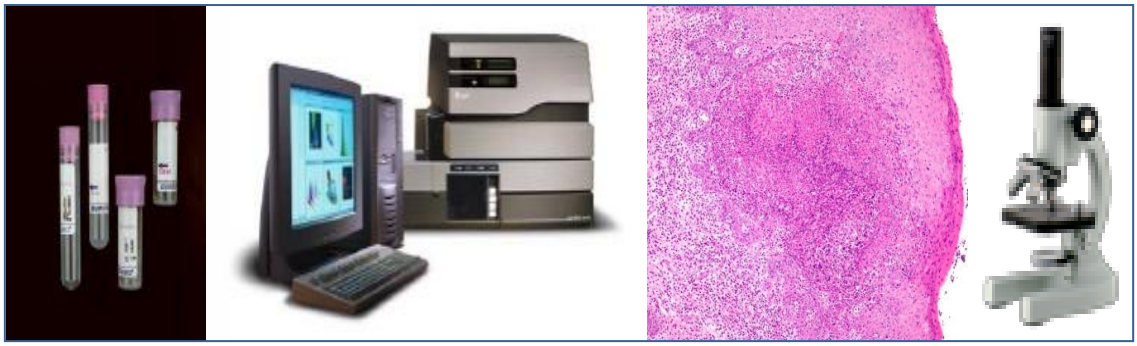
The overall aim of this work was to deepen in the study of the pathogenesis of BVDV by means of the characterization of the systemic and local cell-mediated immune response in calves experimentally infected with ncp BVDV-1, and evaluating the consequences of the immunosuppression associated with acute BVDV infections, especially the exacerbated effect developed in co-infections with BHV-1.1 in cattle.

In order to achieve this general objective, the following partial objectives were proposed:

1. To examine the systemic changes of immunocompetent cell populations, acute phase proteins, cytokines, viremia and specific antibodies in experimentally infected calves with non-cytopathic BVDV-1. **CHAPTER 1**

2. To evaluate the systemic changes of immunocompetent cell populations in calves with subclinical BVD and in healthy calves, both challenged with BHV-1.1. **CHAPTER 2**

3. To characterize the quantification and biosynthetic activity of immunocompetent cells in the nasal mucosa and palatine tonsil of calves pre-infected with BVDV and healthy calves, both challenged with BHV-1.1, with the aim of evaluate their potential role as primary replication organs located in the oronasal route of entry of both BVDV and BHV-1.1. **CHAPTER 3**



MATERIALS AND METHODS

1. Experimental outline

Thirty male Friesian calves (8-9 months old) were purchased from a conventional dairy farm declared free of tuberculosis, brucellosis and bovine leucosis, in accordance with the rules of Spanish programmes for the eradication of these diseases.

On arrival, calves were housed at the University of Cordoba Experimental Animal Center (Spain), and were allowed to adapt for one week before the experiment started. BVDV and BHV-1.1 free status was confirmed by Ag and Ab enzyme-linked immunosorbent assays (ELISAs). At the beginning of the experiment, calves were randomly assigned to three different groups called according to the inoculation they were exposed to (Figure 1).

- BVDV/BHV1 group: 14 calves were infected by intranasal inoculation with 10 ml (5 ml per nostril) of a suspension containing 10^5 tissue culture infective dose 50% ($TCID_{50}$)/ml of ncp BVDV genotype-1 7443 strain (subtype 1a), courtesy of the Institut für Virologie TiHo (Hannover, Germany). Ncp BVDV-1 7443 is a field strain of low-virulence isolated from a PI calf (McClurkin et al., 1979), which has been extensively used in previous *in vitro* and *in vivo* studies that gives reproducible infection profiles (Bolin et al., 1985b; Greiser-Wilke et al., 1991; Grummer et al., 2001; 2002; Pedrera et al., 2009b; Risalde et al., 2011b; 2013).

Twelve days later (0 dpi BHV1, i.e., 12 dpi BVDV), when calves did not show neither clinical signs nor viremia against BVDV, 12 of them were challenged receiving an intranasal inoculation with a total of 2 ml (1ml per nostril) containing 10^7 $TCID_{50}$ of BHV-1 subtype 1 (BHV-1.1) virulent Iowa strain. Calves were sedated and sacrificed in batches of two at 1, 2, 4, 7 and 14 dpi BHV1. The other two animals inoculated with BVDV but BHV-1 free were killed before BHV-1 infection (0 dpi BHV1, i.e., 12 dpi BVDV) and used as BVDV infection controls.

- BHV1 group: 12 calves were inoculated with the same amount and concentration of BHV-1 Iowa strain and euthanized in batches of two at 1, 2, 4, 7 and 14 dpi BHV1.

- Negative control group: 4 calves received 1 ml of tissue culture fluid free of viruses in each nostril and were killed at the end of the study (14 dpi BHV1).

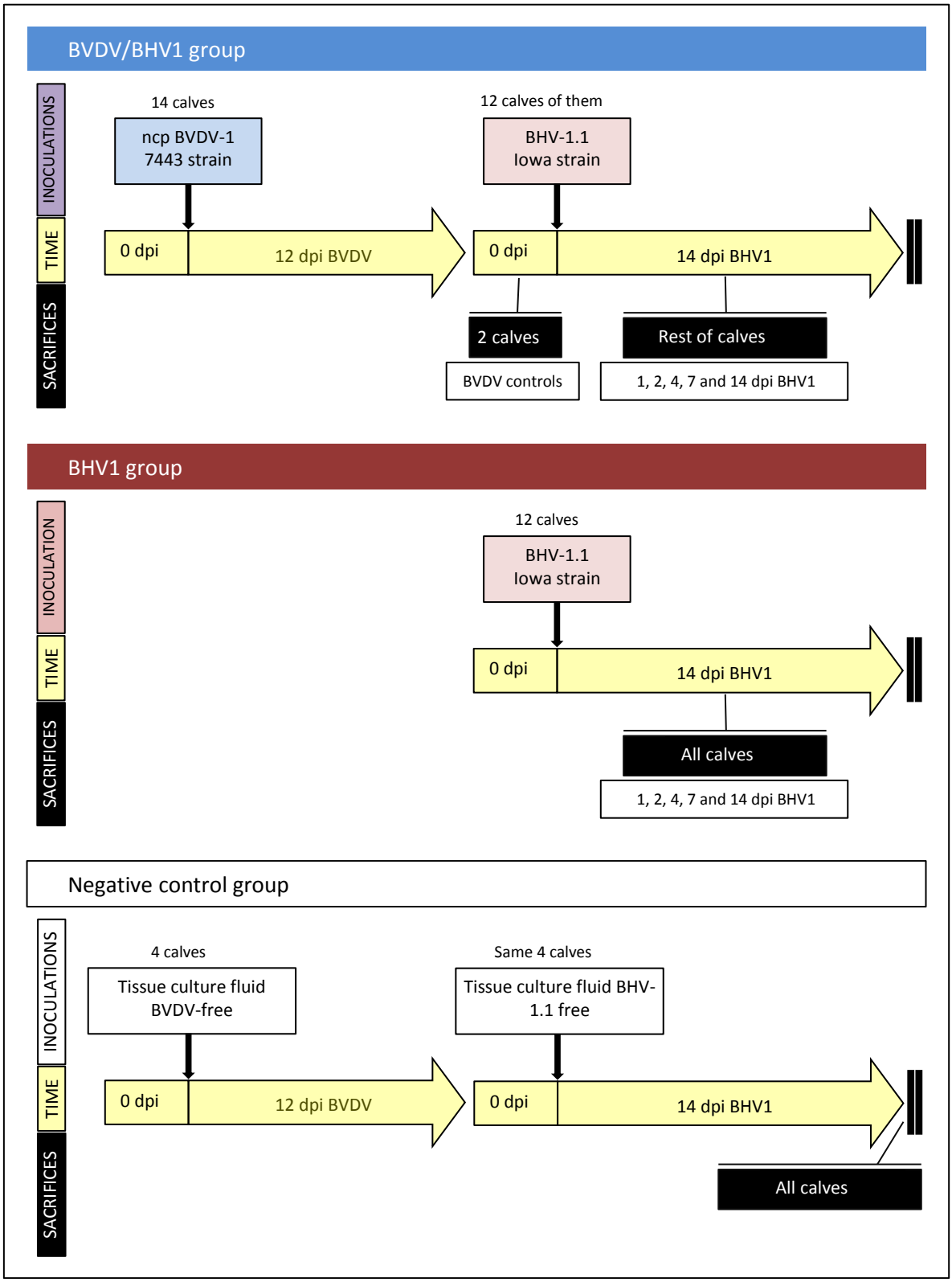


Figure 1. Schematic summary of experimental outline.

Rectal temperatures and clinical signs (anorexia, depression, hypersalivation, lacrimation, nasal discharge, cough, dyspnea, accessible mucosal surface lesions and diarrhea) were monitored daily prior to virus inoculations and throughout the study. Clinical evaluation was based on an assessment of the severity of the clinical response as follows: -, absence of clinical signs; +, mild; ++, moderate; and +++, severe intensity of each clinical sign.

The whole experimental procedure was carried out in accordance with the Guidelines for accommodation and care of animals, approved by the European Economic Community (Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, as amended by Directive 2003/65/EC).

2. Samples collection

2.1. Blood, serum and plasma samples

EDTA and without additives blood samples were taken. Whole EDTA-blood samples were collected and used fresh for flow cytometry analysis and measurement of total and differential leukocyte counts, and frozen at -80°C until viral detection. EDTA-blood samples and samples without additive were centrifuged at 4000 rpm for 10 min to obtain plasma and serum, respectively. All serum and plasma samples were frozen at -80°C until the day of APP, cytokine and viral Ab analysis.

Blood, serum and plasma samples were obtained twice from each calf prior to BVDV and BHV-1 inoculations in order to obtain basal values: 0 dpi BVDV and 0 dpi BHV-1 (i.e. 12 dpi BVDV) respectively, for each analytical procedure.

After BVDV inoculation, blood and serum samples were collected at 1, 2, 3, 6, 9 and 12 dpi BVDV from BVDV and control group calves; just as after BHV-1 inoculation at 6, 12 and 18 hpi BHV1 and 1, 2, 4, 5, 7 and 14 dpi BHV-1 in all calves (n) of each group for several different assays (Table 1).

Table 1. Number of calves bleed per group in each time-point.

Time-Point	Animals bleeded per group (n)		
	BVDV/BHV1	BHV1	Control
0 hpi	14	12	4
6 hpi	12	12	4
12 hpi	12	12	4
18 hpi	12	12	4
1 dpi	12	12	4
2 dpi	10	10	4
4 dpi	8	8	4
5 dpi	6	6	4
7 dpi	6	6	4
9 dpi	4	4	4
14 dpi	4	4	4

2.2. Tissue samples

Animals were sedated with xylazine (Rompun 2% solution; Bayer Healthcare, Kiel, Germany) and euthanized by overdosing with thiopental-sodium (Thiovet; Vet Limited, Leyland, Lancashire, UK), being object of necropsy examination.

Nasal mucosa tissue samples were collected from ventral nasal concha (Figure 2) and palatine tonsil tissue samples were colleted as indicated in Casteleyn et al., 2011 (Figure 3a).

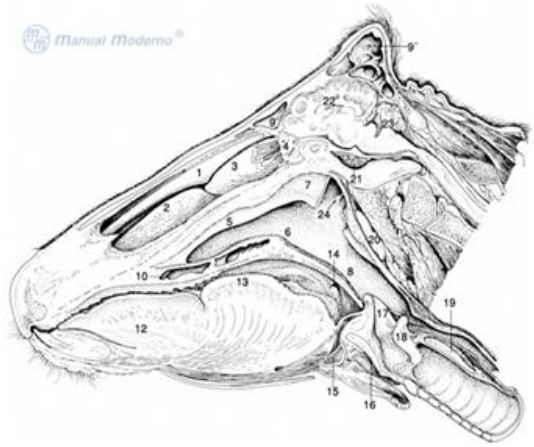


Figure 2. Anatomical localization of bovine nasal mucosa samples collected from ventral concha (2). *Source: www.manualmoderno.com/apoyos_electronicos/9707292539/cap25.swf*

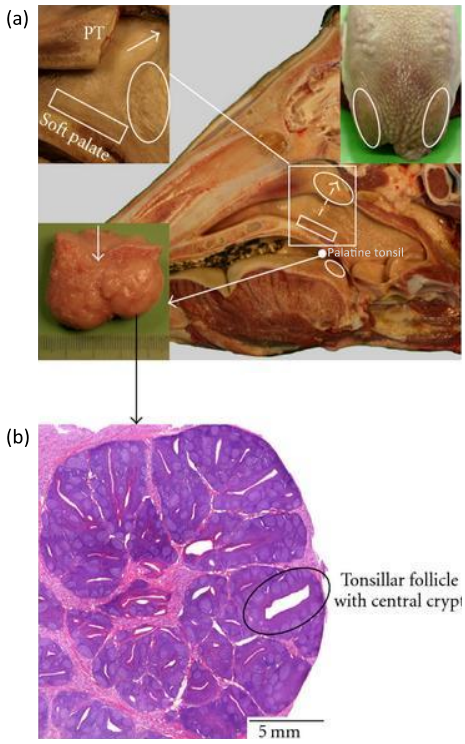


Figure 3. Anatomical localization of bovine palatine tonsil samples collected. (a) Median section through a bovine head. The palatine tonsil is shown in the lower left insert. Its sinus is indicated by the arrow. (b) Histological section through part of the palatine tonsil. *Adapted from Casteleyn et al., 2011*

Further tissue slices placed immediately in each one of freshly prepared fixative buffers: 10% neutral buffered formalin solution (10%F) for 24h, Bouin's solution (BS) for 8h and Zinc buffer salts fixative (ZSF) for 24h. Afterwards, the tissue samples were processed routinely for paraffin embedding. Nasal mucosa and palatine tonsil sections (3-4 µm) were stained with haematoxylin and eosin (HE) for histological study and sections of 2-3 µm thick were cut, mounted on Vectabond® (VectorLaboratories, Burlingame, CA, USA) coated slides and dried overnight at 37°C for immunohistological study in order to identify and quantify different lymphocyte populations and cytokine markers.

3. Methods and techniques

3.1. Viruses detection by PCR or virological examination

By PCR

High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany) was used to extract BVDV RNA from EDTA-blood samples, according to manufacturer's instructions. Samples were analyzed by RT-PCR in one-step real-time PCR. Primers and TaqMan probes (at the same concentration) based on the highly-conserved 5´-untranslated region (UTR) of BVDV-1 were used as previously described by Letellier and Kerkhofs, 2003 and in combination with the Real Time Ready RNA Virus Master (Roche, Mannheim, Germany), according to manufacturer's instructions. The PCR conditions and the used reagent concentration followed manufacturer's protocol of Real Time Ready RNA Virus Master. Reactions were carried out in a LightCycler. Any sample that had a cycle threshold (Ct) value less than or equal to 45 was considered positive. The positive control was the ncp BVDV-1 strain 7443 at 10^5 $TCID_{50}$/ml, which was used to make the standard curve by serial log10 dilutions of RNA. Ct values measured were plotted against the log of the input BVDV RNA dilution. Viral titres were expressed as $TCID_{50}$/ml.

BHV-1 DNA was extracted from EDTA blood, using Genomic DNA Purification kits (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). Reactions were carried out in a LightCycler®. Any sample that had a Ct value less than or equal to 45 was considered as positive. The positive control was the BHV-1.1 Iowa strain at $10^{8.3}$ $TCID_{50}$/ml.

By immunohistochemistry (IHC)

Detection of BVDV and BHV-1.1 Ags from tissue samples was performed by means of identification of Erns (gp48)-BVDV and gC-BHV-1.1 surface glycoproteins with 15c5 (provided by Dr. Dubovi, Cornell University, NY, USA) and F2 (VMRD Inc., Pullman, WA, USA) primary mAbs by IHC (*see section 4. Histology and Immunohistochemical Study of Materials and Methods, pp. 69-70*).

3.2. Antibodies detection

The Ab response to BVDV NS3 (p80) was determined from serum samples using a commercially-available competitive ELISA test (INGEZIM BVD Compac; Ingenasa, Madrid, Spain), following manufacturer's protocol. ELISA values under 0.9 were considered positive, while values between 1.1 and 0.9 were deemed doubtful and values above 1.1 were considered negative.

Virus neutralization (VN) was applied for evaluation of the Ab response against BHV-1.1 strain Iowa, previously used for challenge 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 is considered to be positive.

3.3. Total and differential leukocyte counts

Total leukocyte, lymphocyte, monocyte and neutrophil counts from EDTA blood samples were carried out using a Cell-Dyn 3700 Automatic Hematology Analyzer (Abbott Laboratories, Abbott Park, IL, USA).

3.4. Flow cytometric analysis of lymphocyte subpopulations

Indirect immunofluorescence staining and flow cytometric analysis for detection of lymphocyte subset surface antigens on EDTA blood samples was performed. Briefly, 100 µl of fresh EDTA-blood diluted 1:1 with PBS were incubated with 1 µl of each bovine-specific primary mAb: CD4+ T cells (CACT138A), CD8+ T cells (CACT80C), B cells (BAQ44A) or γδ T cells (CACTB6A), all from VRMD, Pullman, WA (Table 2). After incubation for 30 min at 4°C in darkness, cells were washed with PBS and 200 µl of FITC-conjugated goat anti-mouse IgM (Sigma-Aldrich, St. Louis, MO, USA) or R-phycoerythrin-conjugated goat anti-mouse IgG_1 (Invitrogen, San Diego, CA) diluted 1:500 with PBS were added, following incubation in the dark for a further 30 min at 4°C. After being labeled, cells were resuspended in 2 ml of FACS Lysing Solution (Becton Dickinson, San José, CA, USA) for 10 min at Rt to lyse erythrocytes. The lysing process was stopped by adding 2 ml of PBS. Finally, cells were fixed by adding 200 µl of 1X formaldehyde-buffered solution (CellFix 10X Concentrate, Becton Dickinson, San José, CA, USA) to each sample and were

stored at 4°C in the dark until flow cytometric analysis. Specificity controls of the labeling procedures included the omission of specific mAbs in the first step of the procedure and the use of isotype negative controls. Differences in cell-surface-molecule expression were detected using a FACScan Flow Cytometer (Becton Dickinson Immunochemistry Systems, San José, Puerto Rico).

Table 1. Bovine-specific primary monoclonal antibodies used for flow cytometric analyses.

Surface Ag	FC region	mAb*	Isotype
CD4	Lymphocytes	CACT138A	Ig G_1[a]
CD8	Lymphocytes	CACT80C	Ig G_1[a]
γδ	Lymphocytes	CACTB6A	Ig M[b]
B cells	Lymphocytes	BAQ44A	Ig M[b]

* Source VMRD, Pullman, WA, USA

[a]R-PE-conjugated goat anti-mouse IgG1 (Invitrogen, San Diego, CA)

[b]FITC-conjugated goat anti-mouse IgM (Sigma-Aldrich, St. Louis, MO, USA)

All data were further analyzed with CELLQuest (Becton Dickinson, San José, CA, USA). Twenty thousand events were collected from each sample. The following parameters were collected: forward light scatter (FSC), side light scatter (SSC), FITC fluorescence (FL1) and PE fluorescence (FL2). Lymphocytes were differentiated by their size (FSC) and granularity (SSC), and further evaluated for lymphocyte subpopulation by FL1 and FL2 (Figure 4). The proportions of lymphocytes positive for each of the mAb were determined after subtraction of the corresponding control.

Results were expressed as the absolute numbers of each lymphocyte subpopulation reacting to each mAb, calculated using the total lymphocyte number and the proportion of the subpopulation given by flow cytometric analysis at each time-point.

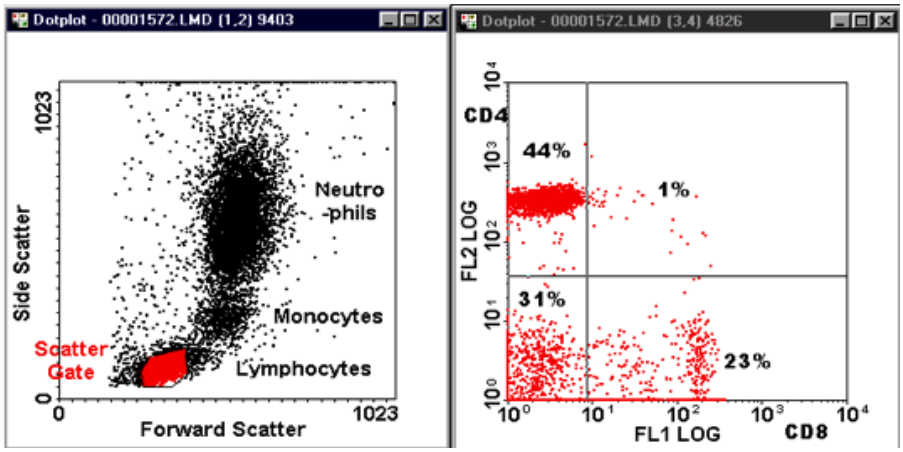


Figure 4. Dot plot with a gate emcompassing the lymphocyte population.

3.5. Acute phase protein assays

Haptoglobin, SAA and albumin were measured in duplicate serum samples, and fibrinogen levels were measured in plasma samples. Haptoglobin levels were measured according to prevention of the peroxidase activity of hemoglobin, which is directly proportional to haptoglobin, by using a commercial spectrophotometric assay (PHASE RANGE Haptoglobin Assay, Tridelta Ltd., Ireland). SAA concentrations were determined by a commercially-obtained solid-phase sandwich ELISA kit (PHASE RANGE Serum Amyloid A Assay, Tridelta Ltd., Ireland). Under normal conditions, haptoglobin and SAA are absent in serum or present at very low levels, ranging from less than 50 mg/l and 20 mg/l in cattle, respectively. Fibrinogen concentrations were measured according to the colorimetric Biuret method by using a commercial kit (Total protein Kit, Spinreact SA, Spain), calculating the difference between total plasma protein and serum protein. In healthy cattle, fibrinogen values range from 5 to 7 g/l. Serum albumin was measured by Bromocresol Green method using a commercial test kit (Albumin Kit, Spinreact SA, Spain). The reference range for albumin is 25 to 35 g/l in bovine species. All analyses were performed following manufacturers' instructions.

3.6. Cytokine assays

Cytokine concentrations (IL-1β, TNF-α, IFN-γ, IL-12, IL-4 and IL-10) were determined by specific sandwich ELISA protocols in duplicate serum samples making use of commercially-available mAb pairs (Serotec). All cytokines were measured in microtitre plates (Nunc Maxisorb, Roskilde, Denmark) previously coated with each one of highly purified anti-cytokine Abs (Table 3) at 1 µg/mL (2 mg/ml for bovine IL-1β), diluted in PBS, 2% Tween 20 (PBST) and 3% BSA, and incubated overnight at 4°C. After a blocking step with PBS, 2% Tween-20 and BSA 3% for 1 h at Rt with constant stirring, plates were washed 3 times with PBST and incubated with the serum diluted 1:50 for 1 h at Rt. After three washes with PBST, plates were incubated with each secondary mAb pairs (Table 3) at 1 µg/mL (2 mg/ml for bovine IL-1β) for 1 h at Rt in agitation. After a new washing cycle, 100 µL of streptavidin-horseradish peroxidase conjugate (Sigma–Aldrich Química, Spain) diluted 1/1000 in blocking solution were added to each well and incubated for 45 min at Rt. After a final wash, chromogenic substrate (OPD) was added, the reaction was stopped by the addition of H_2SO_4 and the absorbance values were read with an ELISA-plate reader (Bio-Rad Laboratories, Spain) at 450 nm. Standard curves to calculate cytokine concentrations were generated using recombinant bovine cytokines such as IL-1β, TNF-α, IFN-γ and IL-4 (Serotec). Due to the lack of an appropriate standard curve for IL-10 and IL-12, the results of these cytokines were presented as optical density (O.D.) values.

Table 2. Antibodies pairs to bovine cytokines for ELISA assays.

Clone*	Specificity†	Position
PBP008	IL-1β boCK	Capture
AHP851Z	IL-1β boCK	Detection
CC327	TNFα boCK	Capture
CC328	TNFα boCK	Detection
CC330	INFγ boCK	Capture
CC302	INFγ boCK	Detection
	IL-12 boCK	Capture
CC326	IL-12 boCK	Detection
CC313	IL-4 boCK	Capture
CC314	IL-4 boCK	Detection
CC318	IL-10 boCK	Capture
CC320	IL-10 boCK	Detection

*Source Serotec
†Capture and detection concentration: 1mg/ml, except for IL-1β 2mg/ml
boCK means bovine cytokine

4. Histology and immunohistochemical study

For histology or immunohistochemical study, fixed samples were placed in plastic cassettes and dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax. Sections 2-4 μm thick were cut on a microtome, mounted on Vectabond® (VectorLaboratories, Burlingame, CA, USA) and stained with hematoxylin and eosin (HE) for light microscopy histopathological study or processed for IHC by the avidin-biotin-peroxidase complex (ABC) method.

The ABC method for IHC was performed on serial sections of fixed samples as described previously (Pedrera et al., 2007; 2009a,b). Briefly, endogenous peroxidase activity was exhausted by incubation with 3% hydrogen peroxide in methanol for 30 min at Rt. The samples were subjected to different methods for Ag retrieval (Table 3). After pretreatment, sections were rinsed three times in 0.01 M PBS, pH 7.2, for 10 min each. For the mAbs, tissue sections were covered with 1% normal horse serum (Pierce-Endogen, Woburn, USA) in 0.05M Tris buffer (TBS), pH 7.6, for 30 min at Rt. For the policlonal antibodies (pAbs), 20% normal goat serum replaced to normal horse serum. After this blocking stage, sections were incubated with primary mAbs or pAbs at 4°C overnight. Details of the commercial primary Abs used in this study, including dilutions and pretreatments, are summarized in Table 3. After primary incubation, the slides were washed in PBS (three times for 5 min each) and then incubated with the secondary Abs for 30 min at Rt. Biotinylated horse anti-mouse IgG secondary Ab (Pierce-Endogen), diluited 1:200 in TBS containing 1% normal horse serum was used for the mAbs. Biotinylated goat anti-rabbit IgG secondary Ab (VectorLaboratories, Burlingame, CA, USA) diluited 1:200 in TBS containing normal goat serum 1.5% was used for the pAbs. 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 Rt. 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.

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

Internal positive controls consisted of tissues samples obtained from calves experimentally infected with BVDV where cytokines and cellular markers detection was previously carried out by IHC (Pedrera et al., 2007; 2009b). Mouse and rabbit non-immune sera (DakoCytomation, Glostrup, Denmark) were used in place of specific mAbs and pAbs respectively as internal negative controls, and omission of the primary Ab was used to provide an additional negative control.

4.1. Evaluation of immunostaining

To evaluate the number of immunostained cells and to correlate the results obtained using different Abs, two paraffin-wax blocks from nasal mucosa and palatine tonsil and of each animal were selected. On tissue sections from these blocks, cell counts were carried out in 25 fields of 0.2 mm^2 chosen randomly of epithelium and lamina propria. Identification of different kind of immunostained cells was based on morphological features, location, and size of the cells.

Table 4. Specificity of primary antibodies for immunohistochemical study.

Antibody†	Specificity	Fixation*	Retrieval	Dilution	Source
15c15, mAb	BVDV protein Erns (gp48)	10%F	Proteinase K[g]	1:75	Cornell University[‡]
F2, mAb	BHV-1 protein gC	10%F	TC pH 6 MW[a]	1:1000	VMRD
CD4, mAb	CD4+ T lymphocytes	ZSF	TC pH 9 37°C[b]	1:10	Serotec
CD8, mAb	CD8+ T lymphocytes	ZSF	TC pH 3.2 37°C[b]	1:25	Serotec
WC1, mAb	γδ T lymphocytes	ZSF	TC pH 3.2 37°C[b]	1:100	Serotec
CD79αcy, mAb	B lymphocytes	10%F	TC pH 6 MW[c]	1:25	Dako
MCA874G, mAb	Monocytes-macrophages	10%F	Protease[d]	1:100	Serotec
HLA-DR, mAb	MHC-II	10%F	TC pH 3.2 MW[a]	1:100	Dako
TNFα, pAb	TNFα cytokine	BS	TC pH 3.2 37°C[a]	1:25	Serotec
IL-1α, pAb	IL-1α cytokine	BS	Tween 20[f]	1:100	Endogen
IFNγ, mAb	INFγ cytokine	BS	TC pH 3.2 MW[g]	1:10	Serotec

† Monoclonal antibody (mAb) or policlonal antibody (pAb).
* 10% neutral buffered formalin solution (10%F), Bouin's solution (BS) and Zinc buffer salts fixative (ZSF).
‡ Courtesy of Dr. E. Dubovic, Cornell University, Ithaca, NY.
[a] Incubation with 0.1M citric acid (pH 3.2 or 6), microwave for 5 min at sub-boiling temperature.
[b] Incubation with 0.1M citric acid (pH 3.2 or 9) for 30 min at 37°C in oven.
[c] Incubation with 0.1M citric acid (pH 6), microwave for 20 min at sub-boiling temperature.
[d] Incubation with 0.1% protease type XIV (Sigma-Aldrich) in 0.01M PBS, pH7.2 for 10 min at Rt.
[f] Incubation with 0.1% Tween 20 (Merck) in 0.01M PBS, pH7.2 for 10 min at Rt.
[g] Incubation with 0.2% Proteinase K (Sigma-Aldrich) in Tris-HCl buffer, pH7.2 for 20 min at Rt.

5. Statistical analyses

Statistical analyses were performed using SAS (v9.1 for Windows, SAS Institute, Cary, NC, USA), and GraphPad InStat (v3.05, San Diego, CA, USA) softwares.

In Chapter 1, non-paired Student's t-test was used to compare the measured parameters between the control and BVDV groups at the same time point for all analytical procedures (*).

In Chapter 2, Duncan's Multiple Range Test was performed for BVDV/BHV1, BHV1 and negative control groups to analyze significant differences of the values in each group at various time points (*). *P* values < 0.05 were considered significant. Non-paired Student's t-test was used to determine differences between both BVDV/BHV1 and BHV1 groups at the same time point (letters "a" and "b", respectively), as well as to establish significant differences between BVDV/BHV1 or BHV1 group and negative control group at the same time point (letters "c" and "d", respectively). *P* values < 0.05 were considered significant.

In Chapter 3, Shapiro-Wilk test was used to evaluate mean of numbers of positive cells for determining the normality of distribution. Differences between the means of controls and inoculated animals were assessed by the Mann–Whitney U nonparametric test (*).

All values were expressed as mean ± standard error of mean (SEM), and *P* <0.05 was considered as statistically significant.

CHAPTER 1

Cell-mediated immune response during experimental acute infection with bovine viral diarrhea virus (BVDV): evaluation of blood parameters

Transboundary and Emerging Diseases
(doi: 10.1111/tbed.120002)

Cell-mediated immune response during experimental acute infection with bovine viral diarrhea virus (BVDV): evaluation of blood parameters

Transboundary and Emerging Diseases, 2012

(doi: 10.1111/tbed.120002)

Abstract

BVDV, a major pathogen of cattle, are often asymptomatic or produce only mild clinical symptoms. However, they may play an important role in the bovine respiratory disease complex by exerting a marked immunosuppressive effect, as a result of the death of the immunocompetent cell populations involved in controlling innate and adaptive immune responses, together with a marked reduction of both cytokine expression and co-stimulatory molecule synthesis. Although experimental research and field studies have shown that acute BVDV infection enhances susceptibility to secondary infection, the precise mechanism involved in BVDV-induced immunosuppression remains unclear. The present study is aimed at measuring a range of blood parameters in a single group of fourteen calves infected with ncp BVDV-1. Focus has been put on those related to the cell-mediated immune response just as leukocyte populations and lymphocyte subpopulations, serum concentrations of cytokines (IL-1β, TNF-α, IFN-γ, IL-12, IL-4 and IL-10) and APPs (Hp, SAA, fibrinogen and albumin), as well as BVDV specific Abs and viremia. After ncp BVDV-1 infection, clinical signs intensity was never more than moderate coinciding with the presence of viremia and leukocyte and lymphocyte depletion. An early increase in TNF-α, IFN-γ and IL-12 levels in contrast to IL-1β was observed in line with a raise in Hp and SAA levels on the latest days of the study. As regerds IL-4 levels no evidence was found of any changes. However, a slight increase in IL-10 was observed, matching the TNF-α decline during the APR. These findings would help to increase our knowledge of the immune mechanisms involved in acute infection with ncp BVDV-1 strains, suggesting the existence of a clear tendency towards a type 1 immune response thereby enhancing resistance against viral infections.

1. Introduction

Cell-mediated immune responses including phagocytes and T lymphocytes responses play a critical role in fighting viral infections (Zajac and Harrington, 2008). Virally-infected cells interact with several populations of T lymphocytes, including CD4+, CD8+ and γδ T cells by means of specific engagement of viral-peptide MHC complexes. In contrast, humoral immune responses of B lymphocytes are characterized by the presence of soluble Abs that are capable of binding intact viral particles. T lymphocytes, together with B lymphocytes, form the adaptive immunity (Srikumaran et al., 2007).

CD4+ lymphocytes have a primary role in the release of cytokines such as IFN-γ, which stimulates innate immunity cells including macrophages and neutrophils, and assists CD8+ T lymphocytes and B lymphocytes in the development of adaptive immune responses. In addition CD8+ T lymphocytes are potent antiviral effector cells due to their ability to produce both inflammatory mediators and cytotoxic effector molecules (Doherty et al., 1997). However, other subpopulation of T cells, known as γδ T lymphocytes, have a role in the highly complex mechanism involved in cell-mediated immune responses. These γδ T cells have a wide range of functions, including cytokine production, immunomodulation and regulation of inflammation (Kaufmann et al., 1993; Welsh et al., 1997; Carding and Egan, 2000; Price et al., 2007). It is known that γδ T cells promote a cytotoxic activity in a non Ag-dependent stimulation form supporting a role in the innate immunity, in addition to their role in Ag-dependent responses distinctive of adaptive immunity. Because of these features, γδ T lymphocytes play an important role by linking innate and adaptive immune response (Endsley et al., 2004; Guzman et al., 2012).

Non-specific responses of innate immunity are the first reaction of the host against viral infections, preceding the specific immune responses of acquired immunity (Gruys et al., 2005). Linkages between these two systems are proving instrumental in explaining the initial steps in the inflammatory process, as well as the stimulation and activation of immune cells (Lippolis, 2008). One way of evaluating the host inflammatory response during infection is to determine the non-specific reaction, so-called APR, by measuring serum concentrations of different APPs (Baumann and Gauldie, 1994; Murata et al., 2004; Petersen et al., 2004; Orro et al., 2011). Hp and SAA are considered major positive APPs in cattle, since serum levels increase during viral diseases (Murata et al., 2004; Petersen et al., 2004). Plasma fibrinogen levels also increase during viral infection; indeed, fibrinogen was the first positive APP used to evaluate

inflammatory disease in cattle (McSherry et al., 1970; Eckersall and Conner, 1988). Albumin is a negative APP widely used as a biomarker, since serum albumin levels decline during the inflammatory process (Eckersall and Bell, 2010). Changes in serum APP composition are mediated by pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) released by activated monocytes-macrophages, which are rapidly induced and expressed in a disease process (Petersen et al., 2004; Gruys et al., 2005; Eckersall and Bell, 2010). In order to balance the effect of the pro-inflammatory cytokines, it is necessary a group of anti-inflammatory cytokines including IL-10, which dampens the immune response to prevent injury to the host by its own immune system (Lippolis, 2008). Moreover, both type 1 and type 2 immune responses have been reported in cattle, depending on the cytokines secreted by the two T helper cell subsets (Th1 and Th2) and on the involvement of cytotoxic T cells and natural killer cells (Brown et al., 1993; 1994; Abbas et al., 1996; Mosmann and Sad, 1996). Specifically, the type 1 response (IFN-γ and IL-12) is relates to cell-mediated immunity, contributing to inflammation process; whereas the type 2 response (IL-4) stimulates the production of neutralizing Abs associated with humoral responses. A balance between type 1 and type 2 bias seems to be necessary for efficient elimination of infectious agents (Collins et al., 1999; Waldvogel et al., 2000). In mice and humans, it has been proven that defence against viruses requires a predominance of type 1 immune response (Mosmann and Coffman, 1989; Romagnani, 1994).

BVDV is a positive single-stranded RNA virus belonging, like classical swine fever virus and border disease virus, to the genus *Pestivirus,* a member of the *Flaviviridae* family (Calisher and Gould, 2003). BVDV, a major pathogen of cattle, comprises two recognized genotypes, BVDV-1 and BVDV-2; and strains vary from avirulent to highly virulent. BVDV strains of either genotype 1 or 2 may exist as one of two biotypes: a widely-distributed ncp biotype in nature, and a rare cp biotype associated predominantly with outbreaks of mucosal disease (Ridpath, 2010). Acute infections with low-virulence ncp BVDV are often asymptomatic or produce only mild clinical symptoms (Marshall et al., 1996; Liebler-Tenorio et al., 2004); however, they may play an important role in the BRDC by exerting a marked immunosuppressive effect that predisposes affected cattle to other infections (Potgieter et al., 1984a; Castrucci et al., 1992; Kelling, 2007; Srikumaran et al., 2007). The mechanism of immunosuppression induced by BVDV has not been fully determined, although reports highlight the ability of BVDV to infect cells that play a critical role in the host immune system, and are crucial to regulating the innate and acquired immune

responses. These cells include granulocytes, monocytes-macrophages, DCs, CD4+, CD8+ and γδ T lymphocytes, and B cells (Sopp et al., 1994; Chase et al., 2004). Indeed, the cell-mediated immune response is postulated as an important effector mechanism in the recovery from primary BVDV infections (Howard et al., 1992; Larsson and Fossum, 1992). In parallel, as a BVDV infection becomes established in the host, a series of molecular and cellular signals are initiated, responsible for activating cell-mediated immune responses. These signals include the production of interferons, other cytokines, and inflammatory mediators, in addition to the mobilization of local DCs (Brackenbury et al., 2003; Glew et al., 2003; Peterhans et al., 2003; Al-Haddawi et al., 2007; Zajac and Harrington, 2008). The type of immune response against BVDV has yet to be clearly defined. Charleston et al. 2002 established that during acute infections with ncp BVDV infections, there are no evidences of a type 2-like response.

This study is aimed at characterizing the cell-mediated immune response in calves experimentally infected with ncp BVDV-1. With this purpose, we have examined the systemic changes of immunocompetent cell populations, APPs and cytokines; as well as, viremia and specific Abs. Several *in vivo* and *in vitro* studies have been reported following BVDV infections on a range of individual changes, but to our knowledge, no previously-published studies have examined all these changes together, in a single group of experimentally-infected calves. The findings of the present research will help to increase our knowledge of the complex immune mechanisms involved in acute infections with ncp BVDV-1 strains; highlighting the contribution of the systemic cytokines results.

2. Materials and Methods

2.1. Experimental design

Eighteen male Friesian calves aged approximately 8-9 months were purchased from a conventional dairy farm declared free of tuberculosis, brucellosis and bovine leucosis, in accordance with the rules of Spanish programmes for the eradication of these diseases.

On arrival, calves were housed at the University of Cordoba Experimental Animal Center (Spain), and were allowed to adapt for one week before starting the experiment. BVDV-free status was confirmed by commercial ELISA tests.

Fourteen calves were assigned to the BVDV group, and were infected by intranasal inoculation with 10 ml (5 ml per nostril) containing 10^5 $TCID_{50}$/ml of ncp BVDV 7443 (subtype 1a). And four calves were housed separately as the control group, and received intranasally an identical amount of virus-free cell culture fluid.

Rectal temperatures and clinical signs (anorexia, depression, hypersalivation, lacrimation, nasal discharge, cough, dyspnea, accessible mucosal surface lesions and diarrhea) were monitored daily prior to virus inoculation and throughout the study. Severity of clinical signs was assessed as follows: -, absence of clinical signs; +, mild; ++, moderate and +++, severe intensity of each clinical sign.

Blood, serum and plasma samples were obtained twice from each calf prior to BVDV inoculation in order to obtain basal values (0 dpi) for each analytical procedure. After BVDV inoculation, blood and serum samples were collected at 1, 2, 3, 6, 9 and 12 dpi from BVDV and control group calves.

Whole EDTA-blood samples were collected and used fresh for flow cytometry analysis and measurement of total and differential leukocyte counts, and frozen at -80°C until viral detection. EDTA-blood samples and samples without additive were centrifuged at 4000 rpm for 10 min) to obtain plasma and serum, respectively. All serum and plasma samples were frozen at -80°C until the day of APP, cytokine and viral Ab analysis.

The whole experimental procedure was carried out in accordance with the Guidelines for accommodation and care of animals, approved by the European Economic Community (Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, as amended by Directive 2003/65/EC).

2.2. Virus and specific antibody detection

High Pure Viral Nucleic Acid Kit was used to extract BVDV RNA from EDTA-blood samples, according to manufacturer's instructions. Samples were analyzed by RT-PCR in one-step real-time PCR. Primers and TaqMan probes (at the same concentration) based on the highly-conserved 5´-untranslated region (UTR) of BVDV-1 were used as previously described by Letellier and Kerkhofs, 2003 and in combination with the Real Time Ready RNA Virus Master, according to manufacturer's instructions. The PCR conditions and the used reagent concentration followed

manufacturer's protocol of Real Time Ready RNA Virus Master. Reactions were carried out in a LightCycler. Any sample that had a Ct value less than or equal to 45 was considered positive. The positive control was the ncp BVDV-1 strain 7443 at 10^5 $TCID_{50}$/ml, which was used to make the standard curve by serial log10 dilutions of RNA. Ct values measured were plotted against the log of the input BVDV RNA dilution. Viral titres were expressed as $TCID_{50}$/ml.

The Ab response to BVDV NS3 (p80) was determined from serum samples using a commercially-available competitive ELISA test (INGEZIM BVD Compac; Ingenasa, Madrid, Spain), following manufacturer's protocol. ELISA values of under 0.9 were considered positive, while values between 1.1 and 0.9 were deemed doubtful and values of above 1.1 were considered negative.

2.3. Leukocyte counts and lymphocyte subset analyses

Total leukocyte, lymphocyte, monocyte and neutrophil counts were carried out from EDTA-blood using a Cell-Dyn 3700 Automatic Hematology Analyzer.

Indirect immunofluorescence staining and flow cytometric analysis for detection of lymphocyte subset surface antigens was performed. Briefly, 100 µl of fresh EDTA-blood diluted 1:1 with PBS were incubated with 1 µl of each bovine-specific primary mAb: CD4+, CD8+ and γδ T cells, and B cells (*see Table 2 in Materials and Methods section, p. 66*). After incubation for 30 min at 4°C in darkness, cells were washed with PBS and 200 µl of FITC-conjugated goat anti-mouse IgM or R-phycoerythrin-conjugated goat anti-mouse IgG_1 diluted 1:500 with PBS were added, following incubation in the dark for 30 min at 4°C. After being labeled, cells were resuspended in 2 ml of FACS Lysing Solution for 10 min at Rt to lyse erythrocytes. The lysing process was stopped by adding 2 ml of PBS. Finally, cells were fixed by adding 200 µl of 1X formaldehyde-buffered solution to each sample and were stored at 4°C in the dark until flow cytometric analysis. Specific controls of the labeling procedures included the omission of specific mAbs in the first step of the procedure and the use of isotype negative controls. Differences in cell-surface-molecule expression were detected using a FACScan Flow Cytometer. All data were further analyzed with CELLQuest. Results were expressed as the absolute numbers of each lymphocyte subpopulation reacting to each mAb, calculated by using the total lymphocyte number and the proportion of the subpopulation given by flow cytometric analysis at each time-point.

2.4. APP assays

Hp, SAA and albumin were measured in duplicate serum samples, and fibrinogen levels were measured in plasma samples. Hp levels were measured according to prevention of the peroxidase activity of hemoglobin, which is directly proportional to Hp, by using a commercial spectrophotometric assay (PHASE RANGE Haptoglobin Assay, Tridelta Ltd., Ireland). SAA concentrations were determined by a commercially obtained solid-phase sandwich ELISA kit (PHASE RANGE Serum Amyloid A Assay, Tridelta Ltd., Ireland). Under normal conditions, Hp and SAA are absent in serum or present at very low levels, ranging from less than 50 mg/l and 20 mg/l in cattle, respectively. Fibrinogen concentrations were measured according to the colorimetric Biuret method by using a commercial kit (Total protein Kit, Spinreact SA, Spain), calculating the difference between total plasma protein and serum protein. In healthy cattle, fibrinogen values range from 5 to 7 g/l. Serum albumin was measured by Bromocresol Green method using a commercial test kit (Albumin Kit, Spinreact SA, Spain). The reference range for albumin is 25 to 35 g/l in bovine species. All analyses were performed following manufacturers' instructions.

2.5. Cytokine assays

Cytokine concentrations (IL-1β, TNF-α, IFN-γ, IL-12, IL-4 and IL-10) were determined by specific sandwich ELISA protocols in duplicate serum samples making use of commercially-available mAb pairs (Serotec). All cytokines were measured in microtitre plates previously coated with each one of highly purified anti-cytokine Abs (*see Table 3 in Materials and Methods section, p. 70*) at 1 µg/mL (2 mg/ml for bovine IL-1β), diluted in PBS, 2% Tween 20 (PBST) and 3% BSA, and incubated overnight at 4°C. After a blocking step with PBS, 2% Tween-20 and BSA 3% for 1 h at Rt with constant stirring, plates were washed 3 times with PBST and incubated with the serum diluted 1:50 for 1 h at Rt. After three washes with PBST, plates were incubated with each secondary mAb pairs (*see Table 3 in Materials and Methods section, p. 70*) at 1 µg/mL (2 mg/ml for bovine IL-1β) for 1 h at Rt in agitation. After a new washing cycle, 100 µL of streptavidin-horseradish peroxidase conjugate diluted 1/1000 in blocking solution were added to each well and incubated for 45 min at Rt. After a final wash, chromogenic substrate (OPD) was added, the reaction was stopped by the addition of H_2SO_4 and the absorbance values were read with an ELISA-plate reader at 450 nm. Standard curves to calculate cytokine concentrations were

generated using recombinant bovine cytokines such as IL-1β, TNF-α, IFN-γ and IL-4 (Serotec). Due to the lack of an appropriate standard curve for IL-10 and IL-12, the results of these cytokines were presented as optical density (O.D.) values.

2.6. Statistics

Statistical analyses were performed using SAS software. Non-paired Student's t-test was used to compare the measured parameters between the control and BVDV groups at the same time point for all analytical procedures. All values were expressed as mean and SEM, and $P < 0.05$ was considered as statistically significant (*).

3. Results

3.1. Assessment of clinical symptoms

Calves in the BVDV group displayed an initial elevation of rectal temperature to around 39.5°C at 3 dpi (Figure 1). This was followed by a second episode of more pronounced hyperthermia (up to 40°C) at 7 dpi; difference in temperature was significant with respect to control calves at those time points.

BVDV-infected calves exhibited generally mild to moderate clinical symptoms (Table 1). Slight clinical signs first became apparent at 3 dpi, and included nasal discharge and diarrhea. From 4 to 7 dpi, slight lacrimation and moderately-intense nasal discharge and diarrhea were observed in the BVDV group. Between 8-9 dpi, nasal discharge diminished and diarrhea and lacrimation disappeared. None of these clinical signs were observed from 10 dpi to the end of the study. The other assessed clinical symptoms were practically nonexistent. The development of clinical symptomatology was similar in most BVDV-infected animals. Calves in the control group had rectal temperatures within the normal range, around 38.6°C (Figure 1) and remained clinically unaffected throughout the study.

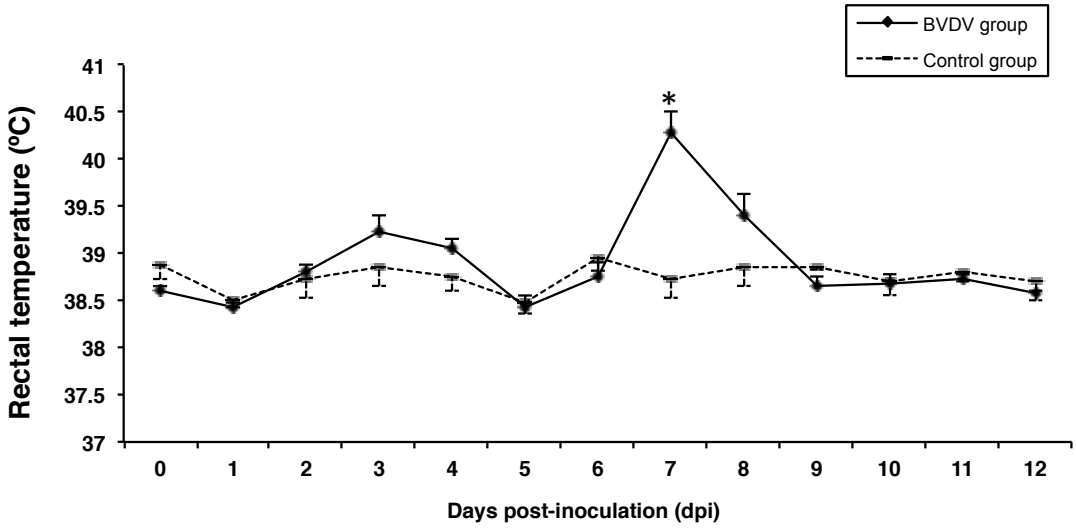


Figure 1. Rectal temperatures following intranasal BVDV inoculation (BVDV group) versus uninfected calves (control group). Results are shown as means ± SEM. *Significant (*P* <0.05) values between groups at the same time point.

Table 1. Evaluation of the severity of the clinical response in calves intranasally infected with BVDV.

Clinical signs	Clinical response† - Days post-inoculation (dpi)					
	0-2	3	4	5-7	8-9	10-12
Lacrimation	-	-	++ (9/14)	+ (12/14)	-	-
Nasal discharge	-	+ (10/14)	++ (12/14)	++ (13/14)	+ (11/14)	-
Diarrhea	-	+ (9/14)	++ (11/14)	++ (10/14)	-	-

†Clinical response: -, absence of signs; +, mild; ++, moderate; +++, severe intensity.
Figures in brackets: Number of affected calves as proportion of total

3.2. Viremia and anti-BVDV antibodies

The presence of BVDV was confirmed in BVDV-infected blood samples by PCR (Figure 2), viremia being detected from 3 to 9 dpi, and peaking at 6 dpi. There was no evidence of BVDV infection at 12 dpi neither in BVDV-infected blood samples nor in those from control calves.

BVDV-specific serum Abs were not detected during the study neither in BVDV nor in control groups (Figure 2).

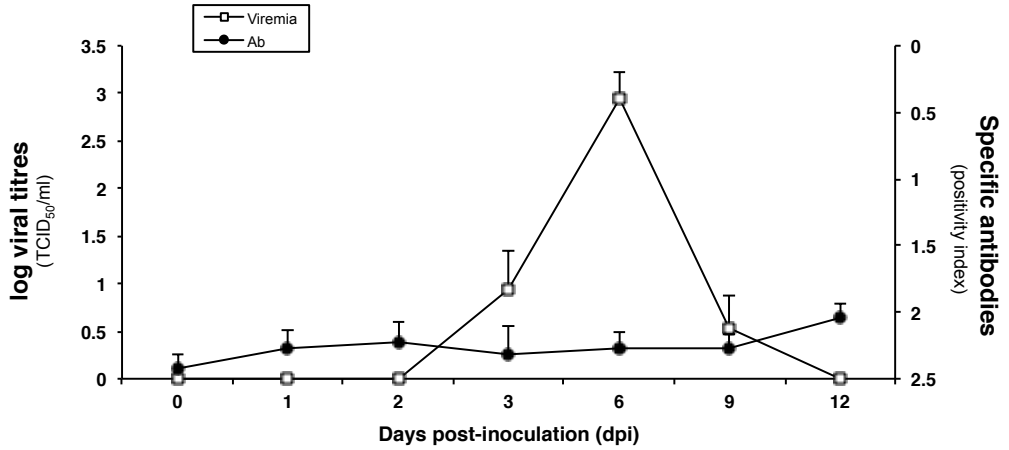


Figure 2. Mean ± SEM of BVDV viremia and BVDV-specific antibodies in samples from calves experimentally infected with BVDV (BVDV group).

3.3. Leukocyte populations

Total leukocyte counts declined significantly in the BVDV group compared to control animals as from 3 dpi. Thereafter, counts progressively increased, though remaining significantly below those of controls until the end of the study (Figure 3A). This leukopenia was characterized by significant lymphocyte, monocyte and neutrophil depletion compared with controls (Figure 3A to D). Lymphocyte counts (Figure 3B) dropped to significantly low levels between 3-9 dpi, and although increased slowly at 12 dpi, remaining lower until the end of the study. Monocyte counts (Figure 3C) fluctuated somewhat, falling to their lowest levels at 3 and 9 dpi, and returning to values close to those of control calves the last day. Neutrophil counts (Figure 3D) displayed a significant decline, reaching minimum values at 3 dpi. Values then increased somewhat, but remained significantly below those of control calves until 12 dpi. No changes were observed in total or differential leukocyte counts in control calves throughout the experiment (Figure 3A to D).

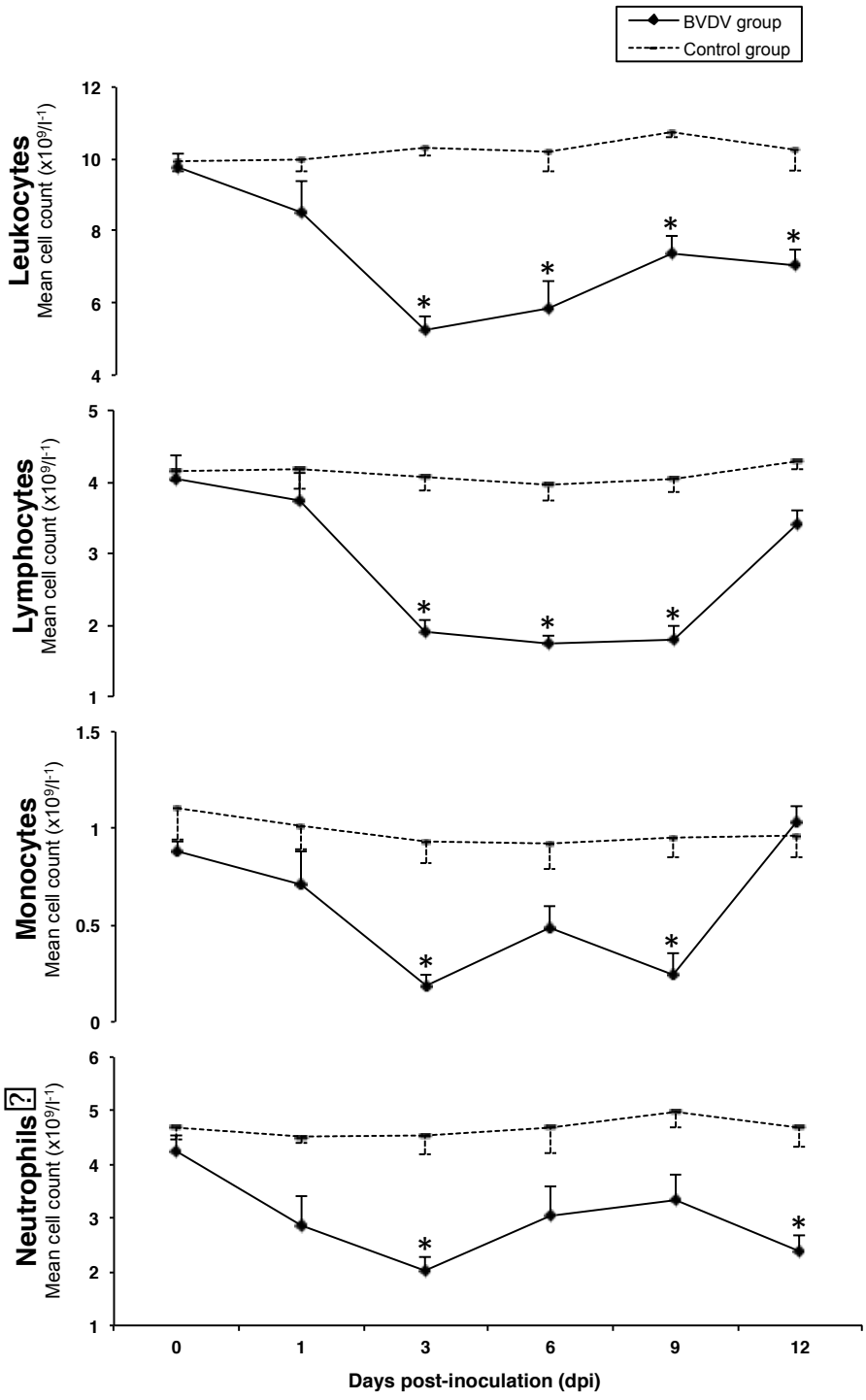


Figure 3. Cell counts ($x10^{9}l^{-1}$) expressed as mean ± SEM for total leukocytes (A), lymphocytes (B), monocytes (C) and neutrophils (D) in blood samples from calves experimentally infected with BVDV (BVDV group; —♦—) versus uninfected calves (Control group; ----------). *Significant differences ($P < 0.05$) between groups at the same time point.

3.4. Lymphocyte subpopulations

Kinetic changes in absolute counts for each lymphocyte subpopulation were charted as shown in Figures 4A to D.

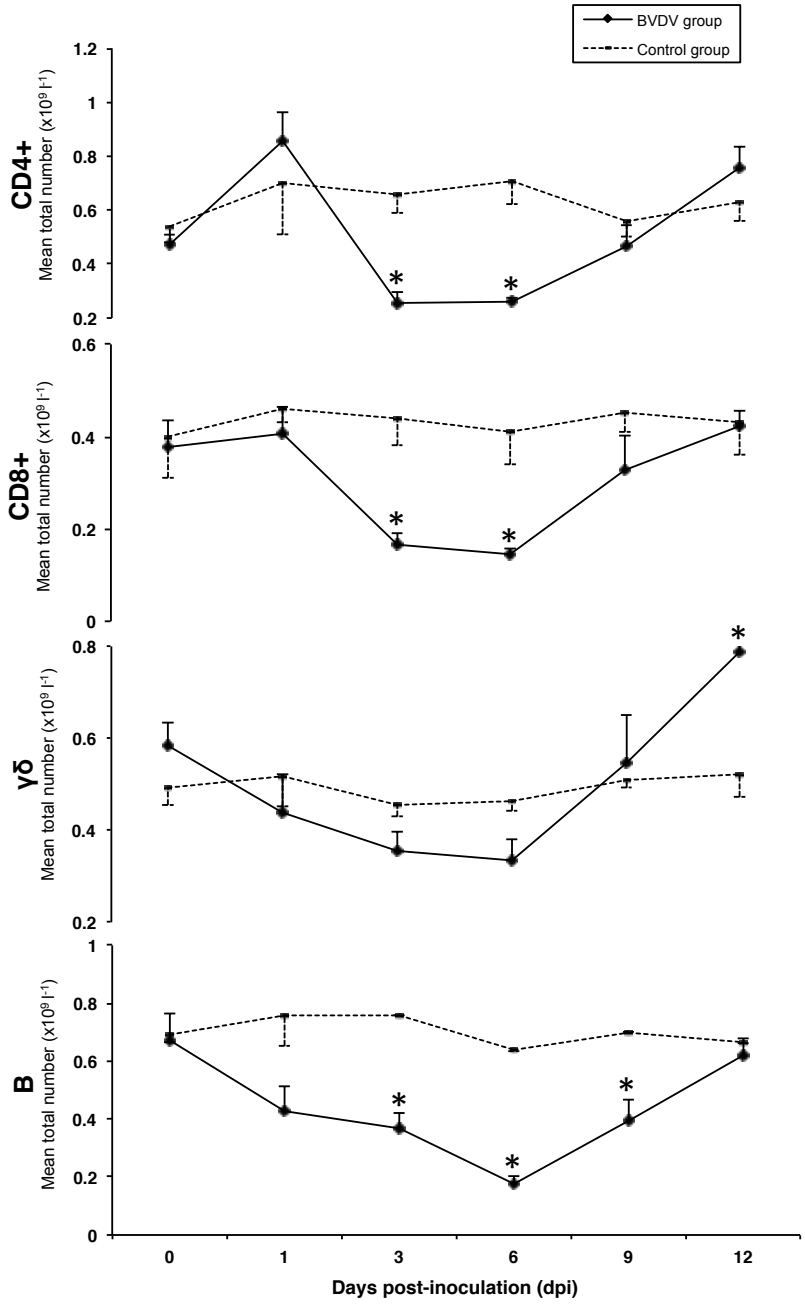


Figure 4. Cell counts ($x10^{9}l^{-1}$) expressed as mean ± SEM for CD4+ (A), CD8+ (B), γδ (C) and B (D) lymphocytes in blood samples from calves experimentally infected with BVDV (BVDV group; —♦—) versus uninfected calves (Control group; ----------). *Significant differences ($P < 0.05$) between groups at the same time point.

In the BVDV-infected group, CD4+ and CD8+ T lymphocyte kinetics (Figures 4A and B) displayed similar profiles: although the CD4+ lymphocyte count showed a transient increase at 1 dpi, both declined significantly after BVDV inoculation, reaching minimum values between 3-6 dpi. Thereafter, CD4+ T lymphocyte counts (Figure 4A) started to rise gradually reaching values close to control group at 9 dpi; thenceforth they remained higher than control group ones at 12 dpi. However, CD8+ T lymphocyte counts (Figure 4B) approached those of control calves at 12 dpi. γδ T lymphocyte counts (Figure 4C) declined slowly from BVDV infection until 6 dpi but did not differ significantly from those of control animals. Thereafter, they rised and peaked significantly at 12 dpi. B lymphocyte numbers (Figure 4D) fell sharply following BVDV infection, attaining levels significantly lower than those of control group between 3-9 dpi; the lowest count was recorded at 6 dpi. It then displayed a recovery, remaining close to those of the control group until the end of the experiment.

In the control group, lymphocyte subpopulation counts displayed no significant change throughout the study (Figures 4A to D).

3.5. APP analysis

Serum Hp and SAA protein levels (Figure 5) increased significantly in BVDV infected animals with respect to controls, peaking at 9 dpi (1175 mg/l and 29 mg/l, respectively). At 12 dpi, the afore-mentioned levels remained somewhat above control values. However, fibrinogen and albumin protein levels (Figure 5) displayed nonsignificant changes over the study period.

Serum APP concentrations (Figure 5) remained low in control animals, and exhibited no changes during the experiment.

3.6. Cytokine analysis

ELISA cytokine results for BVDV and control groups are shown in Figures 6, 7 and 8. Levels of the cytokines evaluated in the control group remained consistently low throughout the study.

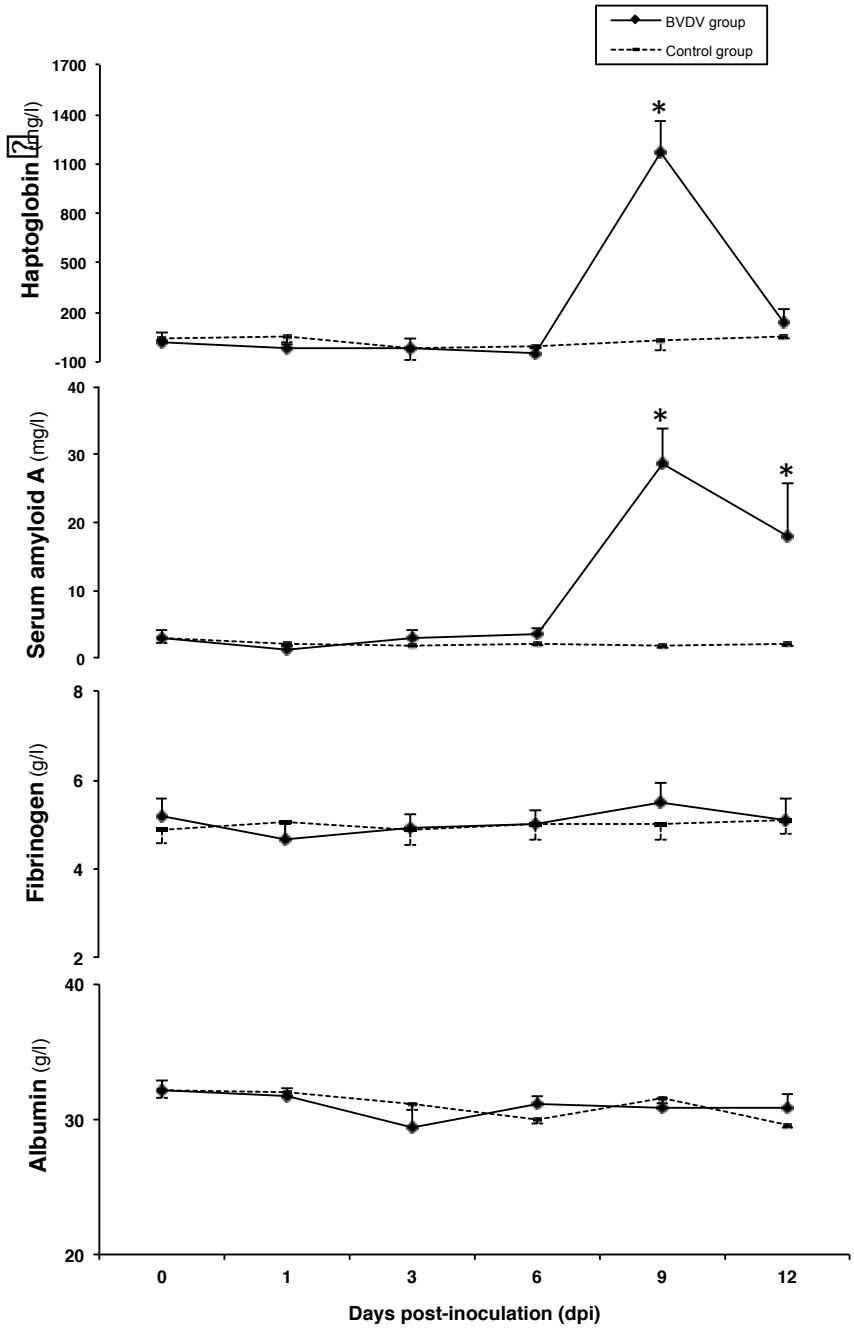


Figure 5. Serum APPs (haptoglobin, serum amyloid A, fibrinogen and albumin) levels in calves infected with BVDV (BVDV group; —♦—), versus uninfected calves (Control group; --------). Results are shown as means ± SEM. *Significant (*P* <0.05) differences between groups at the same time point.

3.6.1. Proinflammatory cytokines

Significant differences in both magnitude and kinetics were observed between the proinflammatory cytokines, IL-1β and TNF-α (Figure 6) in serum samples from BVDV-infected calves. Serum IL-1β levels peaked at 9 dpi (1.21 ng/ml), being significantly higher than those of controls, and remaining higher at 12 dpi. By contrast, serum TNF-α levels displayed a slight, non-significant, increase at 1 dpi; thenceforth, these levels declined as from 3 dpi and remained lower than those of controls until the end of the study.

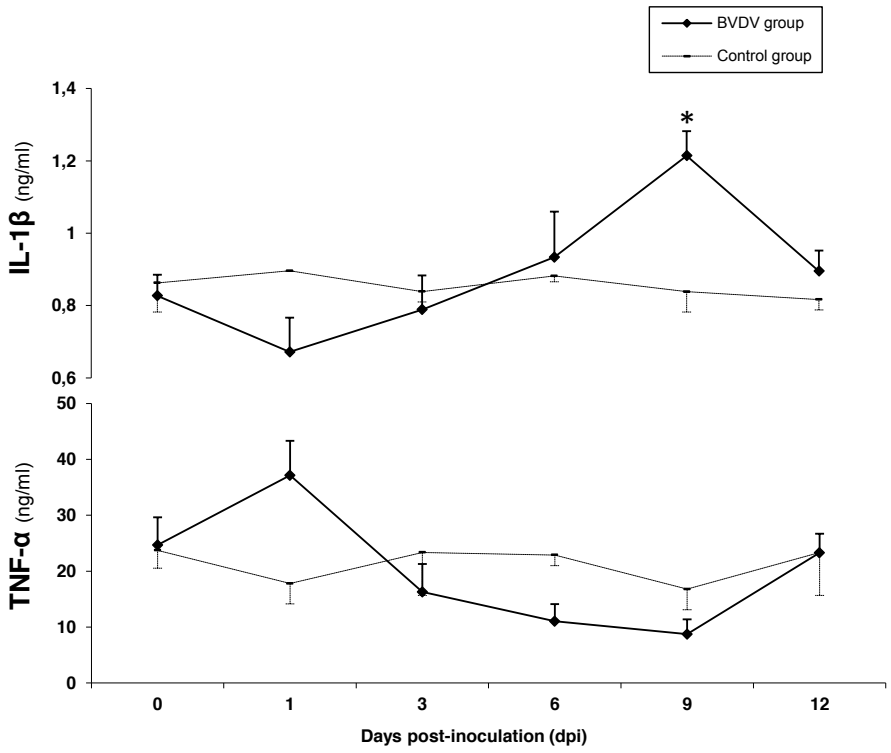


Figure 6. Serum proinflammatory cytokine (IL-1β and TNF-α) levels in calves inoculated with BVDV (BVDV group ; —♦—), versus uninfected calves (Control group; ---------). Results are shown as means ± SEM. *Significant (*P* <0.05) differences between groups at the same time point.

3.6.2. Type 1 and type 2 cytokines

Levels of type 1 cytokines IFN-γ and IL-12 (Figure 7) rose from an early stage. IFN-γ levels displayed a significant early increase following BVDV infection with maximum values at 1 and 3 dpi, and remained at higher levels than those of control calves from 6 dpi until the end of the

study. IL-12 serum also changed but somewhat later than IFN-γ levels, showing higher values than control animals between 3 to 6 dpi, although these levels then dropped to almost match those of the control group from 9 dpi until the end of the study.

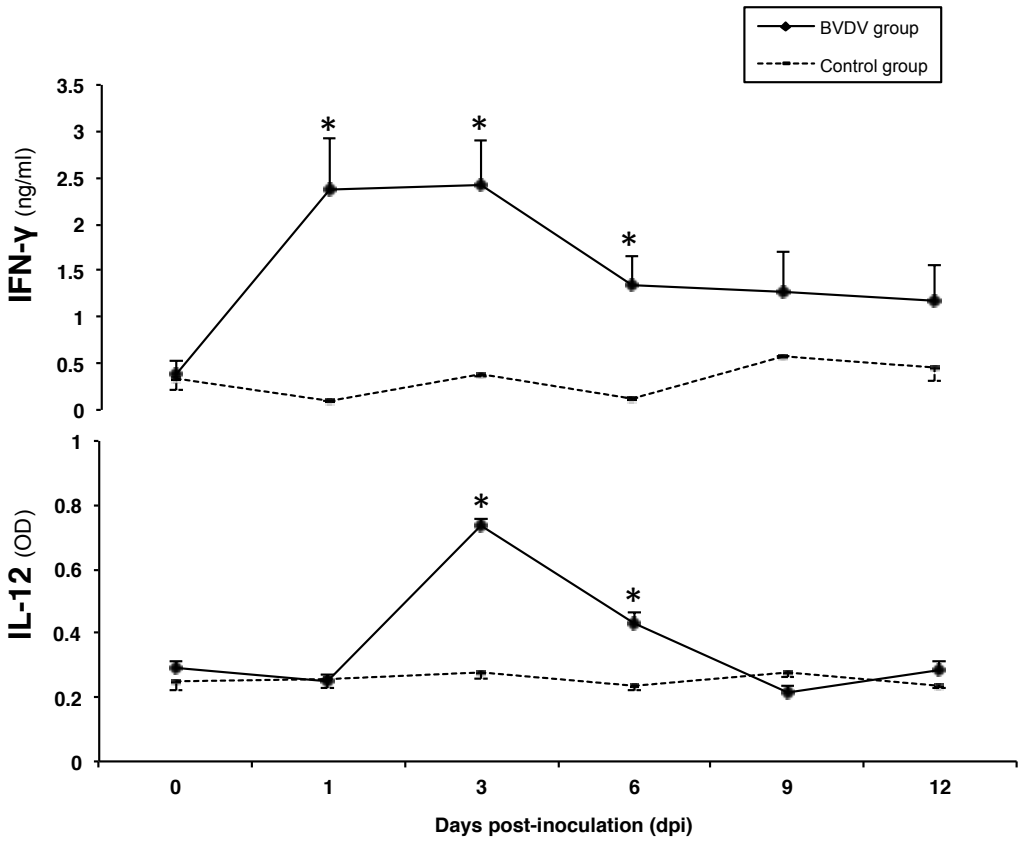


Figure 7. Serum type 1 cytokine (INF-γ and IL-12) levels in calves inoculated with BVDV (BVDV group; —♦—), versus uninfected calves (Control group; ----▪----). Results are shown as means ± SEM. *Significant (*P* <0.05) differences between groups at the same time point.

Serum levels of the type 2 cytokine IL-4 (Figure 8) remained consistently low throughout of the study.

Levels of the anti-inflammatory cytokine IL-10 (Figure 8) displayed a slightly-significant increase from 3 dpi in BVDV-infected group, and remained significantly higher than those of control calves until 12 dpi.

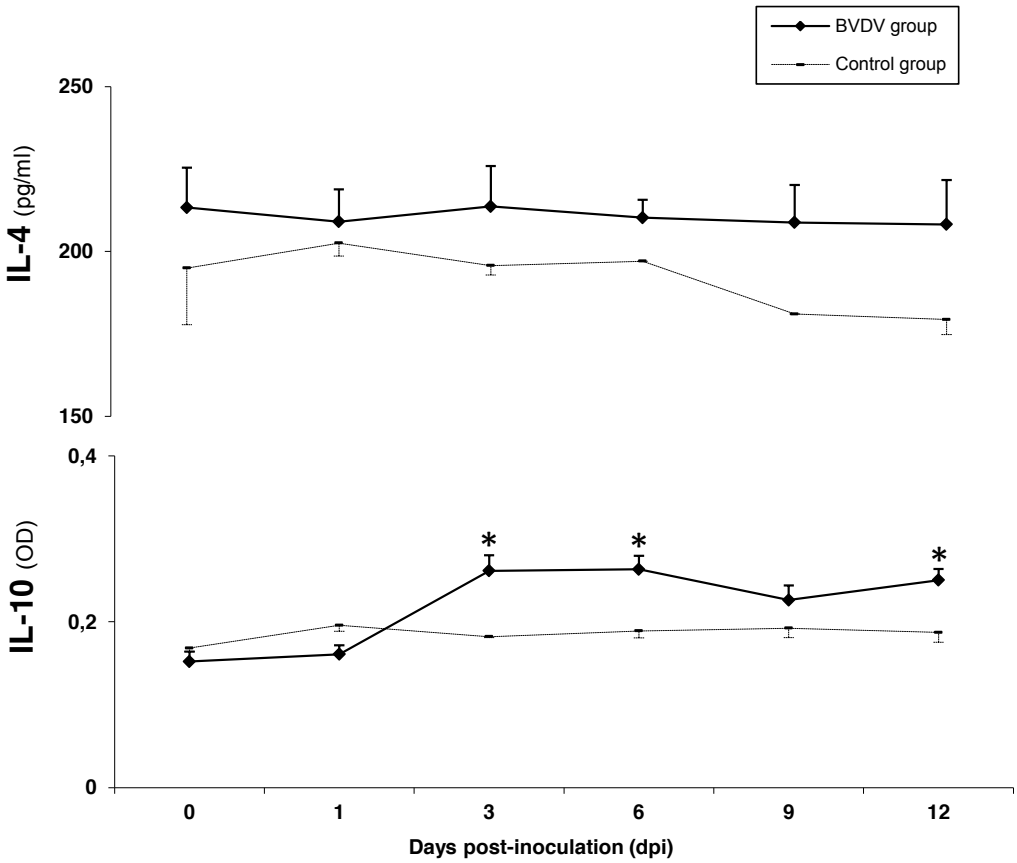


Figure 8. Serum type 2 cytokine (IL-4) and anti-inflammatory (IL-10) level in calves inoculated with BVDV (BVDV group ; —♦—), versus uninfected calves (Control group; --------). Results are shown as means ± SEM. *Significant (P <0.05) differences between groups at the same time point.

4. Discussion

Consequences of the transient immunosuppression reported in acute ncp BVDV-1 infection include the death of immunocompetent cell populations involved in controlling the innate and adaptive immune responses, and a marked reduction in both cytokine expression and co-stimulatory molecule synthesis (Bolin et al., 1985a; Wilhelmsen et al., 1990; Potgieter, 1995; Walz et al., 2001; Chase et al., 2004; Muylkens et al., 2007). In the present study, a wide range of blood parameters has been measured in a single group of calves infected with ncp BVDV-1, focussing on those related to the cell-mediated immune response, in order to contribute to a better understanding of the immune mechanisms involved in BVDV infections.

Following acute infection with the ncp BVDV-1 strain 7443, calves displayed biphasic temperature elevation, with a slight increase at 3 dpi and a more pronounced hyperthermia peak at 7 dpi (Ellis et al., 1988; Traven et al., 1991; Bolin and Ridpath, 1992; Kelling et al., 2002; Liebler-Tenorio et al., 2003a,b; 2004; Muller-Doblies et al., 2004; Pedrera et al., 2009b). Clinical symptoms were more intense – though intensity was never more than moderate – during this period. While a number of authors reported that infection with low-virulence ncp BVDV-1 strains, similar to that used here, generally prompts mild-to-moderate clinical signs (Muller-Doblies et al., 2004; Ganheim et al., 2005); other experimental studies with low-virulence ncp BVDV-1 (Wilhelmsen et al., 1990) and ncp BVDV-2 (Liebler-Tenorio et al., 2003a,b; 2004) did not describe clinical symptoms. Moreover, studies of acute infections with ncp BVDV-1 7443 showed changes in clinical presentation that may be due to age, virus-administration procedure or immune status of host, among others (Bolin et al., 1985b; Pedrera et al., 2009b; Ridpath, 2010). Viremia was detected between 3 and 9 dpi, and peaked at 6 dpi in parallel with hyperthermia and the highest intensity of clinical signs, highlighting the pathogenic action of the virus. Most authors report that, as observed here, experimental infection of calves with ncp BVDV-1 gives rise to transient viremia, which resolves about 12 days after infection (Nuttall et al., 1980; Castrucci et al., 1992; Howard et al., 1992; Bruschke et al., 1998b; Archambault et al., 2000; Charleston et al., 2002; Brackenbury et al., 2003; Kelling et al., 2007).

Moreover, an inverse relationship has been observed between the degree of viremia and leukocyte counts (Walz et al., 2001; Muller-Doblies et al., 2004; Ganheim et al., 2005; Pedrera et al., 2009a; 2011). A marked decrease was observed in total leukocyte counts between 3 and 9 dpi; lymphocyte, monocyte and neutrophil levels were all depleted, thus confirming the tropism of BVDV on immunocompetent cell populations (Ellis et al., 1988; 1998; Chase et al., 2004; Muller-Doblies et al., 2004; Ganheim et al., 2005; Pedrera et al., 2009b). Analysis of lymphocyte subpopulations revealed that the decline in total lymphocyte counts was due to a sharp decrease in CD4+ and CD8+ T cells, and also in B lymphocytes, between 3 and 6 dpi; levels of all three subpopulations subsequently recovered at 12 dpi, coinciding with no BVDV detection from blood samples at this time point (Ellis et al., 1988; Wilhelmsen et al., 1990; Archambault et al., 2000; Chase et al., 2004; Brewoo et al., 2007). The drop in CD4+ T cell numbers critically impairs calf's ability to deal with a primary BVDV infection, pointing out the crucial importance of these cells in the control mechanisms against BVDV, since this population is responsible for

the cell-mediated response during the early infection. However, CD8+ T lymphocyte depletion appears to have no effect in the length or intensity of viremia (Howard et al., 1992; Chase et al., 2004). Nevertheless, BVDV viremia has been observed in parallel with both CD4+ and CD8+ T cell depletion, subsequently coinciding their numerical recovery with non-BVDV haematological detection, which implies that CD8+ T cells depletion is actually associated with the presence of viremia. Ganheim et al. 2005 observed a decrease in γδ T cell numbers, but no significant changes were recorded here in early stages of the study, displaying a significant increase at 12 dpi. Due to the fact that mAb CACT6A used in this study does not label the entire γδ T cells population (Parsons et al., 1993), the results obtained on this particular issue must be cautiously interpreted. Although this sub-population of γδ T cells does not show meaningful changes until the end of the experiment, a significant increase has been noticed at 12 dpi compared to the control group. Although it has been suggested that γδ T cells do not play a major role in resisting BVDV infection (Howard et al., 1992), these cells may be involved in early stages of infection by means of production of IL-12, TNF-α and IFN-γ (Sopp and Howard, 2001; Baldwin et al., 2002), as well as during later stages of infection, controlling the resolution of pathogen-mediated inflammation by compensating CD4+ and CD8+ T lymphocyte subsets depletion (Pollock and Welsh, 2002). The reported effects of BVDV on circulating B+ lymphocyte numbers vary considerably: whilst some authors note a transient increase (Brodersen and Kelling, 1999), others report no change at all (Archambault et al., 2000; Ganheim et al., 2005). However, our results showed a significant decrease of B lymphocytes between 3-9 dpi, reaching a recovery at 12 dpi. No BVDV-specific Abs were detected throughout the study, given that Abs usually appear from 12 dpi as reported by other authors (Nuttall et al., 1980; Castrucci et al., 1992; Müllier-Doblies et al., 2004; Pedrera et al., 2009b). Thus, the lack of the BVDV-specific Abs detection was most likely to be due to the short time period of the present study, rather than to the sharp drop of the B lymphocyte counts.

It has been suggested that APPs could be used as indicators of the presence and severity of pathological states in both humans and animals. Changes in serum APP levels occur in response to the release of a number of inflammatory mediators, including the pro-inflammatory cytokines TNF-α and IL-1β (Baumann and Gauldie, 1994; van Miert, 1995; Murtaugh et al., 1996; Petersen et al., 2004; Gruys et al., 2005; Eckersall and Bell, 2010). Positive APPs in cattle include Hp, SAA and fibrinogen, although fibrinogen alone is not considered a reliable indicator of the

APR in this species (Gruys et al., 2005), and no significant changes in serum fibrinogen levels were observed throughout the present study. The absence of changes in fibrinogen levels may be due to the fact that they are considerered as moderate positive APPs, together with the low injury caused by ncp BVDV-1 7443 strain. Moreover, viral infections generally cause a mild APR (Alsemgeest, 1994; Höfner et al., 1994). However, Ganheim et al. 2003 reported an increase in fibrinogen levels between 8 and 9 dpi in experimentally BVDV-infected calves. Negative APPs in cattle include albumin, whose levels remained unchanged throughout the present study. The lack of fall in albumin levels may be due to the fact that ncp BVDV-1 7443 did not develop a severe enough injury, leading to a low vascular permeability that diminish the changes in the distribution of fluid and plasma proteins in the body (Fleck, 1989). As it has been reported, major APPs (SAA and Hp) are characterized by showing a dramatical increase, thus they may be considered as good indicators of acute inflammation during viral infections (Höfner et al., 1994; Godson et al., 1996; Heegaard et al., 2000; Ganheim et al., 2003). An increase was observed in these major bovine APPs (Hp and SAA); peak concentrations in serum were observed at 9 dpi, coinciding with an increase in IL-1β, a key player in the process of inflammation (Murtaugh et al., 1996). The kinetics of major APPs observed here were similar to those reported in other *in vivo* studies of experimental BVDV infection (Ganheim et al., 2003; Muller-Doblies et al., 2004; Risalde et al., 2011b). In contrast to IL-1β, serum TNF-α levels, after a slight increase at 1 dpi that coincides with a slight rise in CD4+ T lymphocyte counts, declined slightly in the course of the APR. According to *in vitro* research (Adler et al., 1996), the drop in TNF-α level was probably linked to both a decrease in circulating monocyte numbers due to ncp BVDV infection and to IL-10 production, being IL-10 a cytokine displaying anti-inflammatory activity in cattle (Adler et al., 1996; Chase et al., 2004). This would suggest that TNF-α does not play a key role in anti-BVDV action, and indeed that this pro-inflammatory cytokine may be directly or indirectly inhibited by the virus, at least during the acute phase of infection.

An early increase was observed in both IFN-γ and IL-12 levels; IL-12 started to rise at 3 dpi and by 9 dpi had returned to pre-inoculation values, remaining without changes until the end of the study. However, IFN-γ suffered a significant rise as from 1 dpi that coincided with a slight increase in TNF-α and CD4+ T lymphocyte numbers, the major producers of IFN-γ; indicating that type 1 cytokines levels were not affected by BVDV infection and asserting the strength of the IFN-γ response to infection (Biron, 1994; Collins et al., 1999; Charleston et al., 2002; Lee et al.,

2008). Despite this, viremia peaked at 6 dpi showing an IFN-γ failure in resisting BVDV infection. In the present study, analysis of cytokines related to a type 2 response, such as IL-4, revealed no systemic changes following infection. A slight increase in IL-10, a cytokine involved in regulating the pro-inflammatory response, was observed between 3-12 dpi, matching the decline in TNF-α. Pattern of cytokine production by T cells is known to be influenced by the cytokine background existing during early stages of activation of the T cell response (Abbas et al., 1996). Rhodes et al. 1999, in an *in vitro* study of differential cytokine responses of CD4+ and CD8+ cells following BVDV infection, found that CD4+ T cells produced IL-4 but not IFN-γ, whilst CD8+ T cells produced IFN- γ but not IL-4. Here, however, although CD8+ T cell numbers were depleted over a longer period, the release of IFN-γ was stronger than that of IL-4 by CD4+ T cells. These results suggest that following an acute ncp BVDV infection there is a clear tendency towards a type 1 cytokine response in accordance with the findings of Charleston et al., 2002.

CHAPTER 2

Effect of infection with BHV-1 on peripheral blood leukocytes and lymphocyte subpopulations in calves with subclinical BVD

Research in Veterinary Science
(doi: 10.1016/j.rvsc.2013.02.018)

Effect of infection with BHV-1 on peripheral blood leukocytes and lymphocyte subpopulations in calves with subclinical BVD

Research in Veterinary Science, 2013

(doi: 10.1016/j.rvsc.2013.02.018)

Abstract

BVDV and BHV-1 are important cattle pathogens that induce a broad immunosuppression on cell-mediated immune response on its own participating in the BRDC. The aim of our study was to evaluate the quantitative changes in immunocompetent cells in healthy calves and calves with subclinical BVD, both inoculated with BHV-1. Total leukocyte counts exhibited changes mainly in neutrophils and lymphocytes that can contribute to the BVDV immunosuppression, thus accounting for some of the intergroup differences. Monocytes did not display numerical changes in either group. Regarding lymphocyte subpopulations, even though CD4+ T lymphocytes and B cells were depleted around 4 dpi in both infected groups, the main difference observed between both groups was in CD8+ T cells which displayed an earlier depletion in BVDV inoculated calves that can promote a greater BHV-1 dissemination, thus aggravating the course of the disease.

1. Introduction

BRDC is a major cause of economic losses in the cattle industry, weakening the immune system and increasing the susceptibility of the infected animals to secondary infections. Several infectious agents have been associated with BRDC, including BVDV and BHV-1. Frequently, severe respiratory tract disease in cattle is associated with concurrent infections of these pathogens (Srikumaran et al., 2007). Although it is known that both BVDV and BHV-1 display tropism for the respiratory tract on its own, their participation in the BRDC is mainly due to their enhancer role in mixed infections derived from their significant immunosuppressive effect (Potgieter et al., 1984a; Castrucci et al., 1992; Kelling, 2007).

Changes related to the immunosuppressive action of BVDV and BHV-1 include a transient leucopenia in calves, characterized by lymphopenia (Griebel et al., 1987-1988; Müller-Doblies et al., 2004; Pedrera et al., 2009b; Molina et al., 2012) with decreases in absolute numbers of B lymphocytes (Bolin et al., 1985a; Brewoo et al., 2007) and T lymphocytes, mainly of both CD4+ and CD8+, and γδ T cells to a lesser extent (Bolin et al., 1985a; Griebel et al., 1987-1988; Winkler et al., 1999; Archambault et al., 2000; Chase et al., 2004), as well as monocytopenia (Van Drunen Little-van den Hurt, 2007; Pedrera et al., 2009b) and neutropenia (Filion et al., 1983; Chase et al., 2004; Pedrera et al., 2009b), with functional impairment of monocytes, neutrophils and lymphocytes (Griebel et al., 1987-1988; Brown et al., 1991; Potgieter, 1995; Tikoo et al., 1995b; Glew et al., 2003; Chase et al., 2004). It is known that the cell-mediated immune response plays a key role in countering both BVDV and BHV-1 infections (Howard et al., 1992; Babiuk et al., 1996; Van Drunen Littel-van den Hurk, 2007), since the humoral response is not sufficient to eliminate infected cells.

However, to our knowledge, no studies have been made comparing the leukocyte populations and in particular the changes in lymphocyte subpopulations, during dual BVDV and BHV-1 respiratory infections in peripheral blood of calves. Studies on kinetics of the cellular response may give valuable information for understanding the cell-mediated immune response during co-infections with these major viral agents of BRDC.

The immunosuppression induced by an acute BVDV infection has been related in previous histopathological studies to an exacerbation of the effects developed during co-infections with BHV-1 (Potgieter et al., 1984a; Castrucci et al., 1992). Thus, the aim of this work was to deepen in the systemic consequences of a BVDV pre-infection in calves subjected to a secondary viral

infection. Thereby, we compared systemic quantitative changes in leukocyte populations and lymphocyte subpopulations of healthy calves and calves apparently recovered from an experimental BVDV infection, both groups challenged with BHV-1.

2. Materials and methods

2.1. Experimental procedure

A detailed description of the experimental procedure is given in Risalde et al. 2011b. In brief, thirty male Friesian calves (8-9 months old) were obtained from a herd free of tuberculosis, brucellosis and bovine leucosis virus. Calves were housed in the Animal Experimental Centre of Cordoba University and had an adaptation period of one week before the experiment started, being controlled daily for apparent clinical signs. The animals were tested to confirm their BVDV and BHV-1 Ags and Abs free status. At the beginning of the experiment, calves were randomly assigned and separated to three different groups and housed indoor under strict sterile conditions in isolated rooms during the study to avoid cross-contamination. The different groups were called according to the inoculation they were exposed to: fourteen calves belonged to the BVDV/BHV1 group, twelve calves to the BHV1 group and four belonged to the negative control group (*see Figure 1 in Materials and Methods section, p. 60*).

The animals of the BVDV/BHV1 group were infected by intranasal inoculation with 10 ml (5ml per nostril) of a suspension containing 10^5 tissue culture infective dose 50% ($TCID_{50}$)/ml of ncp BVDV genotype-1 strain 7443. Twelve days later, when the calves did not show neither clinical signs nor viremia against BVDV, the animals of both BVDV/BHV1 and BHV1 groups were challenged receiving an intranasal inoculation with a total of 2 ml (1ml per nostril) containing 10^7 $TCID_{50}$ of BHV-1.1 virulent strain Iowa. The calves of the negative control group received 1 ml of tissue culture fluid free of viruses in each nostril. Clinical examinations were performed daily and EDTA blood samples were taken at 0, 6, 12 and 18 hpi BHV1, as well as at 1, 2, 4, 5, 7, 9 and 14 dpi BHV1 (*see Table 1 in Materials and Methods section, p. 62*).

2.2. Evaluation of calves status

A detailed description of the procedure for the evaluation of the status of calves was published by Risalde et al. 2011b. Briefly, calves were examined daily with respect to rectal

temperature, general condition, oculonasal discharge, conjunctivitis, cough, dyspnea, nasal lesions and diarrhea throughout the study. Blood and nasal swabs were collected at several days previous and after BHV-1 inoculation in order to confirm and quantify the viral infections by PCR. BVDV and BHV-1 specific Abs detection were carried out by ELISA and VN, respectively.

2.3. Blood leukocyte counts

Total and differential leukocyte counts were performed on EDTA-blood samples using a Cell-Dyn 3700 Automatic Hematology Analyzer, as previously described Molina et al., 2012.

2.4. Immunolabelling of lymphocyte differentiation antigens

A panel of primary mAbs specific for bovine CD4+, CD8+ and γδ T cells, and B cells were used to examine lymphocyte subsets (*see Table 1 in Materials and Methods section, p. 62*). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (μ-chain specific) and R-phycoerythrin (PE)-conjugated goat anti-mouse IgG_1 were used as secondary Abs. Specificity controls of the labelling procedures included the omission of specific mAbs in the first step of the procedure and the use of isotype negative controls. Fresh EDTA blood samples were collected and stored at Rt, performing the surface labelling within 1 h after blood extraction as follows. A quantity of 100 μl of whole blood was diluted 1:1 with PBS and incubated with 1 μl of each primary mAb enumerated above. Following 30 min of incubation at 4°C in the dark, cells were washed with 2 ml of PBS. Afterwards, 200 μl of secondary Ab diluted 1:500 with PBS were added and cells were incubated for 30 min at 4°C in the dark. After labelling, erythrocytes were lysed by using 2 ml of lysing solution for 10 min at Rt. The lysing process was stopped by adding 2 ml of PBS. Finally, cells were fixed by adding 200 μl of formaldehyde buffered solution to each sample and were stored at 4°C in the dark until flow cytometric analysis within 48 h.

2.5. Flow cytometric analysis of lymphocyte subpopulations

The absolute number of each lymphocyte subpopulation reactive with each mAb was calculated using the total lymphocyte number and the proportion of the subpopulation given by flow cytometric analysis at each time point. Indirect immunofluorescent stained cells were analyzed using a FACScan Flow Cytometer. The software used for data collection and analyses was CELLQuest. Twenty thousand events were collected from each sample. The following

parameters were collected: forward light scatter (FSC), side light scatter (SSC), FITC fluorescence (FL1) and PE fluorescence (FL2). Lymphocytes were differentiated by their size (FSC) and granularity (SSC), and further evaluated for lymphocyte subpopulation by FL1 and FL2 (*see Figure 4 in Materials and Methods section, p. 67*). The proportions of positive lymphocytes for each of the mAb were determined after subtraction of the corresponding control.

2.6. Statistical analyses

Values of total and differential leukocyte counts and absolute number of each lymphocyte subpopulation were assessed and expressed as mean ± SEM. Duncan's Multiple Range Test was performed for BVDV/BHV1, BHV1 and negative control groups to analyze significant differences of the values in each group at various time points (*). *P* values < 0.05 were considered significant. Non-paired Student's t-test was used to determine differences between both BVDV/BHV1 and BHV1 groups at the same time point (letters "a" and "b", respectively), as well as to establish significant differences between BVDV/BHV1 or BHV1 group and negative control group at the same time point (letters "c" and "d", respectively). *P* values < 0.05 were considered significant.

3. Results

3.1. Clinical, virological and serological findings

The clinical signs, and the virological and serological status of calves before and after BHV-1 inoculation has been previously published by Risalde et al. 2011b. Briefly, in both BVDV/BHV1 and BHV1 groups, animals showed elevated rectal temperature and their general appearance was affected in varying degrees after BHV-1 inoculation. However, the calves of BVDV/BHV1 group showed more severe clinical symptoms compared with those of BHV1 group, mainly between 4 to 11 dpi. In the BHV-1 group, the animals had a slight affected general appearance during the study. The uninfected control group remained clinically unaffected throughout the study.

The detection of BHV-1 was confirmed in nasal swabs samples from 1 dpi onwards in both BVDV/BHV1 and BHV1 groups. However, viremia was only detected in the BVDV/BHV1 group from 4 dpi until the end of the study. After BHV-1 inoculation, BVDV was isolated from blood of

BVDV/BHV1 group since 1 until 5 dpi in lower amounts; despite to BVDV was not detected at the moment of BHV-1 inoculation. Control group was free of both antigens throughout study.

All animals were negative for BVDV or BHV-1.1 specific Abs at the beginning of the study and the animals of the uninoculated control group remained seronegatives until the end of the study. After BHV-1.1 inoculation, all inoculated animals showed a similar rise of neutralizing serum Abs at 14 dpi. BVDV-specific serum antibodies were only observed in the BVDV/BHV1 group from 4 dpi.

3.2. Blood leukocyte count

In the BVDV/BHV1 group, the total leukocyte count starts with significantly lower levels than in the BHV1 and negative control groups and remained in this way until 2 dpi (Figure 1). From this day, a significant increase in the total cell count was observed, peaking at 5 dpi. Thereafter, the cell count plummeted, reaching the lowest levels at 9 dpi for this group, recovering values close to pre-inoculation levels the last day. However, in the BHV1 group a minor transient increase not significant with respect to negative control group in leukocyte number lasted for 2 days after BHV-1 inoculation, which was followed by leucopenia between 4 and 9 dpi, remaining lower for the rest of the study compared with pre-inoculation values.

Parallel to the leukocytes profile, the neutrophil count was significantly different between BVDV/BHV1 and both BHV1 and negative control groups before BHV-1 inoculation (0 dpi) and remained so during the first stages of the study (Figure 1). In the BVDV/BHV1 group, after a slight initial increase at 12 hpi, neutrophils had a significantly pronounced rise, peaking at 5 dpi. After that, the cell count fell sharply, reaching the lowest level at 9 dpi and recovering the last day the initial values of the study. In the BHV1 group, the neutrophil counts had a moderate increase at 12 hpi and levelled out until 2 dpi. However, from this day onward the neutrophil number underwent a significant decline at 4 dpi and remained with similar low values until the end of the study.

In general, the dynamic of the lymphocyte population was essentially the same in both BVDV/BHV1 and BHV1 groups after BHV-1 inoculation except for some differences. There were no significant differences in pre-inoculation levels between both inoculated groups. However, in the BVDV/BHV1 group, after a slight increase at 6 hpi, which differed in a significative way from the BHV1 group, the lymphocyte count dropped significantly with respect to negative control

group from 1 dpi, which was significantly more pronounced at 4 dpi, recovering values close to pre-inoculation towards the last days of the study. In the BHV1 group, the lymphocyte count decreased from BHV-1 inoculation and, although fluctuated somewhat, stayed low during the whole study period with significant differences between this group and the negative control group, reaching the lowest value at 5 dpi (Figure 2).

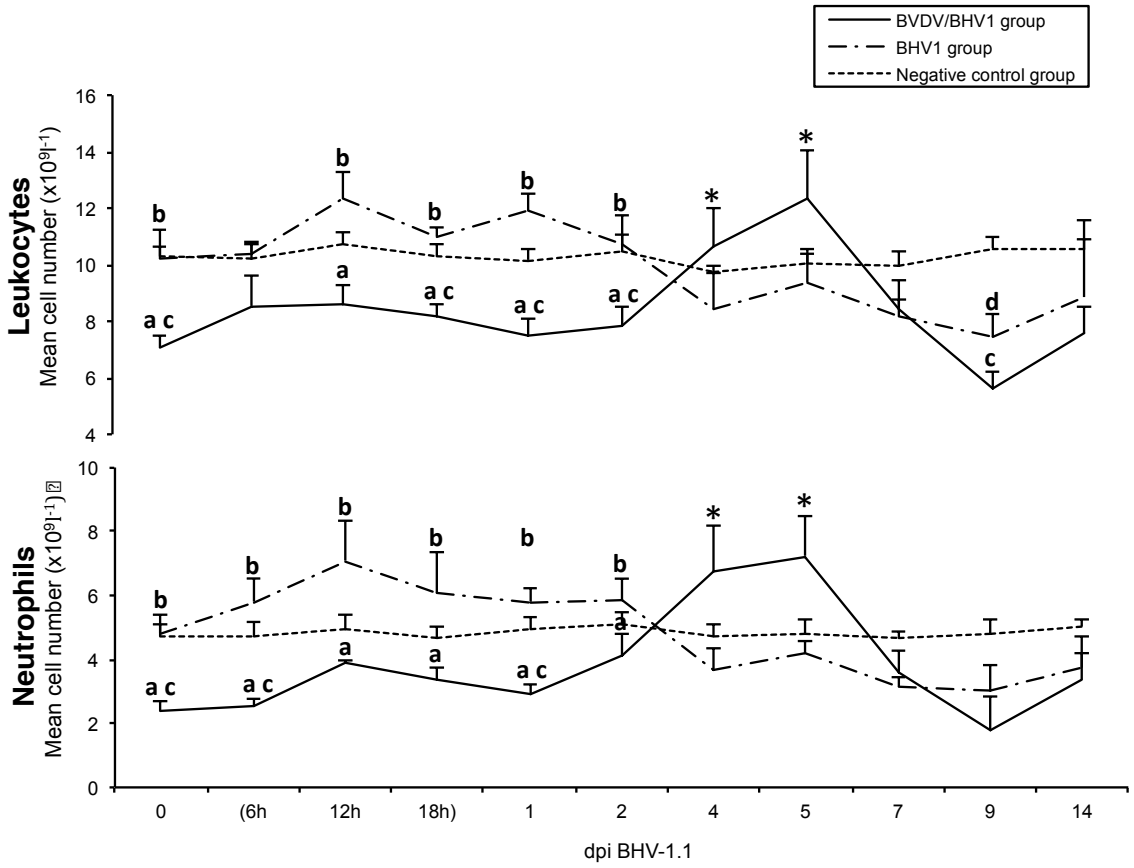


Figure 1. Mean number ($x10^{9}l^{-1}$) ± standard error of total leukocyte and neutrophil counts in blood samples from calves experimentally inoculated with bovine herpesvirus-1 (BHV1 group) versus calves inoculated with both bovine viral diarrhea virus and bovine herpesvirus-1 (BVDV/BHV1 group). *****Significant differences ($p < 0.05$) within each group at various time points. **[a,b]**Significant differences ($p < 0.05$) between both inoculated groups at the same time point. **[c,d]**Significant differences ($p < 0.05$) between BVDV/BHV1 or BHV1 group and the negative control group at the same time point, respectively. (dpi BHV-1.1, means days post-inoculation with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h, means hours post-inoculation with BHV-1.1).

In the BVDV/BHV1 group, the monocyte count did not change throughout the study. However, in the BHV1 group, the profile fluctuated somewhat, showing a descent between 2-4 dpi remaining at low levels until 9 dpi and overcoming on the last day the initial values of the study (Figure 2).

In the negative control group, the total leukocyte count and the number of neutrophils, lymphocytes and monocytes did not change throughout the study (Figures 1 and 2).

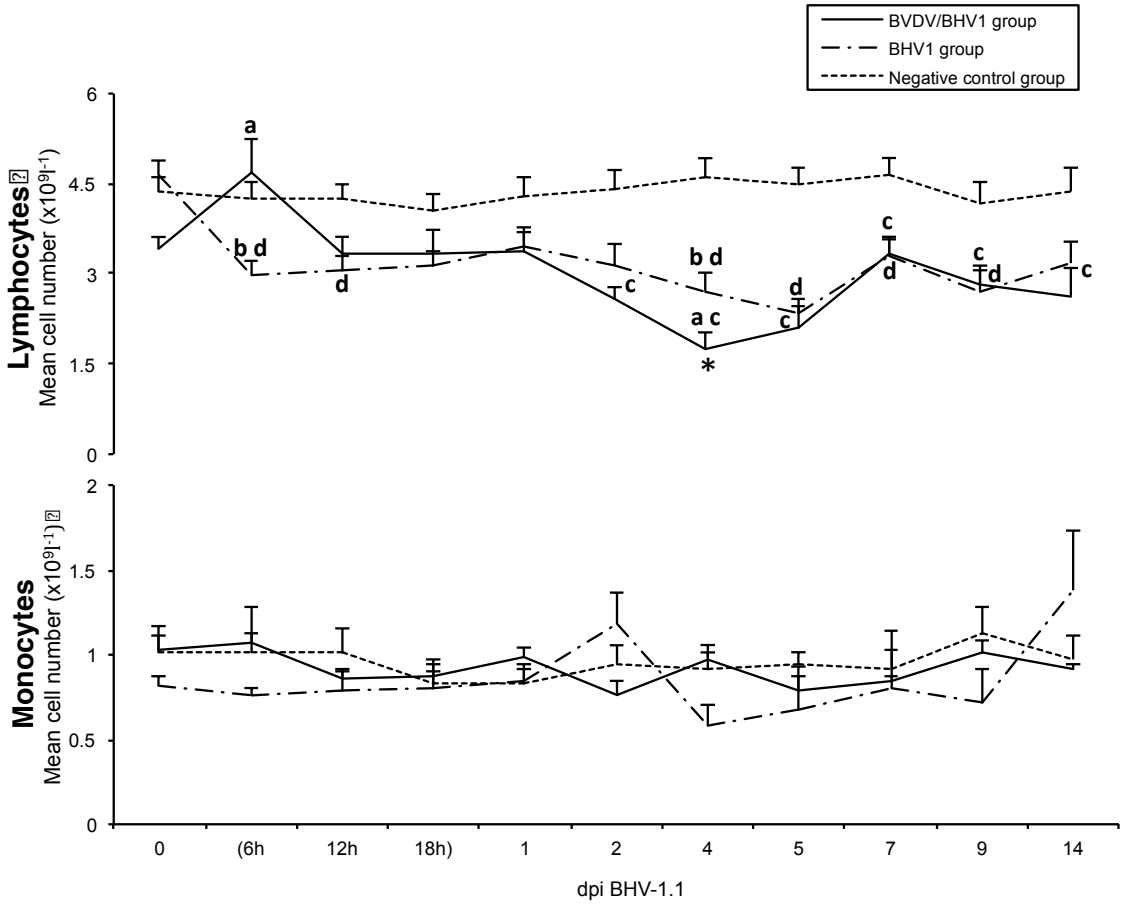


Figure 2. Mean number ($x10^{9}l^{-1}$) ± standard error of lymphocyte and monocyte counts in blood samples from calves experimentally inoculated with bovine herpesvirus-1 (BHV1 group) versus calves inoculated with both bovine viral diarrhea virus and bovine herpesvirus-1 (BVDV/BHV1 group). *Significant differences ($p < 0.05$) within each inoculated group at various time points. [a,b]Significant differences ($p < 0.05$) between both inoculated groups at the same time point. [c,d]Significant differences ($p < 0.05$) between BVDV/BHV1 or BHV1 group and the negative control group at the same time point, respectively. (dpi BHV-1.1, means days post-inoculation with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h, means hours post-inoculation with BHV-1.1).

3.3. Lymphocyte subpopulations

In the BVDV/BHV1 group, the kinetics of CD4+ and CD8+ lymphocyte subsets were essentially the same, which showed significant differences with respect to negative control group throughout the study. The levels in both subsets decreased gradually from 1 to 4 dpi, being significantly the lowest values of the study at this time point. Then, CD4+ and CD8+ cell counts soared at 9 dpi, remaining close to pre-inoculation levels towards the end of the study (Figure 3).

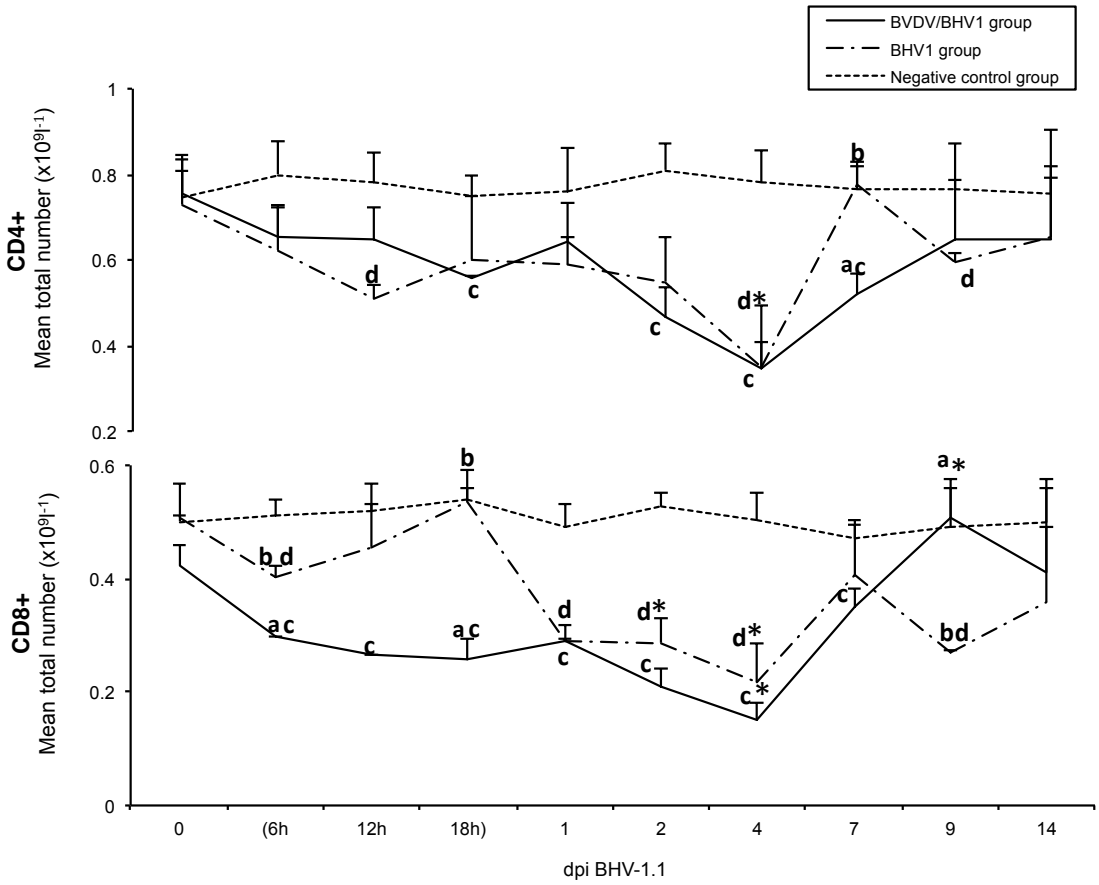


Figure 3. Mean ± standard error of absolute numbers ($x10^{9}l^{-1}$) of CD4+ and CD8+ lymphocytes in blood samples from calves experimentally inoculated with bovine herpesvirus-1 (BHV1 group) versus calves inoculated with both bovine viral diarrhea virus and bovine herpesvirus-1 (BVDV/BHV1 group). *Significant differences ($p < 0.05$) within each inoculated group at various time points. [a,b]Significant differences ($p < 0.05$) between both inoculated groups at the same time point. [c,d]Significant differences ($p < 0.05$) between BVDV/BHV1 or BHV1 group and the negative control group at the same time point, respectively. (dpi BHV-1.1, means days post-inoculation with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h, means hours post-inoculation with BHV-1.1).

In the BHV1 group, CD4+ values suffered a descent at 12 hpi and after a slight increase, levels dropped again at 4 dpi reaching the lowest values of the whole study period. After a short recovery at 7 dpi, CD4+ values dropped and stayed lower than pre-inoculation levels during the rest of the study. The CD8+ subset had significant differences in comparison with the BVDV/BHV1 group during the first 18 hpi. In fact, the CD8+ subset remained with values close to pre-inoculation levels until this time point, and then the cell number gradually showed significant differences against negative control group, reaching the lowest level at 4 dpi. CD8+ cells increased transiently at 7 dpi, but the values remained low for the rest of the study compared with the pre-inoculation levels (Figure 3).

Unlike the others lymphocyte subpopulations studied, the γδ T cell subpopulation showed pre-inoculation values significantly different between the groups, being much higher in the BVDV/BHV1 group. However, from 12 hpi, the dynamics in the BVDV/BHV1 and BHV1 groups were essentially the same where the values fluctuated somewhat for the rest of the study and did not have significant differences among time points (Figure 4).

The B lymphocyte subset presented a parallel profile in both BVDV/BHV1 and BHV1 groups characterized by a significant abrupt decrease with respect to negative control group from 1 dpi until the end of the study. From that time onward, B cell counts in the BVDV/BHV1 group remained below the BHV1 group values until the end of the experiment (Figure 4).

The lymphocyte subpopulations analysed in the negative control group did not show changes until the end of the experiment (Figures 3 and 4).

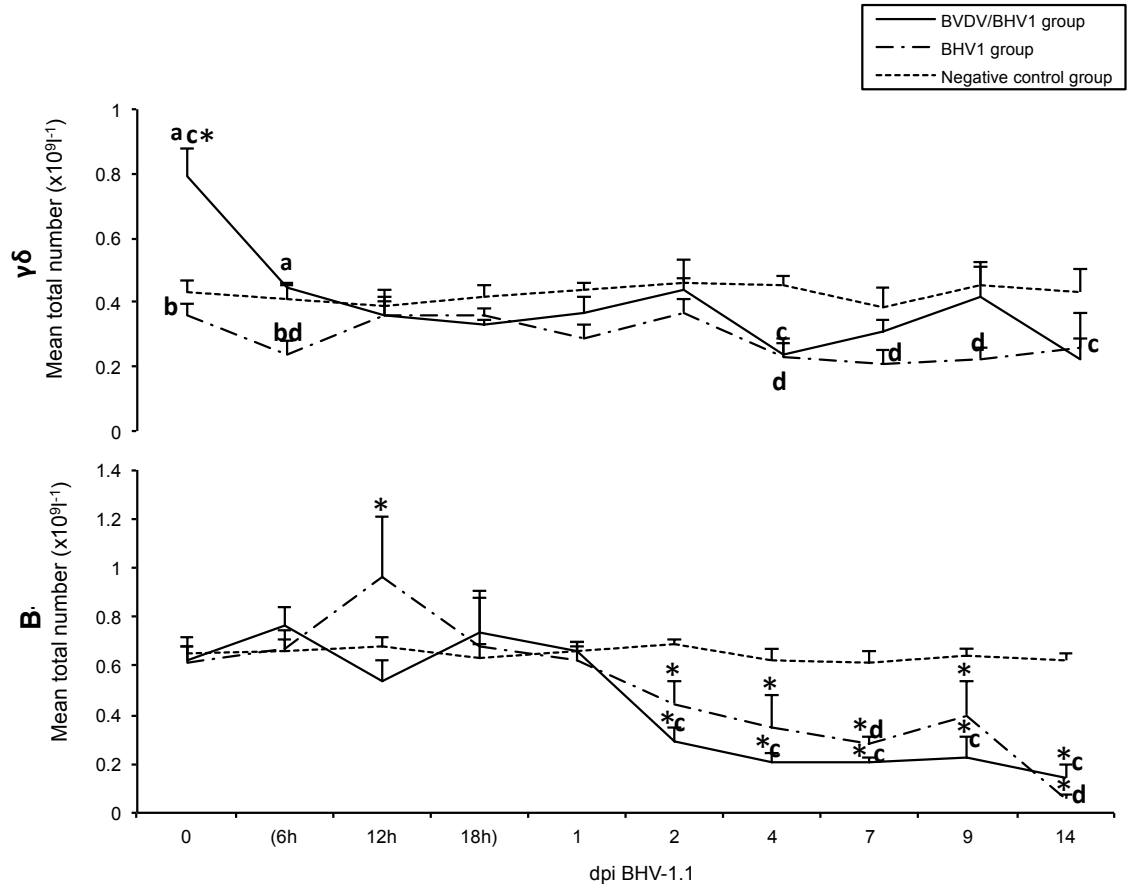


Figure 4. Mean ± standard error of absolute numbers ($x10^{9}l^{-1}$) of γδ T cells and B lymphocytes in blood samples from calves experimentally inoculated with bovine herpesvirus-1 (BHV1 group) versus calves inoculated with both bovine viral diarrhea virus and bovine herpesvirus-1 (BVDV/BHV1 group). *****Significant differences ($p < 0.05$) within each inoculated group at various time points. **[a,b]**Significant differences ($p < 0.05$) between both inoculated groups at the same time point. **[c,d]**Significant differences ($p < 0.05$) between BVDV/BHV1 or BHV1 group and the negative control group at the same time point, respectively. (dpi BHV-1.1, means days post-inoculation with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h, means hours post-inoculation with BHV-1.1).

4. Discussion

This study evaluated quantitative changes in circulating leukocytes and lymphocyte subpopulations in healthy calves and calves with subclinical BVD, both inoculated with BHV-1, with the aim of ascertaining the effect of BVDV on these immunocompetent cells response and on the development of BRDC involving a BHV-1 infection.

The analysis of peripheral blood mononuclear cells was carried out on whole blood lysis, being this method as effective as a density gradient centrifugation for lymphocyte subsets study but with shorter sample preparation time and less handling for the whole blood (Ashmore et al., 1989; Jackson, 1990; Romeu et al., 1992; Molina et al., 2012). Moreover, different studies have also shown that the lysed whole blood method is less likely to show loss of lymphocyte subsets and is more reproducible than other methods (Landay and Muirhead, 1989; Jackson, 1990).

Intergroup differences in the severity of clinical symptoms between healthy calves and calves with subclinical BVD, both inoculated with BHV-1, were accompanied by differences in the viremia (Risalde et al., 2011b). Whilst leukocyte populations such as monocytes displayed no significant numerical alteration, lymphocyte subpopulations and neutrophils exhibited behaviour changes which probably enhanced the immunosuppressive effect of BVDV, thus accounting for some of the observed intergroup differences.

In BVDV-infected calves, leukocyte numbers are reported to return to normal levels between 7 and 12 dpi (Wilhelmsen et al., 1990; Archambault et al., 2000; Pedrera et al., 2009b). In the present study, at 0 dpi (12 dpi BVDV) calves inoculated with BVDV displayed significantly lower leukocyte levels than healthy calves, though neither viremia nor clinical symptoms were apparent; this suggested a status difference prior to the secondary pathogen (BHV-1.1) inoculation. Moreover, the analysis of various leukocyte populations indicated that this difference was mainly due to a significant lower number of neutrophils, which constitute the first line of cellular defence against invading pathogens (Paape et al., 2003). Following inoculation with BHV-1, a significant difference in leukocyte counts was observed between BVDV/BHV1 and BHV1 groups, mainly affecting neurophils and, to a lesser extent, lymphocytes and monocytes. BHV-1 is known to induce a state of leukopenia associated with lymphopenia (Griebel et al., 1987-1988), neutropenia (Filion et al., 1983) and monocytopenia (Van Drunen Littel-van den Hurk, 2007). BVDV/BHV1 group calves – despite presenting a lower leukocyte number, probably due to the immunodepression induced by BVDV inoculation (Chase et al.,

2004; Pedrera et al., 2009b; Molina et al., 2012) – displayed leukocytosis resulting from an increase in circulating neutrophils between 4 and 5 dpi, in spite of the severe lymphopenia detected. This fact contrasted with the neutropenia observed from this moment in the animals only infected with BHV-1.1, which could be indicating the continuing ability of BVDV-infected calves to react against infection by recruiting neutrophils coinciding with an increased evidence of inflammatory signs (Risalde et al., 2011b).

Lymphocytes and monocyte-macrophages are the primary target cells for BVDV replication (Bruschke et al., 1998a; Glew et al., 2003; Pedrera et al., 2009b; Risalde et al., 2011a; Raya et al., 2012), therefore it was somewhat surprising that initial levels of these cell populations were similar in healthy and BVDV-infected calves prior to BHV-1 inoculation. In fact, after the secondary infection, monocytes continued without showing numeric changes in both inoculated groups, despite the observed differences in their biosynthetic activity between these groups through a variable production of proinflammatory mediators and Th1 response-inducing molecules, such as IL-12 (Risalde et al., 2011b). In this regard, previous *in vitro* studies using also a non-cytopathic BVDV strain described the survival of monocytes susceptible to infection, in contrast to the lysis observed in these cells against cytopathic BVDV; however, monocytes infected with non-cytopathic BVDV strain showed a reduced capacity to stimulate T-cell proliferation (Glew et al., 2003).

A key role in the antiviral capacity impairment may be played by circulating T lymphocyte subpopulations (CD4+, CD8+ and γδ T cells) and B cells, involved among others in the cell-mediated immune response, since they are capable of interacting with infected cells (Srikumaran et al., 2007). In general, lymphoid populations were more affected in the co-infected group, which presented a decrease in their number after BHV-1.1 inoculation. However, this was not reflected in the total lymphocyte counts at early time-points, despite the declines in both CD4+ and CD8+ T lymphocyte subpopulations, a fact that could be associated with the absence of changes in B lymphocytes number at the beginning of the study. The drop in the number of CD4+ and CD8+ T lymphocytes has been correlated in previous studies with an increase in apoptosis due to a tropism of BHV-1 for CD4 but not CD8 T cells (Eskra and Splitter, 1997; Winkler et al., 1999). Thus, the process of apoptosis observed in CD8+ cells was related to an indirect mechanism of the virus infection (Winkler et al., 1999), which should be studied in further *in vitro* research.

Analysis of lymphocyte subpopulations showed that CD4+ T cell levels were similar in both groups prior to inoculation with BHV-1, reflecting normal values in contrast to the characteristic CD4+ T cell depletion induced by BVDV (Howard et al., 1992; Collen et al., 2002). Following inoculation with BHV-1, both groups displayed a slight decline in CD4+ T cell numbers from 6 hpi, being the lowest counts recorded at 4 dpi. Despite this decline, numbers in both groups had returned to normal levels by the end of the study, what would suggest that BVDV does not impair the regenerative capacity of circulating CD4+ T cells. Thus, the similarity in CD4+ lymphocyte kinetics indicates that the changes observed were probably induced by BHV-1, which can specifically infect this subpopulation (Babiuk et al., 1996; Winkler et al., 1999; Van Drunen Littel-van den Hurk, 2007).

Although CD8+ T cell levels were similar before BHV-1 inoculation, significant differences in cell counts between BVDV/BHV1 and BHV1 groups were apparent from 6 hpi. The constant decrease observed in the co-infected group was not seen in the BHV1 group, suggesting that it could be attributable not to the infection of these cells by BHV-1 but rather to the BVDV-induced migration of CD8+ T cells to the infection site (Winkler et al., 1999; Van Drunen Littel-van den Hurk, 2007). Between 1 and 7 dpi, CD8+ T cell counts behaved in a similar manner in both groups, being the most marked depletion observed at 4 dpi. This decrease was more pronounced in the BVDV pre-infected calves, where the BHV-1 was detected in blood from 4 dpi (Risalde et al., 2011b). These results may indicate an impairment of the cytotoxic action of CD8+ T cells as a defence against the cell-to-cell dissemination characteristic of BHV-1 prior to blood-borne dissemination (Van Drunen Little-van den Hurt, 2007).

Numbers of γδ T lymphocytes were higher in the BVDV/BHV1 group prior to infection with BHV-1. This initial difference might have affected the response to secondary infection, although the systemic role of this lymphocyte subpopulation in countering pathogenic agents has not yet been clearly established. Amadori et al. 1995 reported that in BHV-1 infection the inhibition of virus replication is among the antiviral activities attributed to circulating γδ T cells. In the present study, γδ T cells reached normal levels in the BVDV/BHV1 group at 6 hpi. Thereafter, and throughout the study, kinetics and cell counts remained similar in both groups. This would suggest that, although circulating γδ T cell levels were initially higher in the BVDV/BHV1 group, these cells did not play a major systemic role in containing BHV-1 dissemination, since viremia was detected in this group, but not in the BHV1 group (Risalde et al., 2011b). Likewise, γδ T cells

did not show numeric changes along the study, as occur in monocyte count, therefore these cells could be resistant to the virus attack or cells for which it does not have a special tropism. In this respect, further *in vitro* studies are required in order to clarify the possible resistance of these cells to the infection.

Reports regarding changes in circulating B lymphocyte counts following BVDV infection are contradictory; some authors have noted a decline (Molina et al., 2012), others have observed no effect (Archambault et al., 2000), whilst still others have recorded a transitory increase (Brodersen and Kelling, 1999). BVDV exerts its greatest effect on thymic lymphocytes and follicular B lymphocytes in lymph nodes and Peyer's patches (Brodersen and Kelling, 1999; Pedrera et al., 2009a,b; Raya et al., 2012). Although BHV-1 pre-inoculation counts were similar in both groups, B lymphocyte counts after BHV-1 infection were lower in the BVDV/BHV1 group than in the BHV1 group from 1 dpi onwards. In this regard, these animals also showed an intense lymphoid depletion associated with BVDV infection in B-dependent areas of lymph nodes and ileal Peyer patches (Risalde et al., 2013), which could contribute to the recruitment of circulating lymphocytes at the site of lymphoid depletion, thus favoring the severe decrease of these cells in blood. This migration of B lymphocytes to lymphoid tissues and the differentiation of some of them into plasma cells would explain the increase of BHV-1 neutralizing antibodies at 14dpi.

CHAPTER 3

Immunohistochemical characterization of inflammatory cells in the oronasal route of BVDV and BHV1 entry from calves dually infected

Immunohistochemical characterization of inflammatory cells in the oronasal route of entry of BVDV and BHV1 in calves dually infected

Abstract

Nasal mucosa and palatine tonsils are located at the entry of oronasal route of natural infection where both inhalated and ingested antigens can induce an immune response. An immunohistochemical study of the nasal mucosa and palatine tonsil was carried out to gain further insight in the pathogenesis of dually BVDV and BHV-1 infections in cattle. Twelve calves were inoculated intranasally with noncytopathic BVDV-1a; 12 days later, ten of these calves were challenged intranasally with BHV-1 subtype 1. Two of the BVDV infected calves were euthanized before BHV-1 inoculation and the rest ones at 1, 2, 4, 7 and 14 days after BHV-1 inoculation, at same time that another ten calves only inoculated with BHV-1. Two calves were inoculated intranasally with virus-free tissue culture fluid and euthanized as negative controls at the end of the study. This study was conducted to determine an immunophenotypic characterization and quantification of the immunocompetent cells in those tissues. Overall, after BHV-1 infection the lamina propria of the nasal mucosa was more infiltrated than the epithelium. The CD8+ and γδ T cells and B lymphocytes appeared earlier than macrophages, showing a delay in the BVDV/BHV1 group compared with the BHV1 group. However, in the palatine tonsil showed high number of immunocompetent cells in both epithelium and lamina propria, and displayed differences between both groups. In the BHV1 group, the immune response was characterized by the presence of CD4+ and γδ T lymphocytes and B cells, and to a lesser extent by CD8+ T cells. In the BVDV/BHV1 group, after BHV-1 infection the γδ T lymphocytes showed an earlier influx coupled with a rise in the macrophage counts, compared with the BHV-1 group. However, CD4+ and CD8+ lymphocytes exhibited an abrupt and progressive drop and B lymphocytes displayed no remarkable changes.

1. Introduction

It is well known that human and animals palatine tonsils are sites for the replication of several pathogens entering through either the oral or nasal cavities (Brandtzaeg and Halstensen, 1992; Nave et al., 2001; Liebler-Tenorio and Pabst, 2006; Palmer et al., 2009). Specifically, in cattle, palatine tonsils have been investigated due to their important role in the immunopathogenesis of several infectious diseases. During BHV-1 infection, tonsils are infected early in the course of the disease (Schuh et al., 1992; Egyed et al., 1996; Winkler et al., 1999; 2000). Viral antigen is present in the epithelium and lymphoid tissue of tonsils and causes necrosis/apoptosis of these structures in neonates, calves and adult cows (Narita et al., 1982; Schuh et al., 1992; Winkler et al., 1999). Indeed, biopsies of the palatine tonsils can be used in the epidemiological study of BHV-1 infections in cattle (Egyed et al., 1996; Winkler et al., 2000). Tonsils are also important for diagnosing asymptomatic BHV-1 carriers, since the virus becomes latent in the lymphoid tissue of tonsils and can be reactivated by immunosuppression thus causing renewed shedding (Winkler et al., 1999; Perez et al., 2005). In acute postnatal BVDV infections, tonsils are the route of entry for the virus (Liebler-Tenorio et al., 1997; 2002), and very high replication rates take place in these organs (Bruschke et al., 1998a). Both BVDV and BHV-1 are important cattle pathogens which induce a broad immunosuppression on cell-mediated immune response, and are involved in the bovine respiratory disease complex, among other infectious agents (Castrucci et al., 1992; Kelling et al., 2007; Srikumaran et al., 2007). Interactions between immunocompetent cells, cytotoxic mechanisms and soluble factors i.e. cytokines help to limit the replication of microorganisms in the upper respiratory tract (Kaashoek et al., 1998).

Tonsils are strategically located at the pharinx entry, forming the Waldeyer's ring, which consist of mucosal associated lymphoid tissue (MALT) of the pharyngeal wall mucosa (Schuh and Oliphant, 1992; Palmer et al., 2011). In bovine and other species, it is composed of five distinct tonsils, i.e. the palatine and lingual tonsils and the rudimentary tonsil of the soft palate in the oropharynx; and the pharyngeal tonsil and the tubal tonsil, in the nasopharynx (Casteleyn et al., 2011). The luminal surface of the palatine tonsils is covered by a stratified, squamous, non-keratinised epithelium that deeply invaginates the tonsil, forming blind-ended crypts that greatly expand the surface area for direct antigenic stimulation (Velinova et al., 2001). The epithelial layer lining the crypts is composed by a modified, so-called reticular epithelium, that also

overlays lymphoid follicles and is characterized by the presence of epithelial cells altered in shape, content and function, infiltrative non-epithelial cells (e.g. lymphocytes, macrophages and DCs), intraepithelial vasculature, and a discontinuous basement membrane (Perry and Whyte, 1998; Cocquyt et al., 2008). This reticular epithelium provides a venue for intimate contact between Ags, specialized epithelial cells, intraepithelial lymphocytes and APCs, with subsequent Ag uptake and transport inducing an immune response (Croitoru and Bienenstock, 1994; Bernstein et al., 1994). In contrast, the non-reticular epithelium is not associated with lymphoid follicles and is characterized by squamous epithelium, rare non-epithelial cells and infrequent vascular structures (Perry and Whyte, 1998).

Additionally, it is known that human nasal mucosa is extremely rich in APCs such as DCs and macrophages that play a key regulatory role in the adaptive immune response, pointing to the nasal mucosa as an efficient defence against viral pathogens and in the maintenance of immunologic homeostasis (Jahnsen et al., 2004). Hence, we consider the bovine nasal mucosa as a site of great interest for studying the pathogenesis of respiratory diseases such as BVD and IBR after natural infections or intranasal inoculations.

A large amount of immunological investigations are focused on providing the anatomical location and histological characterization of the bovine tonsils in healthy calves (Schuh and Oliphant, 1992; Manesse et al., 1998; Rebelatto et al., 2000; Velinova et al., 2001; Yasuda et al., 2006; Cocquyt et al., 2008; Palmer et al., 2009; 2011). However, very few studies describe the immunological characteristics of the tonsils during viral infections in cattle (Schuh et al., 1992; Liebler-Tenorio et al., 1997; Winkler et al., 1999; Perez et al., 2005). To our knowledge, no studies have been carried out focused on immunological characteristics of bovine nasal mucosa and palatine tonsil during dual BVDV and BHV-1 infections. Thus, the aim of the present work was to evaluate the implication and the potential role of these organs in the pathogenesis of dual BVDV and BHV-1 infections from calves pre-infected with BVDV compared with healthy calves both challenged with BHV-1. For this purpose, immunophenotypic characterization and quantification of subpopulations of immune effector cells and their biosynthetic activity was performed by means of immunohistochemical techniques.

2. Materials and methods

2.1. Animals, virus and experimental design

Thirty male Friesian calves (8-9 months old) from farms free of tuberculosis, brucellosis and bovine leucosis, were housed in the Animal Experimental Centre of Cordoba University (Spain) and had an adjustment period of one week before the experiment started, being controlled daily for clinical signs of disease. They were tested to confirm their BVDV and BHV-1 free status by Ag and Ab ELISAs.

At the beginning of the experiment, the calves were randomly assigned to three different groups called according to the inoculation they were exposed to (*see Figure 1 in Materials and Methods section, p. 60*). Fourteen calves belonged to the BVDV/BHV1 group, twelve calves to the BHV1 group and four belonged to the negative control group. The animals of BVDV/BHV1 group were infected by intranasal inoculation with 10 ml (5 ml per nostril) of a suspension containing 10^5 $TCID_{50}$/ml of ncp BVDV genotype-1 strain 7443. Twelve days later, when the calves did not show neither clinical signs nor viremia against BVDV, twelve calves of the BVDV/BHV1 group and all animals of the BHV1 group were challenged receiving an intranasal inoculation with a total of 2 ml (1 ml per nostril) containing 10^7 $TCID_{50}$ of BHV-1 subtype 1 (BHV-1.1) virulent strain Iowa. The animals of the negative control group received 1 ml of tissue culture fluid free of viruses in each nostril. Clinical examinations were performed daily in all calves of each group.

Animals were sedated with xylazine and euthanized by overdosing with thiopental-sodium in batches of two at 1, 2, 4, 7 and 14 dpi with BHV-1. Two animals of the BVDV/BHV1 group were killed prior to the BHV-1 inoculation and used as control of the BVDV/BHV1 group, called BVDV controls. Two animals of the negative control group were euthanatized at the end of the experiment and used as controls of the BHV1 group, called negative controls (*see Figure 1 in Materials and Methods section, p.* 60).

The whole experimental procedure was carried out in accordance with the Guidelines for accommodation and care of animals, approved by the European Economic Community (Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, as amended by Directive 2003/65/EC).

2.2. Histology and immunohistochemical study

Necropsy examination was performed on all calves. Tissue samples were collected from nasal mucosa and palatine tonsil and immediately immersed in three different fixatives: 10% neutral buffered formalin solution (10%F), Bouin's solution (BS) and Zinc buffer salts fixative (ZSF). Fixed samples were routinely processed and embedded in paraffin wax (*see 2.2. Tissue samples in Materials and Methods section, pp. 62-63*).

Tissue sections were stained with HE for the histological study and mounted on Vectabond-coated slides and dried overnight at 37°C for the IHC study.

The ABC technique was used for the IHC study (*see 4. Histology and immunohistochemical study section in Materials and Methods section, pp. 69-70*). Briefly, tissue sections were dewaxed and rehydrated through graded ethanol and the endogenous peroxidase activity was exhausted by incubation with 3% hydrogen peroxide in methanol for 30 min at Rt. After this blocking stage, sections were subjected to one of the different Ag-retrieval methods. After pretreatment, sections were rinsed 3 times PBS for 10 min each. For the mAbs, tissue sections were covered with 1% normal horse serum in Tris buffered saline (TBS) for 30 min. For the pAbs, 20% normal goat serum replaced to normal horse serum. Sections were then incubated with the primary mAbs and pAbs at 4°C overnight. Details of the primary mAbs used in this study, including dilutions and antigen-retrieval pretreatments are summarized in Table 3 in *Materials and Methods section, p. 82*. After primary incubation, the slides were washed in PBS (3 times for 5 min each) and incubated with the secondary Abs for 30 min at Rt. Biotinylated horse anti-mouse IgG secondary Ab, diluted 1:200 in TBS containing 1% normal horse serum for 30 min at Rt was used for the mAbs. Biotinylated goat anti-rabbit IgG secondary Ab diluted 1:200 in PBS containing 1.5% normal goat serum was used for the pAbs. After 3 further 5-min washes in PBS, samples were incubated with the ABC for 1 h at Rt. All tissue sections were finally rinsed in TBS, incubated with a chromogen solution, and counterstained with hematoxylin.

Mouse and rabbit non-immune sera were used in place of specific mAbs and pAbs, respectively as internal negative controls, and omission of the primary Abs was used to provide an additional negative control. Internal positive controls consisted of tissues samples obtained from calves experimentally infected with BVDV where cytokines and cellular markers detection was previously carried out by IHC (Pedrera et al., 2007; 2009b). Additionally, positive control

tissues from calves persistently infected with BVDV were used for the Erns (gp48) detection and positive controls from abortion fetus positive to BHV-1.1 were employed for the gC detection.

2.3. Evaluation of immunostaining

To evaluate the number of immunostained cells and to correlate the results obtained with the different Abs, paraffin-wax blocks from nasal mucosa and palatine tonsil of each animal were selected. Positively labeled cells of epithelium and lamina propria were counted in 25 fields of 0.2 mm^2 randomly chosen. Cellular identification was based on morphological features, location, and cell size. The results were given as the mean of the number of positive cells per 0.2 mm^2 ± SEM.

2.4. Statistical analyses

Differences between the means were assessed by the Shapiro-Wilk test for determining the normality of the distribution, followed by the Mann–Whitney U non-parametric test. $P < 0.05$ represented statistically significant differences between inoculated calves of each infected group (BVDV/BHV1 or BHV1 groups) and their control group in each time point (*). The statistical evaluation was made using the software GraphPad InStat software.

3. Results

3.1. Histologic findings

The main lesions found in the nasal mucosa were characterized by mild hyperemia and infiltrate in lamina propria, displaying more intensity in the BVDV/BHV1 group than in the BHV1 group. Mononuclear infiltrate was the more extended microscopic lesion from 1 dpi until the end of the study in both BVDV/BHV1 and BHV1 groups. Occasional eosinophilic intranuclear inclusion bodies and syncytia appeared in the epithelium in the BVDV/BHV1 group (Figure 1).

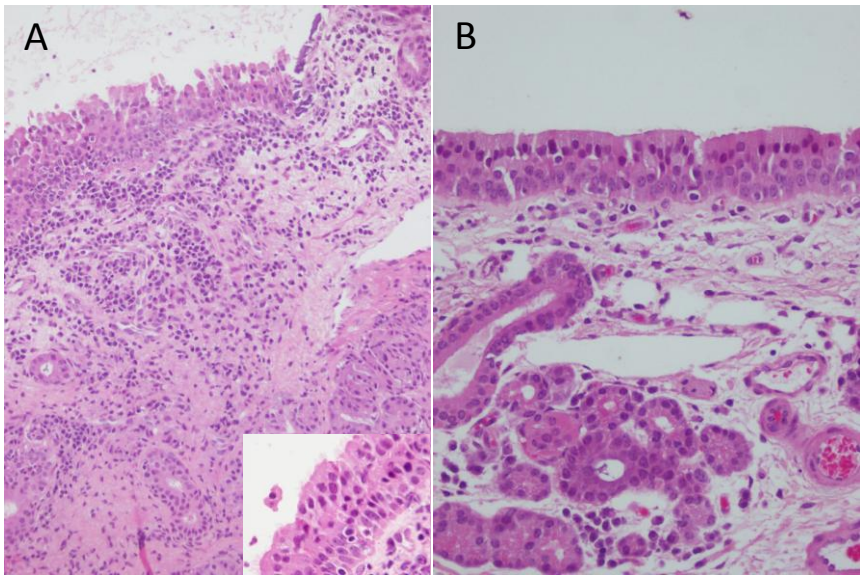


Figure 1. (A) Nasal mucosa from a calf dually infected with BVDV and BHV-1 (BVDV/BHV1 group) at 4 dpi. HE; 20X. Inset: magnification of epithelium containing eosinophilic inclusion bodies. (B) Nasal mucosa from a calf infected only with BHV-1 (BHV1 group) at 4 dpi. HE; 40X.

The histopathological study of the palatine tonsil showed that only calves of the BVDV/BHV1 group displayed focal necrosis mainly in the tonsillar crypts epithelium between 4 to 7 dpi (Figure 2), and petechial hemorrhages within lymphoid follicles from 2 to 4 dpi. Focal necrosis was characterized by the presence of mononuclear and PMN cell infiltrates. The subepithelial connective tissues were edematous and also infiltrated with mononuclear and PMN cells. The necrotic debris and PMN cells generally remained attached to underlying tissues. Moreover, crypts contained cellular debris, PMN cells, degenerated epithelial cells, and small inflammatory-necrotic foci (Figure 2). Common lesions to both BVDV/BHV1 and BHV1 groups consisted of hyperemia and depletion of the lymphoid follicles with pyknosis and karyorrhexis suggestive of apoptosis and were found between 4 to 7 dpi, being hyperemia more intense in the BHV1 group and depletion in the BVDV/BHV1 group.

No histopathological lesions were observed in the nasal mucosa and palatine tonsil of control calves.

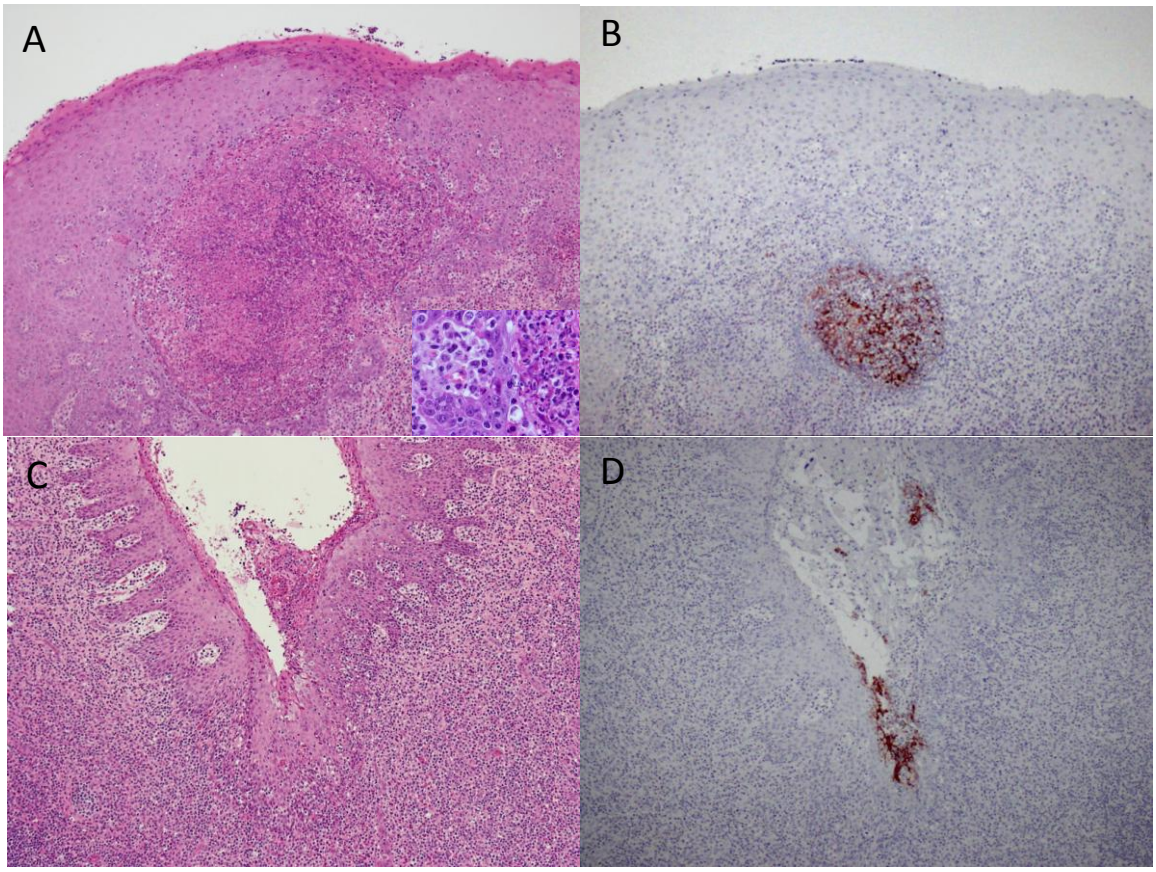


Figure 2. Palatine tonsil from a calf dually infected with BVDV and BHV-1 (BVDV/BHV1 group) at 4 dpi. (A) Focal necrosis in epithelium and underlying subepithelial connective tissue. HE; 20X. Inset: magnification of eosinophilic inclusion bodies. (B) Similar area than (A) section, stained by IHC with an antibody directed against BHV-1 gC. 20X. (C) Cellular necrotic debris inside a tonsillar cript. HE; 20X. (D) Similar area than (C) section, stained by IHC with an antibody directed against BHV-1 gC. 20X.

3.2. Virological localization

BVDV and BHV-1 antigens were detected by IHC in the respective positive control tissues, but not in the negative control tissues. There was no labelling of any tissue when non-immune serum replaced BVDV or BHV-1 specific Ab.

BHV-1 Ag (gC) was located in necrotic areas of crypts epithelium and underlaying lamina propria and in the contents within crypts of the palatine tonsil from 4 to 7 dpi, mainly in the BVDV/BHV1 group being scarce in the BHV1 group (Figure 2). However, BHV-1 Ag was not

detected in the nasal mucosa of neither BVDV/BHV1 nor BHV1 groups. The immunolabeled cells were epithelial cells, and lymphocytes and macrophages to a lesser extent.

BVDV Ag (gpErns) was not detected neither in the nasal mucosa nor the palatine tonsil throughout the study.

Neither BVDV nor BHV-1 Ags were detected in tissues of negative control group calves.

3.3. Inflammatory cell counting

3.3.1. T lymphocyte subpopulations

- ***CD4+ lymphocytes***

The profile of CD4+ lymphocytes is shown in figures 3A and 3B.

In the nasal mucosa (Figure 3A), the quantity of CD4+ cells in the epithelium was scarce with no significant changes being observed either in the BVDV/BHV1 or the BHV1 group. In the lamina propria, the BHV1 group displayed a significant decline at 1 dpi, remaining at low values until the end of the study compared with the negative controls; in the BVDV/BHV1 group, despite falling at 1 dpi, reached similar values to the BVDV controls between 2-4 dpi and then dropped significantly again until 14 dpi.

In the palatine tonsil (Figure 3B), CD4+ lymphocyte counts were higher in the BVDV controls than in the negative controls in both epithelium and lamina propria. After BHV-1 inoculation, CD4+ cells peaked at 2 dpi in the epithelium of the BHV1 group, remained in high number at 4 dpi, and then dropped reaching levels similar to the negative controls. However, in the BVDV/BHV1 group CD4+ lymphocytes displayed an abrupt drop, remaining significantly low between 1 and 4 dpi compared with BVDV control counts and increasing moderately at 7-14 dpi. In the lamina propria, the profile exhibited in the BHV-1 group was similar to that described in the epithelium although to a lesser extent. In the BVDV/BHV1 group, an abrupt significant descent of CD4+ cells was observed from 1 dpi until the end of the study, similar to the epithelium profile.

- ***CD8+ lymphocytes***

In the nasal mucosa, the CD8+ cells (Figure 3C) of BHV1 group peaked at 2 dpi in both epithelium and lamina propria, remaining at low number the rest of the study with similar values to the negative controls. In the BVDV/BHV1 group, the positive cell counts in the epithelium

were significantly high at 1 and 2 dpi, then descended gradually until the end of the study towards values close to the BVDV controls. In the lamina propria of the BVDV/BHV1 group, a significant decline of CD8+ cell counts was detected at 1 dpi in comparison with the BVDV controls.

In the palatine tonsil, the CD8+ cells (Figure 3D) of the BVDV/BHV-1 group tended to gradually descend since the BHV-1 inoculation until the end of the study in both epithelium and lamina propria compared with the BVDV controls count. In the BHV1 group, the number of CD8+ cells in the epithelium was significantly low at 1 dpi, reaching counts close to negative control group between 2-4 dpi, and descending abruptly again from 7 to 14 dpi. In the lamina propria of the BHV1 group, the number of positive cells remained similar to the negative control counts except for 7 and 14 dpi, in which scarce immunolabelled cells were observed, as occur in the epithelium.

- ***WC1+ lymphocytes***

The WC1+ lymphocytes profile (Figure 3E) in the epithelium of the nasal mucosa is parallel in both BVDV/BHV1 and BHV1 groups peaking significantly at 2 dpi; however, the BHV-1 group displayed significant decrease between 7 and 14 dpi compared with negative controls. In the lamina propria, the positive cells of the BVDV/BHV1 group were significantly lower than the BVDV controls at 1 dpi, displaying counts similar to control between 2-4 dpi, and descending significantly again from 7 to 14 dpi. In the BHV1 group, positive cells showed a significant drop at 1 dpi compared with the negative controls and, after a recovery, the values reached the lower significant counts at 7 dpi.

In the palatine tonsil (Figure 3F), the epithelium of both BVDV/BHV1 and BHV1 groups displayed a significant descent at 1 dpi compared with each control group. Then, in the BVDV/BHV1 group, WC1+ cells were recovered at 2 dpi and, in the BHV1 group at 4 dpi. After this, the number of positive cells dropped significantly to levels below control animals in both infected groups. In the lamina propria, the number of positive cells in the BVDV/BHV1 group displayed similar values to the BVDV control animals until reaching a significant drop at 7 and 14 dpi. In the BHV1 group, the WC1+ cell number displayed significant descent at 1, 7 and 14 dpi.

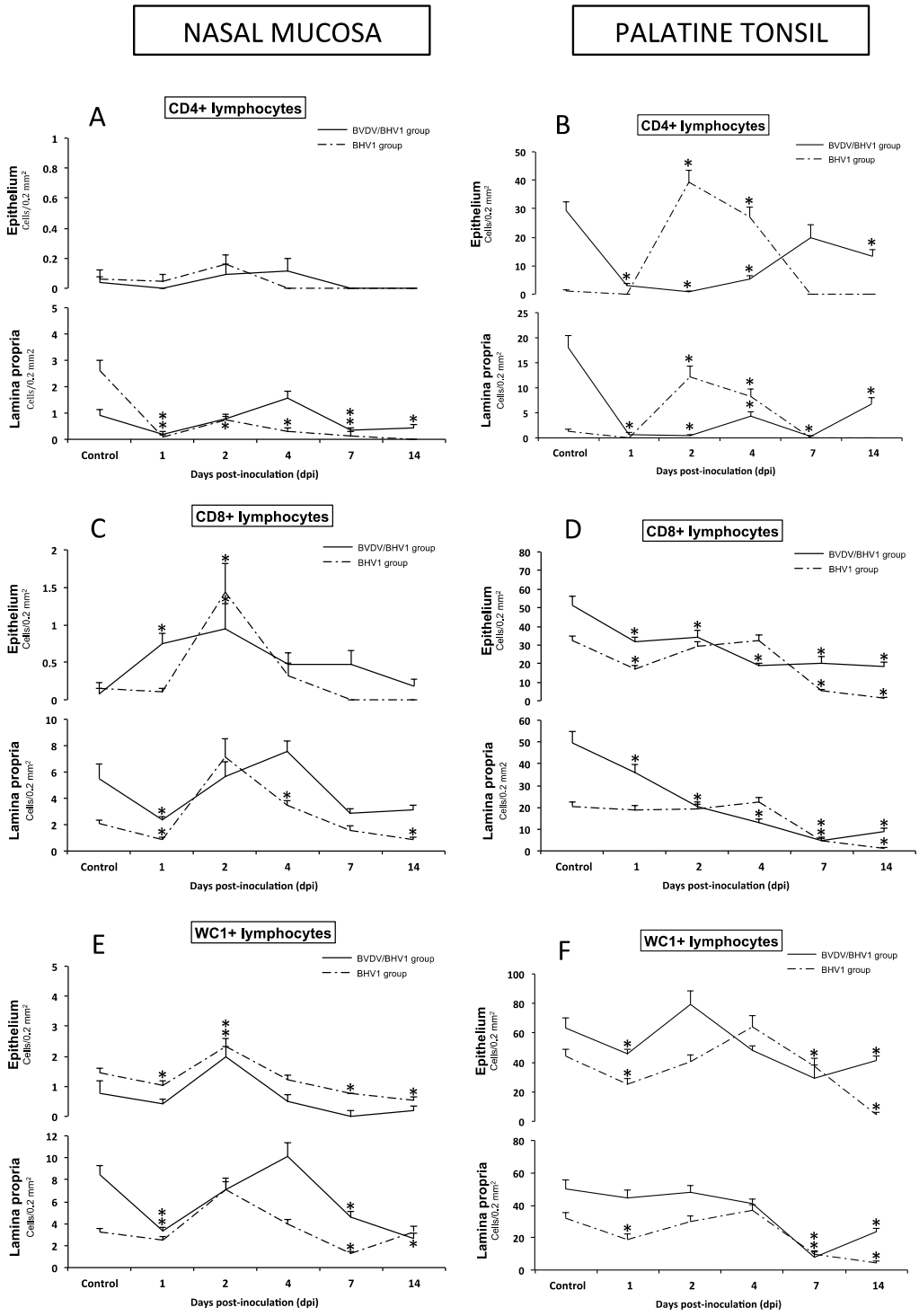


Figure 3. Counts of CD4+ T lymphocytes in the nasal mucosa (A) and palatine tonsil (B); Counts of CD8+ T lymphocytes in the nasal mucosa (C) and palatine tonsil (D); Counts of WC1+ T lymphocytes in the nasal mucosa (E) and palatine tonsil (F). From calves experimentally inoculated with BVDV and BHV-1 (BVDV/BHV1 group) versus calves infected only with BHV-1 (BHV1 group). Results are expressed as mean of cell counts ± SEM. *Significant differences ($P < 0.05$) between each infected group and their control group in each time point.

3.3.2. Macrophages

The kinetics of the macrophages is shown in figures 4A and 4B.

In the nasal mucosa, macrophage counts showed a slight descent the day after BHV-1 infection in both infected group and in both epithelium and lamina propria, being significantly lower in the lamina propria; and reaching values close to each control group again at 4 dpi. After that, in both BVDV/BHV1 and BHV1 groups cell counts peaked at 7 dpi in both epithelium and lamina propria.

In the palatine tonsil, macrophages of the BHV1 group displayed a similar profile in both the epithelium and lamina propria peaking significantly at 4 dpi and remaining close to negative control group the rest of the study, and showing the lamina propria a higher cell density. In the BVDV/BHV1 group, macrophage counts in the epithelium were significantly higher than the BVDV controls between 1 and 7 dpi; macrophage counts in the lamina propria were significantly higher than the BVDV controls between 1 and 4 dpi.

3.3.3. Cells expressing MHC-II molecules

In the nasal mucosa, number of MHC-II+ cells (Fig. 4C) in the epithelium was scarce in both BVDV/BHV1 and BHV1 groups, but the BVDV/BHV1 group rised dramatically peaking significantly at 14 dpi. In the lamina propria, the BVDV/BHV1 group fluctuated somewhat without significant changes, compared with the BVDV control animals. However, in the BHV1 group, a significant decline in the MHC-II+ cell counts was observed from 1 dpi until the end of the study compared with the negative controls.

In the palatine tonsil, the MHC-II+ cells (Fig. 4D) displayed a profile similar in both epithelium and lamina propria within each infected group. In the BVDV/BHV1 group, after a significant descent at 1 dpi, positive cells rose at 2 dpi, leveling out until 4 dpi. Then, the MHC-II+ cells dropped slightly, and rised again until reaching the maximum significant number at 14 dpi compared with the BVDV control group. In the BHV1 group, there was a significant drop at 1 dpi, subsequently started to increase gradually, peaking significantly at 4 dpi, and then dropped significantly at 7 dpi, reaching levels close to negative control group values at 14 dpi.

3.3.4. B lymphocytes

In the nasal mucosa, B lymphocytes (Figure 4E) displayed in the BHV1 group a similar kinetic in both epithelium and lamina propria. After BHV-1 inoculation, cell counts peaked at 1 dpi, being significant in the lamina propria with respect to the negative control, and then dropped at 2 dpi and again started to rise gradually until the end of the study, reaching values significantly higher than negative control group in the lamina propria at 7-14 dpi. In the epithelium of the BVDV/BHV1 group, B lymphocyte counts peaked significantly at 2 dpi, showing values similar to those of the BVDV controls during the rest of the days. However, in the lamina propria of the BVDV/BHV1 group, B lymphocyte counts augmented at 1 and 2 dpi after BHV-1 infection and then plummeted, reaching significantly low values at 7 dpi which were recovered to values close to BVDV control group at the end of the study.

In the palatine tonsil, B lymphocytes (Figure 4F) were numerically higher than in the nasal mucosa. In the epithelium of the BVDV/BHV1 group, B lymphocytes fluctuated somewhat, displaying a significant slight drop at 1 dpi that changed to a significant slight increase at 2 dpi, compared with the BVDV controls. However, in the BHV-1 group, the cell count peaked at 1 dpi, diminishing gradually until values close to the negative controls the following days, and then B cells displayed a significant abrupt rise at 14 dpi. In the lamina propria, B lymphocytes of both BVDV/BHV-1 and BHV-1 group showed a profile similar to the epithelium.

3.3.5. Cytokines

- **IL-1α**

In the epithelium of the nasal mucosa, there were not IL-1α+ cells (Figure 5A). In the lamina propria of the nasal mucosa, in the BHV1 group there was a significant biphasic increase at 1 dpi and at 4 dpi compared with the negative control group values, remaining the number of positive cells close to zero the rest of the days of the study. The BVDV/BHV1 group did not display significantly different counts to those of the BVDV control during the study.

In the epithelium of the palatine tonsil (Figure 5B), only the BVDV/BHV1 group showed significantly increased levels of IL-1α at 14 dpi, with no differences being observed in the BHV1 group during the whole study. In the lamina propria of the palatine tonsil, the number of positive cells in the BVDV/BHV1 group was significantly higher to the BVD controls from 2dpi until the end of the study and no differences were observed in the BHV1 group.

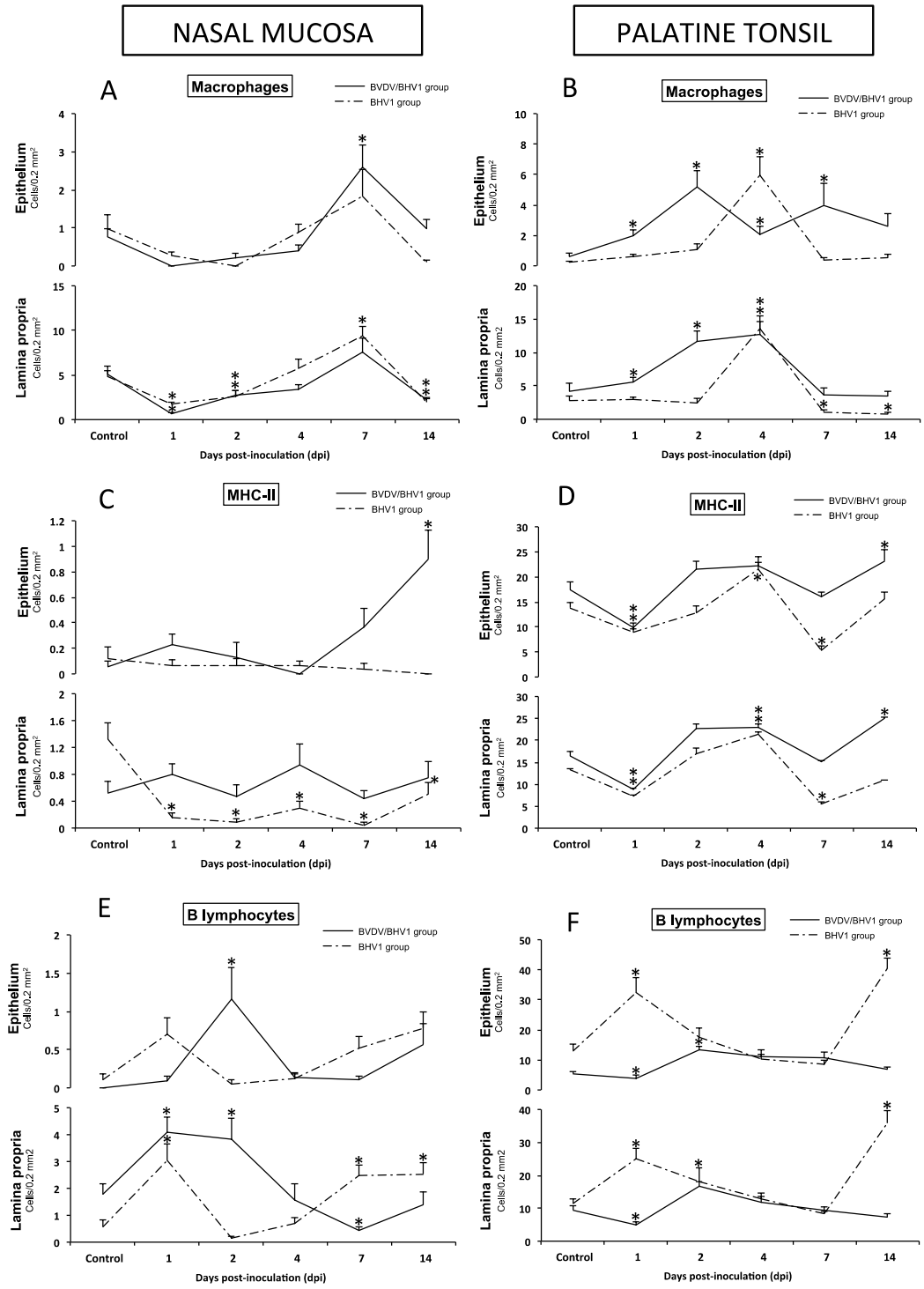


Figure 4. Counts of macrophages in the nasal mucosa (A) and palatine tonsil (B); Counts of cells expressing MHC-II molecules in the nasal mucosa (C) and palatine tonsil (D); B lymphocyte counts in the nasal mucosa (E) and palatine tonsil (F). From calves experimentally inoculated with BVDV and BHV-1 (BVDV/BHV1 group) versus calves infected only with BHV-1 (BHV1 group). Results are expressed as mean of cell counts ± SEM. *Significant differences ($P < 0.05$) between each infected group and their control group.

- **TNF-α**

In the epithelium of the nasal mucosa (Figure 5C), the number of TNFα+ cells in the BHV1 group was low and fluctuated throughout the study with significant low levels at 1, 4 and 14 dpi with respect to the negative controls , while in the BVDV/BHV1 group the only significant difference observed was the peak reached at 14 dpi. In the lamina propria of the nasal mucosa, a significant drop at 1 dpi was displayed in both groups, and then the values in the BHV1 group remained significantly lower than negative control group; while the BVDV/BHV1 group, showed a significant abrupt rise at 2 dpi that remained in values close to the BVDV control animals until the end of the study.

In the epithelium of the palatine tonsil (Figure 5D), in the BHV1 group the TNFα+ cells showed counts significantly higher than negative control group from 2 to 14 dpi; while in the BVDV/BHV1 group, after a slight significant decrease at 1 dpi, the values increased gradually between 2 to 4 dpi, displaying an abrupt significant increase at 14 dpi. In the lamina propria, the number of TNFα+ cells in the BVDV/BHV1 group showed a significant drop between 1 and 4 dpi with respect to the BVDV control animals; while in the BHV1 group, the counts displayed a significant and gradual increase from 2 dpi until the end of the study compared with the negative control.

- **IFN-γ**

A very scarce quantity of IFNγ+ cells was observed in the epithelium of both organs (Figures 5E and 5F) and in the lamina propria of the nasal mucosa, with no differences being observed in these regions between both infected groups and their respective control animals. Only a significant increase of IFNγ+ cells was observed in the lamina propria of the palatine tonsil in the BVDV/BHV1 group at 1dpi.

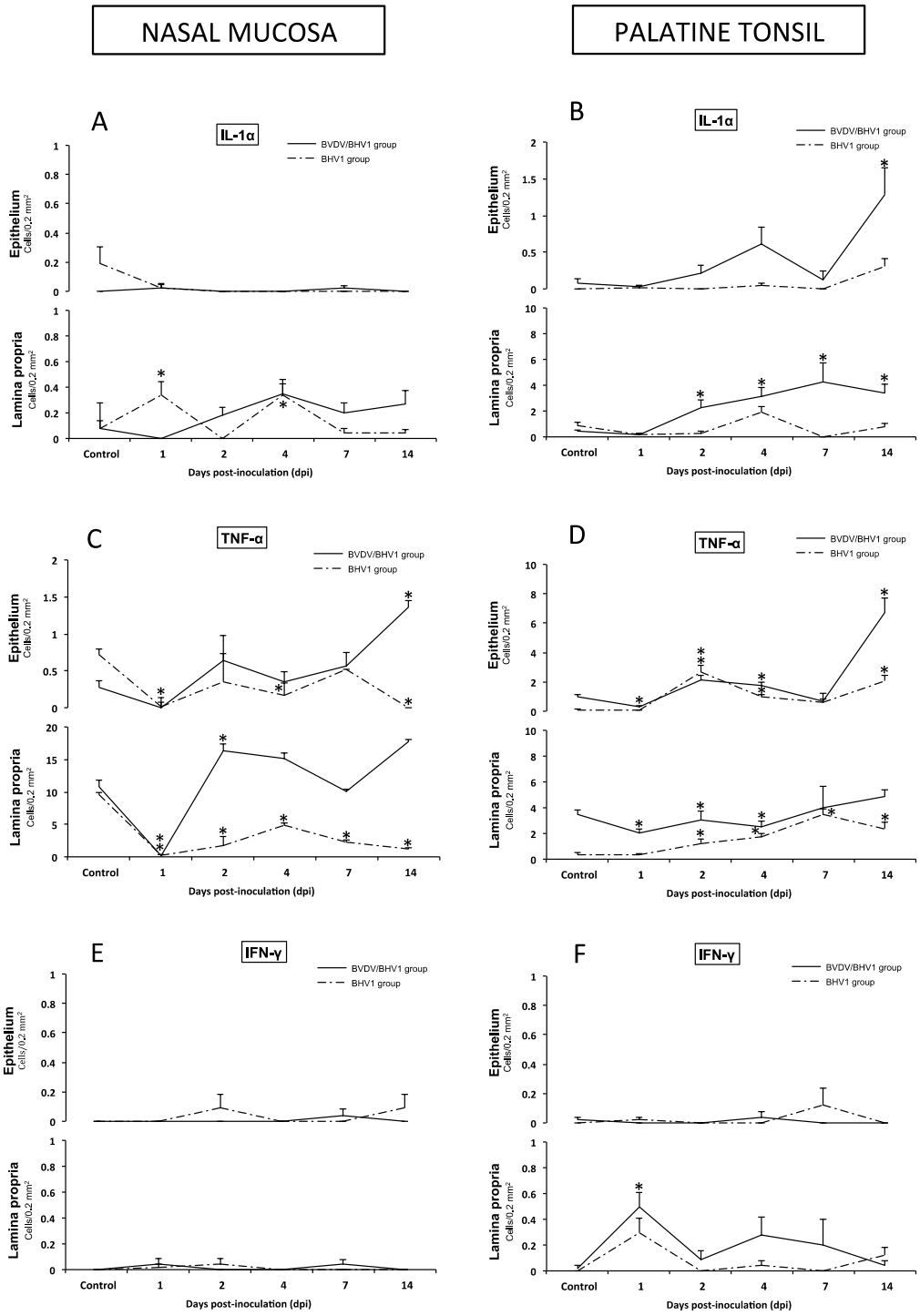


Figure 5. Counts of IL-1α positive cells in the nasal mucosa (A) and palatine tonsil (D); Counts of TNFα positive cells in the nasal mucosa (B) and palatine tonsil (E); IFNγ+ cell counts in the nasal mucosa (C) and palatine tonsil (F). From calves experimentally inoculated with BVDV and BHV-1 (BVDV/BHV1 group) versus calves infected only with BHV-1 (BHV1 group). Results are expressed as mean of cell counts ± SEM. *****Significant differences ($P < 0.05$) between each infected group and their control group.

4. Discussion

An immunological detailed knowledge of the primary organs of entry and replication of BVDV and BHV-1 such as nasal mucosa and palatine tonsil is very important since they represent the first defence line against foreign Ags entering by either respiratory or digestive routes. The presence of BHV-1 Ag was confirmed in both BVDV/BHV1 and BHV1 inoculated groups by PCR in nasal swabs samples from 1 dpi onwards, detecting viraemia only in the BVDV/BHV1 group from 4 dpi until the end of the experiment (Risalde et al., 2011b). Despite this fact, neither BVDV nor BHV-1 Ags have been detected in the present work from nasal mucosa tissues by IHC. However, eosinophilic intranuclear inclusion bodies have been identified in epithelial cells of the nasal mucosa mainly at 4 dpi by light microscopy, coinciding with the detection of BHV-1 Ag from palatine tonsil tissues by IHC. This absence of BHV-1 Ag in the nasal mucosa might be the result of evasion mechanisms of this virus exerted by gC that helps the virus to circumvent an important defence system of the host and allows an immune evasion early in infection, prior to Abs development by means of the cell-to-cell dissemination characteristic of BHV-1 (Harris et al, 1990; Friedman et al, 1996; Lubinski et al, 1998; Van Drunen Little-van den Hurt, 2007). Previous works pointed out that BHV-1 is not usually recovered from tissues others than those of the respiratory tract after experimentally induced respiratory tract infection (Gibbs and Rweyemamu, 1977; Potgieter et al., 1984a). Our studies indicate that BHV-1 detection was difficult by IHC also from tissues of the respiratory tract, as nasal mucosa. As to the palatine tonsil, the structural gC was only seen in the BVDV pre-infected calves subsequently challenged with BHV-1, and localized in necrotic areas of epithelial tonsillar crypts and underlining lamina propria. This inter-group difference might be due to the immunosuppressive effect exerted by BVDV, being that previous studies have suggested that BVDV may have a direct effect on lymphocytes, leading to the death of these cells (Marshall et al., 1996; Liebler-Tenorio et al., 2003a; 2004; Pedrera et al., 2009a). It seems that BVDV previous infection allows the dissemination of BHV-1 by impairing the local immune response due to cell-mediated immunity is the prime mechanism of containing BHV-1 infection in calves (Davies and Carmichael, 1973; Potgieter et al., 1984a).

Macrophages are a cell population widely distributed in most tissues and represent the body's first line of defence against infections and comprise a critical limb of the innate immune system exerting their function *in situ* (Jahnsen et al., 2004; Lebre and Tak, 2010). But in the nasal

mucosa of both BVDV/BHV1 and BHV1 infected groups, macrophages showed only a high count at 7 dpi. However, the lymphocytes subpopulations displayed in the nasal mucosa an earlier rise than macrophages, so also occur inter-group differences. The lamina propria of nasal mucosa was mainly infiltrated by CD8+ and γδ T lymphocytes and by CD4+ T lymphocytes to a lesser extent, between 2-4 dpi, being epithelium poorly infiltrated by immunocompetent cells. This could be due to the fact that BHV-1 can only cause a productive infection in certain cell types as epithelial cells of the upper respiratory tract and tonsils (Tikoo et al., 1995a), as well as CD4+ T cells (Lovato et al., 2003), monocytes and macrophages (Nyaga and McKercher, 1979; Forman et al., 1982). In contrast, palatine tonsil displayed an earliest increase and a delay between groups in the macrophage counts, peaking at 2 dpi in the BVDV preinfected calves, and at 4 dpi in the group only infected with BHV-1. Moreover, we observed necrosis foci only in palatine tonsil, displaying different effect and response between both tissues. Also, the profile of CD4+, CD8+ and γδ T lymphocytes was quite different in palatine tonsil compared with the nasal mucosa. In the palatine tonsil, the CD4+ T lymphocytes displayed inter-group differences, showing a large influx in the BHV1 group between 2 to 4 dpi, while in the BVDV/BHV-1 group exhibit at the same time-point an abrupt drop. Moreover, the CD8+ T lymphocyte counts in the BVDV/BHV1 group diminished progressively from 1 dpi until the end of the study, and in the BHV1 group, the CD8+ positive cells remained constant until 4 dpi, and from that day showed a marked decline. The early decline of CD4+ and CD8+ T lymphocytes in the BVDV/BHV group promotes greater BHV-1 spread and detection in the palatine tonsil, in contrast to the BHV1 group. This fact reflects that the effect caused by BVDV infection promotes a minor cell-mediated immune response, thus leading to a major spread of secondary viral infections like BHV-1 infection (Potgieter et al., 1984a; Castrucci et al., 1992; Risalde et al., 2011b; 2013). This fact also highlights the importance of these two lymphocyte subpopulations in the local immune response against secondary pathogens in primary replication organs sited in the first line of defence. Hence, containment of the virus in these organs of the upper respiratory and digestive tracts is crucial to prevent virus spread to deeper organs. The γδ T lymphocytes in the palatine tonsil were more numerous in the epithelium than in the lamina propria and displayed a delay between both groups, peaking at 2 dpi in the BVDV/BHV1 group and at 4 dpi in the BHV1 group. The number and distribution of γδ T lymphocytes in the bovine palatine tonsil is consistent with the proposed function for such cells of providing protection at epithelial surfaces (Hein and Mackay, 1991). Moreover, the γδ T

lymphocytes were more numerous in the palatine tonsil than in the nasal mucosa, highlighting the immunomodulatory character of the palatine tonsil since γδ T cells play an important role in bridging innate and adaptive cell-mediated immune responses (Guzman et al., 2012). Although the antiviral effect of γδ T cell populations has been previously described Guzman et al. 2012, and despite the fact that palatine tonsil is highly infiltrated by these cell type, it seems to be insufficient for the containment of the virus spread in the BVDV/BHV1 group, coupled to the numerical decline of CD4+ and CD8+ T lymphocytes. However, in the BHV1 group the γδ T lymphocytes increased with a delay compared with BVDV/BHV1 group and despite this, Ag was not detected in the palatine tonsil, highlighting the crucial role of CD4+ and CD8+ T lymphocytes. This also suggests that the BVDV, despite not being detected either in nasal mucosa or palatine tonsil, must have exerted its action in other organs of cell proliferation or lymphocyte maturation such as bone marrow or thymus in order for these animals to produce a lesser numerical response or by direct virus action on own cells by apoptosis (Spagnuolo et al., 1997; Wood et al., 2004; Pedrera et al., 2009a, Raya et al., 2012).

Bovine palatine tonsil and nasal mucosa is an important site for Ag uptake and initiation of the immune response (Jahnsen et al., 2004; Palmer et al., 2009). Both CD4+ and CD8+ T lymphocytes interact with APCs (i.e. DCs, macrophages and B lymphocytes). The Ag presentation process depends on the interaction of the T lymphocyte receptor with Ag-loaded MHC-II molecules on the surface of APCs resulting in the induction of an immune response (Banchereau and Steinman, 1998; Underhill et al., 1999; Ozbilgin et al., 2004; Chase, 2013).

The nasal mucosa, displayed higher B lymphocytes counts in the lamina propria than in the epithelium like occurs with other immunocompetent cells, showing a response at early time-points in both BVDV/BHV1 and BHV1 groups. In the palatine tonsil, the BHV1 group showed similar kinetic than in the nasal mucosa but with increased cell numbers, especially in the epithelium; however, the BVDV/BHV1 group fluctuated somewhat but did not show remarkable changes. Thus, the presence of MHC-II+ cells was very scarce in the nasal mucosa. However, the palatine tonsil exhibited an elevated amount of immunorreactive cells in both epithelium and lamina propria, being the profile similar in both structures, mainly at 2-4 dpi in both BVDV/BHV1 and BHV1 groups.

The absence of cellular expression of MHC-II observed in the nasal mucosa confirms that there is no subsequent Ag processing nor presentation via MHC-II, until the last days of the

experiment coinciding with the influx of macrophages. Thus, CD4+ T cells would be activated and not attracted to the site of infection justifying the shortage of this subpopulation in the nasal mucosa. The action against foreign pathogens in the nasal mucosa fall on CD8+ and γδ T cells through its cytotoxic action, which does not require prior Ag presentation. In the palatine tonsil, greater cell counts expressing MHC-II coincides with the BVDV/BHV1 group influx of macrophages and to a lesser extent with γδ T cells and viral Ag detection. However, in the BHV1 group coincides with the increased presence of all immunocompetent cell populations studied.

The study of proinflammatory cytokines such as IL1α, TNF-α and IFN-γ showed that TNF-α was the most expressed cytokine in the nasal mucosa, mainly in the lamina propria. However, the palatine tonsil displayed certain expression of IL1α and TNF-α, while IFN-γ was scarce chiefly in epithelium.

GENERAL CONCLUSIONS

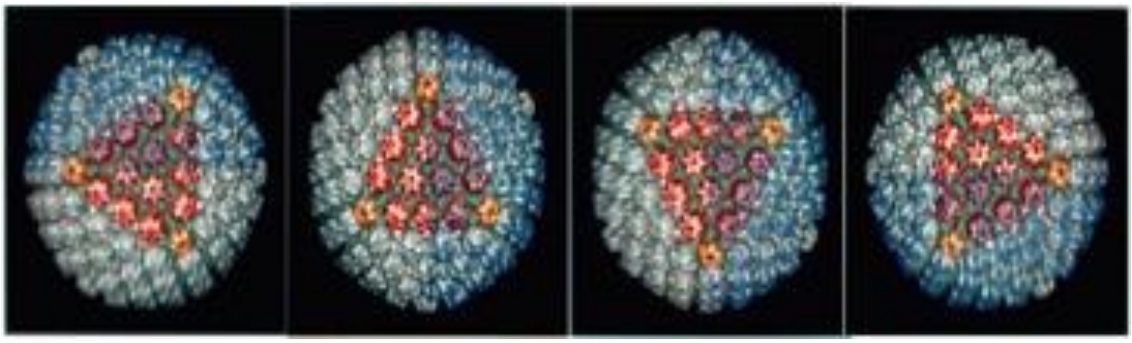
1. Calves experimentally infected with the ncp BVDV-1 strain 7443 present a subclinical manifestation of the disease characterized by a marked suppressive effect on CD4+, CD8+ and B lymphocyte subpopulations, associated with the degree of viremiaand an increase in acute phase proteins as haptoglobin and serum amyloid A. **CHAPTER 1**

2. Ncp BVDV-1 infection promotes a reduced Th1 immune response, which may result in a decreased ability to control infection, thus enhancing cattle susceptibility to secondary infections. **CHAPTER 1**

3. The failure of cell-mediated antiviral mechanisms developed against BHV-1 in previously BVDV infected calves could be partially due to the significant changes observed in CD8+ T lymphocytes and neutrophils, compared with healthy calves infected with BHV-1, resulting this subclinical BVDV infection in a less effective cell-mediated immune response against BHV-1 secondary infections. **CHAPTER 2**

4. γδ T lymphocytes, a critical component of the mucosal immunity, which are increased in the palatine tonsil after BHV-1 infection, seems to be insufficient for the containment of this pathogen in calves previously infected with BVDV. **CHAPTER 3**

5. The early decline of CD4+ and CD8+ T lymphocytes in the BVDV-infected calves promotes greater BHV-1 spread and detection in the palatine tonsil, highlighting the importance of these subpopulations in the local immune response against secondary pathogens in primary replication organs sited at the first line of defence. **CHAPTER 3**



SUMMARY

Bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) are important cattle pathogens, which induce a broad immunosuppression on cell-mediated immune response. Both BVDV and BHV-1 are involved in participation of bovine respiratory disease complex (BRDC) among others several microbial agents, being BRDC a major cause of economic loss in the cattle industry. BVDV infection of immunocompetent cattle can cause acute bovine viral diarrhea (BVD). Acute infections with low-virulence ncp BVDV are often asymptomatic or produce only mild clinical symptoms. However, their marked immunosuppressive effect predisposes affected cattle to other infections. Cell-mediated immune response plays a key role in countering infection both in BVDV and in BHV-1, since the humoral response is not sufficient to eliminate infected cells. In this way, the general aim was deepen in the study of systemic and local cell-mediated immune response in calves with subclinical BVD and challenged with BHV-1.

Based on these premises we design an experimental model in which thirty male Friesian calves (8-9 months old) were randomly assigned to three different groups. Fourteen calves were infected intranasally with ncp BVDV genotype 1 strain 7443. Twelve days later, when the calves did not show neither clinical signs nor viremia against BVDV; twelve of them (BVDV/BHV1 group) and twelve calves belonging to BHV1 group were challenged with BHV-1.1 Iowa strain. The other two animals inoculated with ncp BVDV-1 and BHV-1.1-free, were killed before BHV-1.1 inoculation and used as BVDV infection controls. The animals of the negative control group received dosis of tissue culture fluid free of viruses and were sacrified at the end of the study.

Rectal temperatures and clinical signs were monitored daily prior to virus inoculation and throughout the study. Blood, serum and plasma samples were obtained twice from each calf prior to BVDV inoculation in order to obtain basal values, for each analytical procedure. After BVDV inoculation, blood, serum and plasma samples were collected at 1, 2, 3, 6, 9 and 12 dpi BVDV from BVDV and control group calves; just as after BHV-1 inoculation at 6, 12 and 18 hpi BHV1 and 1, 2, 4, 5, 7 and 14 dpi BHV-1.1 for several assays. The presence of BVDV and BHV-1 in

blood was assessed by PCR and BVDV specific Abs were measured by ELISA. The systemic changes in immunocompetent cell populations were assessed by flow cytometry. The systemic inflammatory and immune response was analyzed through the study of serum concentrations of cytokines (IL-1β, TNF-α, IFNγ, IL-12, IL-4 and IL-10) measured by ELISA, and serum APPs levels (Hp, SAA, albumin and fibrinogen) determined by ELISA and colorimetric methods. After sacrificing the calves in batches of two at 1, 2, 4, 7 and 14 dpi and post-mortem examination, samples collected from nasal mucosa and palatine tonsil were fixed and routinely processed for histopathological and for immunohistological study in order to identify and quantify locally different lymphocyte populations and cytokine markers.

In order to characterize the systemic cell-mediated immune response implicated in acute BVD, our first objective (CHAPTER 1) was to study several blood parameters in a single group of experimentally infected calves with ncp BVDV-1. For this purpose we have examined the systemic changes of immunocompetent cell populations, APPs and cytokines, as well as, viremia and BVDV specific antibodies.

Following acute infection with the ncp BVDV-1 strain 7443, calves displayed biphasic temperature elevation, with a slight increase at 3 dpi and a more pronounced fever peak at 7 dpi at the same period of the most clinical symptoms appeared – though never more than moderate intensity. Additionally, viremia was detected between 3 and 9 dpi, and peaked at 6 dpi in parallel with hyperthermia and the highest intensity of clinical signs, highlighting the pathogenic action of the virus. Moreover, an inverse relationship has been observed between the degree of viremia and leukocyte counts. A marked decrease was observed in total leukocyte counts between 3 and 9 dpi; lymphocyte, monocyte and neutrophil levels were all depleted, thus confirming the tropism of BVDV on immunocompetent cell populations. Analysis of lymphocyte subpopulations revealed that the decline in total lymphocyte counts was due to a sharp decrease in CD4+ and CD8+ T cells, and also in B lymphocytes, between 3 and 6 dpi; levels of all three subpopulations subsequently recovered at 12 dpi, coinciding with no BVDV detection from blood samples at this time point. The drop in CD4+ T cell numbers critically impairs calf's ability to deal with a primary BVDV infection, pointing out the crucial importance of these cells in the control mechanisms against BVDV, since this population is responsible for the cell-mediated response during the early infection. However, CD8+ lymphocyte depletion appears to have no

effect in the length or intensity of viremia. Nevertheless, BVDV viremia has been observed in parallel with both CD4+ and CD8+ T cell depletion, subsequently coinciding their numerical recovery with non-BVDV haematological detection, which implies that CD8+ T cells depletion is actually associated with the presence of viremia. No significant changes were recorded in γδ T cell numbers in early stages of the study, displaying a significant increase at 12 dpi compared to the control group. Although it has been suggested that γδ T cells do not play a major role in resisting BVDV infection, these cells may be involved later stages of infection, controlling the resolution of pathogen-mediated inflammation by compensating CD4+ and CD8+ T lymphocyte subsets depletion. The circulating B lymphocyte numbers showed a significant decrease between 3-9 dpi, reaching a recovery at 12 dpi. No BVDV-specific Abs were detected throughout the study, given that Abs usually appear from 12 dpi. Thus, the lack of the BVDV-specific Abs detection was most likely to be due to the short time period of the present study, rather than to the sharp drop of the B lymphocyte counts after the BVDV infection.

Positive APPs in cattle include Hp, SAA and fibrinogen are considered as indicators of the acute phase response (APR) in this species. Major APPs in cattle (SAA and Hp) are characterized by showing a dramatical increase, thus they may be considered as good indicators of acute inflammation during viral infections. An increase was observed in these major bovine APPs (Hp and SAA); peak concentrations in serum were observed at 9 dpi, coinciding with an increase in IL-1β, a key player in the process of inflammation. In contrast to IL-1β, serum TNF-α levels, after a slight increase at 1 dpi that coincides with a slight rise in CD4+ lymphocyte counts, declined slightly in the course of the APR. The drop in TNF-α level was probably linked to both a decrease in circulating monocyte numbers due to ncp BVDV infection and to IL-10 production, being IL-10 a cytokine displaying anti-inflammatory activity in cattle. This would suggest that TNF-α does not play a key role in anti-BVDV action, and indeed that this pro-inflammatory cytokine may be directly or indirectly inhibited by the virus, at least during the acute phase of infection. Additionally, an early increase was observed in both IFN-γ and IL-12 levels; IL-12 started to rise at 3 dpi and by 9 dpi had returned to pre-inoculation values, remaining without changes until the end of the study. However, IFN-γ suffered a significant rise as from 1 dpi that coincided with a slight increase in TNF-α and CD4+ lymphocyte numbers, the major producers of IFN-γ; indicating that type 1 cytokines levels were not affected by BVDV infection and asserting the strength of the IFN-γ response to infection. Despite this, viremia peaked at 6 dpi showing an IFN-γ failure in

resisting BVDV infection. The analysis of cytokines related to a type 2 response, such as IL-4, revealed no systemic changes following infection. A slight increase in IL-10, involving in regulating the pro-inflammatory response, was observed between 3-12 dpi, matching the decline in TNF-α. Pattern of cytokine production by T cells is influenced by the cytokine background existing during early stages of activation of the T cell response. Here, however, although CD8+ T cell numbers were depleted over a longer period, the release of IFN-γ was stronger than that of IL-4 by CD4+ T cells. These results suggest that following an acute ncp BVDV-1 infection there is a clear tendency towards a type 1 cytokine response that should prevent the development of pathological effect, but a reduced response would likely result in a decreased ability to control infection, enhancing susceptibility to secondary infections.

Our second objective (CHAPTER 2) was to deep in the systemic consequences after the immunosuppression associated to an acute BVDV infection, especially the susceptibility of BVD calves against secondary infections with BHV-1. For that, we examine systemic changes in leukocyte counts and lymphocyte subpopulations appeared in apparently recovery calves of an experimental bovine viral diarrhea (BVD) and in healthy calves, both challenged with BHV-1.1. Parallel studies on kinetics of the cellular response may give valuable information for the understanding of the cell-mediated immune response during co-infections with these viral agents in BRD.

The results displayed inter-group differences of immunocompetent cells. Whilst leukocyte populations such as monocytes displayed no significant alteration, lymphocyte subpopulations and neutrophils exhibited changes in behaviour. In BVDV-infected calves, leukocyte numbers displayed significantly lower leukocyte levels than healthy calves at 0 dpi, i.e., 12 dpi BVDV calves inoculated with BVDV; this suggested a difference in status prior to inoculation of the secondary pathogen BHV-1 due to the immunosuppressive effect of BVDV. Moreover, analysis of the various leukocyte populations indicated that this difference was due mainly to a significant lower number of neutrophils, which constitute the first line of cellular defence against invading pathogens. Since lymphocytes and monocyte-macrophages are the primary target cells for BVDV replication, it was somewhat surprising that initial levels of these cell populations should be similar in healthy and BVDV-infected calves prior to inoculation with BHV-1.

Following inoculation with BHV-1, a significant difference in leukocyte counts was observed between the BVDV/BHV1 and BHV1 groups, mainly affecting neutrophils and, to a lesser extent, lymphocytes and monocytes. In the BHV1 group, leukocyte depletion was noted from 4 dpi. Paradoxically, calves in the BVDV/BHV1 group – despite to present a lower leukocyte number, due probably to immunodepression induced by BVDV inoculation – displayed leukocytosis resulting from an increase in circulating neutrophils, with no significant change in monocyte numbers even though lymphopenia was more severe than in the BHV1 group. The neutropenia prompted by BHV-1 in the previously healthy group contrasted with the neutrophilia observed in the BVDV/BHV1 group, which had initially exhibited BVDV-induced neutropenia. These findings could be indicating the continuing ability of calves with BVDV to react to infection by recruiting neutrophils.

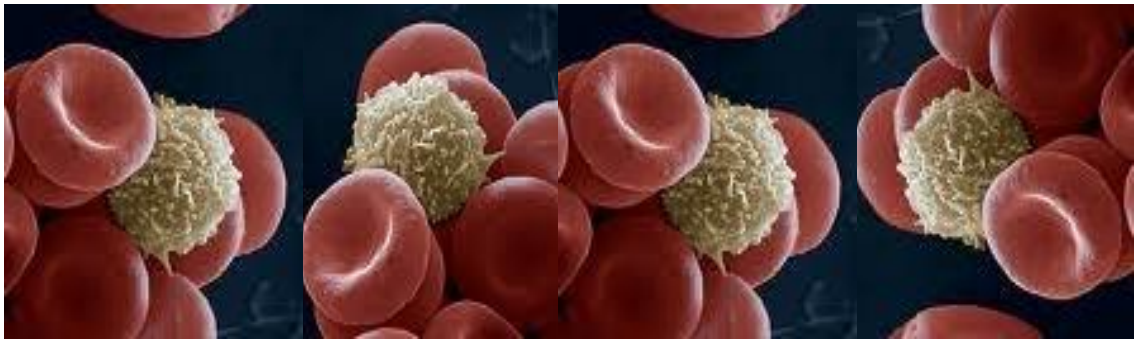
A key role in the impairment of antiviral capacity may be played by circulating T lymphocyte subpopulations (CD4+, CD8+ and γδ) and B cells, involved among others in the cell-mediated immune response, which are known to be capable of interacting with infected cells. Analysis of lymphocyte subpopulations showed that CD4+ T cell levels were similar in healthy calves and calves with subclinical BVD, prior to inoculation with BHV-1, reflecting normal values. Following inoculation with BHV-1, both groups displayed a slight decline in CD4+ T cell numbers at 4 dpi, reaching normal levels by the end of the study in both groups. This would suggest that BVDV does not impair the regenerative capacity of circulating CD4+ T cells. The similarity in CD4+ lymphocyte kinetics indicates that the changes observed were induced probably by BHV-1. Although CD8+ T cell levels were similar between BVDV/BHV1 and BHV1 groups prior to BHV-1 inoculation, significant differences in cell counts were apparent from 6 hpi with BHV-1. The early constant decline observed in the BVDV/BHV1 group was not seen in the BHV1 group, suggesting that it was attributable not to infection of these cells by BHV-1 but rather to BVDV induction at this time point. Between 1 and 7 dpi, CD8+ T cell counts behaved in a similar manner in the two groups, being the decline in numbers more pronounced in the BVDV/BHV1 group. The third lymphocyte subpopulation studied, γδ T lymphocytes displayed the highest count in the BVDV/BHV1 group prior to infection with BHV-1. This initial difference might have affected the response to secondary infection. γδ T cells reached normal levels in the BVDV/BHV1 group at 6 hpi; and from this time point, kinetics and cell counts remained similar in both groups throughout the study. About B lymphocyte counts, pre-inoculation counts were similar in both

groups; but from 1 dpi onwards the BVDV/BHV1 group showed lower number than the BHV1 group, possibly reflecting the more reduced ability to produce B cells. In conclusion, calves infected previously with BVDV were more affected concerning immunocompetent cells counts than healthy animals, after BHV-1 infection. The early decline of CD8+ T lymphocyte subpopulation coupled with significant numerical changes found in the neutrophil population, may contribute partially to poor cell-mediated immune response against secondary viral infections with BHV-1, specially in BVDV-infected calves.

Frequently, BVDV and BHV-1 cause severe respiratory and digestive tract disease in cattle associated with concurrent infections. Although it is known that both viruses can colonize the respiratory tract on its own, their participation in the BRD is mainly due to their enhancer role in mixed infections derived from their significant immunosuppressive effect.

It known that human nasal mucosa is extremely rich in antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages that play a key regulatory role in the adaptive immune system, pointing to the nasal mucosa as an efficient defence against viral pathogens and in the maintenance of immunologic homeostasis. So, the bovine nasal mucosa may represent an attractive site in order to study the pathogenesis of respiratory diseases such as BVD and IBR after experimental intranasal infections. Additionally, it is well known that human and animals palatine tonsils are sites for replication of several pathogens entering through either the oral or nasal cavities. Specifically, in cattle, palatine tonsils have a crucial role in immune responses and in the pathogenesis of several infectious diseases due to represent the first line of specialized-defence in oro-nasal route infections. Indeed, in both BHV-1 and acute BVDV infections, the tonsils are the initial sites of replication. With the purpose of describe local cell-mediated immunity in the nasal mucosa and palatine tonsil after mixed respiratory infections, the third objective (CHAPTER 3) was carried out the immunophenotypic characterization and quantification of immune effector cells and their biosynthetic activity from calves pre-infected with BVDV comparing with healthy calves both challenged with BHV-1. Overall, after BHV-1 infection the lamina propria of the nasal mucosa was more infiltrated than the epithelium. The CD8+ and γδ T cells and B lymphocytes appeared earlier than macrophages, showing a delay in the BVDV/BHV1 group compared with the BHV1 group. However, in the palatine tonsil showed high number of immunocompetent cells in both epithelium and lamina propria, and displayed

differences between both groups. In the BHV1 group, the immune response was characterized by the presence of CD4+ and γδ T lymphocytes and B cells, and to a lesser extent by CD8+ T cells. In the BVDV/BHV1 group, after BHV-1 infection the γδ T lymphocytes showed an earlier influx coupled with a rise in the macrophage counts, compared with the BHV-1 group. However, CD4+ and CD8+ lymphocytes exhibited an abrupt and progressive drop and B lymphocytes displayed no remarkable changes.



RESUMEN

El virus de la diarrea vírica bovina (vDVB) y el herpesvirus bovino tipo 1 (HVB-1) son patógenos importantes del ganado bovino, los cuales inducen una profunda inmunosupresión que afecta a la respuesta inmune celular. Ambos virus destacan por su participación en el síndrome respiratorio bovino (SRB), entre otros muchos agentes patógenos, tanto víricos como bacterianos, siendo este SRB una de las mayores causas de pérdidas económicas en la industria del vacuno. Entre los cuadros clínicos que aparecen tras la infección de terneros inmunocompetentes con el vDVB nos centraremos en la diarrea vírica bovina (DVB) en su proceso agudo. Las infecciones agudas con cepas ncp del vDVB de baja virulencia se presentan frecuentemente asintomáticas o producen sólo una sintomatología clínica leve. Sin embargo, su marcado efecto inmunosupresor predispone a los animales afectados a padecer otras infecciones secundarias. La respuesta inmune celular juega un papel muy importante en la resolución de estas infecciones, ya que la respuesta inmune humoral no es capaz de eliminar las células que han sido infectadas, por sí sola. En este sentido, el objetivo general que planteamos fue profundizar en el estudio de la respuesta inmune celular sistémica y local en terneros con DVB subclínica y su respuesta ante una infección secundaria con el HVB-1.

En base a estas premisas diseñamos un modelo experimental en el que 30 terneros macho de raza Frisona de entre 8-9 años, se distribuyeron al azar en tres grupos. Infectamos 14 terneros intranasalmente con la cepa ncp 7443 del vDVB genotipo 1. Pasados doce días, cuando los animales no presentaron sintomatología clínica ni viremia, 12 terneros de este grupo (grupo BVDV/BHV1) y 12 terneros del grupo BHV1 aún sin infectar, se inocularon con la cepa Iowa del HVB-1 subtipo 1 (HVB-1.1). Los otros 2 animales, inoculados con el vDVB y libres de HVB-1, se sacrificaron previamente a la infección con HVB-1 y se usaron como controles de la infección del vDVB. Los animales del grupo control negativo recibieron medio de cultivo libre de virus y se sacrificaron al final del experimento.

La temperatura rectal y los signos clínicos se controlaron diariamente, en días previos a las infecciones y durante el experimento. Las muestras de sangre, suero y plasma se obtuvieron

de cada uno de los terneros, en dos ocasiones antes de la infección con el vDVB para obtener los valores basales y tras la infección con el vDVB en los 1, 2, 3, 6, 9 and 12 dpi de los terneros de los grupos BVDV y control; así como justo después de la infección con HVB-1 a las 6, 12 and 18 horas post-infección (hpi BHV-1.1) y 1, 2, 4, 5, 7 and 14 dpi BHV-1.1 para varios de los ensayos. La presencia del vDVB y el HVB-1 en sangre se obtuvo mediante PCR y los anticuerpos (Abs) específicos para el vDVB se cuantificaron por ELISA. El estudio de las poblaciones celulares inmunocompetentes se realizó mediante citometría de flujo. La respuesta inmune inflamatoria sistémica se analizó a través del estudio de las concentraciones de citoquinas (IL-1β, TNF-α, IFNγ, IL-12, IL-4 y IL-10) medidas por ELISA y la determinación de los niveles de las proteínas de fase aguda (PFAs): haptoglobina (Hp), proteína amiloide A sérica (SAA), albumina y fibrinógeno mediante ELISA y métodos colorimétricos. Tras el examen post-mortem de los animales sacrificados en grupo de dos a los 1, 2, 4, 7 y 14 dpi de la infección con el HVB-1, se tomaron muestras de tejidos de la mucosa nasal y la tonsila palatina que se fijaron y se procesaron rutinariamente para realizar el estudio histopatológico e inmunohistoquímico, con el fin de identificar y cuantificar a nivel local diferentes subpoblaciones linfocitarias y sus marcadores específicos para citoquinas (IL-1α, TNF-α e INF-γ).

Con la finalidad de caracterizar la respuesta inmune sistémica celular implicada en la DVB aguda, nuestro el primer objetivo (CAPÍTULO 1) fue estudiar varios parámetros sanguíneos en un único grupo de terneros infectados experimentalmente con una cepa ncp del vDVB. Para ello, nos propusimos examinar los cambios sistémicos de las poblaciones inmunocompetentes, PFAs y citoquinas, así como, la viremia y los Ab específicos contra el vDVB.

Tras la infección aguda con la cepa ncp 7443 del vDVB, los terneros presentaron un aumento bifásico de la temperatura: primero, un ligero incremento en el 3 dpi y, segundo, otro pico de fiebre más pronunciado en el 7 dpi, coincidiendo en el tiempo con la aparición de la mayoría de síntomas clínicos, que fueron de intensidad moderada. La viremia se detectó entre el 3 y el 9 dpi, mostrando el valor máximo el 6 dpi, paralelamente a la fiebre y a la sintomatología clínica más intensa, revelando la acción patógena del virus. Además, se observó una relación inversa entre el grado de viremia y el recuento de leucocitos total. El recuento total de leucocitos presentó un marcado descenso entre el 3 y el 9 dpi; mostrando la depleción en las poblaciones de linfocitos, monocitos y neutrófilos, confirmando el tropismo del vDVB por las

poblaciones de células inmunocompetentes. El análisis de las subpoblaciones de linfocitos reveló que el descenso de los linfocitos totales se debió a un descenso acusado en los linfocitos T CD4+ y CD8+, y también en los linfocitos B, entre los 3 y 6 dpi; recuperándose los niveles de las tres subpoblaciones en el 12 dpi, coincidiendo con la ausencia del vDVB en sangre. La caída numérica de los linfocitos T CD4+ debilita la capacidad de los terneros de superar una infección primaria con el vDVB, señalando la crucial importancia de estas células en el control de los mecanismos contra el vDVB, ya que esta subpoblación es la responsable de la respuesta inmune celular durante la fase temprana de la infección. Sin embargo, la depleción de los linfocitos T CD8+ parece no tener efecto en la duración o intensidad de la viremia. No obstante, la viremia del vDVB se observó en paralelo con la depleción de los linfocitos T CD4+ y CD8+, coincidiendo su recuperación numérica posterior con la ausencia de detección sanguínea del vDVB, lo que implicaría que la depleción de los linfocitos T CD8+ está realmente asociada con la viremia. No se detectaron cambios en el recuento de los linfocitos T γδ en estadios tempranos del estudio, mostrando un posterior aumento significativo en el 12 dpi, comparado con el grupo control. Aunque se ha sugerido que las células T γδ no juegan un papel importante en la resistencia a la infección con el vDVB, estas células pueden estar implicadas en estadios tardíos de la infección, controlando la resolución de la inflamación mediada por patógeno de manera que compensaría la depleción de las subpoblaciones de linfocitos T CD4+ y CD8+. Por otro lado, no se detectaron Abs específicos frente al vDVB a lo largo del estudio, dado que suelen aparecer a partir de los 12 dpi tras la infección. Así, la ausencia de Abs se debió más al hecho de que el periodo del estudio fue corto para poder detectarlos, más que al descenso acusado que sufrieron los linfocitos B tras la infección con el vDVB.

Las PFAs positivas en bovino incluyen la Hp, SAA y fibrinógeno y se consideran indicadores de la respuesta de fase aguda en esta especie. Las PFAs mayores en bovino (SAA y Hp) se caracterizan por un incremento dramático, considerándose muy buenos indicadores de la inflamación aguda durante las infecciones víricas. En nuestro estudio observamos un pico significativo en las concentraciones séricas de estas PFAs el 9 dpi, coincidiendo con un incremento de la IL-1β, un componente clave del proceso inflamatorio. A diferencia de la IL-1β, los niveles de TNF-α en suero, tras un ligero incremento el 1 dpi que coincidió una una ligera subida en el recuento de los linfocitos T CD4+, experimentaron un descenso durante el curso de la respuesta de fase aguda. La caída de los niveles de TNF-α estuvieron probablemente unidos

tanto al descenso del número de monocitos circulantes, como consecuencia de la infección con el vDVB, como a la producción observada de IL-10, siendo la IL-10 una citoquina que promueve la acción anti-inflamatoria. Esto podría sugerir que el TNF-α no juega un papel importante en la lucha contra el vDVB; de hecho, esta citoquina pro-inflamatoria podría ser inhibida directa o indirectamente por el virus, al menos durante la fase aguda de la infección. Además, observamos incremento temprano en los niveles de IFN-γ e IL-12. El IFN-γ coincidió con el ligero aumento experimentado por el TNF-α y el número de linfocitos T CD4+, los mayores productores de IFN-γ; indicando que los niveles de citoquinas de tipo 1 no se vieron afectadas por la infección con el vDVB, lo que constata la fuerza de la respuesta del IFN-γ frente a la infección. A pesar de esto, la viremia tuvo un pico a los 6 dpi mostrando la debilidad del IFN-γ en la resistencia a la infección por el vDVB. La IL-4, relacionada con la respuesta inmune de tipo 2, no mostró cambios significativos tras la infección. Estos resultados sugieren que tras una infección aguda con una cepa ncp del vDVB hay una clara tendencia hacia una respuesta de citoquinas de tipo 1, que podría prevenir del desarrollo de efectos patológicos, aunque una respuesta reducida podría resultar en un descenso de la capacidad para controlar la infección, aumentando la susceptibilidad de infección de otros agentes patógenos secundarios.

Nuestro segundo objetivo (CAPÍTULO 2) fue profundizar en las consecuencias sistémicas tras la inmunosupresión asociada a la infección aguda con el vDVB, especialmente la susceptibilidad de los terneros con DVB frente a infecciones secundarias con el HVB-1. Para ello, examinamos los cambios numéricos de los leucocitos y las subpoblaciones de linfocitos circulantes en terneros aparentemente recuperados de la DVB inducida experimentalmente y en terneros sanos, que son infectados con el HVB-1.1. Los estudios en paralelo de la cinética de la respuesta de las células inmunocompetentes desarrollada en ambos grupos podrían aportar información valiosa para entender la respuesta inmune celular durante infecciones mixtas con estos agentes víricos en el SRB.

Las células inmunocompetentes presentan diferencias entre ambos grupos BVDV/BHV1 y BHV1. Mientras que los monocitos no muestran una alteración significativa, los linfocitos y los neutrófilos exhibieron cambios en su comportamiento. Los terneros infectados con el vDVB presentaban un menor recuento de leucocitos total que los animales sanos en el 0 dpi, que corresponde con el 12 dpi tras la infección con el vDVB. Estas diferencias previas a la infección

secundaria con el HVB-1, mostrando al efecto inmunosupresor del vDVB, se debieron principalmente al descenso numérico significativo de los neutrófilos, los cuales constituyen la primera línea de la defensa celular contra patógenos. Por el contrario, los linfocitos y los monocitos-macrófagos muestran valores iniciales similares en los animales sanos y los infectados con el vDVB, previamente a la inoculación con el HVB-1, lo que sorprende dado que representan las principales células diana de replicación el vDVB.

Tras la inoculación con el HVB-1, se continúa observando la diferencia significativa de los leucocitos totales entre grupos, afectando principalmente a los neutrófilos y, en menor medida, a los linfocitos y los monocitos. En el grupo de animales sanos infectados con el HVB-1 (grupo BHV1), la depleción de los leucocitos se notó a partir del 4 dpi. Paradójicamente, los terneros con DVB (grupo BVDV/BHV1), a pesar de presentar un menor número de leucocitos antes de la infección con el HVB-1, mostraron una leucocitosis resultado de un incremento en los neutrófilos circulantes, sin experimentar cambios significativos en el número de monocitos a pesar de que la linfopenia fue más severa que en el grupo BHV1. La neutropenia provocada por el HVB-1 en los terneros sanos contrasta con la neutrofilia observada en el grupo terneros expuestos al vDVB. Estos resultados podrían indicar la continua capacidad de reacción de los terneros con DVB frente a la infección secundaria, a través del reclutamiento de neutrófilos.

Las subpoblaciones de linfocitos T circulantes (CD4+, CD8+ y γδ) y las células B juegan un papel clave en la capacidad antiviral, ya que participan en la respuesta inmune celular, interactuando con las células infectadas. El análisis de estas subpoblaciones mostró que los niveles de linfocitos T CD4+ fueron similares en los animales sanos y los animales con DVB, antes de la inoculación con el HVB-1, reflejando valores normales. Tras la inoculación con el HVB-1, ambos grupos de animales presentaron un descenso en el número de células T CD4+ el 4 dpi, alcanzando los valores normales hacia al final del experimento en ambos grupos. Esto podría sugerir que el vDVB no debilita la capacidad de regeneración de los linfocitos T CD4+ circulantes. La similitud en la cinética de esta subpoblación linfocitaria entre ambos grupos podría indicar que los cambios observados fueron inducidos probablemente por el HVB-1. Aunque el recuento de linfocitos CD8+ fue similar entre los grupos BVDV/BHV1 and BHV1 antes de la inoculación del HVB-1, sin embargo se observaron diferencias significativas en el recuento celular a partir de 6 hpi tras la infección del HVB-1. Este descenso temprano observado en el grupo BVDV/BHV1 no se observó en el grupo BHV-1, sugiriendo que no se debió solo a la infección de estas células por

el HVB-1 sino también al efecto que habría ejercido ya el vDVB. Entre el 1 y el 7 dpi, los linfocitos T CD8+ se comportaron de manera similar en los dos grupos, siendo el descenso más pronunciado en el grupo BVDV/BHV1. La tercera subpoblación de linfocitos estudiada, los linfocitos T γδ mostraron el mayor recuento en el grupo BVDV/BHV1 antes de la infección con el HVB-1. Esta diferencia inicial podría afectar a la respuesta frente a la infección secundaria. Las células T γδ del grupo BVDV/BHV1 alcanzaron los valores normales a las 6 hpi y a partir de ese momento, las cinéticas y el recuento celular fueron similares en ambos grupo a lo largo del estudio. En cuanto al recuento de los linfocitos B, los valores previos a la inoculación con el HVB-1 fueron similares en ambos grupos y, a partir del 1 dpi en adelante, mostraron un descenso que fue más acusado en el grupo BVDV/BHV1 que en el grupo BHV1, reflejando posiblemente una capacidad más reducida de producir células B. En general, los terneros infectados previamente con el vDVB estuvieron más afectados en cuanto al recuento de células inmunocompetentes que los animales sanos, tras la infección con el HVB-1. El descenso temprano en el número de linfocitos T CD8+ junto con los cambios numéricos significativos de la población de neutrófilos podría contribuir parcialmente a una respuesta inmune celular pobre contra infecciones víricas secundarias con el HVB-1, especialmente en terneros infectados con el vDVB.

Frecuentemente, el vDVB y el HVB-1 causan enfermedades severas del tracto respiratorio y digestivo bovino, asociadas con infecciones concurrentes. Aunque ambos virus colonizan por sí mismos ambos tractos, su participación en el SRB se debe principalmente a su papel potenciador en infecciones mixtas, consecuencia de su efecto inmunosupresor.

La mucosa nasal humana es extremadamente rica en células presentadoras de antígenos (APCs) como son las células dendríticas (DCs) y los macrófagos, que juegan un papel clave regulador en la respuesta inmune adaptativa, señalando la mucosa nasal como un lugar eficiente de defensa contra ptógenos víricos y de mantenimiento de la homeostasis inmunológica. En homología, la mucosa nasal del bovino podría representar un sitio atractivo de estudio de la patogénesis de enfermedades respiratorias como la DVB y la rinotraqueítis infecciosa bovina (IBR), causada por el HVB-1, tras infecciones intranasales experimentales. Además, en el ganado bovino, la tonsila palatina tiene un papel fundamental en la respuesta inmune y en la patogénesis de varias enfermedades infecciosas debido a que representan la primera línea de defensa especializada en infecciones por la ruta oro-nasal. De hecho, en infecciones con el vDVB

y el HVB-1, las tonsilas son sitios de replicación primaria. Con el propósito de describir la respuesta inmune celular local en la mucosa nasal y la tonsila palatina tras infecciones respiratorias mixtas, el tercer objetivo (CAPÍTULO 3) que nos planteamos fue llevar a cabo una caracterización inmunofenotípica, así como la cuantificación de las células efectoras del sistema inmune y su actividad biosintética en tejidos de terneros infectados con el vDVB comparando con terneros sanos, ambos grupos inoculados con el HVB-1. En general, tras la infección con el HVB-1, la lamina propia de la mucosa nasal estuvo más infiltrada de células inmunocompetentes que el epitelio. Los linfocitos T CD8+ y γδ y los linfocitos B aparecieron antes que los macrófagos, los cuales muestran un retraso en el grupo BVDV/BHV1 comparado con el grupo BHV1. Sin embargo, la tonsila palatina presentó un elevado número de células inmunocompetentes tanto en el epitelio como en la lámina propia, mostrando diferencias entre ambos grupos tras la infección con el HVB-1. En el grupo BHV1, la respuesta inmune se caracterizó principalmente por la presencia de linfocitos T CD4+ y γδ, así como de linfocitos B, y en menor medida de linfocitos T CD8+. El grupo BVDV/BHV1 mostró una gran afluencia de linfocitos T γδ, que fue anterior en el tiempo al aumento del número de macrófagos, en comparación con el grupo BHV1. Sin embargo, los linfocitos T CD4+ y CD8+ exhibieron un descenso acusado, no mostrando cambios importantes en la subpoblación de linfocitos B.

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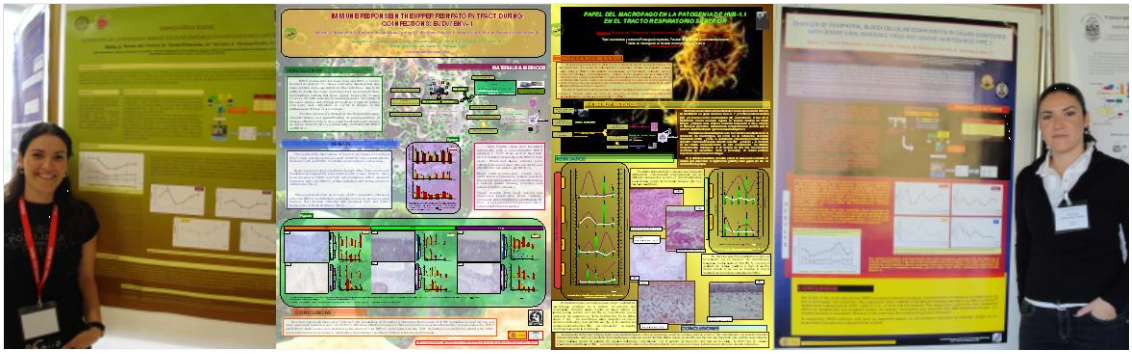

ANNEX

Scientific contributions

PUBLICATIONS

Molina, V., M.A. Risalde, P.J. Sánchez-Cordón, F. Romero-Palomo, M. Pedrera, B. Garfia and J.C. Gómez- Villamandos, 2012: Cell-mediated immune response during experimental acute infection with bovine viral diarrhea virus (BVDV): evaluation of blood parameters. Transbound. Emerg. Dis. (doi: 10.1111/tbed.120002)

Área: Ciencias Veterinarias. Índice de impacto: 1.809. Posición: 22 de 143.

Molina, V., M.A. Risalde, P.J. Sánchez-Cordón, M. Pedrera, F. Romero-Palomo, C. Luzzago and J.C. Gómez- Villamandos, 2013: Effect of infection with BHV-1 on peripheral blood leukocytes and lymphocyte subpopulations in calves with subclinical BVD. Res. Vet. Sci. (doi: 10.1016/j.rvsc.2013.02.018.)

Área: Ciencias Veterinarias. Índice de impacto: 1.649. Posición: 27 de 143.

Molina, V., M.A. Risalde, F. Romero-Palomo, P.J. Sánchez-Cordón, M. Pedrera and J.C. Gómez-Villamandos, 2012: Enfermedades respiratorias bovinas: vías de entrada, respuesta inmune y medidas profilácticas. Tierras (ganadería) 189, pp 62-68.

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COMMUNICATIONS AT MEETINGS

Molina, V., 2012: Respuesta inmune sistémica y local en terneros con DVB subclínica enfrentados a una infección secundaria con el HVB-1. In: I Congreso Científico de Investigadores en Formación en Agroalimentación de la eidA3 y II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba, Córdoba (Spain). Oral communication

Molina V., M.A. Risalde, M. Pedrera, F. Romero-Palomo, P.J. Sánchez-Cordón and J.C. Gómez-Villamandos, 2011: Valoración del papel inmunosupresor del virus de la Diarrea Vírica Bovina en infecciones agudas. In: XXXVI Congreso de la Sociedad Española de Inmunología, Pamplona (Spain). Poster communication

Molina V., M.A. Risalde, M. Pedrera, P.J. Sánchez-Cordón, B. Rodríguez-Sánchez and J.C. Gómez-Villamandos, 2010: Inmune response in the upper respiratory tract during coinfections BVDV/BHV1. In: 28th Meeting of the European Society of Veterinary Pathology and European College of Veterinary Pathologsits, Belgrade (Serbia). Poster communication

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Molina V., M.A. Risalde, M. Pedrera, P.J. Sánchez-Cordón, F. Romero-Palomo and J.C. Gómez-Villamandos, 2010: Papel inmunomodulador de la tonsila faríngea y mucosa nasal en la patogénesis del herpesvirus bovino-1. In: XXXV Congreso de la Sociedad Española de Inmunología, San Sebastián (Spain). Poster communication

Molina V., P.J. Sánchez-Cordón, J.C. Gómez-Villamandos, M.A. Risalde, B. Rodríguez-Sánchez and M. Pedrera, 2010: Detección y cuantificación mediante RT-PCR en tiempo real del virus de la Diarrea Vírica Bovina en sangre y tejidos de terneros infectados experimentalmente. In: XXXV Congreso de la Sociedad Española de Inmunología, San Sebastián (Spain). Poster communication

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ACKNOWLEDGEMENTS

A mi director de Tesis Prof. Dr. José Carlos Gómez Villamandos, al que estaré eternamente agradecida por confiar en mí y darme la oportunidad de cumplir mi deseo de realizar el doctorado. Por hacernos ver la luz en la oscuridad, por apoyarnos incondicionalmente en todos nuestros proyectos, por dejarnos volar y a la vez hacernos crecer. Por decir siempre: "Sí, adelante". Sin ti nada habría sido posible.

A mi co-director Dr. Pedro José Sánchez Cordón, por enseñarme a dar los primeros pasos en este mundo, a veces complicado.

A la ya Dra. Mª Ángeles Risalde Moya. Mi compañera de fatigas pero mucho más de risas. Ha sido un placer recorrer este arduo camino junto a ti, porque a tu lado todo es más fácil. Gracias por ser tan generosa, por lo que me has enseñado a nivel profesional y personal. Gracias Amiga.

A Fernando Romero y Miriam Pedrera por su apoyo y por compartir tantos momentos juntos, con nuestros terneros y sin ellos. A José Lorenzo Romero "Pachi", Antonio Da Silva, Fran Silva y Ana Raya. Por su participación de una u otra forma, este trabajo también es suyo. A Gema Muñoz, nuestra joya más oculta, por ser indispensable en nuestro trabajo. A Esther López, por todas las gestiones, que no son pocas.

A los profesores, becarios y personal del Dpto. de Anatomía y Anatomía Patológica Comparadas, por su buena acogida e inmejorable estancia. Con mención especial a la Dra. Mª José Bautista, por ofrecer desinteresadamente su sabiduría y por el enorme cariño que emana, y al Dr. Rafa Zafra, por compartir estos años el mismo despacho, por sus consejos y por estar siempre dispuesto a echar una mano.

Al Dpto. de Genética de la UCO, por abrirnos las puertas de par en par. Especialmente a la Dra. Gema García , por su introducción a la citometría de flujo y al Dr. Andrés Muñoz, por asesorarnos en el campo de la estadística.

A los Dres. José Manuel Sánchez Vizcaíno, Belén Rodríguez Sánchez, Deborah Kukiellka, Ana Cristina Pérez de Diego y al resto del personal del Dpto. de Sanidad Animal de la Universidad Complutense de Madrid, que nos hicieron sentir parte del equipo. Y a Amalia Vázquez del Centro de Citometría y Microscopía de Fluorescencia (UCM), por su disposición y disponibilidad.

A los Profs. Sandor Bélak, Anders Johannisson, Neil LeBlanc y Karl Ståhl, por acogerme y poner todo a mi disposición, introduciéndome en el mundo del Luminex xMap en el Departament of Virology of The National Veterinary Institute (SVA) and Department of Anatomy, Physiology and Biochemistry of the Swedish University of Agricultural Sciences (SLU) in Uppsala (Sweden).

Al personal de las Rozuelas del Valle de Torrecampo y de la Granja Experimental de Rabanales, por su ayuda con nuestros terneros, los protagonistas de esta historia. Al Laboratorio Veterinario Garfia y a todas aquellas empresas y entidades que han colaborado en la realización de este trabajo.

A mi familia. Lo que soy se lo debo a ellos. Por ser un ejemplo de trabajo incesante, superación y coraje. Gracias por animarme en todo momento a seguir adelante. A Pedro, por estar siempre ahí.

A todos los que emprendieron o emprenderán este camino, animándolos a concluirlo aunque flaqueen las fuerzas.