UNIVERSIDAD DE CÓRDOBA

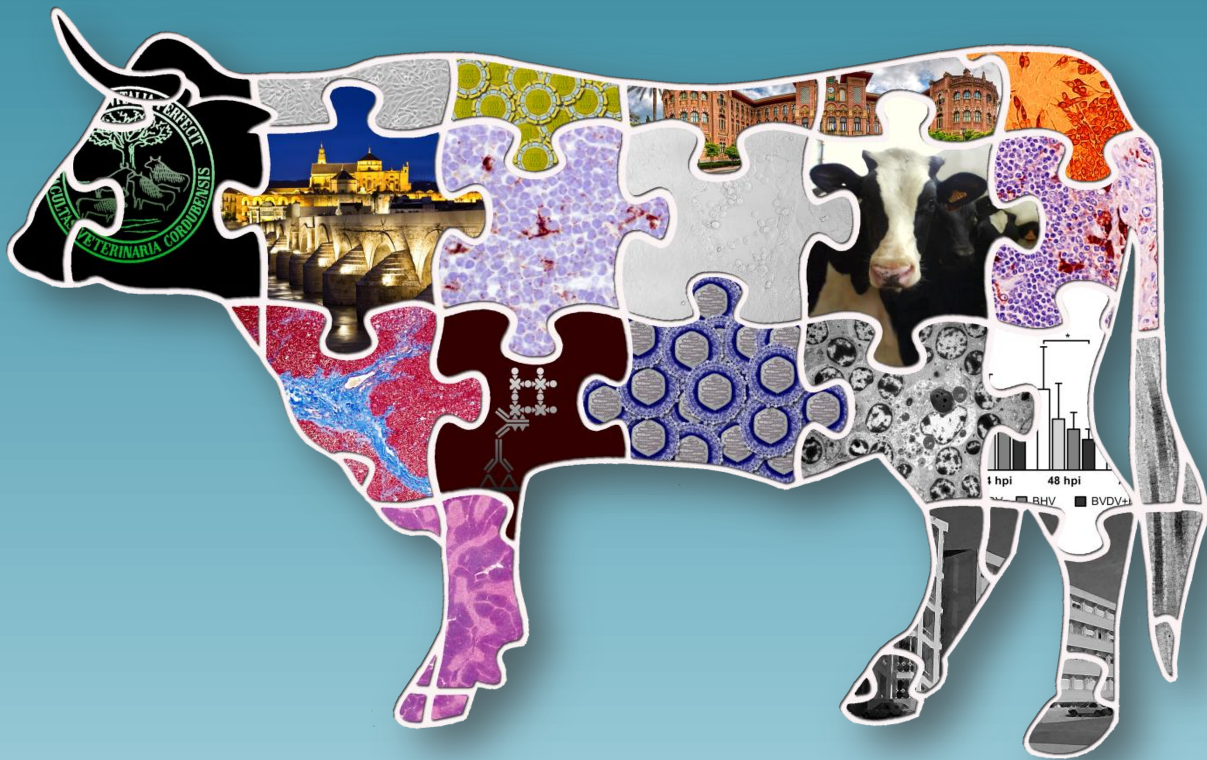
Facultad de Veterinaria

Departamento de Anatomía y
Anatomía Patológica Comparadas

TESIS DOCTORAL

STUDY OF THE EFFECTS CAUSED BY BVDV AND BHV-1 ON ANTIGEN-PRESENTING CELLS BY MEANS OF *IN VIVO* AND *IN VITRO* EXPERIMENTAL MODELS

ESTUDIO DE LOS EFECTOS CAUSADOS POR EL VDVB Y EL HVB-1 EN CÉLULAS PRESENTADORAS DE ANTÍGENO MEDIANTE MODELOS EXPERIMENTALES *IN VIVO* E *IN VITRO*



Fernando Romero Palomo

Córdoba, febrero 2015

TITULO: *Study of the effects caused by BVDV and BHV-1 on antigen-presenting cells by means of in vivo and in vitro experimental models.*

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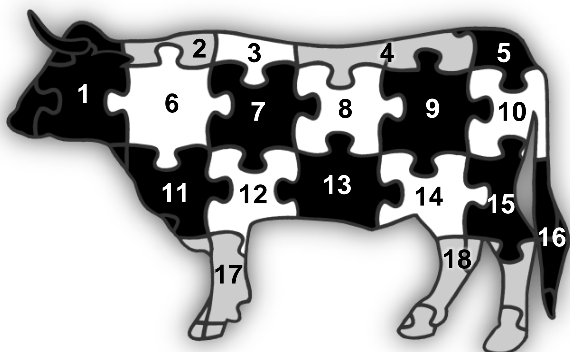

IMAGEN DE PORTADA. (1) Escudo Facultad de Veterinaria de Córdoba, **(2)** Cultivo de células epiteliales de bovino (MDBK), **(3)** Virus de la Diarrea Vírica Bovina (VDVB), **(4)** Rectorado de Córdoba (Antigua Facultad de Veterinaria), **(5)** Inmunoperoxidasa con marcaje de células MDBK infectadas (VDVB+), **(6)** Mezquita y Puente Romano de Córdoba, **(7)** Célula dendrítica inmunomarcada (CD208+), **(8)** Foco de efecto citopático producido por Herpesvirus bovino typo 1 (HVB-1) en células MDBK, **(9)** Animales del estudio *in vivo,* **(10)** Células inmunomarcadas infectadas con VDVB en timo, (11) Tinción Tricrómico de Masson (colágeno en azul), **(12)** Esquema técnica del ABC (Inmunohistoquímica), **(13)** Herpesvirus bovino (HVB), **(14)** Microscopía electrónica de transmisión (MET). Apoptosis, **(15)** Gráfica de resultados de estudio *in vitro,* **(16)** MET (colágeno de tipo I), **(17)** Timo (H&E), **(18)** Edificio de Sanidad Animal (Universidad de Córdoba).

UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

Tesis Doctoral con Mención Internacional

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D. Fernando Romero Palomo
Departamento de Anatomía y Anatomía Patológica Comparadas
Córdoba, febrero 2015

TÍTULO DE LA TESIS:

Study of the effects caused by BVDV and BHV-1 on antigen-presenting cells by means of *in vivo* and *in vitro* experimental models.

Estudio de los efectos causados por el VDVB y el HVB-1 en células presentadoras de antígeno mediante modelos experimentales *in vivo* e *in vitro*.

DOCTORANDO: Fernando Romero Palomo

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

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

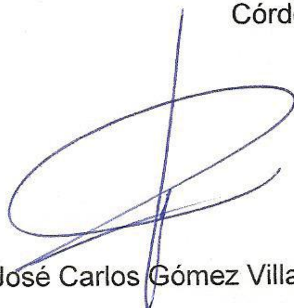
Este trabajo se ha realizado dentro del proyecto de investigación P09-AGR-4671, titulado "Respuesta inmune en la diarrea vírica bovina. Papel de las células dendríticas y células estromales de las estructuras linfoides frente a virus patógenos y vacunales" (Proyecto de excelencia).

El objetivo de esta tesis fue contribuir al conocimiento de las estrategias inmunopatogénicas por las que infecciones por el virus de la Diarrea vírica bovina predisponen a otras infecciones secundarias, como las producidas por el herpesvirus bovino 1. Para alcanzar este objetivo general se plantearon diferentes objetivos específicos que se han cumplidos con éxito al finalizar el trabajo.

Los resultados obtenidos han permitido realizar cuatro capítulos que serán enviados como artículos a revistas científicas indexadas, uno de los cuales ya ha sido publicado en *Veterinary Pathology* y otro se encuentra en estado de revisión en *Veterinary Microbiology*. Estos resultados han sido además expuestos en diferentes congresos nacionales e internacionales (ver anexo).

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

Córdoba, 18 de diciembre de 2014



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TÍTULO DE LA TESIS: Study of the effects caused by BVDV AND BHV-1 on antigen-presenting cells by means of *in vivo* and *in vitro* experimental models

DOCTORANDO: Fernando Romero Palomo

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María de los Ángeles Risalde Moya, investigadora contratada de la Università degli studi di Milano a través de la Fundación Alfonso Martín Escudero, informa que:

Este trabajo se ha realizado dentro del proyecto de investigación P09-AGR-4671, titulado "Respuesta inmune en la diarrea vírica bovina. Papel de las células dendríticas y células estromales de las estructuras linfoides frente a virus patógenos y vacunales" (Proyecto de excelencia).

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

Milán, 12 de diciembre de 2014

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A mi familia

“La curiosidad es hija de la ignorancia y madre de la ciencia”

Giambattista Vico

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LIST OF ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
Abs	antibodies
AEC	3-Amino-9-ethylcarbazole
ALDC	afferent lymph dendritic cells
APCs	antigen-presenting cells
BALT	bronchus-associated lymphoid tissue
BHV-1	bovine herpesvirus-1
BHV1 group	inoculated only with BHV-1
BRDC	bovine respiratory disease complex
BVD	bovine viral diarrhea
BVDV	bovine viral diarrhea virus
BVDV/BHV1 group	inoculated with BVDV and BHV-1
CCM	complete culture medium
CD	cluster of differentiation
cDC	conventional dendritic cell
CP	cytopathic
CPDA-1	citrate phosphate dextrose adenine
CTL	cytotoxic T lymphocyte (CD8+)
DC-LAMP	dendritic cell-lysosomal associated membrane protein
DCs	dendritic cells
DNA	deoxyribonucleic acid
dpi	days post-inoculation
dUTP	desoxiuridin tri-phosphate nucleotide
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FDC	follicular dendritic cells
FL-	fluorescence channel
FSC	forward scatter channel
GALT	gut-associated lymphoid tissue
GCDC	germinal center dendritic cells
gp	glycoprotein
HE	hematoxylin and eosin
HIER	heat–induced epitope retrieval
hpi	hours post-inoculation
IBR	infectious bovine rhinotracheitis
IDC	interdigitant dendritic cells
IFA	interfollicular areas
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IPMA	immunoperoxidase monolayer assay
LCM	leukocyte culture medium
LF	lymphoid follicle
mAbs	monoclonal antibodies
MD	mucosal disease
MDBK	Madin-Darby bovine kidney cells
MEM	minimum essential medium
MFI	mean fluorescence intensity
MHC-I/II	major histocompatibility complex type I/II
min	minutes
m-MØs	monocytes/macrophages
moDC	monocyte-derived dendritic cell
moi	multiplicity of infection
NCP	non-cytopathic
pAbs	polyclonal antibodies
PAMP	Pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PI	persistently infected animals
PnII	type II pneumocytes
PP	Peyer's patches
PRR	pattern (pathogen) recognition receptor
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RSP	red splenic pulp
RT	room temperature
silane	3- (triethoxysilyl)-propylamine
SSC	side scatter channel
TBM	tingible body macrophages
TBS	tris-buffered saline
$TCID_{50}$	tissue culture infective dose 50%
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TEC/ERT	thymic epithelial cells/epithelial reticular cells
TEM	transmission electron microscopy
TGF-β	transforming growth factor beta
Th	T helper lymphocyte (CD4+)
TNC	thymic nurse cells
TNF	tumor necrosis factor
Tris	tris (hydroxymethyl) aminomethane
TUNEL	**T**dT-mediated d**U**TP **N**ick **e**nd **l**abeling
UI	un-infected animals
UTR	untranslated regions
VNT	virus neutralization test
vWF	Von Willebrand factor/ coagulation factor VIII
WC	workshop cluster

PREFACE

Aim and scope of the thesis

Aim and scope of the thesis

The impairment of the immune system arisen during bovine viral diarrhea virus (BVDV) infections is one major paradigm of the immunology in the modern research of cattle diseases. In this sense, several efforts are being conducted to elucidate the complex mechanisms used by BVDV to evade the host immune response. Although BVDV is not a primary agent in the pathogenesis of bovine respiratory disease complex (BRDC), its suppressive effects on the host immune system can increase the risk of secondary infections, thus enhancing pulmonary colonization by other pathogens such as bovine herpesvirus type 1 (BHV-1).

Due to the important effects that viral pathogens can induce on the immune system, this work was firstly focused on a cell type of outstanding relevance for the immune system, the dendritic cell (DC). These cells are the most effective antigen-presenting cells (APC), and modulate both innate and adaptive immune responses. The absence of a solid literature detailing DC marker candidates for cattle gave rise to the first objective (CHAPTER 1), focused on finding potential DC markers for the bovine species, standardize their immunohistochemical protocol and describe their histological distribution, being considered this study as a tool to further investigate this important cell type in cattle diseases, including BRDC.

Secondly, an *in vivo* experimental model was established aimed at consistently reproducing the pathologic condition developed during the BRDC (CHAPTER 2). The experimental design consisted of a primary BVDV respiratory infection followed by a challenge with BHV-1 in order to examine the specific mechanisms by which a primary BVDV infection favors the dissemination and worsening of BHV-1 infection. The thymus was the focus of attention since it is considered a target organ for BVDV and because it remains as an active organ also throughout the adult life, allowing lymphocyte supply to secondary lymphoid organs when required. CHAPTER 2A focuses on the characterization of the BVDV-induced atrophy observed in this calves. On the other hand, CHAPTER 2B describes

from an immunopathologic point of view the thymus immune cells, including the main APCs (dendritic cells and macrophages) and lymphocyte subpopulations they can interact with. To do so, some of the DC markers described in CHAPTER 1 were used. These data contribute to the knowledge on the lesional and immunopathologic alterations of the thymus during BVDV infections, and its importance in the development of secondary infections.

Several papers with results from the animals in this experiment have been published by the author's research group, mainly focused on systemic cytokines *(Risalde et al., 2011)*, gross pathology, histopathology and viral antigen distribution *(Risalde et al., 2013b)*, systemic cell-mediated immune response *(Molina et al., 2013)*, systemic cell-mediated immune response during the acute phase of BVDV-infected calves *(Molina et al., 2014)*, as well as vascular and immunopathologic changes occurring in the lung *(Risalde et al., 2013a; Risalde et al., 2014)*. These papers, together with the findings observed in the thymus in this thesis, encompass the complete and extensive set of results obtained from this experimental study so far, to the benefit of a wider knowledge of these complex diseases.

These *in vivo* studies revealed not only the immunosuppressive features of BVDV but also a synergic action of the pathogenic mechanisms between both viruses. In an attempt of shedding light on these mechanisms, an *in vitro* experimental model was established, where peripheral blood mononuclear cells (PBMCs) from calves free of antibodies and antigen for both viruses were subjected to single or dual infections with BVDV and BHV-1. CHAPTER 3 analyses by flow cytometry the effects of these viral infections and is part of an ongoing broader project.

Published articles based on the same *in vivo* experimental model:

Molina V, Risalde MA, Sanchez-Cordon PJ, Pedrera M, Romero-Palomo F, Luzzago C and Gomez-Villamandos JC, **2013**. Effect of infection with BHV-1 on peripheral blood leukocytes and lymphocyte subpopulations in calves with subclinical BVD. *Research in Veterinary Science*: 95 (1), 115-122.

Molina V, Risalde MA, Sanchez-Cordon PJ, Romero-Palomo F, Pedrera M, Garfia B and Gomez-Villamandos JC, **2014**. Cell-mediated immune response during experimental acute infection with bovine viral diarrhoea virus: evaluation of blood parameters. *Transboundary and Emerging Diseases*: 61 (1), 44-59.

Risalde MA, Molina V, Sanchez-Cordon PJ, Pedrera M, Panadero R, Romero-Palomo F and Gomez-Villamandos JC, **2011**. Response of proinflammatory and anti-inflammatory cytokines in calves with subclinical bovine viral diarrhea challenged with bovine herpesvirus-1. *Veterinary Immunology and Immunopathology*: 144 (1-2), 135-143.

Risalde MA, Molina V, Sanchez-Cordon PJ, Romero-Palomo F, Pedrera M, Garfia B and Gomez-Villamandos JC, **2013a**. Pathogenic mechanisms implicated in the intravascular coagulation in the lungs of BVDV-infected calves challenged with BHV-1. *Veterinary Research*: 44 (1), 20.

Risalde MA, Molina V, Sanchez-Cordon PJ, Romero-Palomo F, Pedrera M and Gomez-Villamandos JC, **2014**. Effects of preinfection with bovine viral diarrhea virus on immune cells from the lungs of calves inoculated with bovine herpesvirus 1.1. *Veterinary Pathology*: Epub ahead of print.

Risalde MA, Molina V, Sonchez-Cordon PJ, Pedrera M, Romero-Palomo F, et al., **2013b**. Comparison of pathological changes and viral antigen distribution in tissues of calves with and without preexisting bovine viral diarrhea virus infection following challenge with bovine herpesvirus-1. *American Journal of Veterinary Research*: 74 (4), 598-610.

LITERATURE REVIEW

I. Bovine Viral Diarrhea Virus (BVDV)

BVDV is responsible for the most prevalent infectious disease of cattle. It causes financial losses from a variety of clinical manifestations and is the subject of a number of mitigation and eradication schemes around the world. Various species and biotypes of BVDV producing complex pathogenic mechanisms exist, with infection pre- and post-gestation leading to different outcomes. Infection of the dam during gestation can result in the birth of persistently infected (PI) calves that shed BVDV in their excretions and secretions throughout life and are the primary route of transmission of the virus. Acute infection with BVDV results in transient viremia prior to seroconversion and can lead to reproductive dysfunction and immunosuppression leading to an increased incidence of secondary disease. Understanding of the host defense mechanisms of innate and adaptive immunity is one of the most challenging fields of BVDV research.

The disease complex of Bovine Viral Diarrhea/Mucosal Disease looks back on a short (compared to some other viral diseases) but busy **history**. Olafson et al. of Cornell University described the disease in 1946 for the first time *(Olafson et al., 1946)*. Although with variable severity, the "new" disease was mainly characterized by diarrhea, depression, anorexia, and ulceration of oral mucosa, as well as respiratory signs, leukopenia, drop in milk production, and increased abortion rates. Since no bacteria could be isolated, Olafson suspected a viral etiology, and this reproducible disease, with its varying severity, became known as virus diarrhea (VD) of cattle. In the fifties, a special form of disease characterized principally by hemorrhages and intestinal erosions_was reported for the first time in Iowa-USA in 1953 and it was named Mucosal Disease (MD) *(Ramsey and Chivers, 1953; 1957)*. After an intensive period of research and despite their unequal epidemiology, VD and MD were suspected to be etiologically related. At the end of the sixties, both pathologies were considered a single disease complex commonly called BVD-MD, and BVDV was classified into CP and NCP biotypes. After several

studies, a special form of disease was discovered, produced by an intrauterine infection with NCP BVD-MD virus, with calves affected being persistently infected and displaying the astonishing immunological peculiarity of being unable to produce antibodies against the BVD-MD virus *(Johnson and Muscoplat, 1973)*. This was the discovery of immunotolerance in persistently infected animals. It took almost twenty more years to resolve the observation that MD was not transmissible, when two different research groups reported the first experimental demonstration of MD by inoculation of a CP virus which was antigenetically homologous to the NCP virus causing the persistent infection *(Brownlie et al., 1984; Bolin et al., 1985)*. During the late eighties and early nineties, cases of "Severe Acute BVD" and "BVD Hemorrhagic Syndrome" occurred *(Corapi et al., 1990; Pellerin et al., 1994; Carman et al., 1998)*. The genetic dissimilarities between the initial BVDV isolates and the newly found viral isolates associated with severe acute disease, promoted the identification of BVDV genotype 2 *(Ridpath et al., 1994)*. For more details on the history of BVD, see reviews by Goens *(2002)* and Deregt *(2005)*.

1. Etiology

BVDV is classified as a member of the genus *Pestivirus* within the ***Flaviviridae*** family *(Table 1)*. Members of the Flaviviridae family 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, including the following: a) a single-stranded, positive-sense RNA genome that encodes a single large polyprotein; b) the polyprotein is post-translationally processed by both cellular and viral proteases to yield the final, mature viral proteins; and c) an outer lipid membrane that carries viral glycoproteins, and is derived from budding through cellular membranes during assembly and maturation of the virus particle *(Bowen, 2011)*.

Unlike the other two genera, the ***Pestivirus*** genus encodes two unique proteins, N^{pro} and E^{rns} (described below). Pestiviruses are currently divided into four species: *Classical swine fever virus* (CSFV, formerly *hog cholera virus*), *Bovine viral diarrhea virus 1* and *2* (BVDV-1 and BVDV-2), and *Border disease virus* (BDV) of sheep *(Simmonds et al., 2012)*. BVDV was formerly classified as a single species with two genotypes (genotype 1 and 2) *(Pellerin et al., 1994; Ridpath et al., 1994)*,

but detailed analysis of the genomic RNA sequence, as well as antigenic characteristics demonstrated that these viruses constituted two distinct species *(Neill, 2013)*.

Table 1. Taxonomy of Bovine Viral Diarrhea Virus

Group	Family	Genus	Species
Single-Stranded Positive-Sense RNA Viruses (Group IV)	*Flaviviridae* →	*Pestivirus* → *Flavivirus* · *West Nile virus* · *Yellow fever virus* · *Dengue virus* ... *Hepacivirus* · *Hepatitis C virus* *Pegivirus* (new)	· **BVDV-1** · **BVDV-2** · CSFV · BDV · Giraffe pv* · Pronghorn pv* · Bungowannah pv* · HoBi-like pestiviruses*

*Putative (not recognized yet as species by ICTV [International Committee on the Taxonomy of Viruses]). For a direct link to http://viralzone.expasy.org/, press on each word on the table. [Etymology: *Flavi* (from Latin *flavus*, "yellow"); *Hepaci* (from Greek *hepar*, *hepatos*, "liver"); *Pesti*: from Latin *pestis*, "plague" *(Simmonds et al., 2012)*]

In addition to the 4 recognized species, 4 additional *Pestivirus* species have been proposed but remain officially unrecognized. These putative species include Giraffe pestivirus *(Becher et al., 1997)*, Pronghorn pestivirus *(Vilcek et al., 2005b)*, Bungowannah pestivirus in pigs *(Finlaison et al., 2009)*, and a varied group of viruses referred to as HoBi-like viruses, named after their similarity to the first isolate called HoBi_D32/00 *(Schirrmeier et al., 2004)*. The term "atypical pestiviruses" has also been used for HoBi-like viruses; however, this term could also be applied to any of the 3 other putative pestivirus species *(Bauermann et al., 2013)*. Some authors also refer to these viruses as BVDV-3 due to the similarity of clinical presentation in cattle following infection with BVDV and HoBi-like viruses; in fact, the presence of PI and even MD in association with HoBi-like viruses has been recently described for the first time *(Decaro et al., 2013; Decaro et al., 2014; Weber et al., 2014)*. However, there is resistance to declaring HoBi-like viruses a third species of BVDV *(Bauermann et al., 2013)*.

BVDV virions are spherical, pleomorphic (40-60 nm in diameter), and consist of a tightly adherent lipid envelope displaying indistinct glycoprotein spikes (arranged in an icosahedral-like symmetry), surrounding a spherical-to-icosahedral nucleocapsid which contains the viral genome *(Figure 1A)*. The single-stranded RNA molecule (approximately 12.3 kb in length) contains a single, large open reading frame (ORF) that encodes all viral proteins, flanked on the 5' and 3'

ends by untranslated regions (UTR) *(Figure 1B)*. The 5' UTR extreme is the most conserved region in pestiviruses, and is widely used for detection and genotyping of BVDV *(Letellier and Kerkhofs, 2003)*. The ORF of BVDV can be divided into distinct regions that encode the individual viral proteins *(Nettleton and Entrican, 1995)*. With the exception of N^{pro}, the first coding region of the ORF encodes the **structural** proteins forming the capsid (core protein, C) and the envelope (glycoproteins E^{rns} (gp48), E1 (gp33) and E2 (gp53)). The **E^{rns}** protein is attached weakly to the viral surface and has a unique characteristic in that possesses RNase activity, a feature of important implications at inducing tolerance of the innate immune response *(Iqbal et al., 2004; Peterhans and Schweizer, 2013)*. The E^{rns} protein possesses a minor neutralizing epitope, is highly conserved among BVDV strains, and is a very common target for antigen-based tests *(Ridpath, 2010b; Dubovi, 2013)*. The other envelope glycoproteins, E1 and E2, are, in contrast, integral membrane proteins arranged as heterodimers *(Ronecker et al., 2008)*. The **E2** glycoprotein possesses the major neutralizing epitopes for inducing humoral response and its genomic sequence displays the greatest variability of all viral proteins, thus being widely used for classification of BVDV isolates *(Deregt et al., 1998; Vilcek et al., 2005a; Ridpath, 2010b)*. Due to the E2 antigenic variability, there is potential for a lack of cross-protection against wild-type BVDV viruses, even though cross-neutralization studies have shown reactivity to different genotypes *(Loy et al., 2013)*. The first **non-structural** protein is an autoprotease called **N^{pro}**, unique to the pestivirus genus, and its sequence is frequently used in phylogenetic comparisons *(Ridpath, 2010b)*. N^{pro} cleaves itself from the polyprotein and is now recognize as having a role in blocking interferon (IFN) production *(Peterhans and Schweizer, 2013)*. Different functions in the replication cycle of BVDV have been attributed to the rest of non-structural proteins (p7, NS2/3, NS4a, NS4b, NS5a, NS5b) (see review by Neill *(2013)*).

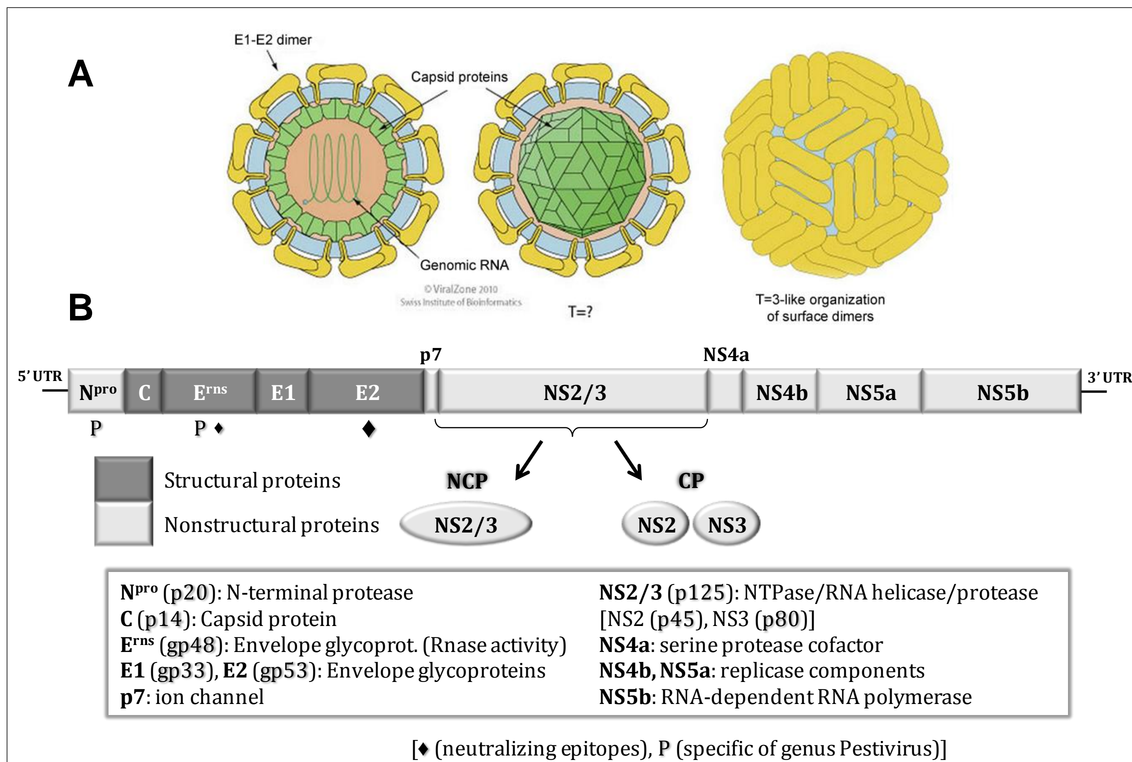

Figure 1. Structure (A) and genome organization (B) of BVDV. [Source of figure (A): S*wiss Institute of Bioinformatics (http://viralzone.expasy.org)*]

NS2/3 protein (along with its cleaved forms) deserves a special mention since they play an outstanding role in the pathogenesis of this complex disease, determining the occurrence of cytopathic (CP) and noncytopathic (NCP) **biotypes** of BVDV *(Brownlie, 1991)*. These features are based on the lytic activity on cultured epithelial cells, with CP biotypes inducing cytoplasmic vacuolization and death, and NCP biotypes replicating in these cells without causing morphological changes (not to be confused with pathogenicity *in vivo*) *(Kummerer et al., 2000)*. The CP biotype invariably expresses high levels of NS3 as a free protein, either by proteolytic cleavage in the NS2/3 protein expressed by NCP biotypes, by genome duplication of the NS2/3 sequence encoding the NS3 protein or by genetic deletion of the NS2 genomic sequence *(Donis and Dubovi, 1987; Deregt and Loewen, 1995; Kummerer et al., 2000)*. It has been demonstrated that cleavage of NS2/3 is necessary for replication of NCP viruses very early in the infection, with cleaved NS2/3 dropping off drastically later in the infection process resulting in primarily NS2/3 being present in the infected cells *(Lackner et al., 2004)*. NS2/3-NS3 proteins are highly conserved among all BVDV strains, are highly immunogenic (although generated antibodies are not neutralizing), and are also targeted for antigen-based tests

(Ridpath, 2010b; Dubovi, 2013; Ridpath, 2013). It must be pointed out that the CP or NCP nature of BVDV strains does not correlate with their virulence *in vivo*, as their name would suggest *(Fulton et al., 2002; Ridpath, 2005)*. A third biotype (lymphocytopathic) inducing cell death in cultured lymphoid cells but not in cultured epithelial cells has also been proposed *(Ridpath et al., 2006a)*, being correlated with high virulence in acute infections *in vivo*.

As it has been stated, BVDVs can be divided according to their genome (BVDV-1 and BVDV-2, still frequently called genotypes) and to their phenotype (CP and NCP), with any combination of them being observed. However, the most common biotype in nature is the NCP (>70%) *(Fulton et al., 2000b; 2005b)*, which is responsible for the more severe clinical forms of acute BVDV infection and the establishment of persistent infection. BVDV-1 is considered the *Pestivirus*-type species and is reportedly the most prevalent genotype *(Fulton et al., 2005b)*, being generally associated with milder symptoms than its counterpart BVDV-2. The analysis of the genomic RNA has led to the current genetic division of BVDV-1 into at least 17 subtypes (1a, 1b, 1c, 1d…), and BVDV-2 into three subtypes (2a-c), revealing the considerable increasing genetic diversity of BVDV *(Strong et al., 2013; Giammarioli et al., 2014; Luzzago et al., 2014)*. This genetic diversity that occurs among BVDV isolates is a common feature among RNA viruses, that exist in nature as quasispecies (a swarm of viral mutants) *(Bolin and Grooms, 2004)*. Due to the antigenic cross-reactivity observed among pestiviruses, it is not possible to differentiate them based on serology alone *(Ridpath, 2003)*, with a single serotype being recognized for BVDV.

2. Clinical features and lesions

BVDV has been associated with a complex of disease syndromes. Although the term *diarrhea* is prominent in the name, respiratory and reproductive disease associated with BVDV infection are more commonly reported *(Ridpath, 2010b)*. BVDV has been described as affecting the reproductive, respiratory tract, gastrointestinal, circulatory, immunologic, lymphatic, musculoskeletal, integumentary, and the central nervous system. Therefore, this multi-purpose

pathogen has been described as having "many faces" *(Brock, 2004)*. The wide variety of clinical forms is highly dependent on the interaction of several factors at the time of infection: genotype, biotype, and virulence of the virus; age, stage of gestation, and immune status of the animal, and the interplay of stressors. All these factors lead to a complicate classification of clinical forms, which is not always equal. Three situations are considered here: postnatal infection in non-pregnant cattle (acute infections), infection in pregnant cows, and development of mucosal disease *(Figure 2)*. Beside the clinical signs occurring after BVDV infection, some aspects of the pathogenesis will be discussed in parallel in this section.

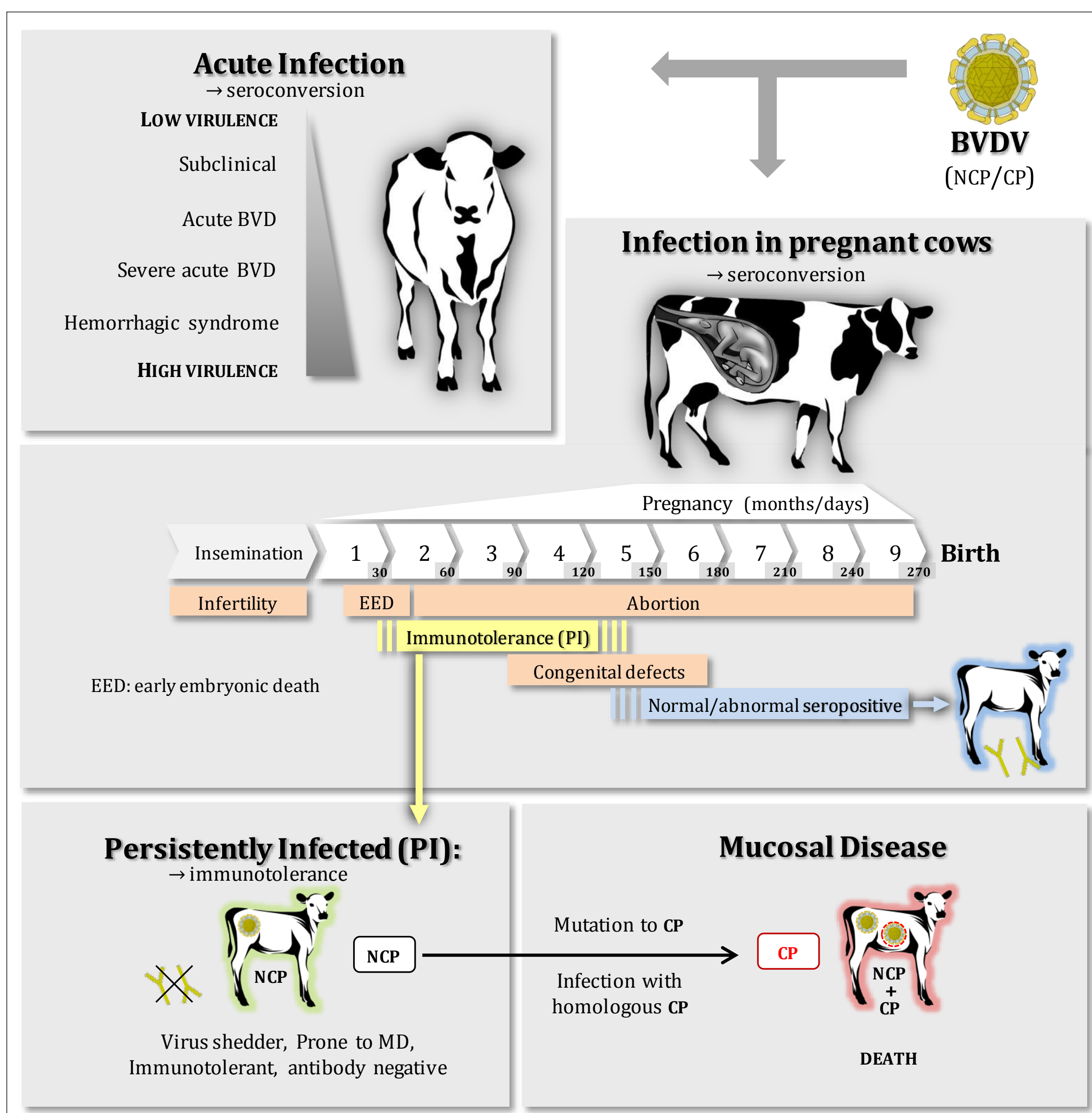

Figure 2. Schematic representation of the clinical manifestations after BVDV infection.

2.1. Postnatal infection (acute infections)

Susceptible (seronegative), immunocompetent cattle of all ages may contract a primary, transient BVDV infection, termed *acute infection*. Seropositive cattle, dependent on the levels of antibody titers, are usually not susceptible. Depending on the clinical course, acute infections can develop as several clinical variants: asymptomatic acute infections, symptomatic acute infections, severe acute infections, and hemorrhagic syndrome.

2.1.1. Subclinical infections

The majority of BVDV infections in immunocompetent seronegative cattle proceed as **asymptomatic acute infections**. However, close observation of infected animals usually reveals mild signs including hyperthermia, leukopenia, and decreased milk production. This decrease of white cells induces an state of immunosuppression that may favor the emergence of opportunistic infections *(Potgieter, 1995)*, which are more easily detected.

2.1.2. Acute BVD

Symptomatic acute infections (frequently termed as "acute BVD"), are most commonly observed in 6–24-month-old cattle following waning of maternal immunity, in colostrum-deprived calves, or in seropositive cattle as a result of infection with a heterologous BVDV strain *(Evermann and Barrington, 2005)*. These clinical manifestations are normally moderate, including fever, anorexia, lethargy, leukopenia, ocular and nasal discharge, and mild diarrhea *(Muller-Doblies et al., 2004; Pedrera et al., 2009b; Molina et al., 2014)*. During infections with more virulent strains, the aforementioned signs may get worse and even be accompanied by epithelial erosions and ulcers in the muzzle, oral cavity or gastrointestinal track *(Blowey and Weaver, 2011a)*; in dairy cows there may be a considerable decrease in milk yield. Acute symptomatic infections are also associated with immunosuppression *(Chase et al., 2004)*, frequently resulting in increased incidence of opportunistic respiratory and intestinal infections. These processes have a high morbidity, and uncomplicated cases show very low or no

mortality. Any genotype and biotype can be isolated in acute BVD cases, but NCP BVDV-1 strains (and some BVDV-2 of low virulence) are the most frequent.

2.1.3. Severe acute BVD and Hemorrhagic syndrome

In the early 1990s, an atypical and significantly more severe form of BVDV infection was recognized in the United States and Canada (now known as severe acute BVD) *(Carman et al., 1998)*, which was frequently presented with profuse hemorrhages and thrombocytopenia (being denominated as hemorrhagic syndrome) *(Corapi et al., 1990; Pellerin et al., 1994; Carman et al., 1998)*. **Severe acute** outbreaks were unusual and characterized by a peracute course with high rates of morbidity and mortality in all ages of cattle (in contrast to most previous descriptions of transient BVDV infections). The described clinical signs included high fever, severe diarrhea and leukopenia, respiratory disorders and erosions in the oral cavity *(Carman et al., 1998)*. Postmortem findings included a dramatic lymphocytolysis and lymphoid depletion of Peyer's patches, necrosis of intestinal crypt epithelium, and diffuse ulcerative lesions in the upper alimentary tract resembling those of mucosal disease. The viral isolates from all reports of severe acute BVD were determined to be caused by BVDV-2, which at that time was differentiated for the first time from BVDV-1 *(Pellerin et al., 1994; Ridpath et al., 1994)*. **Hemorrhagic syndrome** is a variant form of the severe acute BVD, which is accompanied by marked thrombocytopenia that contributes to the appearance of hemorrhagic signs as petechiation/ecchymoses of mucosal surfaces, epistaxis and bloody diarrhea *(Corapi et al., 1990; Walz et al., 1999a; Stoffregen et al., 2000)*.

As stated before, severe acute forms and their hemorrhagic variants were originally described in North America and Canada and attributed to NCP BVDV-2 strains of high virulence *(Corapi et al., 1990; Pellerin et al., 1994; Ridpath et al., 2006b)*; however, this situation has changed from that period to nowadays, as described below. BVDV-2 was subsequently detected in European countries albeit at a significantly lower percentage compared to North America *(Lindberg et al., 2006; Letellier et al., 2010; Ridpath, 2010b)*. A rising occurrence of severe outbreaks produced by BVDV-2 in Europe in recent years is being responsible for an increasing concern in the European countries, some of which had already eradicated BVDV-2 *(Astiz-Blanco, 2013; Anonymous, 2014b; a; Polak et al., 2014)*.

Additionally, it must be reminded that not all BVDV-2 outbreaks are necessarily associated with severe disease *(Marshall et al., 1996; Ridpath et al., 2000)*. Similarly, a severe outbreak of disease should not be assumed to have been caused by BVDV-2 *(David et al., 1994; Liebler-Tenorio et al., 2006; Ridpath et al., 2007)*; in fact, recent cases of severe acute hemorrhagic diseases have been attributed to BVDV-1 strains *(Colloff et al., 2012; Yesilbag et al., 2014)*.

2.2. Reproductive disorders and congenital infection

2.2.1. Reproductive disorders

BVDV-related reproductive disorders, together with the immunosuppressive effects, are responsible for the most economically important consequence of BVDV infections *(Astiz-Blanco, 2014a; b)*. Aside from the impact of BVDV in the fetus (see below), acute BVDV infection can have a direct impact on reproductive performance *(Brock et al., 2005; Garoussi and Mehrzad, 2011; Yavru et al., 2013)*, inducing chronic postnatal infections in ‘‘immunoprivileged’’ sites such as testicles and ovaries *(Givens and Marley, 2013)*. As a result of acute infection, there can be prolonged testicular infection and shedding of virus in semen for as long as 2.75 years after infection, giving rise to reductions in male fertility and possible (not frequent) venereal infections *(Paton et al., 1990; Voges et al., 1998; Givens et al., 2009)*. BVDV has been detected in ovarian and oviductal tissues during acute infections, inducing associated pathologies with important reproductive consequences *(Fray et al., 1998; Grooms et al., 1998; Fray et al., 2000a; Brock et al., 2005)*.

2.2.2. Infection of pregnant cattle

Infection of immunocompetent pregnant cattle can result in clinical manifestations in the dam similar to those described above (i.e., subclinical to severe, acute disease, or hemorrhagic syndrome). However, additional clinical outcomes in pregnant cattle are related to the potential transplacental transfer of either biotype or genotype to the fetus *(Fray et al., 2000b; Swasdipan et al., 2002)*, being the NCP biotype the most frequently observed inducing maternal viremia and transplacental infection *(Harding et al., 2002)*. The gestational age of the fetus

is considered the primary determinant of the **fetal infection** outcome. During the first 18 days of pregnancy, BVDV does not penetrate the zona pellucida of the unattached embryo *(Moennig and Liess, 1995)*. Infection between 30 days (when implantation takes place) and ≈45 days can result in **embryonic death** and reduced pregnancy rates *(Grahn et al., 1984; Carlsson et al., 1989; McGowan et al., 1993; Tsuboi et al., 2013)*. **Abortions** can occur at any gestational age, but are most common during the first trimester, frequently resulting in fetal resorption or mummification *(Fray et al., 2000b; Grooms, 2004)*. Fetal death later in gestation generally ends up with expulsion of the fetus *(Murray, 1991; Blanchard et al., 2010)*. Infection of the dam with NCP strains between the 2nd and the 5th month of gestation (i.e. prior to the full development of the fetus immune system) frequently result in the development of **persistent infections** (see below). Fetal infections during midgestation (4th-6th month) may severely affect the process of organogenesis, resulting in **congenital defects** such as CNS abnormalities (cerebellar hypoplasia, porencephaly…), ocular defects (microphthalmia, retinal atrophy), thymic hipoplasia, retarded growth, pulmonary hypoplasia, hypotrichosis or skeletal abnormalities *(Mickelsen and Evermann, 1994; Baker, 1995; Blanchard et al., 2010; Webb et al., 2013)*. When infection is acquired after the 5th month of gestation, the fetus usually survive, whether manifesting tissue injury or not, since is able to mount an effective immune response, thus developing neutralizing antibodies and clearing the virus *(Hansen et al., 2010)*.

2.2.3. Persistent infection (PI)

Although transplacental infection can be produced by any genotype and biotype, only NCP strains can induce the development of **persistently infected (PI)** calves, since CP strains always trigger a strong innate immune response *(Brock, 2003; Peterhans et al., 2010)*. The window for the creation of PI calves varies but is generally accepted to be between 30 and 125 days, when viral antigens are recognized as self antigens as a result of the uncompleted development of the fetus immune system *(Brock, 2003)*. A gestational age of 75 days is commonly used under experimental conditions for inducing persistent infections in up to 100% of the fetuses *(Brock and Cortese, 2001; Charleston et al., 2001a; Webb et al., 2012; Smirnova et al., 2014)*. Due to the absence of an antibody

response *(Coria and McClurkin, 1978)*, PI animals will shed large amounts of virus in all excretions and secretions *(Brock et al., 1998; Brock, 2003)*. Calves with persistent infection are frequently stunted, weak and characterized as "poor-doers", with some other clinical signs being associated *(Bachofen et al., 2010; Webb et al., 2012)*; however, other PI animals may also appear clinically healthy *(Baker, 1995; Voges et al., 2006)*. PI calves are regularly reported to be susceptible to secondary infections *(Voges et al., 2006)*, as a result of their poor immune function. This, combined with susceptibility to mucosal disease, leads to low survivability of most PI animals, most of them dying during the first year of life *(Houe, 1993; Muñoz-Zanzi et al., 2003; Voges et al., 2006)*, although recent data suggest that as many as 28% of PIs in a population may be over 2 years of age *(Booth and Brownlie, 2012)*. Some PI females reaching sexual maturity can even become pregnant and give birth new PI animals. In endemic regions, the frequency of PI animals is very low (less than 2% of animals), although their presence have an enormous epidemiological impact *(Houe, 1999; Fulton et al., 2005a; 2009)*.

2.3. Mucosal disease (MD)

Mucosal disease is a fatal and sporadic disease that only develops in PI cattle after infection with a genetically and antigenically homologous CP virus strain, which can arise either from superinfection *(Brownlie et al., 1984; Bolin, 1995)*, or mutation of NCP BVDV already circulating in the PI animal *(Tautz et al., 1998; Darweesh et al., 2014)*. In general terms, the higher is the homology the earlier and more acutely the symptoms develop. MD also occurs when PI cattle are exposed with a CP BVDV that is antigenically heterologous with the resident noncytopathic BVDV. In those situations, it may be a race between the cytopathic virus and the immune system *(Bolin, 1995)*.

The name of the disease was given for the first time after the observation of the typical severe erosions, ulcers, and hemorrhages of the mucosal surfaces of muzzle and digestive tract *(Ramsey and Chivers, 1953)*. Additionally, animals undergoing MD may also show fever, anorexia, skin lesions in the hoof interdigital space, and profuse watery diarrhea (often with fibrinous casts, blood or foul odor). On histological examination, there is a clear demonstration of destruction of the lymphoid tissues, especially within the GALT and regional lymph nodes

(Wilhelmsen et al., 1991; Liebler et al., 1995). A small proportion of cattle with symptoms of acute MD do not die in the expected time frame, but rather develop signs of a more chronic form of the disease *(Evermann and Barrington, 2005; Grooms et al., 2009)*.

Under experimental conditions, early onset and late onset MD can be distinguished, with MD occurring within 2-3 weeks and months-years, respectively, after exposure to the CP strain *(Fritzemeier et al., 1997; Liebler-Tenorio et al., 2000)*. In early onset MD, CP BVDV reisolated from moribund animals is identical to the persisting NCP. After late onset MD, conversely, reisolated CP BVDV is a recombination of the persisting NCP and the CP BVDV used for inoculation *(Fritzemeier et al., 1997)*. Clinical signs in the end (acute) phase of early and late onset MD are indistinguishable, with only subtle differences in tissue lesions being observed *(Liebler-Tenorio et al., 2000)*.

3. Pathogenesis

The pathogenesis of any disease reveals the complex interaction between the infecting pathogen and the infected host. This process begins with the entry of the pathogen into the host, followed by the subsequent replication and dissemination, having the nature of the pathogen and the immune response of the host a determinant role in the outcome of the infection. Some aspects of the complex pathogenesis of BVDV infection have been described in the previous section. The following sections will focus principally in the distribution of target cells, the immune response and the role of BVDV in the pathogenesis of BRDC, with special emphasis on acute postnatal infections.

3.1. Entry and dissemination to target organs

The main way of entry of BVDV during **acute infections** (irrespective of the clinical course) is the oronasal route. After initial attachment to cellular co-receptors such as CD46 *(Maurer et al., 2004)*, viral particles penetrate susceptible cells and undergo primary non-lytic replication in the nasal mucosa and tonsils *(Bruschke et al., 1998b)*. For more details on the replication cycle of BVDV se reviews by *Hietala and Crossley (2005)* and *Ridpath (2005)*. Through blood and

lymphatic dissemination, the virus spreads to regional lymph nodes *(Figure 3)*, and subsequently to the rest of the organism in a free form or associated with leukocytes, principally lymphocytes and monocytes *(Brownlie, 1990; Sopp et al., 1994; Bruschke et al., 1998b; Archambault et al., 2000)*. Though BVDV can infect a wide variety of tissues, there is an evident predilection for lymphoid and intestinal tissues *(Sopp et al., 1994; Spagnuolo-Weaver et al., 1997; Liebler-Tenorio et al., 2003a; Pedrera et al., 2012a)*. Some differences between NCP and CP biotypes have been described after experimental infections with homologous strains, with a wider spread in the host in the case of the NCP biotype *(Spagnuolo-Weaver et al., 1997)*. However, the most important factor affecting the outcome of acute BVDV infection in susceptible animals is the virulence of the individual BVDV strain, thus determining the wider or more restricted dissemination of the virus and its pathogenic effect *(Liebler-Tenorio, 2005)*.

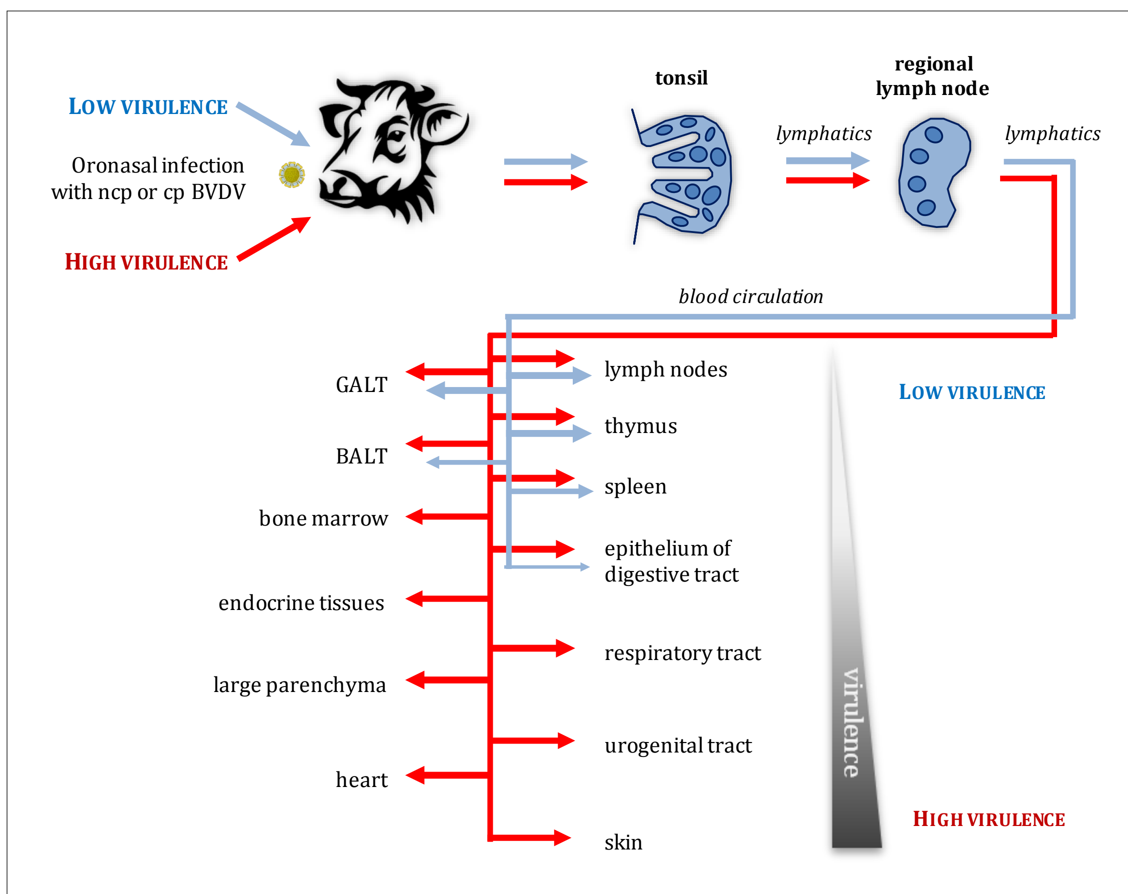

Figure 3. Spread of BVDV of low and high virulence in acute BVDV infections *(Adapted from Liebler-Tenorio, 2005)*.

In experimental infections with low virulence (LV) strains, animals developed mild signs of disease *(Wilhelmsen et al., 1990; Bolin and Ridpath, 1992; Marshall et al., 1996; Bruschke et al., 1998b; Liebler-Tenorio et al., 2003a; Molina et al., 2014)*. These studies revealed the presence of viral antigen in the majority of lymphoid organs (tonsils, lymph nodes, GALT, thymus, BALT and spleen), with the exception of bone marrow *(Liebler-Tenorio et al., 2003a; Pedrera et al., 2009b; Raya et al., 2012)*. Less frequently, the presence of viral antigen has been described in other locations such as liver *(Risalde et al., 2011a)*, and intestinal mucosa *(Liebler-Tenorio et al., 2003a; Pedrera et al., 2009b)*. Cells predominantly expressing BVDV included different types of mononuclear leukocytes, stromal cells and some epithelial cells. In general, the presence of BVDV antigen was not directly associated with the presence of tissue lesions, as observed with other pathogens such as BHV-1 *(Moeller et al., 2013)*.

Following experimental infections with high virulence (HV) strains, clinical signs are severe but are often nonspecific, consisting of high fever, depression, and frequently diarrhea *(Bolin and Ridpath, 1992; Ellis et al., 1998; Archambault et al., 2000; Liebler-Tenorio et al., 2002)*. The initial spread of HV strains is similar to that of LV strains, with initial infections of lymphoid tissues *(Liebler-Tenorio et al., 2002; 2003b)*. However, the amount of viral antigen in tissues rapidly exceeds that caused by LV strains, extending to T-cell–dependent areas or even bone marrow, frequently observed in cases developing thrombocytopenia *(Spagnuolo et al., 1997; Walz et al., 1999a)*. In contrast to BVDV of LV, which is cleared from infected tissues, HV strains keep spreading beyond lymphoid tissues, reaching other regions such as the mucosa of the upper and lower digestive tract, the respiratory tract, endocrine tissues, or even the heart and skin. Additional cell types become infected with HV strains, including endothelial cells, neutrophils, smooth muscle cells, megakaryocytes and platelets *(Walz et al., 1999b; Liebler-Tenorio et al., 2002)*. As in infections with LV strains, the association between presence of viral antigen and lesions is rare, particularly in the initial phase of disease *(Liebler-Tenorio et al., 2002)*.

PI animals are characterized by a generalized distribution of BVDV in all their organ systems, with a wide variety of cell types becoming infected. Several lesions can be observed in different tissues, although they are not directly

associated with the detection of viral antigen *(Fredriksen et al., 1999; Shin and Acland, 2001; Liebler-Tenorio et al., 2004; Hilbe et al., 2007; Montgomery, 2007; Bachofen et al., 2010)*. By using specific monoclonal antibodies for selectively detecting NCP and CP BVDV antigens, it has been demonstrated that the NCP BVDV antigen, present in PI animals in a wide distribution, is still found in cases of **mucosal disease** *(Liebler et al., 1991)*. However, CP BVDV is predominantly detected in sites where tissue destruction is observed *(Liebler et al., 1991; 1997)*, consistently found in lymphoid and intestinal tissues, and mucosa of the upper digestive tract *(Liebler et al., 1995; Hilbe et al., 2013)*. These studies revealed that the spreading pattern for the CP BVDV in MD is similar to the one described for acute BVDV infections either with CP or NCP BVDV *(Liebler-Tenorio, 2005)*.

As stated before, BVDV can establish prolonged infection by means of persistent infections of the fetus during pregnancy, being these PI animals unable to mount an effective immune response to the infecting BVDV. However, there is a second form of maintaining prolonged replication, which may occur in some animals following acute infections, specifically in immunoprivileged sites that can support **chronic infections** such as ovarian tissues, testicular tissues, CNS tissues and circulating white blood cells *(Givens and Marley, 2013)*. Unlike classical persistent infections, animals undergoing these chronic infections do mount a significant immune response *(Givens and Marley, 2013)*.

3.2. BVDV and Immunity

Viruses have a low capacity for survival outside their hosts, and they use two different strategies to remain associated with their hosts, consisting on "hit-and-run" or "infect-and-persist". Along with other pestiviruses, the success of BVDV survival in its host population is based on a combination of the afore-mentioned strategies *(Peterhans and Schweizer, 2010)*. Thus, BVDV has the ability to infect its hosts transiently, resulting in a short duration of infection in the individual host and rapid transfer to the next host (hit-and-run). The second strategy consists in infecting persistently individual hosts by evading their immune response (infect-and-persist), doing so through mechanisms that differ radically from all other viruses causing persistent infections *(Peterhans and Schweizer,*

2010). The interaction of BVDV with the immune system is complex and multifaceted. This section summarizes the evidences indicating that BVDV interferes with the function of the innate and adaptive immune system which may explain the immunosuppressive effects observed in transiently and persistently infected animals.

3.2.1 Innate immune response to BVDV

The innate/natural (non–antigen-specific) immune response, either with its cellular or non-cellular components, can influence the outcome of BVDV infection. BVDV can infect **cells** of the innate immune system affecting the function of neutrophils, monocytes, macrophages and dendritic cells *(Potgieter, 1995; Lambot et al., 1998a; Glew et al., 2003; Peterhans et al., 2003)*. Infection with BVDV may result in impairment of microbicidal, chemotactic and antibody-dependent cell-mediated citotoxicity of neutrophils *(Potgieter, 1995)*. Many reports have described the different effects of *in vitro* infection with BVDV on macrophages (reviewed by Peterhans *(2003)* and Chase *(2004)*), revealing that certain functions are similarly altered by CP and NCP viruses (e.g. impaired TNFα response, decreased chemotactic activity, inhibited action of IL-1 activity…), while others are influenced in a biotype-specific fashion (e.g. low type I IFN response to NCP strains). It has been suggested that natural killer (NK) cells can become infected with BVDV *(Darweesh et al., 2013)*, although there are no reports on the role of these cells in BVDV pathogenesis and immune response. (More details on monocytes-macrophages and DCs will be discussed below as APCs).

Among the non-cellular components of innate responses, **cytokines** develop an important role, being the IFN family of cytokines one of the most relevant antiviral defense systems of the host *(Samuel, 2001)*. Only NCP BVDVs establish fetal infections and persist in host animals, and in this process, the modulation of the IFN system by BVDV has an exceptional role *(Peterhans and Schweizer, 2013)*. Experiments carried out *in vivo* showed that NCP BVDVs do not induce IFN in the developing fetus when injected during the period when PI is established *(Charleston et al., 2001a)*, whereas during acute postnatal infections it stimulates a vigorous and prolonged type-I (α/β) IFN response *(Charleston et al., 2002; Brackenbury et al., 2005; Smirnova et al., 2008; Palomares et al., 2013)*. These

results indicate that the immunosuppression caused by BVDV may not be associated with low IFN responses, being the lack of IFN production in the fetus an important factor in the establishment of PI. Unlike NCP strains, CP BVDVs induce a strong IFN response *in vitro (Adler et al., 1997; Glew et al., 2003)* and in the fetus *(Charleston et al., 2001a)*, whereas during intranasal infections, CP BVDV may be confined to the route of entry as a result of a rapid and potent induction of IFN *(Lambot et al., 1998b; Brackenbury et al., 2003)*. A wide variety of subsequent studies have revealed that, NCP BVDV not only establishes "self-tolerance" to the infecting strain by avoiding induction of IFN, but also by being resistant to the action of IFN once the infection is established *in utero*, not interfering with IFN action against unrelated viruses ("non-self") replicating in the same host cells *(Schweizer et al., 2006; Schweizer and Peterhans, 2014; Smirnova et al., 2014)*. This novel finding of discrimination between "self" and "non-self" may contribute to the good health status seen in many PI cattle *(Peterhans and Schweizer, 2010)*. Strong evidences exist supporting the involvement of both viral proteins N^{pro} and E^{rns} in the evasion of the host's IFN system *(Meyers et al., 2007; Peterhans and Schweizer, 2010)*.

Other cytokines have been considered of great importance in the innate immune response against BVDV infections, such as the proinflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α), among others. Results in this regard are inconsistent and strongly dependent on the experimental model (*in vivo* or *in vitro*), the organ studied or the viral strain *(Adler et al., 1996; Pedrera et al., 2009a; Risalde et al., 2011b; Raya et al., 2012; Palomares et al., 2014a)*.

During acute infections, changes in the synthesis of certain plasma proteins denominated **acute phase proteins** (APP) can be observed *(Petersen et al., 2004)*. Elevated levels of APP such as Haptoglobin (Hp) and serum amyloid A (SAA) have been described during acute infections with BVDV *(Ganheim et al., 2003; Muller-Doblies et al., 2004; Risalde et al., 2011b; Molina et al., 2014)*.

The last, but not least important, elements of the innate immune system are **antigen presenting cells** (APC), which are in fact considered as pivotal interfaces linking both innate and adaptive responses *(Figure 4)*. "Professional" APC (dendritic cells, macrophages/monocytes and B cells) recognize pathogens (or

more precisely, "pathogen-associated molecular patterns" - PAMP) as BVDV through pattern recognition receptors (PRR), phagocytose them, and accompanied by co-stimulatory signals, present peptides through the MHC-II molecule to T helper cells, which are responsible for subsequent pathogen-specific immune responses *(Werling and Jungi, 2003; Coffey and Werling, 2011; Romero-Palomo et al., 2011)*. Therefore, detrimental effects of BVDV infections on APCs will hamper adaptive immune responses. Studies by Glew *et. al. (2003)* have shown that monocytes and monocyte-derived DCs (moDC) are both susceptible to infection with NCP BVDV and CP BVDV *in vitro*, although striking differences in the response of the two cell types to infection with CP virus were seen. DCs were not susceptible to the cytopathic effect caused by CP BVDV, whereas monocytes were killed. In addition, monocytes infected with NCP BVDV were compromised in their ability to stimulate allogeneic and memory CD4+T cell responses, but DCs were not affected *(Glew et al., 2003)*.

3.2.2. Adaptive immune response to BVDV

Adaptive immunity is an antigen-specific response with immunologic memory regulated by T and B lymphocytes, and the soluble factors produced by them - cytokines and Abs, respectively -, occurring as a result of a previous interaction with APCs *(Murphy et al., 2012)*.

✓ *T cell-mediated immune response*

T-lymphocytes are an important part of the cell-mediated response. T-lymphocytes are divided into three groups: helper (CD4+), cytotoxic (CD8+) and gamma/delta (γδ). The effect of BVDV infection on the number of **circulating** T-lymphocytes is strain dependent and varies from a mild lymphopenia (10-20%) *(Ellis et al., 1988; Brodersen and Kelling, 1999)* to 40-50% *(Ridpath et al., 2007)* or even severe lymphopenia (50-70% decrease) with high virulent strains *(Archambault et al., 2000; Ridpath et al., 2007)*.

BVDV infections have a major impact on **lymphoid organs**, consistently resulting in lymphoid depletion due to apoptosis, whose severity depends on the virulence of the strain or the clinical form, thus causing detrimental effects on T cell populations abundantly found in interfollicular areas of lymph nodes and

tonsils, and in the thymus cortex *(Marshall et al., 1996; Brodersen and Kelling, 1999; Liebler-Tenorio et al., 2003b; 2004; Raya et al., 2012)*.

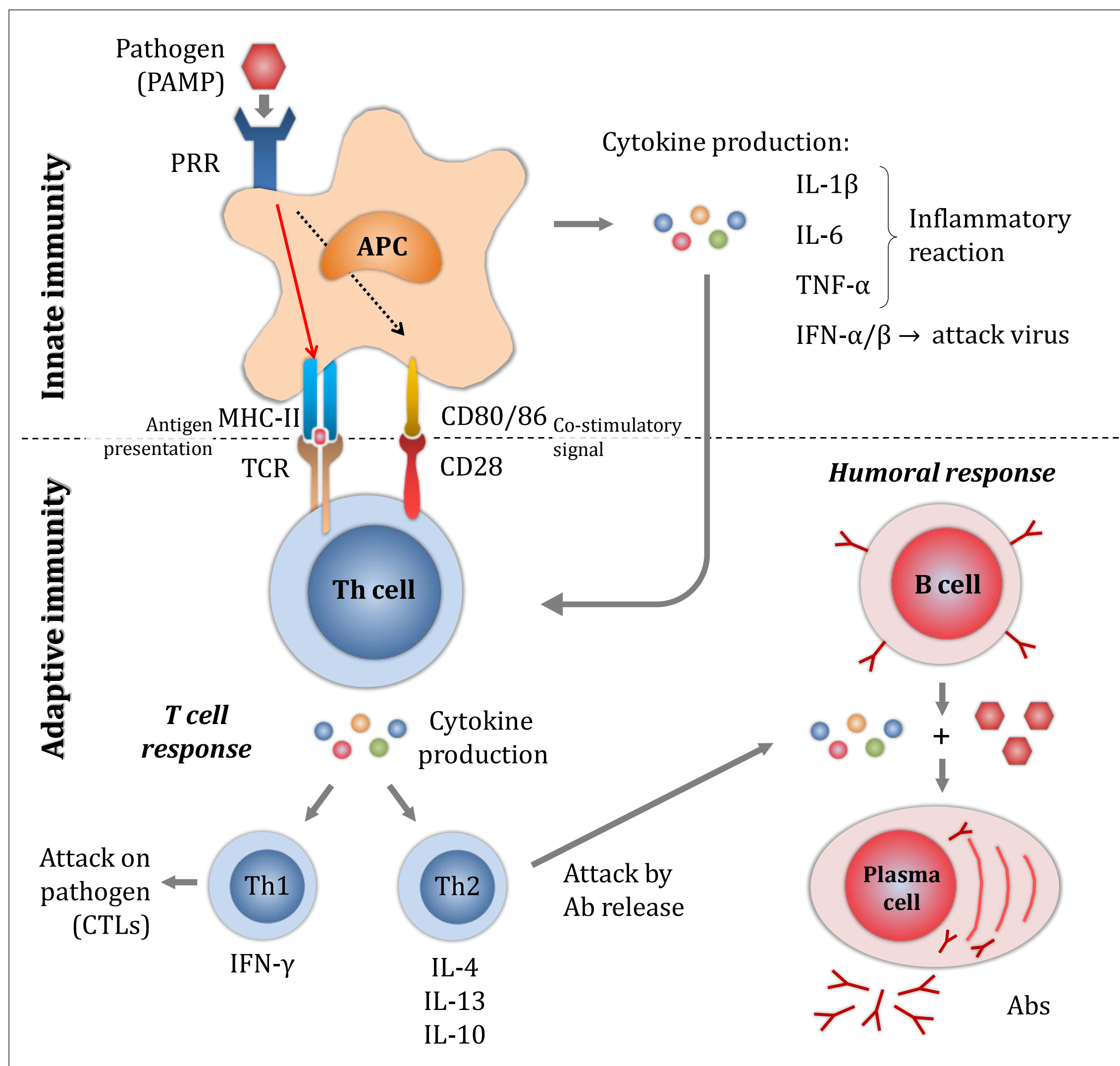

Figure 4. Interaction between innate and adaptive immune responses (adapted from *Chase et al. 2013*). PAMP=pathogen-associated molecular pattern, PRR=pattern recognition receptor, APC=antigen-presenting cell, Th=T helper lymphocytes (CD4+), CTLs=cytotoxic T lymphocytes (CD8+), Abs=antibodies

· T helper lymphocytes (Th/CD4+):

In vivo studies on the role of T-lymphocyte subsets in the response to intranasal NCP BVDV-1 infection revealed that a previous depletion of CD4+ cells with specific mAbs resulted in an extension of the duration of viremia and an increase in the titre of virus in blood, with no effect on nasopharyngeal shedding being noted *(Howard et al., 1992)*. Production of IFN-γ has also been attributed to

CD4 lymphocyte subpopulations after BVDV infection *(Liang et al., 2008)*. These studies demonstrated that CD4+ cells play a pivotal role in coordinating a cell mediated response early in infection *(Howard et al., 1992; Liang et al., 2008)*.

The **Th1/Th2** paradigm postulated by Mosmann *et. al. (1986)* from studies on cytokines produced by murine Th lymphocytes seems to be related with the biotype of BVDV producing infection, being proposed that CP strains tend to shift the immune response towards a pronounced cell-mediated immunity (CMI, or Th1), while NCP strains induce a predominant Th2 immune response *(Lambot et al., 1997)*. Thus, the proliferative T cell response occurring with CP BVDV infections is faster and more prolonged than with NCP BVDV *(Lambot et al., 1997; Collen and Morrison, 2000; Brackenbury et al., 2003)*. Another factor that is seen with **Th1** response is up regulation of IL-2 receptor (IL-2R or CD25) in response to increased levels of IL-2, and intense production of IFNγ, as observed after CP BVDV infection *(Adler et al., 1997; Hou et al., 1998)*, with no production of IL-4 or B-cell stimulatory activity. Conversely, infections with NCP BVDV induce limited cell-mediated immunity, but high levels of antibodies, B cell growth factor and IL-4 activity, with low levels of IL-2/IL-2R and IFN-γ, features more typically observed in **Th2** immune responses *(Lambot et al., 1997; Hou et al., 1998; Rhodes et al., 1999; Burciaga-Robles et al., 2010)*. The down regulation of IFN-γ observed during acute infections with NCP BVDV also inhibited the cell-mediated response against *Mycobacterium bovis* and BHV-1, which could result in reduced host´s ability to contain these pathogens and also in the failure of diagnosis tests to identify cattle with tuberculosis *(Charleston et al., 2001b)*. This Th1/Th2 paradigm attributed to BVDV biotype is not devoid of controversies, since for example, studies with acute ncp BVDV infections show clear tendencies towards a type 1 cytokine response *(Charleston et al., 2002; Molina et al., 2014)*. Differences in the virulence and genotype of BVDV strains have been suggested to be also responsible for differential cytokine expression *(Palomares et al., 2014)*.

· Cytotoxic T lymphocytes (CTL/CD8+):

In vivo depletion of cytotoxic T lymphocytes (CTLs/CD8+), unlike CD4+ depletion, had no demonstrable effect on controlling viremia after BVDV infection, although CD8+ depletion with antibodies did not result as efficient as for CD4+

cells *(Howard et al., 1992)*, therefore, an active role for CD8+ T cells in clearing BVDV should not be ruled out. A specific CD8+ proliferating response against BVDV has been described *in vitro*, appearing to be a Th1-like memory response, with increased IL-2 and IFN-γ but no IL-4 or B cell stimulatory activity *(Rhodes et al., 1999)*. Since CTL cells are MHC-I restricted T-cells *(MacHugh and Sopp, 1991)*, alterations on the surface expression of MHC-I of infected cells will directly affect the CTL response. However, studies on the effect of BVDV infection in this regard do not provide consistent results *(Archambault et al., 2000; Glew et al., 2003; Lee et al., 2009)*.

· Gamma-delta (γδ) T lymphocytes:

As with CD8+ lymphocytes, antibody-mediated depletion of γδ T cells had no demonstrable effect on controlling viremia after BVDV infection *(Howard et al., 1992)*. The role of γδ T cells in BVDV infections has been scarcely investigated, but evidences exist reporting that they may have an important role in preventing MD by controlling CP BVDV infection in PI animals *(Bruschke et al., 1998a)*.

Ruminants have higher levels of γδ T cells than other species. In neonates, up to 60% of lymphocytes can be γδ T cells and levels drop to 30% by a year of age and 5-10% in adults in the peripheral blood and with similar levels in the intestinal epithelium and lamina propria *(Hein and Mackay, 1991)*. The γδ T cells recognize self-determinants on virus infected cells, without the requirement for antigen processing and APCs *(Jutila et al., 2008)*. The function of γδ T cells has been for long a matter of debate *(Guzman et al., 2012)*. These cells have been considered to be more related to natural killer cells in innate immunity rather than adaptive immunity *(Bruschke et al., 1998a; Jutila et al., 2008)*. However, more recent studies consider this cell type the major regulatory T cell subset in cattle *(Hoek et al., 2009; Guzman et al., 2014)*.

✓ *B lymphocyte and humoral response*

The effect of BVDV infection on the number of **circulating B lymphocytes** varies by study from a decrease *(Ellis et al., 1988)* to no effect *(Archambault et al., 2000)* to a transient increase *(Brodersen and Kelling, 1999)*. The major impact on

lymphoid organs occurring after BVDV infection is particularly evident in follicular B lymphocytes, as observed in the depleted lymphoid follicles of lymph nodes, tonsils, and above all of Peyer's patches *(Jubb and Kennedy, 2007a)*, with variations in the severity of the lymphoid depletion depending primarily on the viral strain. Different pathways and mechanisms of apoptosis have been proposed for B cell death *(Stoffregen et al., 2000; Liebler-Tenorio et al., 2003b; Pedrera et al., 2009a; 2009b; 2012b; Brodersen, 2014)*.

After first stimulation, naïve B lymphocytes process and present antigens through surface MHC-II molecules to helper T lymphocytes. Due to this interaction, Th cells further activate B cells to undergo subsequent clonal proliferation and differentiation to memory B lymphocytes and plasma cells, thus producing large amounts of antigen-specific circulating immunoglobulins that, along with maternal passive immunity, constitute the **humoral immunity** *(Mescher, 2013)*.

Intracellular pathogens such as viruses are most effectively eliminated by cell-mediated immune responses. However, extracellular pathogens, like viruses at the portal of entry into the host, are eliminated mostly by neutralizing antibodies *(Srikumaran et al., 2007)*. Therefore, the disappearance of BVDV in acute infections cannot be attributed to the presence of specific antibodies, which have a moderate and delayed response, not being detected until 2-3 weeks post-infection (for diagnostic purposes, paired sera should be taken 3-4 weeks apart) *(Archambault et al., 2000; Muller-Doblies et al., 2004; Lanyon et al., 2014)*.

Maternal BVDV neutralizing Abs provide efficient protection against severe infections. However, high titres of maternal Abs should be take into account during vaccination programs, since although they may generate BVDV specific memory T and B cells, maternal Abs prevent the development of vaccine Ab responses *(Ellis et al., 2001; Endsley et al., 2003; Ridpath et al., 2003)*.

✓ *Adaptive immunity and PI animals*

It has been observed that the leukocyte profile of PI animals can be either altered *(Piccinini et al., 2006)* or remain unaltered *(Brewoo et al., 2007)*. APCs in these animals have been reported to be not compromised in their ability to present viral antigen *(Glew and Howard, 2001)*. The non-responsive character of PI animals has been proved to be very dependent on CD4+ cells and specific to the strain

causing the persistent infection, with single amino acid differences being sufficient for CD4+ T-cell recognition of a heterologous virus *(Collen et al., 2000)*. Due to the mechanisms of immune tolerance occurring in PI animals, exceptionally high viral loads exist in the absence of a humoral response against the persisting viral strain. Thus, the presence of antibodies against BVDV should not be used to rule out persistent infections, since these antibodies can be transmitted via colostrum or be originated in response to heterologous BVDV strains.

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

Interstitial pneumonia has been seen in a limited number of field cases of respiratory disease that occurred as a result of acute BVD and in a few cattle with experimentally induced acute BVD *(Potgieter et al., 1984b; Baszler et al., 1995; Baule et al., 2001; Risalde et al., 2014)*. Conclusive experimental evidence that uncomplicated infection with BVDV causes severe respiratory disease in cattle is lacking, with BVDV eliciting in most cases only mild respiratory tract disease in susceptible cattle. Difficulties in reproduction of severe respiratory disease with BVDV under experimental conditions may reflect choice of viral strain, age of experimental animal, method of viral exposure, or absence of environmental stressors present under field conditions *(Potgieter et al., 1985; Potgieter, 1997; Bolin, 2002)*.

Despite the controversies and inconsistent results considering BVDV as a pathogen inducing lung pathology on its own, BVDV is frequently isolated in outbreaks of bovine respiratory disease complex (BRDC) *(Fulton et al., 2000a; Fulton et al., 2005b)*, giving this virus an outstanding role in the pathogenesis of this disease *(Loneragan et al., 2005; Hessman et al., 2009)*. BRDC, sometimes called *shipping fever* pneumonia of beef calves or *enzootic pneumonia* of dairy calves *(Caswell, 2014)*, is the leading cause of morbidity and mortality in feedlot cattle *(Fulton, 2009)*, and is considered as a multifactorial disorder characterized by a primary active viral infection with bovine respiratory viruses as BVDV, BHV-1, bovine respiratory syncytial virus (BRSV) and parainfluenza-3 virus (PI-3V), that favors secondary bacterial infections produced principally by *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni* (formerly *Haemophilus*

somnus), *Mycoplasma bovis*, and *Trueperella* (formerly *Arcanobacterium) pyogenes (Confer, 2009; Ellis, 2009; Woolums et al., 2009; Caswell, 2014).* The greatest impact of BVDV to the development of bovine respiratory disease lies on the immunosuppression that accompanies acute BVDV infections and predisposes animals to secondary infections, along with the synergy resulting in increased virulence occurring in coinfections of BVDV with other pathogens *(Ridpath, 2010a)*. In this regard, there is evidence that combined infections with BVDV have a potentiating effect on several pathogens, increasing the respiratory disease in a more severe form compared to calves infected only with BHV-1 *(Potgieter et al., 1984a; Risalde et al., 2013)*, BRSV *(Elvander et al., 1998; Brodersen and Kelling, 1999)*, or *Mannheimia haemolytica (Potgieter et al., 1984b; Burciaga-Robles et al., 2010).*

Beside the generalized immune alterations described in section 3.2., BVDV may also contribute to BRDC by different local mechanism, including inhibited ciliary activity of tracheal epithelial cells *(Rossi and Kiesel, 1977)*, immune impairment of the airway epithelium and bronchoalveolar defenses *(Silflow et al., 2005; Al-Haddawi et al., 2007)*, or alterations in the inflammatory response of pulmonary macrophages *(Welsh et al., 1995; Liu et al., 1999)*, among others.

Taken together, field and experimental data indicate that BVDV contribute to BRDC primarily by altering or impairing systemic and local defense mechanisms, rather than acting as a pneumopathogenic virus. By doing this, BVDV allows other pathogens to propagate to higher numbers and to persist for an extended period, leading to severe disease *(Bolin, 2002)*.

4. Epidemiology, diagnosis and control.

Infections with BVDV are not limited to cattle, but may be detected in various species in the mammalian order Artiodactyla (even-toed ungulates). Despite epidemiological evidence of **BVDV infections in species other than cattle**, current knowledge regarding the impact of BVDV on heterologous species is incomplete. In heterologous hosts, BVDV infections with clinical signs analogous to those in cattle have been described and include disease of multiple organ systems, most notably the reproductive tract and immune system. Clinical infections may

negatively impact the health and well-being of heterologous species, including camelids and captive and free-ranging wildlife. Of additional importance are BVDV infections in small ruminants and swine where difficulties arise in laboratory testing for BDV and CSFV, respectively. Pestiviruses are antigenically closely related and their cross-reactivity requires additional efforts in virological testing. As in cattle populations, persistent infections have also been detected in heterologous species, which could facilitate reservoirs for BVDV that may be of great importance when control programs are in progress *(Ames, 2005; Vilcek and Nettleton, 2006; Passler and Walz, 2010; Ridpath, 2010b; Henningson et al., 2013; Passler et al., 2014)*.

Cattle persistently infected with BVDV shed large amounts of virus their entire life and are the major source of BVDV **transmission** both within and among herds. Acutely infected cattle are also an important source of BVDV transmission, but the level of virus shed is considerably lower and the length of shedding is limited. Oronasal route is the most common route of infection, and the most efficient mode of transmission is direct contact with body fluids, although indirect transmission can occur through mechanical vectors. Vertical transmission has an indispensable role in the development of PI animals *(Lindberg and Houe, 2005; Thurmond, 2005)*.

Extended reviews on the techniques and considerations for **diagnosis** of BVDV infections can be found in the literature, standing out the following: Saliki and Dubovi *(2004)*, Goyal *(2005)*, OIE *(2008)*, Dubovi *(2013)*, Lanyon *et al. (2014)*.

The wide range of diagnostic tools available has allowed successful BVDV **control and eradication** schemes to become a reality *(Brownlie and Booth, 2014)*. Successful control and eventual eradication of BVDV requires a multidimensional approach, involving vaccination (reviewed by Fulton *(2005)* and Ridpath *(2013)*), biosecurity *(Smith and Grotelueschen, 2004)*, and continuous surveillance for rapid detection of reinfection and PI animals. With the understanding that PI individuals are the primary transmission source, these animals naturally become the target for eradication *(Lindberg and Houe, 2005)*. Test and cull schemes have successfully been applied in many countries, including all or regions of Austria, Scotland, The

Netherlands, Norway, Denmark, Sweden, Switzerland, Italy, Slovenia, Germany, France, Ireland and Finland *(Astiz-Blanco, 2013; Lanyon et al., 2014)*. These schemes have been thoroughly reported, with common features identified and reviewed *(Lindberg and Alenius, 1999; Sandvik, 2004; Houe et al., 2006; Barrett, 2012; Stahl and Alenius, 2012; Loken and Nyberg, 2013)*. The Scandinavian countries, considered the pioneers in control programs *(Stahl and Alenius, 2012)*, along with the dairy industry in Switzerland, are now largely regarded as BVDV-free, due to the implementation of successful systematic eradication programs *(Lanyon et al., 2014)*. In **Spain** the virus is endemic, with herd prevalences varying among regions from 50 to 100% *(Arnaiz et al., 2012)*. In this country, control programmes exist in some regions, with the aim of reducing economic losses due to BVDV outbreaks *(Dieguez et al., 2009)*. Some of the key points that might success for controlling and eradicating BVDV according to the current status in Spain are proposed by Arnaiz *et al. (2012)*.

II. Bovine Herpesvirus type 1 (BHV-1)

Bovine herpesvirus 1 (BHV-1), isolated for the first time in the USA in 1956 *(Madin et al., 1956)*, is one of the major pathogens affecting cattle, being responsible for important economic losses worldwide *(Sanchez-murillo, 1996; Deregt, 1998; Bowland and Shewen, 2000)*. BHV-1 is the causative agent of a variety of clinical syndromes, including infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB), and it is also involved in the multi-factorial bovine respiratory disease complex (BRDC) *(Jones and Chowdhury, 2010)*.

1. Etiology

BHV-1 is a member of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae*, which belongs to the *Herpesviridae* family, order *Herpesvirales* *(Pellett et al., 2012) (Table 1)*. Herpesviruses are enveloped, spherical to pleomorphic, of 120-200 nm diameter, composed of an icosahedral nucleocapsid of 162 capsomers surrounded by a proteinaceous tegument and an outer envelope *(Figure 1A)*. The viral genome consists of linear double-stranded DNA (125-290 kbp) that encodes for about 70 structural and non-structural proteins. The viral genomic sequence can be divided into a unique long (UL) segment and a unique short (US) segment flanked by two repeated and inverted sequences (internal repeat, IR; terminal repeat, TR) *(Figure 1B)*. This genome encloses 10 genes encoding glycoproteins (GP), six of them located in the UL segment and the other four in the US segment. gB, gC, and gD are considered 'major' or more abundant GPs, and others (e.g. gE and gH) as 'minor' GPs. A segment located in the IR inverted sequence is actively transcribed during latency giving rise to the Latency Related Transcripts (LRT). Information on the proteins encoded by BHV-1 genome can be found extensively detailed by Levings and Roth *(2013a)* and Biswas *et al. (2013)*. DNA replication and encapsidation occur in the nucleus and immature virions acquire their envelope by budding through the inner layer of the nuclear membrane *(Knowles, 2011)*. BHV-1 virus can be grown in a wide variety of cells

from different species, where it produces a characteristic cytopathic effect *(Wilkes, 2013)*.

Table 1. Taxonomy of Bovine herpesvirus type 1

Group	Family	Subfamily	Genus	Species
Double-Stranded DNA Viruses (Group I) Order *Herpesvirales*	*Herpesviridae* →	*Alphaherpesvirinae* → *Betaherpesvirinae* *Gammaherpesvirinae*	*Varicellovirus* → *Iltovirus* *Simplexvirus* *Mardivirus*	· **BoHV-1**, 5 · SuHV-1 · CaHV-1 · EHV-1, 3, 4, 8, 9 · FeHV-1 ...

For a direct link to http://viralzone.expasy.org/, press on each word on the table. [Etymology: *Herpes* (from Greek *herpes*, "creeping"); *Ilto (*from "infectious laryngotracheitis"); *Mardi* (from "Marek's disease"); *Simplex* (from Latin *simplex*, "simple"); *Varicello* (derived from Latin *varius*, "spotted", and its diminutive variola, "smallpox")*(Pellett et al., 2012)*]

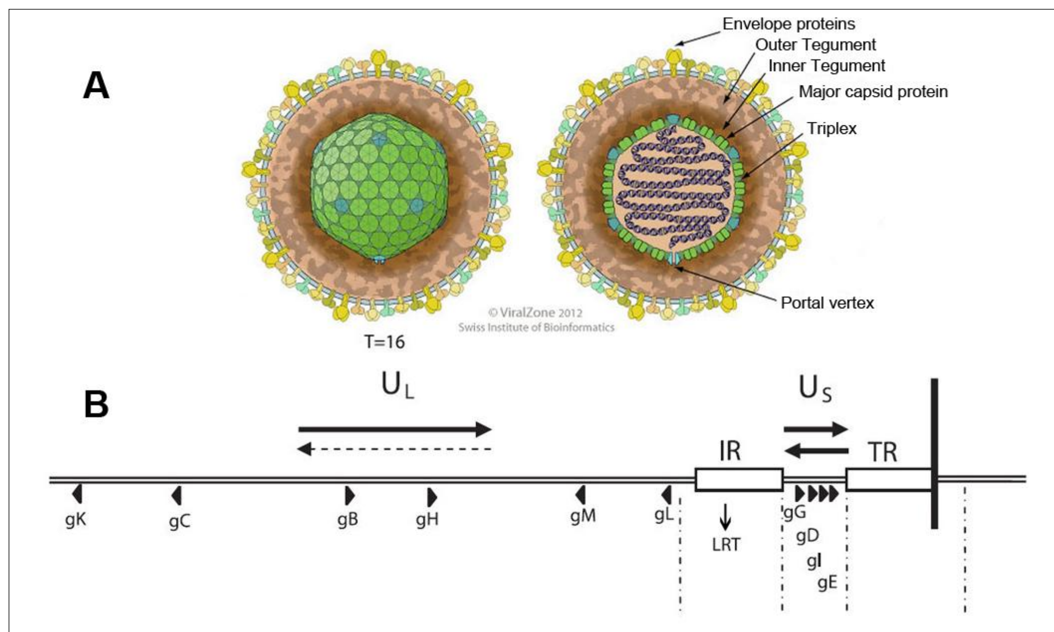

Figure 1. Structure (A) and genome organization (B) of BHV-1 (Adapted from *Swiss Institute of Bioinformatics (http://viralzone.expasy.org)* and *Muylkens et al., 2007*)

Only a single serotype of BHV-1 is recognized; however, based on the genomic analysis and viral peptide patterns, BHV-1 can be divided into several subtypes: BHV-1.**1**, BHV-1.**2a** and BHV-1.**2b** *(Miller et al., 1991)*. BHV-1.1 mostly is related to the respiratory syndrome while BHV-1.2 subtypes are related to genital infections, being considered BHV-1.2 subtypes of less virulence than subtype 1.1 *(Edwards et al., 1990)*. The former BHV-1.3, previously considered responsible for a neurologic form of BHV-1 infection, has been reclassified as BHV-5 (bovine encephalitis virus) *(Roizmann et al., 1992; Del Medico Zajac et al., 2010)*.

2. Clinical forms and lesions

The clinical signs may vary widely and have been grouped as respiratory-conjunctival forms and genital forms. Mortality is low and severity of disease depends on the immune status of the animal and the possible appearance of secondary bacterial infections. Induction of abortion has also been frequently associated to BHV-1 infection, described for the first time 50 years ago *(Kennedy and Richards, 1964; Graham, 2013; O'Toole et al., 2014)*, as well as the appearance of fatal multisystemic infection in neonates *(Mechor et al., 1987; Moeller et al., 2013)*. The incubation period for the respiratory and genital forms of BHV-1 is 2–6 days *(Yates, 1982)*. Uncomplicated cases of respiratory or genital disease caused by BHV-1 last about 5-10 days and the animals recover rapidly, although they remain as latent carriers.

Although most BHV-1.1 strains have been isolated from respiratory tract diseases or abortion cases and BHV-1.2 strains from genital organ lesions, the only reliable distinctive criterion is the viral DNA analysis. Indeed, calves infected experimentally by the nasal route with BHV-1.2 strains showed respiratory clinical signs and were able to transmit the respiratory infection to control calves. Likewise, reproductive tract lesions in heifers were observed after intrauterine inoculation with BHV-1.1 *(Muylkens et al., 2007)*.

2.1. Respiratory form (IBR)

IBR occurs as a subclinical, mild or severe disease. In mild cases, clinical signs may be limited to a serous nasal discharge and conjunctivitis with profuse lacrimation, with the hair beneath the eye becoming heavily soiled. Classical IBR is characterized by pyrexia (40.5-42ºC), inappetence, increased respiratory rate, dyspnea, persistent harsh cough and depression *(Blowey and Weaver, 2011b)*. Loss of body weight and severe drop in milk production in milking cows can also be observed. There is bilateral nasal discharge that is initially serous and later muco-purulent. The nasal mucosa is hyperemic and lesions can progress to pustular necrosis or large hemorrhagic and ulcerated areas covered by a cream colored diphtheritic membrane. Mouth breathing, salivation and a deep bronchial cough are common. The lesions extend to trachea and sinuses if complicated with

secondary bacterial infections; pneumonia is a complication and not part of the primary disease. Abortion is a consequence of a respiratory BHV-1 infection of a seronegative cow, consistently being observed several foci of coagulative necrosis in fetal organs, particularly common in the liver *(Borel et al., 2014; O'Toole et al., 2014)*. Associated with BHV-1 infections, intranuclear inclusion bodies may be present in necrotic epithelial cells or in the periphery of necrotic foci, although these are an inconsistent finding *(Jubb and Kennedy, 2007b)*. Secondary bacterial or viral agents may contribute to severe respiratory disease in the context of the bovine respiratory disease complex (BRDC) *(Fulton, 2009)*.

2.2. Genital form (IPV/IPB)

The BHV-1 genital form is usually transmitted at mating. The names given to the diseases affecting the cow (infectious pustular vulvovaginitis, IPV) and the bull (infectious pustular balanoposthitis, IPB) describe clearly the clinical pictures observed following the primary infection. Frequent urination and tail swishing are characteristic signs noticed initially. Affected animals develop fever, depression and anorexia; they seek to avoid contact of the tail with the vulva. It is also common to observe swollen vulva or small papules followed by erosions and ulcers on the mucosal surface *(Miller and van der Maaten, 1984)*. Lesions similar to those of IPV develop on the mucosa of the penis and prepuce *(Vogel et al., 2004)*. IPV/IPB are commonly mild or subclinical, being restricted to the genital organs, although more severe infections affecting more organs have been reported. Secondary bacterial infection is common in both genital forms.

3. Pathogenesis

3.1. Entry and dissemination

The natural infection occurs through mucous membranes of the upper respiratory tract or genital tract *(Steukers et al., 2011)*, where BHV-1 undergoes massive lytic replication in epithelial cells, excreting high titres of virus in the nasal exudates and genital secretions. The new progeny also spreads into the infected animal through different routes: (1) by local dissemination (in the extracellular matrix or directly to neighboring uninfected cells); (2) by systemic spread/viremia

(Fuchs et al., 1999), causing other manifestations as abortions or fatal systemic infection in young calves; or (3) by the neuroinvasive route. This latter route is responsible for the establishment of **latent infections** when BHV-1 reaches by neuronal axonal transport the trigeminal or sacral ganglia *(Homan and Easterday, 1980; Ackermann and Wyler, 1984)*, being established a life-long infection. During latent infections, gene expression is restricted to the so-called latency-related transcripts (LRT), which inhibit programmed cell death in latently infected cells *(Ciacci-Zanella et al., 1999; Sinani et al., 2014)*. Stress situations can induce reactivation of the latent infection (detailed below in section Epidemiology). Consequently, the virus may switch between latent and lytic infection and may be shed intermittently into the environment and spread to contact animals. Although establishment of latency in ganglionic neurons is the main site of latency for BHV-1 and other α-herpesvirinae subfamily members, latent or persistent infections also occur in non-neural sites like tonsils, lymph nodes or peripheral blood cells *(Jones et al., 2011)*.

3.2. BHV-1 and immunity

Although BHV-1 can cause transient immunosuppression in cattle (described below), a potent immune response eventually occurs during acute infection, which defeats disease. The host immune response to BHV-1 infection includes innate and adaptive immune responses.

Innate immune responses are the first line of defense against BHV-1 infection. Some of these non-specific mechanisms include the activation of the complement pathway and the antiviral action of IFN *(Campos et al., 1989)*. The production of early cytokines leads to the recruitment and activation of different cells such as macrophages, neutrophils and natural killer (NK) cells. In addition, NK-like cytotoxicity is also associated with a population of γδ T-cells *(Amadori et al., 1995)*. These effectors enhance the first antiviral wave by secreting cytokines in the infected epithelium and killing virus infected cells. The non-specific activated immune cells are also essential in initiating and regulating the specific immune response to BHV-1. For more details on innate immunity during BHV-1 infections, see review by Levings and Roth *(2013a)*.

Cell-mediated immune (CMI) responses play an important role in killing virus-infected cells that express viral antigens on the cell surface. The specific cellular immunity is detected from the 5th day post infection (dpi) and reaches a peak at 7–10 dpi. It generally coincides with the recovery of clinical manifestations *(Babiuk et al., 1996)*. Specific T helper lymphocytes (Th/CD4+) mediate the lysis of BHV-1 infected cells by activating macrophage and NK cells through IFN-γ and IL-2 secretion, and by recruiting and promoting the proliferation of specific cytotoxic T lymphocytes (CTL/CD8+) *(Janssen et al., 2003)*, of huge importance against BHV-1 cell-to-cell spread occurring in upper respiratory epithelium before hematogenous dissemination *(van Drunen Littel-van den Hurk, 2007)*.

While cell-mediated immunity is involved in recovery from infection, the specific humoral immune response is thought to be critical in preventing productive secondary infection and limiting the consequences of reactivation *(Babiuk et al., 1996)*, as well as in protecting the neonate against systemic and lethal disease through passive colostral immunity *(Mechor et al., 1987)*. Envelope glycoproteins gB, gC, gD and gH are the most potent inducers of virus neutralizing antibodies *(Marshall et al., 1988)*. In addition, non-neutralizing antibody may mediate the destruction of enveloped virus or cells expressing viral proteins on the cell membranes, and this process is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). After the acute infection, specific antibodies to BHV-1 can be detected after 7-14 days. BHV-1 usually persists life-long in a latent state in the trigeminal or sacral ganglia and can be subsequently reactivated resulting in virus shedding (re-excretion) without exhibition of clinical disease. Therefore, antibody-positive animals have to be classified as infected with BHV-1 (with two exceptions: serological responses induced by vaccination with an inactivated vaccine or by colostral antibodies) *(OIE, 2010)*. However, this antibody response may fall below the detection limit of some tests after a number of years. So, the serological test must be highly sensitive to detect the low level of antibodies in the serum of latently infected animals *(Nandi et al., 2009)*. Maternal antibodies have a biological half-life of about 3 weeks, but may be detected occasionally in animals up to 9 months old, and rarely in animals over this age. For more details on adaptive immunity during BHV-1 infections, see review by Levings and Roth *(2013b)*.

BHV-1 can transiently suppress the immune system of infected cattle. There is impairment of function of macrophages, PMNs and lymphocytes, decreased expression of interleukin-2 (IL-2) receptor, decreased mitogenic stimulation of peripheral blood mononuclear cells (PBMCs) and a reduced number of circulating T cells *(Hutchings et al., 1990; Tikoo et al., 1995; Winkler et al., 1999)*. There is an impairment of phagocytosis, ADCC and T cell stimulation due to infection of monocytes and macrophages, as well as inhibition of IFN-β-dependent transcription *(Babiuk et al., 1996; Henderson et al., 2005)*. The virus infects CD4+ T cells (but not CD8), inducing a loss of CD4 expression followed by apoptosis of these cells *(Babiuk et al., 1996; Winkler et al., 1999)*. BHV-1 is known to down-regulate the expression of MHC-I molecules on the surface of infected cells *(Nataraj et al., 1997)*, doing so by different mechanism *(Koppers-Lalic et al., 2001; 2005; Wei et al., 2011)*. This MHC-I downregulation compromises the development of a robust CTL response against not only BHV-1 *(Denis et al., 1993)*, but also the other viral pathogens of BRDC.

3.3. Role of BHV-1 in the BRDC

It has been described above that in addition to the clinical symptoms, BHV-1 infection can suppress the immune system of infected cattle, although in a transient manner, since potent immune response eventually occurs during acute infection. With respect to BRDC, this implies that immunosuppression initiated by BHV-1 is short-lived. In addition to the transient immunosuppression described, some other factor associated to BHV-1 infection may contribute to bacterial secondary infections and appearance of BRDC: BHV-1 can induce loss of cilia and goblet cells in the upper respiratory tract, leading to epithelial erosions which could progress to necrosis of epithelium and adjacent lymphoid tissue *(Schuh et al., 1992)*, reducing the mucosal clearance. After this direct CPE, BHV-1 may also reduce the repair of the airway epithelium by inhibiting the migration of new epithelial cells to injured areas *(Spurzem et al., 1995)*. BHV-1 infection of bronchial epithelial cells triggers cytokine overexpression that may contribute to inducing lung injury *(Rivera-Rivas et al., 2009)*. This sequence of events favors the migration and colonization of the lower respiratory tract by bacterial respiratory pathogens *(Yates, 1982; Confer, 2009; Jones and Chowdhury, 2010).*

4. Epidemiology, diagnosis and control.

BHV-1, unlike some other alphaherpesviruses such as suid herpesvirus 1 (Aujeszky's disease virus), does not commonly or stably cross species barriers and has restricted cattle and buffalo **host range** *(Brake and Studdert, 1985; Knowles, 2011)*. Direct nose to nose contact is the preferential way of **transmission** of BHV-1. However, airborne transmissions by the aerosol route were demonstrated on short distances *(Mars et al., 2000)*. Genital infection requires direct contact at mating. Genital transmission also occurs through virus contaminated semen *(Kupferschmied et al., 1986)*. The **latency** reactivation cycle has a deep epidemiological impact since it is responsible for the maintenance of BHV-1 in a cattle population. Reactivation may be triggered by stress associated with parturition, transport, animal movement and mixing, inclement weather, concomitant infections, poor husbandry or diet, overcrowding or following treatment with corticosteroids *(Raaperi et al., 2014)*.

BHV-1 infection may be suspected on the basis of clinical, pathological and epidemiological findings. However, to make a definite **diagnosis**, laboratory examinations (serology and/or virus detection) are required. Extended reviews that include different techniques and considerations for diagnosis of BHV-1 infections can be found in the literature *(OIE, 2010; Fulton and Confer, 2012; Biswas et al., 2013; Mahajan et al., 2013)*.

BHV-1 is endemic in cattle populations worldwide, although there are significant differences in prevalence and incidence. By implementing **control** measures, the virus has been **eradicated** in several European countries (Austria, Denmark, Finland, Sweden, Switzerland and Norway), as well as in the Federal State of Bavaria in Germany and the Province of Bolzano in Italy *(Arnaiz et al., 2012; Thiry and Casademunt, 2012; Raaperi et al., 2014)*. Several ruminant alphaherpesviruses have been shown to form a cluster of viruses closely related to BHV-1, sharing common antigenic properties. Therefore, the serological relationships between them can be considered as a threat to BHV-1 eradication programmes *(Thiry et al., 2006)*. A number of reasons speak in favor of IBR

eradication. However, the price of such an achievement is unfortunately very high and the costs of such campaigns have to be weighed against the benefits *(Zimmerman et al., 2007)*.

Most vaccines are very efficacious at preventing the clinical signs after the challenge with highly virulent strains (for more details on BHV-1 vaccines, see reviews by *van Drunen Littel-van den Hurk (2006)*, *Ruiz-Saenz et al. (2009)*, and *Levings and Roth (2013b)*). However, no one is able to fully prevent the infection by that challenge strain, which establishes a latent infection, and might be reexcreted under reactivation stimulus. For this reason, culling of seropositive animals without vaccination has been the most successful method for eradicating BHV-1 in those regions where the seroprevalence is relatively low *(Ackermann and Engels, 2006)*.

In **Spain** the virus is endemic, with herd seroprevalences of 60% *(Yus et al., 2014)* (70.4% in Andalusia *(Gonzalez-Garcia et al., 2009)*. In this country, voluntary regional BHV-1 control programmes in specified herds are ongoing *(Dieguez-Casalta, 2012; Yus et al., 2014)*. The reasons for including BHV-1 in control and eradication programmes in Spain, unlike BVDV, are principally promoted for the trade restrictions in the EU rather than the direct economic losses due to the disease itself *(Arnaiz et al., 2012)*.

III. Thymus

The thymus is a primary lymphoid organ in which bone marrow-derived T cell precursors undergo differentiation, ultimately leading to migration of positively selected thymocytes to the T cell-dependent areas of peripheral lymphoid tissues.

1. Development and anatomy

The mammalian thymus is located in the cranial mediastinum with variable extension into the cervical region. Embryologically, it develops as a network (thymic epithelial reticulum) from the endoderm of the third pharyngeal pouch, which is invaded by blood vessels from the surrounding mesenchyme and infiltrated by large numbers of lymphocyte precursors from the bone marrow. In the calf, the thymus is particularly large and extends from the larynx to the pericardium *(König and Liebich, 2009)*. It is distinctively divided into a paired cervical and an unpaired thoracic part, which are connected by a narrow isthmus ventral to the trachea *(Figure 1)*. The cervical part consists of a body that divides into two tapering horns along the trachea. The thoracic part is located in the left half of the dorsal part of the cranial mediastinum.

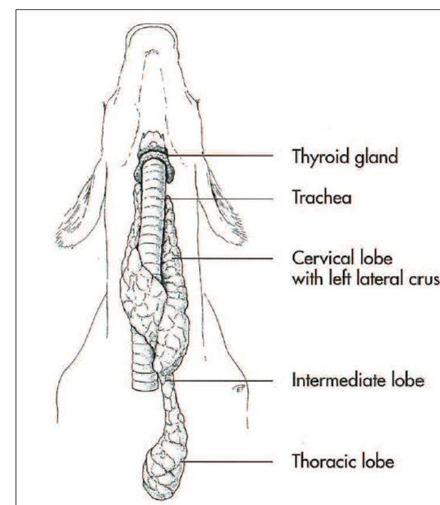

Figure 1. Topography of the thymus of the calf, schematic. *(König and Liebich, 2009)*

The thymus is most prominent in young animals and after sexual maturity, a progressive normal involution of the organ takes place. Thymic involution is characterized by a gradual depletion of lymphocytes (especially from the cortex), enlargement of the epithelial reticular cells, and invasion of the parenchyma by adipocytes originating from the interlobular connective tissue. Despite this progressive physiological involution, the thymus retains even in the adult (although at lesser extent) its ability to form T lymphocytes *(Douek and Koup, 2000)*. In calves, the thymic weight increases up to 12 months of age, and then

begins to decline by the age of 14 months, although no abrupt diminution in thymus weight has been detected at puberty *(Blanco et al., 2000)*.

2. Histology

An heterogeneous population of epithelial reticular cells instead of connective tissue form the framework of this organ which is seeded with developing lymphocytes from the bone marrow. The organ is comprised of **lobes** covered by a connective tissue capsule. The capsule extends into the lobes as septa that further divide the parenchyma into **lobules** *(Gartner and Hiatt, 2013)*. The thymus possesses no lymphoid follicles; instead, each lobule is clearly divided into a dark staining cortex and a lighter medulla, attributable to the much higher density of T-cells in the cortex *(Figure 2)*. Although these thymic lobules are well demarcated by connective tissue, they are actually all interconnected, since the lobulation is not complete, as it is in some other lobulated organs.

2.1. Thymic cortex

The thymic cortex consists mainly of an epithelial reticulum and lymphocytes. **Stellate epithelial reticular cells (ERC)** form a framework in the cortex, and are characterized by having large, pale, ovoid nuclei and long, branching cytoplasmic processes that contain numerous intermediate filaments; their cellular organelles are inconspicuous. Adjacent epithelial reticular cells are connected to each other by desmosomes, thus forming a cellular stromal network which is almost impossible to demonstrate in H&E preparations, being obscured by the lymphocytes that sit on them. At the periphery of the lobules and around the perivascular spaces, a single layer of long, **flattened ERC** forms a continuous lining. ERC cells produce thymosin, thymulin, thymic humoral factor and thymopoietin, which are proteins that influence thymocyte differentiation. Some of the ERC cells in the outer cortex, called **thymic nurse cells** (TNC), have long membrane extensions that surround various numbers of thymocytes, forming large lymphoepithelial complexes *(Savino and Dardenne, 2000)*. **Thymocytes** (maturing T lymphocytes) occupy the space between the epithelial reticular cells. Blast thymocytes migrate from the bone marrow via the blood and locate in the

periphery of the cortex where the cells undergo mitotic division. As the thymocytes continue to mature, they move from the outer thymic cortex toward the medulla, being subjected to a rigorous process of lymphocyte selection *(Figure 3)*. Some **macrophages** can be observed in the cortex, with the role of phagocytose and eliminate dead thymocytes. Due to this process of phagocytosis, macrophages are frequently observed in the cortex containing remnants of apoptotic cells in their cytoplasm, giving rise at this phase to the so-called **tingible body macrophages**.

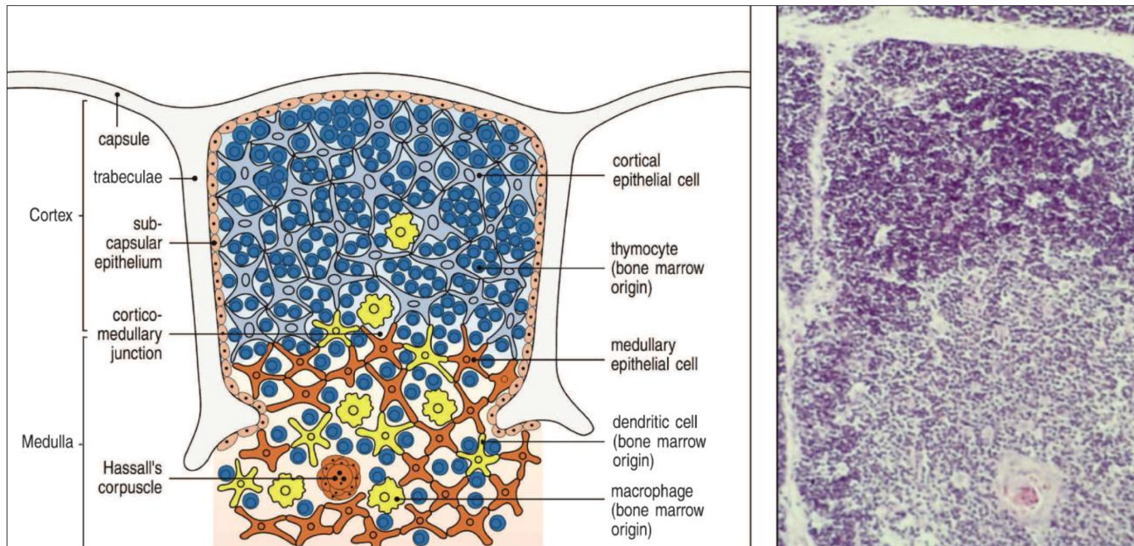

Figure 2. Cellular organization of the thymus *(Murphy et al., 2012)*

2.2. Thymic medulla

The cellular scaffolding of the medulla is mainly composed by **medullary ERC**, which are somewhat larger than their cortical counterparts (and thus more obvious). These larger cells contain more mitochondria, an extensive rough endoplasmic reticulum, well-developed Golgi complex, and granules when compared to cortical epithelial reticular cells. Some medullary ERC form thymic corpuscles, also called **Hassall's corpuscles**, whose function remains unclear. Hassall's corpuscles consist of one to several calcified or degenerated large central cells, which are surrounded by layers of flat keratinized cells in a concentric arrangement. Corpuscle cells are connected by desmosomes and contain bundles of intermediate filaments *(Pierscinski, 1979)*. Interdigitating **dendritic cells**, similar to those present in the T-cell areas of secondary lymphatic organs, are also present in the medulla *(Romero-Palomo et al., 2011)*. The medulla is lighter

staining than the cortex, as fewer small lymphocytes and macrophages predominantly fill the space of the framework.

2.3. Thymic vascularization

The thymic arteries penetrate the organ through the capsule, follow the course of the interlobular connective tissue septa and enter the parenchyma at the corticomedullary junction. The corticomedullary arterioles ramify into capillaries that extend into the cortex and medulla *(Kato, 1997)*. These vessels branch into the cortex as capillaries rarely fenestrated, which are surrounded by sheaths of epithelial reticular cells processes, and perivascular connective tissue, forming all these structures the so-called **blood-thymus barrier** *(Raviola and Karnovsky, 1972)*. The barrier prevents antigens from passing out of the blood and interfering nearby positive selection of maturing lymphocytes within the cortex. Cortical capillaries then empty into postcapillary venules at the corticomedullary junction. By contrast, medullary capillaries are fenestrated and freely permeable, allowing circulating antigens to contribute to the negative lymphocyte selection processes. After looping through the medulla and cortex, the capillaries terminate in the postcapillary venules, located either at the corticomedullary junction or in the medulla; the postcapillary venules join veins in the connective tissue septa. The wall of the postcapillary venules is also highly permeable and allows lymphocytes from the thymus to enter blood circulation at this point. Lymphatics in the thymus are primarily located in the connective tissue septa surrounding lobules, draining into adjacent lymph nodes. No afferent lymphatics are observed in the thymus *(Pearse, 2006)*.

3. Function

The process of T cell differentiation begins when T cell precursors from the bone marrow enter the thymus in the cortico-medullary region and migrate to the cortex *(Figure 3)*, where proliferate extensively. These immature thymocytes begin the process of differentiation in the subcapsular cortical region of the thymic lobules, and are known as double-negative (**DN**, $CD4^{-}CD8^{-}$) cells, since they do not express CD4 or CD8 accessory molecules (nor the CD3/TCR complex),

representing about 5% of total thymocytes. As they progress in differentiation, they begin to acquire CD4 and CD8 markers, becoming double-positive (**DP**, $CD4^{+}CD8^{+}$) thymocytes, which occupy most of the cortical region and account for about 80% of the whole population. At this stage, TCR genes are completely rearranged, yielding the membrane expression of TCRs (complexed with CD3) at low densities (TCR^{low}). These cells are then submitted to the process of **positive selection**, where only those thymocytes bearing TCRs that recognize antigen in combination with self-MHC molecules of thymic microenvironmental cells will continue to mature *(Benoist and Mathis, 1989)*, whereas thymocytes that do not express TCRs die by apoptosis. Positive selection also coordinates the choice of co-receptor expression: CD4 becomes expressed by T cells harboring MHC class II restricted receptors, and CD8 by cells harboring MHC class I restricted receptors. The small percentages of positively selected cells progress in their differentiation, moving towards the medulla and becoming mature single-positive cells (**SP**, $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$), both expressing high densities of CD3/TCR complex, and accounting for nearly 15% of total thymocytes *(Savino and Dardenne, 2000)*. Besides positive selection, an additional process of **negative selection** takes place within the thymus, promoting apoptosis-mediated deletion of self-reactive cells from the lymphocyte repertoire, rendering it tolerant to the antigens of the body *(Nossal, 1994)*. Although this mechanism of deletion controls most autoimmune disorders, the process is not perfect and T lymphocytes reactive to self-antigens do escape the thymus. In this regard, along with differentiation into CD4+ SP cells, some elements do not acquire the functional feature of typical helper cells (that is, cells able to trigger and/or enhance an immune response in the periphery), but rather differentiate into “regulatory” T cells (most of them bearing the phenotype CD4+CD25+Foxp3+), which actually block a given immune response *(Maggi et al., 2005)*. As we can see in *Figure 3*, positive selection events begin earlier in DP cells, whereas negative selection takes place in both DP and SP thymocytes. In parallel with this migration and differentiation, thymocytes interact with various components of the thymic microenvironment, a tridimensional network formed of epithelial cells, macrophages, DCs, fibroblasts, and extracellular matrix (ECM) components *(Anderson et al., 1996)*. According to this interaction, it has been demonstrated that the process of positive selection appears to be essentially

conveyed by cortical thymic epithelial cells (cTEC) *(Anderson and Takahama, 2012)*, whereas professional APCs such as DCs seem to be critical for negative selection of high-affinity developing T cells in the medulla *(Brocker et al., 1997; Derbinski and Kyewski, 2010)*. After this highly stringent process of differentiation, only about 1-3% of total thymocytes exit the thymus (also by blood vessels) as mature naïve T cells, migrating to the peripheral lymphoid organs.

In the thymus, besides functional CD4+ and CD8+ T lymphocytes, dense accumulations of **γδ T lymphocytes** can be observed in the medulla, and much more scarcely in the cortex *(Hein and Mackay, 1991)*, conversely to what happens with CD4 and CD8 molecules, which are present in the vast majority of the cortical thymocytes. γδ T cells in the thymus are frequently observed surrounding Hassal's corpuscles, and some theories claiming for an important role of medullary epithelial cells in the process of γδ T cells differentiation have been proposed *(Hein and Mackay, 1991)*. Additionally, some **B lymphocytes** can also be observed in the thymus, although these cells arrive fully mature from other locations and do not undergo any type of selection.

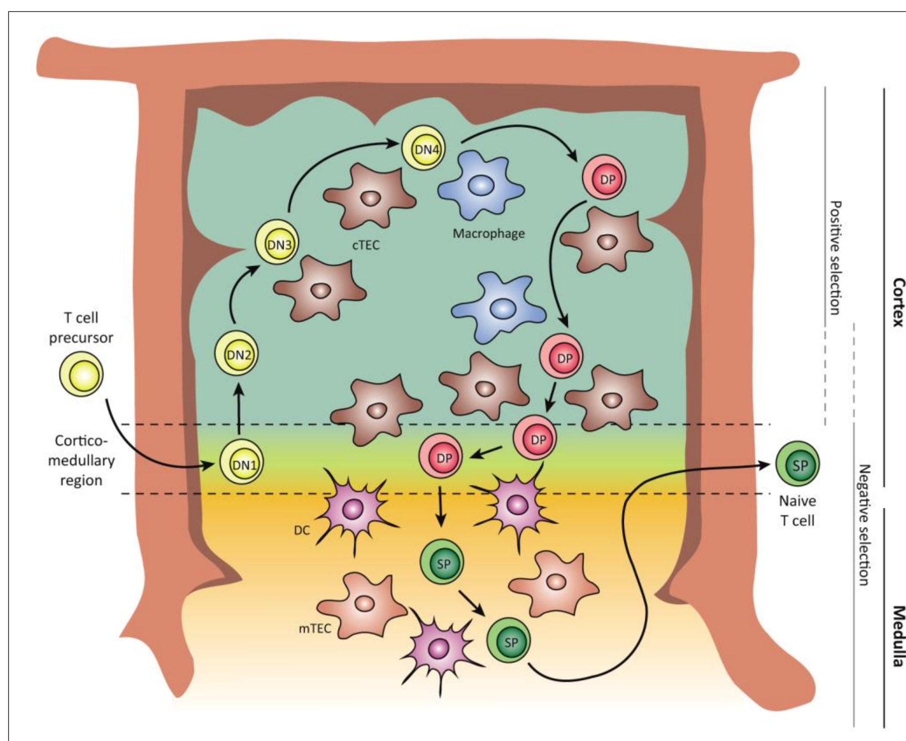

Figure 3. T cell differentiation. Schematic representation of T cell traffic within the thymus and location of the major steps during T cell selection *(Nunes-Alves et al., 2013)*.

4. Thymus and infection

T cell differentiation depends on the thymic microenvironment and the cytokine milieu surrounding the differentiating cells *(Starr et al., 2003)*. This raises the possibility that during infection, changes in soluble factors or antigens present within the thymus alter T cell differentiation. Indeed, certain bacteria, virus, fungi, and parasites can directly invade the thymus, leading to detrimental effects on thymic structure and function *(Savino, 2006; Nunes-Alves et al., 2013)*. Infection-induced alterations include thymic atrophy, modifications in the thymic structure, and alterations in T cells exported to the periphery *(Figure 4)*.

Two scenarios are possible when considering the origin of thymic infection during hematogenous spread of infection. First, circulating pathogens can enter the thymus and infect cells in a targeted manner, as represented by thymotropic variants of HIV *(Calabro et al., 1995)*. Alternately, there is the “Trojan Horse” model. The trafficking of several cell types between the periphery and the thymus makes this possible. T cells circulate from the periphery to the thymus *(Hale and Fink, 2009)*, and if infected, could seed the thymus with pathogens that target T cells. Similarly, certain DC subsets migrate from the periphery to the thymus and modulate T cell tolerance *(Proietto et al., 2009)*, raising the possibility that infected DCs spread the infection.

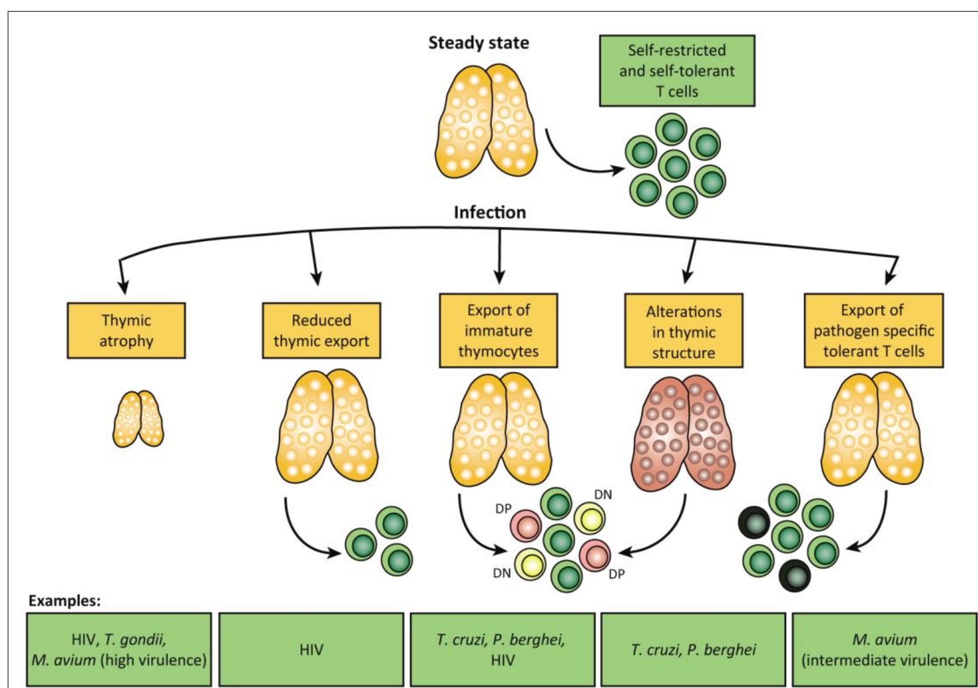

Figure 4. The effects of infection on the thymus *(Nunes-Alves et al., 2013)*

OBJECTIVES

The **general objective** of this PhD Thesis was to contribute to the knowledge of the immunopathogenic strategies by which BVDV infections predispose to secondary infections, as those produced by BHV-1. Therefore, the following **specific objectives** were proposed:

1. To standardize the immunohistochemical method for the detection of different potential dendritic cell (DC) markers in bovine tissues as well as to elucidate the histological distribution of these markers, thus helping to investigate *in vivo* the roles of DCs in cattle diseases: **CHAPTER 1**.

2. To characterize the lesional alterations associated with BVDV-induced thymic atrophy occurring in calves preinfected with BVDV and challenged later with BHV-1: **CHAPTER 2a**.

3. To investigate in the thymus of the aforementioned animals, the immunopathologic changes ensuing after the viral infections, evaluating the main antigen-presenting cells (DCs and macrophages), lymphocyte subpopulations and the proliferative activity of these cells: **CHAPTER 2b**.

4. To examine by flow cytometry the effect of dual and single infections *in vitro* with BVDV and BHV-1 on peripheral blood mononuclear cells (PBMCs): **CHAPTER 3**.

MATERIALS AND METHODS

Materials and Methods

A. *IN VIVO* STUDIES

1. Experimental designs

1.1. Experimental design CHAPTER 1

Six Friesian healthy male calves, aged 8 to 9 months, were obtained from farms free of tuberculosis, brucellosis, bovine leukosis virus, bovine viral diarrhea, and infectious bovine rhinotracheitis and were housed in the Animal Experimental Center of Cordoba University (Spain). Only those animals clinically healthy and with blood parameters within the normal range were included in the study. Parasitic coprological analyses were negative in all animals. Animals were sedated with xylazine (Rompun 2% solution; Bayer Healthcare, Kiel, Germany) and euthanized by overdose with thiopental-sodium (Thiovet; Vet Limited, Leyland, Lancashire, UK). This work was carried out in accordance with the code of Practice for Housing and Care of Animals used in Scientific Procedures, approved by the European Economic Community Union in 1986 (86/609/EEC) amended by the directive 2003/65/EC.

No significant macroscopic pathological lesions were observed at necropsy. Samples from liver, intestine and lung were subjected to microbiological routine cultures by using standard procedures, and no bacteria were isolated beyond the normal intestinal microflora.

1.2. Experimental design CHAPTER 2

The experimental design *(Fig. 1)* has been previously described by Risalde et al. *(2013)*. Briefly, 24 Friesian calves (8-9 months old) were obtained from a herd that was free of tuberculosis, brucellosis, and bovine leucosis virus. The animals were tested via ELISA to confirm their BVDV and BHV-1 antigens and antibodies free status. The calves were housed in the Animal Experimental Center of Cordoba University and had an adaptation period of one week before starting the study. The entire experimental procedure was carried out in accordance with the Code of Practice for Housing and Care of Animals used in Scientific Procedures, approved by the European Economic Community in 1986 (86/609/EEC amended

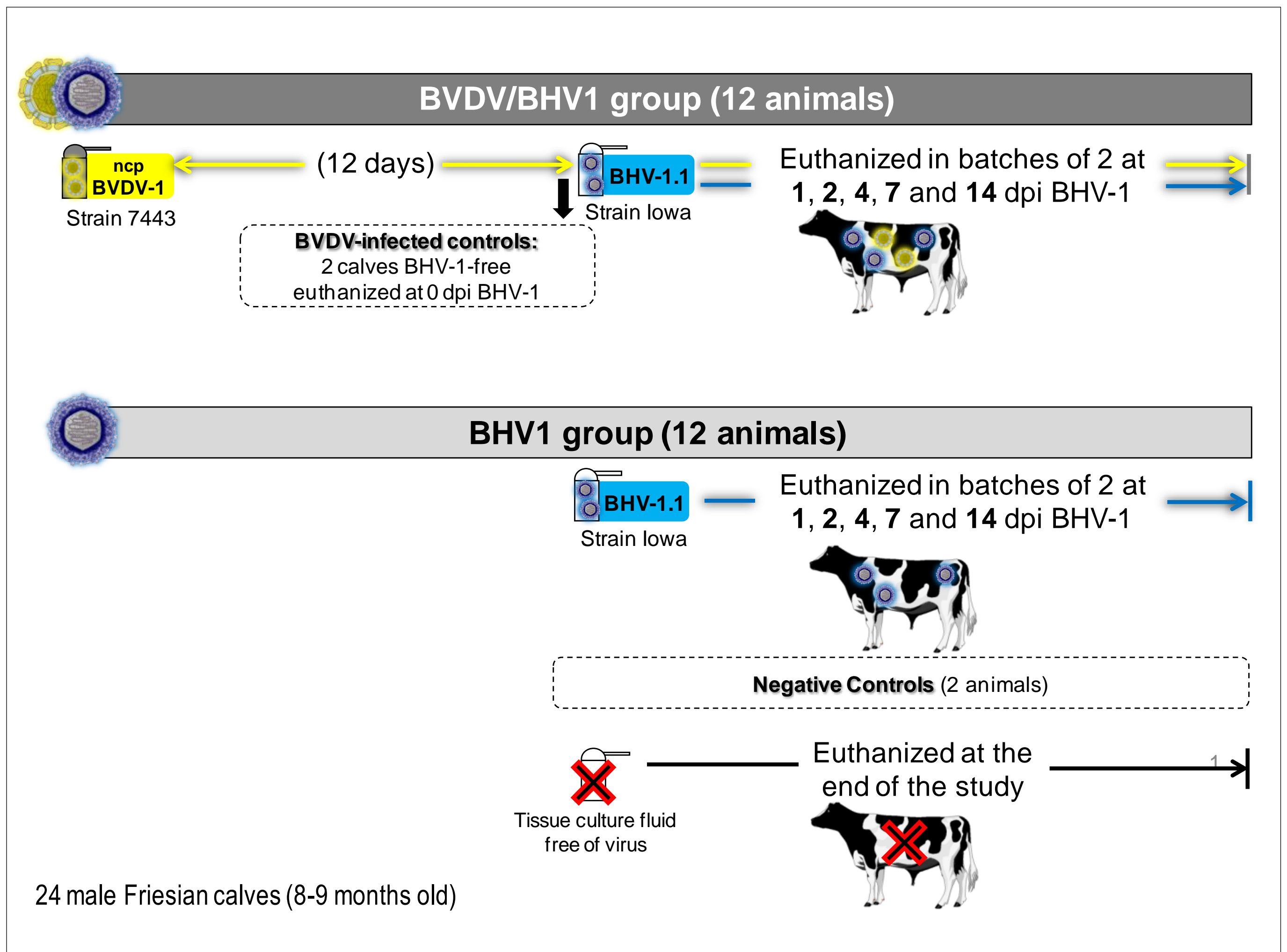

Figure 1. Schematic summary of experimental design (CHAPTER 2)

by the directive 2003/65/EC). The calves were separated in infection groups and inoculated as follows:

BVDV/BHV1 group: Twelve calves were inoculated with 10 ml (5 ml per nostril) of a suspension of non-cytopathic BVDV-1a strain 7443 *(Ridpath et al., 2010)*, containing 10^5 tissue culture infective dose 50% ($TCID_{50}$)/ml (courtesy of the Institut für Virologie, TiHo, Hannover, Germany). Twelve days later, when the calves had no clinical signs or evidence of BVDV-related viremia, 10 of them were challenged with 2 ml (1 ml per nostril) suspension of BHV-1 subtype 1 (BHV-1) strain Iowa *(Lemaire et al., 2000)*, containing 10^7 $TCID_{50}$/ml (courtesy of the Hipra Laboratories, Girona, Spain). At 1, 2, 4, 7, or 14 days post-inoculation (dpi) with BHV-1, calves were sedated with xylazine (Rompun® 2% solution; Bayer Healthcare, Kiel, Germany) and euthanized in batches of two by overdosing with

thiopental-sodium (Thiovet®; Vet Limited, Leyland, Lancashire, UK). The 2 calves that were not inoculated with BHV-1 were similarly sedated and euthanized on the day that the other calves were inoculated with BHV-1 and were used as BVDV-infected, BHV-1-free controls.

BHV1 group: At the time that the calves in the BVDV/BHV1 group were inoculated with BHV-1, another 10 calves were inoculated only with BHV-1 at the same infectious dose. At 1, 2, 4, 7, or 14 dpi with BHV-1, these calves were sedated and euthanized in batches of two, as described. At the time of inoculation with BHV-1, 2 additional separately housed calves were inoculated with 2 ml (1 ml per nostril) of virus-free tissue culture fluid and euthanized as described at the end of the study period (i.e., 14 days after calves in the other 2 groups were inoculated with BHV-1). These 2 calves were used as un-inoculated (UI) controls for the BHV1 group.

2. Methods and techniques

2.1. Tissue sample collection

At postmortem examination, tissue samples (0.5-1 cm thick) from a wide range of organs (CHAPTER 1-Table 1), and samples from the cervical thymus (CHAPTER 2) were immersed in three different fixatives: 10% neutral buffered formalin for 24 hours, Bouin's solution for 8 hours and zinc salts fixative for 24 hours. Tissue samples were routinely processed and embedded in paraffin wax for histopathological and immunohistochemical studies.

Thymus samples were also frozen at -80ºC for the viral genome isolation as well as fixed in 2.5% glutaraldehyde for the ultrastructural analysis (CHAPTER 2).

2.2. Immunohistochemical study

The avidin-biotin-peroxidase complex (ABC) method was used *(Ramos-Vara and Miller, 2014)*. Briefly, tissue sections (3 µm) were placed in silane-coated slides [3-(triethoxysilyl)-propylamine], dewaxed, and rehydrated using graded ethanol series *(Table 1)*. Endogenous peroxidase activity was exhausted by incubation of the sections with H_2O_2 3% in methanol for 45 minutes at room temperature. Tissue sections were subjected to different retrieval pretreatments *(Table 2)*. After

pretreatment, sections were given three 5 minute rinses in PBS. Tissue sections were covered with normal serum (horse, rabbit or goat normal serum, see *Table 3*) for 30 minutes at room temperature and incubated with the primary antibodies (mouse, rat or rabbit primary antibodies, see *Table 3*) at 4ºC overnight. Details of the commercial primary antibodies used in this study are summarized in *Table 2*. After primary incubation, slides were washed in PBS (3 times for 5 minutes each) and then incubated for 30 minutes at room temperature with the secondary antibodies (horse anti-mouse, rabbit anti-rat, or goat anti-rabbit biotinylated antibodies, see *Table 3*). After 3 further 5-minute washes in PBS, samples were incubated with the avidin-biotin-peroxidase complex (Vectastain® Elite ABC Kit, Vector Laboratories) for 1 hour at room temperature in the dark. All tissue sections were finally rinsed in TBS, incubated with a chromogen solution (NovaRED® Substrate Kit, Vector Laboratories), and counterstained with Harris' hematoxylin. Mouse, rat or rabbit non-immune sera were used in place of specific primary antibodies as additional negative controls.

Note on antibodies specificity for the bovine species (CHAPTER 1): Two monoclonal antibodies (CD1b and CD205) directed against bovine antigens were used in this study. Anti-S100 antibody has been raised against S100 protein isolated from cow brain, and CNA.42 monoclonal antibody cross-reacts with FDCs in different species, including cattle *(Raymond et al., 1997)*. Studies by Ababou et al *(1993)* and Grüneberg et al *(1997)* have demonstrated that the clone TAL.1B5 (anti-human HLA-DR α chain) also binds to an intracellular epitope of the BoLA-DR α chain. Given the close phylogenetic relationship between cattle and sheep, an anti-mouse CD208 antibody that cross-reacts with sheep *(Salaun et al., 2004)* was used in this study (Table 2).

Note on infection tissue controls for BVDV and BHV-1 (CHAPTER 2): Tissues from calves persistently infected with BVDV and tissues from aborted fetuses that were positive for BHV-1 *(Fig. S2, p209)* were analyzed as positive controls for the immunohistochemical detection of E^{rns} and gC, respectively. Tissues from specific pathogen–free calves that were not exposed to either BVDV or BHV-1 were analyzed as negative controls.

Table 1. Immunohistochemical ABC protocol for paraffin-embedded tissue sections

Stage	Step
Stage 1 Pre-immunologic procedures	1. Deparaffinize: xylene (x3, 10min each)
	2. Rehydrate: 100° ethanol (x2, 5min each)
	3. Inhibition of endogenous peroxidase: incubation with H_2O_2 3% in methanol (45min at RT)
	4. Rehydrate: 96° ethanol, 70° ethanol, deionized water (5min each)
	5. Rinse in PBS (10min)
	6. Pretreatment-antigen retrieval (heat/enzymatic/detergent-induced epitope retrieval)
	7. Rinse in PBS (x3, 5min each)
	8. Background blocking: incubation with normal serum (30min, RT)*
	9. Remove excess of blocking solution (do not rinse)
Stage 2 Immunologic and histochemical procedures	10. Incubate with primary antibody (overnight (≈18h), 4°C)*
	11. Pre-warm slides for 1 hour at RT
	12. Rinse in PBS (x3, 5min each)
	13. Incubate with biotinylated secondary antibody (30min at RT)*
	14. Rinse in PBS (x3, 5min each)
	15. Incubate with ABC complex (1 hour at RT)*
	16. Rinse in TBS (x3, 5min each)
Stage 3 Antigen-Antibody Reaction Visualization	17. Incubation with chromogen solution: NovaRED Substrate Kit, containing H_2O_2 for peroxidase
	18. Rinse in tap water
	19. Counterstain with Harris' hematoxylin
	20. Rinse in tap water
	21. Dehydrate in ethanol: 70° (2 sec), 96° (2 sec), 100° (x2, 1min)
	22. Wash in xylene (x2, 1min each)
	23. Mount with permanent medium (Eukitt®) and coversilp

*Incubations in opaque humid chamber. ABC: avidin-biotin-peroxidase complex, RT: room temperature

Table 2. Details of the primary antibodies used in the immunohistochemical studies.

	Specificity	Antigen or cell detected	Clone	Dilution	Fixative	Pre-treatment	Source
CHAPTER 1	[1]Anti-human **HLA-DR**	(MHC-II) DCs, B cells, MØ	TAL.1B5	1:100	10%F	TC-microwave [a]	Dako
	[2]Anti-mouse **CD208**	(DC-LAMP) Mature DCs, PnIIs	1010E1	1:100	10%F	TC-microwave [b]	Dendritics
	[1]Anti-bovine **CD1b**	DCs, cortical thymocytes	CC20	1:10	10%F	Protease [c]	Serotec
	[1]Anti-bovine **CD205**	DCs, some B/T cells, some epithelial cells	CC98	1:10	10%F	Tween-20 [d]	Serotec
	[1]Anti-human **FDC**	FDCs	CNA.42	1:50	10%F	TC-microwave [e]	Dako
	[3]Anti bovine-**S100**	Wide (including FDCs)	pAb	1:400	10%F	TC-microwave [a]	Dako
CHAPTER 2A	[1]Anti-**BVDV**	Erns (gp48) viral glycoprotein	15c5	1:75	10%F	Proteinase K [f]	Dr. E. Dubovi, Cornell University
	[1]Anti-**BHV-1**	gC viral protein	F2	1:1000	10%F	TC-microwave [g]	VMRD
	[3]Anti-human **caspase 3**	Caspase 3	pAb	1:400	10%F	TC-microwave [b]	D. BioSystems
	[3]Anti-human **vWF**	Endothelial cells, platelets	pAb	1:800	10%F	Protease [c]	Dako
CHAPTER 2B	[1]Anti-human **MØs**	Monocytes and MØs	MAC387	1:100	10%F	Protease [c]	Serotec
	[1]Anti-human **Ki67**	Cellular proliferation	MIB-1	1:100	10%F	TC-autoclave [h]	Dako
	[1]Anti-bovine **CD8**	CD8+ T lymphocytes	CC63	1:200	ZSF	TC-37ºC [i]	Serotec
	[1]Anti-bovine **WC1**	γδ T lymphocytes	CC15	1:100	ZSF	TC-37ºC [i]	Serotec
	[2]Anti-mouse/rat **Foxp3**	Foxp3+ T lymphocytes	FJK-16	1:100	10%F	TC-autoclave [h]	eBioscience
	[2]Anti-mouse **CD208**	(DC-LAMP) Mature DCs, PnIIs	1010E1	1:100	10%F	TC-microwave [b]	Dendritics
	[1]Anti-bovine **CD1b**	DCs, cortical thymocytes	CC20	1:10	10%F	Protease [c]	Serotec
	[1]Anti-bovine **TGF-β**	TGF-β (isoforms 1, 2 & 3)	1D11	1:50	BS	TC-microwave [a]	Serotec

DCs: dendritic cells, **DC-LAMP**: dendritic cell-lysosomal associated membrane protein, **FDCs**: follicular dendritic cell, **MHCII**: major histocompatibility complex class II molecule, **MØ**: macrophages, **PnIIs**: type II pneumocytes, **pAb**: Polyclonal antibody, **10%F**: 10% neutral buffered formalin, **BS**: Bouin's solution, **ZSF**: zinc salts fixative, **BVDV**: Bovine Viral Diarrhea virus, **BHV-1**: Bovine Herpesvirus type 1, **pAb**: Polyclonal antibody, **vWF**: Von Willebrand Factor/Coagulation factor VIII, **TC**: 0.01M tri-sodium citrate dehydrate.

[1]Mouse monoclonal antibody

[2]Rat monoclonal antibody

[3]Rabbit polyclonal antibody

[a] Incubation with TC (pH 3.2), microwave for 5 min at sub-boiling temperature.

[b] Incubation with TC (pH 6), microwave for 15 min at sub-boiling temperature.

[c] Incubation with 0.1% protease type XIV (Sigma–Aldrich Chemie) in PBS, for 7 min at RT.

[d] Detergent permeabilization with Tween-20 0.1% in PBS for 10 minutes at RT.

[e] Incubation with TC (pH 9), microwave for 30 min at sub-boiling temperature.

[f] Incubation with 0.2% proteinase K (Sigma-Aldrich Chemie) in Tris buffer for 7 min at RT.

[g] Incubation with TC (pH 6), microwave for 5 min at sub-boiling temperature.

[h] Incubation with TC (pH 6) and autoclaved for 10 min at 135 ºC, 2 atm.

[i] Incubation with TC (pH 3.2) for 30 min at 37 ºC in oven.

Table 3. Secondary antibodies and normal serum used according to the animal source of primary antibodies

Animal source of primary Ab:	MOUSE	RAT	RABBIT
Dilution of primary Ab:	depends	depends	depends
Dilution medium of primary Ab:	1% NHS in TBS	1% NRS in TBS	10% NGS in PBS
Background blocking medium:	1% NHS in TBS	1% NRS in TBS	20% NGS in PBS
Secondary (biotinylated) Ab:	Horse anti-MOUSE	Rabbit anti-RAT	Goat anti-RABBIT
Dilution:	1/200	1/100	1/200
Dilution medium:	1% NHS in TBS	1% NRS in TBS	2% NGS in PBS
Primary antibodies:	MHCII, CD1b, CD205, CNA.42, 15c5, F2, MAC387, Ki67, CD8, WC1, TGF-β	CD208 FoxP3	S100 Caspase 3 Factor VIII

Ab: Antibody; PBS: phosphate buffered saline; TBS: tris-buffered saline
NHS: Normal horse serum (Thermo-Pierce, Rockford-IL, USA)
NRS: Normal rabbit serum (Thermo-Pierce)
NGS: Normal goat serum (MP Biomedicals, Santa Ana-CA, USA)
Biotinylated horse anti–mouse IgG secondary antibody (Thermo-Pierce)
Biotinylated rabbit anti–rat Igs secondary antibody (Dako, Glostrup, Denmark)
Biotinylated goat anti–rabbit IgG secondary antibody (Vector Laboratories, Burlingame-CA, USA)

2.3. Cell counting

CHAPTER 1: A semiquantitative assessment of the immunolabeled cells was performed by 2 experienced observers in 25 fields of 0.2 mm^2 randomly chosen. Results were expressed as number of immunolabeled cells per area of 0.2 mm^2: - (none), + (0-5), ++ (0-20), +++ (20-60), ++++ (>60). Intensity and quantity of cells stained with CNA.42 and anti-S100 antibodies were assessed only within lymphoid follicles as + (slight) or ++ (high) *(CHAPER 1-Table 2)*.

CHAPTER 2: To evaluate the number of immunostained cells and to correlate the results obtained with the different antibodies, paraffin-wax blocks from the thymus of each animal were selected. Positively labeled cells of cortex or medulla 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. In the case of collagen and factor VIII evaluation, results were expressed as the percentage of stained

surface based on 10 low-magnification pictures from each paraffin block, comprising both cortex and medulla structures.

2.4. Cortex:medulla ratio assessment (CHAPTER 2A)

The cortex:medulla ratio evaluation was based on the methodology described by Vascellari *et al. (2012)*, with some modifications. Briefly, low magnification pictures (4x) from the slides stained with hematoxylin and eosin were taken to evaluate cortex and medulla thickness. For each slide, 10 functional lobules composed of an outer cortex and inner medulla were randomly selected and the extension of the cortex and medulla were measured by drawing a graduated line, starting and ending at the interlobular connective tissue *(Figure 2)*; a second parallel line was drawn to measure medulla thickness. Measurements were performed using ImageJ software version 1.46f. The cortex thickness was obtained by subtracting the second value from the first one and the cortex:medulla ratio was calculated.

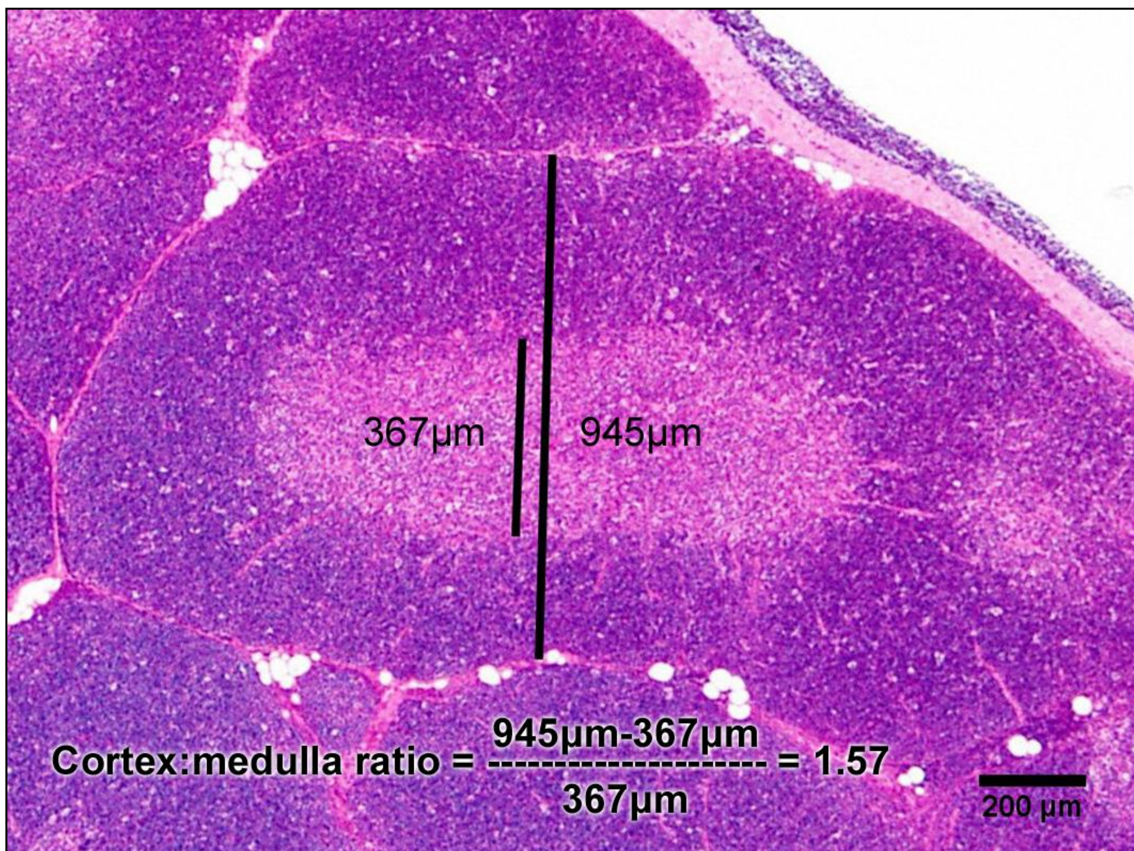

Figure 2. Example of the measurements performed in the thymus lobules for the evaluation of the cortex:medulla ratio. Extension of the cortex was measured against a graduated line, starting and ending at the interlobular connective tissue; a second parallel line was drawn to measure medulla thickness. (CHAPTER 2A)

2.5. In situ identification of nuclear DNA fragmentation (*CHAPTER 2A*)

Presence of apoptosis in formalin-fixed samples was investigated via DNA fragmentation by the TUNEL method (terminal deoxynucleotidyl transferase dUTP nick end labeling; In Situ Cell Death Detection kit, POD, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

2.6. Collagen and Factor VIII quantification (*CHAPTER 2A*)

The thymus sections were subjected to Masson's trichrome stain, which provides an excellent blue color contrast for collagen that differentiates it from other structures. Ten pictures at low magnification comprising both medulla and cortex areas were randomly taken from each slide and analyzed using ImageJ software; by thresholding for blue color, collagen was selected and measured for each picture *(Fig. 4a and 4b)*, and results were expressed as mean percentage of collagen. Factor VIII staining for endothelial cells was similarly assessed, but by thresholding for the red-brown chromogen color.

2.7. DNA extraction and PCR analysis for BHV-1 (*CHAPTER 2A*)

Bovine herpesvirus-1 DNA was extracted from thymus samples with a DNA extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany), in accordance with the manufacturer's instructions. A conventional PCR targeting a fragment of the thymidine kinase region of BHV-1 was performed as previously described *(Alegre et al., 2001)*, with minor modifications. Briefly, primers TK1 (Forward: 5'- AGACCCCAGTTGTGATGAATGC-3') and TK2 (Reverse: 5'-ACACGTCCAGCACGAACACC-3') were used, yielding a 183 bp product. The PCR products were run on a 1.5% agarose gel and visualized by ethidium bromide staining. BHV-1 strain Iowa ($10^{4.76}$ $TCID_{50}$/ml) was used as positive control, as well as tissue samples from tonsils positive for BHV-1 immunohistochemical detection *(Fig. S2, p209)*.

2.8. Ultrastructural study (*CHAPTER 2A*)

Glutaraldehyde-fixed samples were post-fixed in 2% osmium tetroxide, dehydrated in acetone, and embedded in Epon resin. Ultrathin sections (80 nm) placed in copper grids were contrasted with uranyl acetate and lead citrate and

examined with a JEOL JEM-1400 transmission electron microscope equipped with a Gatan ORIUS SC1000 camera.

2.9. Statistical analysis *(CHAPTER 2)*

Statistically significant differences between the means were assessed by the Mann–Whitney U non-parametric test ($P < 0.05$) between inoculated groups at the same time point (*), and between inoculated calves in each infected group (BHV1 and BVDV/BHV1 groups) and their control group at each time point ([a,b] respectively). The statistical evaluation was made using GraphPad Prism version 5 software.

B. *IN VITRO* STUDY (CHAPTER 3)

1. Animals and Virus

For this study, 4 Holstein-Friesian heifers (8-10 months old) were selected from a dairy herd free of BVDV and bovine herpesvirus type 1 (BHV-1) and confirmed to be BVDV and BHV-1 antigen and antibody free. The **immune** status of each animal was confirmed by assaying sera for BVDV antibodies by a commercial competitive ELISA (Ingezim BVD Compac, Ingenasa, Madrid, Spain) and by serum neutralization (SN) test, and for BHV-1 antibodies by SN test. SN test is explained in detail below. BVDV **antigen** free status was confirmed in duplicates by a commercial double antibody Sandwich ELISA for the detection of BVDV p80/p125 protein (Ingezim BVD DAS, Ingenasa) and by PCR based on that described by Letellier et al. *(1999)*

Two **viral strains** were used for *in vitro* infection and serological tests: ncp BVDV-1a strain 7443, (courtesy of the Institute für Virologie, TIHO, Hannover, Germany) and BHV-1 subtype 1 strain Iowa (courtesy of the Hipra Laboratories, Girona, Spain). An additional cp BVDV-1a strain NADL (ATCC® VR-534™; Department of Veterinary Science and Public Health, University of Milan, Italy) was used for serological tests.

2. Virus neutralization test (VNT)

In order to confirm the absence of specific neutralizing antibodies against BVDV and BHV-1, sera from the animals used in this experiment were tested by virus neutralization (VN) tests (also called serum neutralization (SN) tests) *(OIE, 2008; 2010)*. Firstly, sera were heat-inactivated at 56°C for 30 minutes to destroy complement components interfering with serum neutralization. From a starting dilution of 1/2 for BHV-1 or 1/4 for BVDV, serial twofold dilutions of the test sera were made in duplicate in a cell-culture flat-bottomed 96-well microtitre plate, using Minimal Essential Medium with Earle's Salts (MEM)[1] as diluent, with a final volume of 50 µl. An equal volume (50 µl) of cytopathic BVDV (NADL strain) or BHV-1 (Iowa strain) containing 100 $TCID_{50}$ was added to each well and the plates were incubated for 1 hour at 37°C to let the possible antibodies neutralize the virus. For this test and for other virological methods, a continuous line of bovine kidney epithelial cells (Madin Darby Bovine Kidney, MDBK) (ATCC® CCL-22™) were used *(Fig. 3a,b).* After the incubation period, a culture flask of MDBK cells was trypsinized and the cell concentration was adjusted to 1.5×10^5/ml in CCM [MEM supplemented with 10% of heat-inactivated fetal calf serum (FCS)[1], L-glutamine 2mM[1], antibiotics and antifungal agents (100U/ml of penicillin, 100µg/ml of streptomycin, 2.5 µg/ml of fungizone)[1]] with 30% of FCS, and 50 µl of this suspension was added to each well of the microtitre plate, obtaining a final concentration of 10% FCS. A back titration of virus stock was also done in some spare wells to check the potency of the virus (controls for the virus without sera), as well as controls for the cells (cells without sera nor virus). The plate was incubated at 37°C for 3 and 4 days, for BHV-1 and BVDV, respectively. The wells were examined microscopically for cytopathic effect (CPE) *(Fig. 4)*. The VN titre for each serum is the dilution at which the virus is neutralized in 50% of the wells. An animal is considered seronegative when no neutralization (i.e. CPE) is observed at the lowest dilution (1/2 for BHV-1 or 1/4 for BVDV).

[1] Euroclone (Milan, Italy)

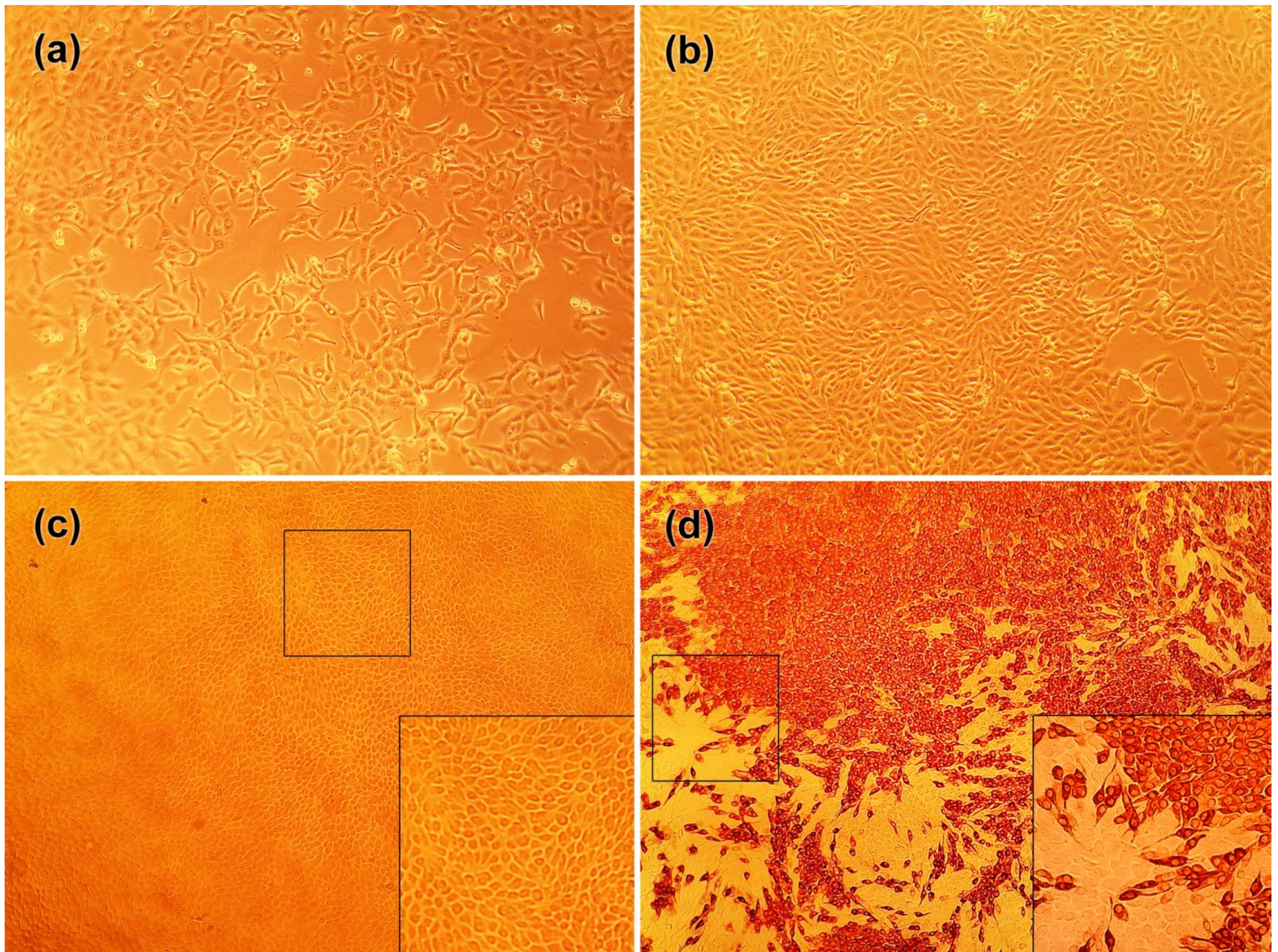

Figure 3. MDBK cells at different growth stages: 24 **(a)** and 48 **(b)** hours of culture. Immunoperoxidase monolayer assay (IPMA) for BVDV (7443 strain): **(c)** Example of a negative well, showing cells uninfected with BVDV; **(d)** Example of a positive well, with BVDV-infected cells. Insets represent higher magnifications of the fields framed in black.

3. Viral replication

For obtaining the viral stocks of each viral strain, viruses were cultured in MDBK cells with CCM (both MDBK cells and FCS were certified free of BVDV, as well as anti–BVDV antibody free for FCS). Firstly, viruses diluted in MEM were added to 75-cm^2 culture flasks containing sub-confluent MDBK cell cultures previously devoid of CCM. After incubation for 2 hours at 37ºC with 5% CO_2, CCM was added and flasks were incubated at different periods depending on the biotypes used for the infection. Flasks infected with ncp BVDV are incubated for 4 days while flasks infected with BHV-1 present cytopathic effect in 70-80% of the cell monolayer at 30 hpi. Following the incubation periods, infected flasks were frozen at -80 ºC in order to lyse the cells and get the virus free in the culture

medium. After thawing the flasks and centrifuge at 1200 g for 10 min, supernatants are aliquoted in vials of 1 ml and frozen again at -80ºC.

4. Viral Titration:

The obtained viral stocks were titrated by microtitre assay in 96-well plates with MDBK cells. Briefly, serial tenfold dilutions (twofold for BHV-1) of the tested sample were made in MEM. 50µl of each dilution are added to each well in quadruplicate and 100 µl of a MDBK cell suspension containing $1.5x10^4$ cells were seeded in each well. 150 µl of cell suspension containing $1.5x10^4$ cells were seeded in the control wells. The plate was incubated at 37ºC in 5% CO_2 for 3 and 4 days, for BHV-1 and BVDV, respectively. For BHV-1, the microtitre assay was performed by evaluating the cytopathic effect *(Fig. 4a,b)*, and for ncp BVDV-1 by immunoperoxidase monolayer assay (IPMA) *(Fig. 3c,d)*. IPMA is explained in detail in the following section. Viral titration was calculated either for BHV-1 or for BVDV by means of the Reed–Muench method, and expressed as Tissue Culture Infectious Dose 50% ($TCID_{50}$).

5. Immunoperoxidase monolayer assay (IPMA)

This technique was performed in order to evidence the presence of the ncp BVDV strain *(Lucchini et al., 2012)*. After a 4 days incubation period of MDBK cells with the virus dilutions, the medium was removed from each well and cells were washed with PBS diluted 1/3 in distilled water (1/3PBS) and fixated at 80ºC for 2 hours. Cells were rehydrated with dilution buffer (DB, PBS with 0.05% of Tween 20) for 5 minutes. After removing DB, each well was incubated for 1 hour at room temperature (RT) with 50µl of a 1:250 dilution of the broad cross-reactivity monoclonal antibody 20.10.06 (Courtesy of Dr. E. Dubovi, Cornell University) directed against the NS2/3 protein of BVDV *(Corapi et al., 1990; Pogranichniy et al., 2008)*. After primary incubation, cells were washed 3 times with washing buffer (WB, DB diluted 1/3 in distilled water); these washing steps were repeated after each incubation period. Wells were then incubated for 1 hour at RT with 50µl of a 1:200 dilution of a biotinylated anti-mouse IgG secondary antibody (Amersham Biosciences). After washing, cells were incubated for 1 hour at 37ºC with 50 µl of a

1:100 dilution of the streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences).

In the meantime, a solution containing the substrate for the peroxidase was prepared, diluting 20mg of AEC (3-Amino-9-ethylcarbazole tablets, Sigma-Aldrich) in 3 ml of *N*,*N*-Dimethylformamide (Sigma-Aldrich); this solution was subsequently diluted at 1:16 in acetate buffer (0.05M, pH 5) and with 0.05% of H_2O_2 30% immediately prior to use. Cells were then incubated with 50 µl of this solution for approximately 15 minutes in the dark.

The positivity of the reaction (i.e. the presence of the viral antigen) was determined by the presence of a reddish color assessed under a light microscope *(Fig. 3d)*.

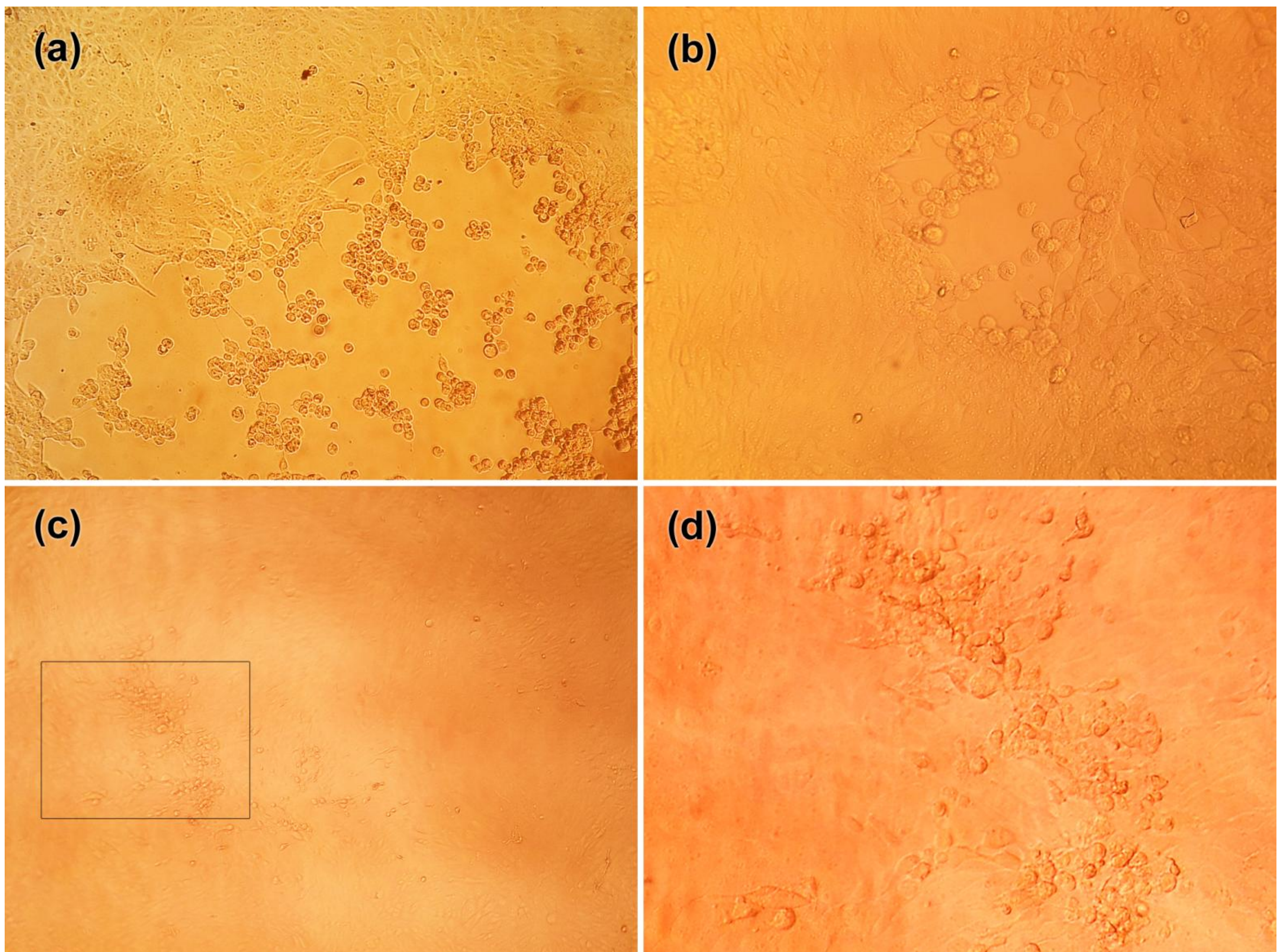

Figure 4. Cytopathic effect (CPE) of BHV-1 (strain Iowa) at different dilutions after 24 hours of infection **(a, b)**; Notice the characteristic formation of grape-like clusters of rounded cells gathered around a hole in the monolayer. CPE produced by BVDV (cp strain NADL) **(c, d)**. Note the shrinking of cells preceding to detachment. (d) represents a higher magnification of the field framed in black in (c).

6. Cell separation, culture and infection of PBMCs

Prior to begin the experiment, 5 ml of peripheral blood in CPDA-1 were separated from each animal and the leukocyte profile was obtained with a hematology analyzer Sysmex XT-2000iV with specific settings for bovine blood (Sysmex Corporation, Kobe, Japan).

Blood was collected into sterile bags containing CPDA-1 by jugular venepuncture. The blood was centrifuged at 1200 g for 30 min and the buffy coat was separated and resuspended 1:2 in PBS. The diluted buffy coat was overlaid 2:1 on Histopaque-1077 (Sigma-Aldrich) and centrifuged at 1200 g for 30 min at RT. After density gradient centrifugation, peripheral blood mononuclear cells (PBMCs) were carefully collected from the interface and washed in PBS. Live cells were counted by means of trypan blue dye exclusion and resuspended in RPMI-1640 medium[1] at 1 x 10^6 cells/ml. Cells from each animal were separated into 4 groups of infection: Uninfected control group (CON), group infected with ncp BVDV (BVDV), group infected with BHV-1 (BHV) and group infected with both BVDV and BHV-1 (BVDV/BHV). After infection at a multiplicity of infection (m.o.i.) of 1 $TCID_{50}$ per cell, cell suspensions were placed in 25-cm^2 culture flasks for 2 hours at 37ºC. After incubation, the inoculum was removed by washing the cells with RPMI-1640 medium in order to eliminate the extracellular virus. Then, the cells were resuspended in leukocyte culture medium (LCM) [RPMI-1640 medium containing 1% L-glutamine, 25 mM Hepes[1] and 10% FCS], placed in 25-cm^2 culture flasks and incubated at 37ºC in 5% CO_2 for 18, 24, 48, and 72 hours.

7. Flow cytometry

To determine the effect of virus infection, PBMCs from the different infection groups were harvested at 18, 24, 48, and 72 hours p.i. Adherent cells (monocyte-macrophages mainly) were removed after 10 minutes of incubation with pre-warmed cell-dissociation solution (Sigma-Aldrich) and washed twice in PBS. PBMCs from each group of infection and time point were divided at 2x10^5 cells/100μl and incubated with optimally diluted mouse monoclonal antibodies (MAb) *(Table 4)*, including isotype-matched control MAb. All primary and

[1] Euroclone (Milan, Italy)

secondary antibodies were optimally diluted and incubated for 30 min at RT. For indirect labeling of B cells, cells were incubated with a FITC-conjugated goat anti-mouse secondary antibody. After two washes, cells were fixed with 1% formaldehyde (CellFix 10x; BD) in PBS and kept at 4ºC in the dark until the analysis by flow cytometry. Cells (1x10^4 events) were analyzed on a FACS Calibur cytometry system (Becton Dickinson, Mountain View, CA, USA) and immunofluorescent staining was analyzed using Flowing Software (version 2.5.0). The following parameters were collected: forward light scatter (FSC), side light scatter (SSC), FITC fluorescence (FL1), RPE fluorescence (FL2), and AlexaFluor647 fluorescence (FL4). Debris were excluded from the analysis by the conventional FSC/SSC gating method and the percentage or mean fluorescence intensity (MFI, geometric mean channel number) of surface molecule staining on the gated cells were expressed *(Figure 5)*. Three, two or single-colour staining of PBMCs for leukocyte differentiation antigens were performed as follows: CD14/CD11b/CD80, CD4/CD8/CD25, MHC-I/MHC-II, CD28 and B-B2.

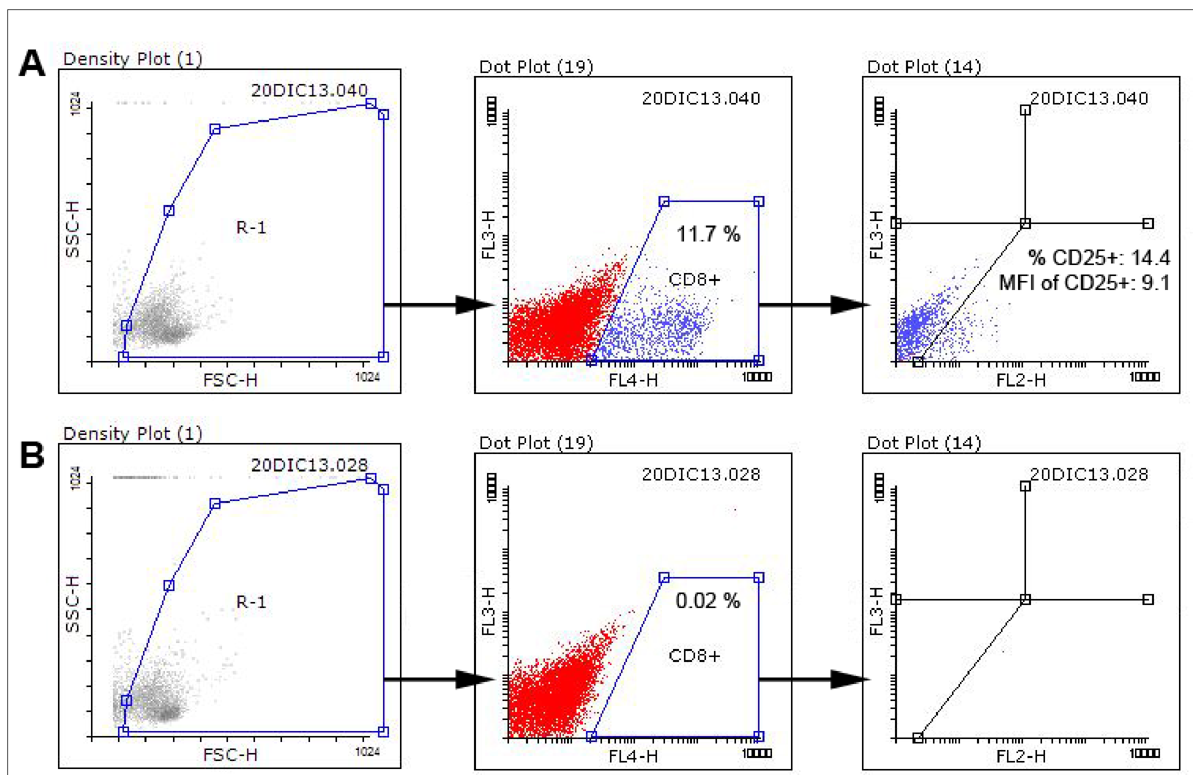

Figure 5. Example of the flow cytometry analysis. PBMCs from animal 2, 24 hpi with BHV-1 **(A)** and unstained control **(B)**. FL4 (AlexaFluor647-CD8), FL2 (RPE-CD25). FL3 (No stain)

Table 4. List of antibodies used in the flow cytometry analysis.

*Clone-Fluorochrome	Specificity	Isotype	Source
TÜK4-AlexaFluor 647	anti-human CD14	IgG2a	Serotec
CC126-FITC	anti-bovine CD11b	IgG2b	Serotec
ILA159-RPE	anti-bovine CD80	IgG1	Serotec
CC8-FITC	anti-bovine CD4	IgG2a	Serotec
CC63-AlexaFluor 647	anti-bovine CD8	IgG2a	Serotec
ILA111-RPE	anti-bovine CD25	IgG1	Serotec
ILA88-FITC	anti-bovine MHC-I	IgG2a	Serotec
CC108-RPE	anti-bovine MHC-II	IgG1	Serotec
CC219-FITC	anti-bovine CD28	IgG1	Serotec
BAQ44A (unconjugated)	anti-bovine B cells (B-B2)	IgM	VMRD
Secondary Ab-FITC	goat anti-mouse IgM	(Secondary Ab)	Sigma-Aldrich

*All primary antibodies are monoclonal mouse antibodies.

8. Statistical analysis

Statistical analyses and graphs were generated using GraphPad Prism version 5.01. Statistically significant differences between the means of different groups of infection at the same time point were assessed by the Mann–Whitney U non-parametric test (P<0.05) (represented with (*)). Statistically significant differences within a group of infection at different time points were assessed by the Kruskal-Wallis non-parametric test (P<0.05)(represented with letters).

CHAPTER 1

Immunohistochemical detection of dendritic cell markers in cattle

Immunohistochemical detection of dendritic cell markers in cattle

Veterinary Pathology 2013, 50(6): 1099-108

Abstract

Dendritic cells (DCs) are “professional” antigen-presenting cells with a critical role in the regulation of innate and adaptive immune responses and thus have been considered of great interest in the study of a variety of infectious diseases. The objective of this investigation was to characterize the in vivo distribution of DCs in bovine tissues by using potential DC markers in order to establish a basis for the study of DCs in diseased tissues. Markers evaluated included MHCII, CD208, CD1b, CD205, CNA.42 and S100 protein, the latter 2 being expressed by follicular dendritic cells whose origin and role are different to the rest of hematopoietic DCs. Paraffin wax-embedded tissues from 6 healthy Friesian calves were subjected to the avidin-biotin-peroxidase method and the most appropriate fixatives, dilutions, and antigen retrieval pretreatments were studied for each of the primary antibodies. The most significant results included the localization of CD208-positive cells not only in the T zone of lymphoid organs but also within lymphoid follicles; CD1b-positive cells were mainly found in thymus and interfollicular areas of some lymph nodes; cells stained with anti-CD205 antibody were scarce, and their location was mainly in non lymphoid tissues; and CNA.42 and S100-protein positive cells localized in primary lymphoid follicles and light zones of germinal centers, although showing differences in the staining pattern. Furthermore, MHCII was established as one of the most sensitive markers for any DC of hematopoietic origin. These results increase our understanding of DC immunolabeling and will help in future DC studies of both healthy and diseased tissues.

Keywords: bovine, CD208, CD205, CD1b, dendritic cells, follicular dendritic cells, immunohistochemistry, MHCII.

Introduction

Dendritic cells (DCs) are “professional” antigen-presenting cells (APCs) and are well known for their unique ability to present processed antigens to naive T lymphocytes *(Knight and Stagg, 1993)*. In addition to their critical role in the regulation of the adaptive immune response, DCs serve as sentinels, recognizing the presence of invading pathogens and secreting proinflammatory cytokines involved in host defense and thereby linking both the innate and adaptive immunity*(Reis e Sousa, 2004)*. These cells have a hematopoietic origin and express the major histocompatibility complex class II (MHCII) molecule. This is contrasted with the so-called follicular dendritic cells (FDCs) present in lymphoid follicles, which have a stromal origin, do not express MHCII, and present intact antigens to B cells in the form of surface immune complexes, being related to B-cell homeostasis *(Tew et al., 1997; van Nierop and de Groot, 2002)*.

Different authors have studied the role of DCs in various cattle diseases by means of in vitro infections of different subtypes of DCs, including monocyte-derived DCs *(Glew et al., 2003; Lei and Hostetter, 2007; Robinson et al., 2011)* and afferent lymph DCs (veiled cells or ALDCs) *(Howard and Hope, 2000; Hope et al., 2012)*. To our knowledge, this report is the first that describes in vivo distribution of DCs in bovine tissues on the basis of their differential expression of potential dendritic cell markers such as MHCII, CD208, CD1b, CD205, CNA.42 and S100 protein.

The MHCII molecule is expressed by professional APCs, which includes all subtypes of DC as well as macrophages and B lymphocytes. APCs are responsible for T helper cell activation by means of a synapse established between the antigen-loaded MHCII molecule and the T-cell receptor. CD208, also known as DC-LAMP (dendritic cell-lysosome-associated membrane protein) was found to be specifically expressed in mature DCs located in T zones of lymphoid tissues, known as interdigitant dendritic cells *(de Saint-Vis et al., 1998)* This molecule is not present in any other cell type, with the exception of type II pneumocytes *(Salaun et al., 2004)*, demonstrating the potential usefulness of this marker in diagnostics of DC related pathology. CD1 molecules are a family of cell surface-associated glycoproteins now recognized as having a role in presentation of lipid antigens to

certain subpopulations of T cells, as MHC molecules do with protein antigens *(Porcelli and Modlin, 1999)*. This family comprises several members, but not all are present in cattle. CD1b has been described as the most important CD molecule in this species *(Rhind, 2001; Van Rhijn et al., 2006)*. The majority of CD1 molecules are strongly expressed in cortical thymocytes as well as in certain other cell types, to include DCs *(Howard et al., 1993)*. CD205, also known as DEC-205, is a C-type lectin that belongs to the same family of the macrophage mannose receptor and is primarily expressed on certain DCs and epithelial cells, acting as an endocytic receptor, and is involved in the capture of antigens from extracellular spaces and transferral to a specialized-antigen processing compartment *(Jiang et al., 1995; Figdor et al., 2002)*. In addition, bovine CD205 has been previously described as the WC6 antigen, showing a strong expression on ALDCs *(Gliddon et al., 2004)*. In this study, we used a monoclonal antibody (clone CNA.42) for the labeling of FDCs *(Raymond et al., 1997)*, and a polyclonal antibody for the detection of S100 protein, which is expressed in a wide variety of cell types, including FDCs *(Ilg et al., 1996; Maeda et al., 2002)*.

Because of the complicated interactions between the distinct cells associated with both normal and pathological mechanisms of action of the immune system, it is of critical interest to examine DCs not only in cell culture but also in vivo. This study aims both to standardize the immunohistochemical method for the detection of different potential DC markers but also to elucidate the histological distribution of these markers, some of which are commonly used in many in vitro studies, thus helping to expand the understanding of the various roles of DCs in different cattle diseases.

Results

Tissues used in this study were assessed by a board veterinary pathologist who certified the absence of histopathological lesions. Signs of inflammation indicative of infectious or toxic agents were not observed in any of the animals included in the study.

Optimization of the Immunohistochemical Method.

Despite using other fixatives such as Bouin's solution or zinc salts fixative, the best results were obtained with buffered formalin. Tissue expression of the MHCII molecule was observed with each of the different antibody concentrations as well as with the different unmasking pretreatments, although the higher dilution (1:100) and the unmasking method with citrate at pH 3.2 during 6 minutes at subboiling temperature were considered the most effective *(Table 1)*. Immunolabeling against CD208 was possible with the higher dilution (1:100), although only when using the HIER methods at pH 6 during 20 minutes at subboiling temperature. This technique appeared to be the most appropriate, since staining using pH 3.2 was too weak, and pH 9 gave rise to intense background staining. The most suitable antigen retrieval method for the CD1b molecule was enzymatic digestion with protease but required the highest concentration of anti-CD1b antibody (1:10) to obtain the best results. In the case of anti-CD205 antibody, the only dilution that yielded positive results was the most concentrated (1:10), with the HIER methods being ineffective and the detergent permeabilization with Tween 20 being considered the most appropriate. For the detection of FDCs with CNA.42 monoclonal antibody, use of the HIER methodology was necessary, with the best results obtained at pH 9 during 30 minutes at subboiling temperature and with a 1:50 antibody dilution. S100 protein expression was observed with all of the unmasking methods except for enzymatic digestion, although the best results were obtained with citrate at pH 3.2 during 6 minutes at subboiling temperature and with a 1:400 antibody dilution.

Quantification and Location of Immunolabeled Cells.

Immunolabeled cells were counted for each animal, with minor differences among animals being observed and included within the stated intervals *(Table 2)*.

MHCII followed by CD208 appeared as the primary molecules expressed in the different tissues, both in intensity of labeling and quantity of labeled cells. All lymphoid organs displayed a high amount of MHCII-positive cells. These variably sized cells were typically stellate or polygonal and had homogeneous cytoplasmic staining. Immunopositive cells in palatine tonsil and lymph nodes could be clearly differentiated from one another in the interfollicular areas *(Fig. 1)*. A general faint

Table 1. Immunoreactivity produced by various primary antibodies on calf tissues fixed in formalin solution and subjected to various antigen-retrieval pretreatments.

Antibody dilutions		Pretreatments					
					Citrate microwave		
		None	Tween20	Protease	pH 3.2	pH 6	pH 9
MHCII	1:10	++	++	++	++	++	++
	1:50	++	++	++	++	++	++
	1:100	++	++	++	+++[a]	++	++
CD208	1:10	-	-	-	Bs	Bs	Bs
	1:50	-	-	-	Bs	Bs	Bs
	1:100	-	-	-	+	++[a]	Bs
CD1b	1:10	-	+	+++[a]	-	-	-
	1:50	-	-	+	-	-	-
	1:100	-	-	+	-	-	-
CD205	1:10	Bs	++[a]	+	-	-	-
	1:50	-	-	-	-	-	-
	1:100	-	-	-	-	-	-
CNA.42	1:10	-	-	-	+	+	++
	1:50	-	-	-	-	+	+++[a]
	1:100	-	-	-	-	-	-
S100	1:10	Bs	Bs	-	Bs	++	-
	1:50	++	++	-	++	++	++
	1:400	++	++	-	+++ [a]	++	+

-, none; + slight positivity and light background; ++, positive reaction and light background; +++, positive reaction without background; Bs, positive reaction but intense background staining. MHCII, major histocompatibility complex class II molecule.
[a] Elected dilution and pretreatment for each antibody.

staining was observed in the majority of lymphoid follicles, and in some of these lymphoid follicles, more strongly immunostained cells were noted and mainly located in the lymphoid follicle light zones *(Figs. 1, 12a)*. Immunolabeled cells were also observed in the medulla of lymph nodes, although to a lesser degree in comparison with the cortex. The cells identified within the medullary regions had a very characteristic dendritic morphology *(Fig. 2)*. Immunostaining observed in the spleen was evident in red splenic pulp and periarteriolar lymphoid sheaths, displaying many immunopositive dendritic-shaped cells *(Fig. 3)*, in contrast to lymphoid follicle staining being weaker if more diffuse. In the thymus, immunolabeling against MHCII was restricted to the medulla, where strong immunoreactivity was observed, including many cells with a dendritic morphology *(Fig. 4)*. Rare immunopositive cells were observed in the pulmonary alveolar septa and periportal areas of the liver. Positive cells were also noted within the tonsillar

Table 2. Distribution of cells immunolabeled with dendritic cell markers in different organs.

	Cell markers					
	MHCII	CD208	CD1b	CD205	CNA42[a]	S100[a]
LYMPHOID TISSUES						
Palatine tonsil						
LF	++++	+++	-	-	+	+
IFA	+++	++	-	+		
Epithelium	++	-	-	-		
Thymus						
Cortex	-	-	++++	-		
Medulla	++++	++	++	-		
Spleen						
LF	++	+++	-	+	++	++
RSP	+++	-	-	-		
Lymph nodes[b]						
LF	++	+++	-	-	++	++
IFA	+++	++	++[c]	-		
Medulla	++	-	-	+		
RESPIRATORY TRACT						
Lung						
Alveolar septa	++	+++	-	++		
BALT	++	++	-	+	+	+
Trachea						
Epithelium	-	-	-	-		
Lamina propria	++	-	-	+		
DIGESTIVE AND TEGUMENTARY SYSTEM						
Liver	+	-	-	++		
Distal ileum						
Lamina propria	+++	-	-	-		
PP follicles	-	+++	-	-	+	+
PP dome regions	++	++	-	-	++	+
Ileocecal valve						
Lamina propria	+++	-	-	-		
LF	++	+++	-	-	+	+
IFA	+++	++	-	-		
Haired skin						
Epidermis	+	-	-	-		
Dermis	+	-	+	-		

Results expressed as number of immunolabeled cells per area of 0.2 mm^2: - (none), + (0-5), ++ (0-20), +++ (20-60), ++++ (>60). BALT: bronchus-associated lymphoid tissue, IFA: interfollicular areas, LF: lymphoid follicles, MHCII: major histocompatibility complex class II molecule, PP: Peyer's patches, RSP: red splenic pulp.
[a]CNA.42 and anti-S100 antibodies were used for the detection of follicular dendritic cells, andthus only cells stained within lymphoid follicles were assessed in this table. Intensity and quantity of cells stained with these antibodies were assessed as + (slight) or ++ (high).
[b]Lymph nodes included in this study were retropharyngeal, submandibular, mediastinal, ileocecal and superficial inguinal lymph nodes.
[c]Positivity to CD1b antibody was mainly observed in superficial inguinal lymph nodes and submandibular lymph nodes.

epithelium and to a lesser degree in the epidermis and dermis of the skin, frequently showing dendritic morphology (mainly in tonsillar crypts epithelium) *(Fig. 5)*. MHCII-positive cells in the distal ileum were located in the lamina propria as well as within the dome region of the Peyer's patches but not within Peyer's patches follicles *(Fig. 6a)*, in contrast to the immunopositivity observed in the ileocecal valve lymphoid follicles.

Staining against CD208 (DC-LAMP) was cytoplasmic and had a dark granular appearance. Positive cells were mainly stellate or polygonal and were widely distributed in lymphoid tissues. A slight generalized staining was observed as tiny dark dots that could be appreciated on the cell surfaces *(Fig. 7, inset)*. This was more evident as the pH of the HIER method was increased. Immunopositive cells were located in the interfollicular areas and, to a greater extent, within the lymphoid follicles *(Fig. 7, 12b)*, where the cells were homogeneously distributed, with no differences observed between the dark and light zones of the germinal center, unlike the differences noted with anti-MHCII and anti-FDC antibodies. In general, not all the lymphoid follicles of the same lymph node presented the same quantity of immunopositive cells, and the medulla of lymph nodes was observed to be free of CD208-positive cells. Immunostained cells against CD208 had a round morphology and were located on the alveolar surfaces in the lung *(Fig. 8)*. A moderate amount of immunolabeled cells that were predominantly stellate were identified in the periarteriolar lymphoid sheaths of the spleen and within the thymic medulla. In addition, numerous large round immunopositive cells were detected within the ileum Peyer's patches and the lymphoid follicles of the ileocecal valve *(Fig. 6b)*.

Very few organs were noted to have CD1b-immunopositive cells. Cortical thymocytes were uniformly immunopositive along with some cells with a dendritic morphology located in the thymic medulla *(Fig. 9)*. Most lymph nodes were immunonegative for anti-CD1b antibody, with the exception of the submandibular and superficial inguinal lymph nodes and, to a lesser extent, the mediastinal lymph nodes, where numerous dendritic-shaped immunolabeled cells were identified in the interfollicular areas. Some scattered immunopositive cells were also demonstrated in the dermis of the skin.

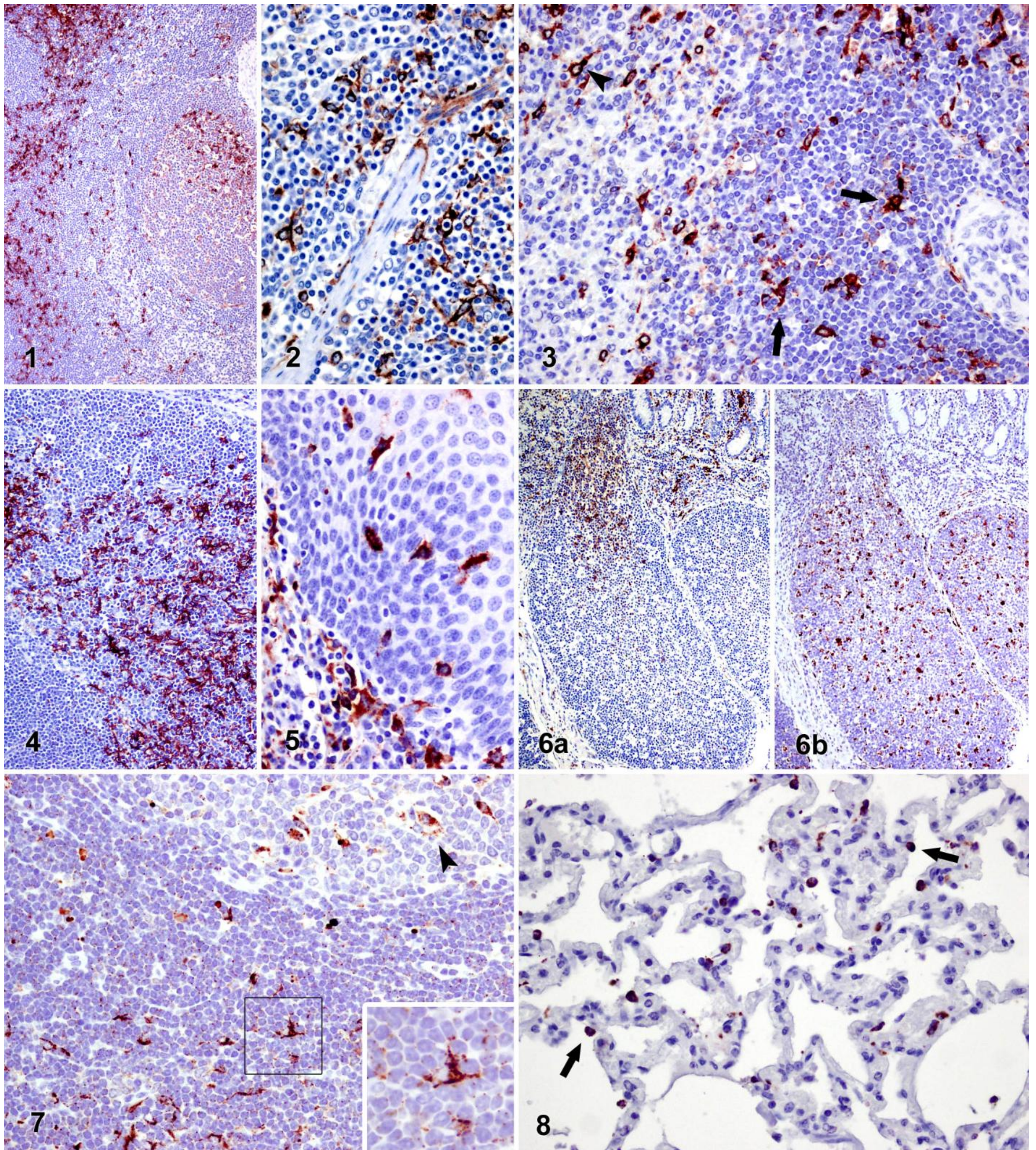

Figure 1. Lymph node; cattle No. 3. Immunostaining against major histocompatibility complex class II (MHCII) demonstrated strong labeling intensity in interfollicular areas and also stained cells within lymphoid follicles. **Figure 2**. Lymph node; cattle No. 2. MHCII immunostaining of medulla, where many positive cells with dendritic morphology can be appreciated. **Figure 3**. Spleen; cattle No. 1. Cells with dendritic morphology and that stained against MHCII can be observed in both the red splenic pulp (arrowhead) and the periarteriolar lymphoid sheaths (arrows). **Figure 4**. Thymus; cattle No. 4. There is strong labeling against MHCII molecule in the thymic medulla, which includes a population of dendritic-shaped cells. **Figure 5**. Palatine tonsil; cattle No. 2. The presence of MHCII-positive cells with dendritic

morphology in the stratified epithelium, compatible with Langerhans cells is demonstrated. **Figure 6.** Serial sections of distal ileum; cattle No. 1. (**a**) Cells staining against the MHCII molecule are restricted to Peyer's patches domes and the lamina propria. (**b**) CD208 positive cells are widely distributed in Peyer's patches domes and follicles. **Figure 7.** Lymph node; cattle No. 1. Cells immunopositive for CD208, having a dendritic cell morphology compatible with interdigitant dendritic cells, are demonstrated (inset). Positive cells are also observed within the lymphoid follicles, having the appearance of tingible body macrophages (arrowhead). **Figure 8.** Lung; cattle No. 6. CD208 immunolabeled cells are found amongst the surface cells of the alveoli, compatible with type II pneumocytes (arrows). IHC (ABC method) counterstained with hematoxylin.

Immunolabeling against CD205 demonstrated a granular appearance, located in the cytoplasm of cells with either round or spindle morphology. In general, few immunolabeled cells were observed by using this antibody, and these were located in uncommon regions such as in tissue adjacent to tonsil mucous glands or to large blood vessels (data not shown), in trabeculae of lymphoid organs, or in the connective tissue of hepatic portal spaces, central veins and Glisson's capsule. It was difficult to find any immunopositive cells within the interfollicular areas or the lymphoid follicles *(Fig.10)*. A greater number of immunolabeled cells were identified in the lung, located in the alveolar septa, the pulmonary pleura, or surrounding bronchioles and arteries *(Fig.11)*.

Use of the CNA.42 antibody yielded a staining pattern in the form of a network distributed among lymphocytes of the primary lymphoid follicles, as well as the light zones and mantle zones of germinal centers *(Fig. 12c)*. Curiously, the lymphoid follicles of palatine tonsil, Peyer's patches and ileocecal valve demonstrated only a scarce staining intensity, unlike the stronger labeling intensity noted in the lymphoid follicles of the examined lymph nodes and the spleen. Expression of S100 also observed in the primary lymphoid follicles and the light zones of the germinal centers, showed organic differences similar to those observed with CNA.42, although staining against S100 was located in both the cell cytoplasm and the nucleus *(Fig. 12d)*. Both antibodies (CNA.42 and anti-S100) yielded immunostaining of cell types other than cells located within the lymphoid follicles, which are not included in the aims of this study.

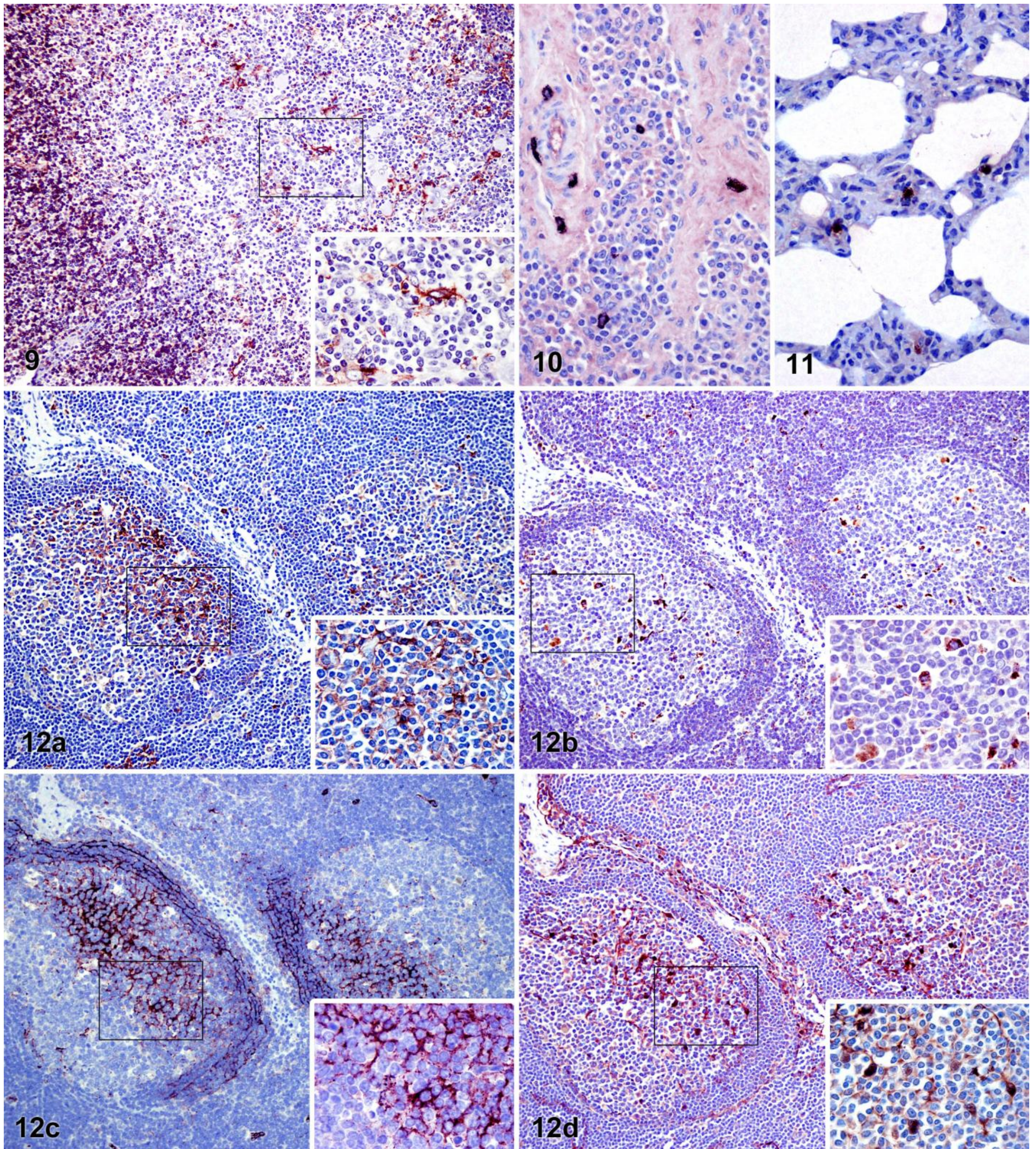

Figure 9. Thymus; cattle No. 3. Anti-CD1b immunolabeling of cortex thymocytes and some dendritic-shaped cells of the medulla are demonstrated (inset). **Figure 10.** Lymph node; cattle No. 1. CD205-positive cells in the medulla, located inside trabeculae, surround a blood vessel and rarely are noted in lymphatic sinuses. **Figure 11.** Lung; cattle No. 3. Cells immunostained against CD205 with round morphology are located inside the alveolar septae. **Figure 12.** Lymph node; cattle No. 4. Serial sections showing the staining pattern within lymphoid follicles with different antibodies; insets represent a higher magnification of the field framed in black. (**a**) Anti-major histocompatibility complex class II (MHCII) immunolabeling of the lymphoid follicle

light zone. (**b**) Anti-CD208 immunolabeling of cells compatible with tingible body macrophages all over the lymphoid follicles. (**c**) CNA.42 antibody staining forming a cytoplasmic pattern restricted to light zone. (**d**) Anti-S100 staining of cells located in the light zone of germinal centers showing the dual cytoplasmic and nuclear staining pattern. IHC (ABC method) counterstained with hematoxylin.

Discussion

It is well known that DCs are a heterogeneous cell population categorized according to their location, functionality, and cell marker expression. The present study has focused on the comparison and characterization of the location of dendritic cells of Friesian calves based on their expression for different cellular markers. This was accomplished by using fixed and paraffin wax-embedded tissues, which preserve an optimal cell morphology, in comparison with frozen tissues, in which there can be a significant loss of morphology, making the identification of a precise location of the antigen-expressing cells within the tissue much more difficult *(Carrasco et al., 2004; Ramos-Vara, 2005; Akesson et al., 2008)*. Furthermore, frozen tissue handling may involve a higher degree of difficulty to produce cryostat sections of consistent quality.

The detection of the MHCII molecule is frequently conducted in tissue sections, although the vast majority of these studies are based on frozen tissue sections and typically for purposes other than detecting DCs *(Manesse et al., 1998; Matsuda et al., 2010)*. The novel information presented by this study employed the MHCII molecule to describe the type of immunolabeled cells within a wide variety of organs, thereby providing a standardization of the immunohistochemical methodology, as well as taking advantage of the fact that DCs are strong expressors of this molecule and that fixed paraffin-embedded tissues preserve the characteristic morphology of these cells, allowing for a mapping of their localization within select organs. To our knowledge, this is the first report of the use of this list of antibodies in the detection of DCs in bovine paraffin-embedded tissues.

This study revealed that of the antibodies examined, the MHCII antibody yielded the greatest quantity of labeled cells in the different tissues analyzed. This is due to the expression of the molecule not only in DCs but also in macrophages and certain populations of B cells, as well as being inducible in endothelial cells.

Despite this variety of positive cells, use of this antibody also permits the detection of all DC populations, unlike other markers that detect only certain subpopulations. A striking finding in the results obtained with anti-MHCII antibody was the marked staining polarization in most of the germinal centers of secondary lymphoid organs, which coincided to a great extent with the immunolabeling observed with the use of CNA.42 and anti-S100 antibodies, which was restricted solely to the light zone (and adjacent mantle zone in the case of CNA.42) of the germinal centers. This observation was possible by using serial sections of the tissue immunostained with the antibodies of interest (Fig. 12a,c,d). It is known that FDCs do not internalize nor present processed protein antigens in the context of MHCII molecules *(van Nierop and de Groot, 2002)*, a fact that would establish as incompatible the coincidence in location of FDCs in light zones with the strong expression of MHCII. However, it has been shown that this cell type can acquire MHCII molecules not expressed by FDCs themselves *(Denzer et al., 2000)*, which may explain why a strong immunostaining against MHCII in light zones can be observed, where FDCs are located. Human studies have demonstrated the CNA.42 antibody as an immunomarker for FDCs for each level of maturation, ranging from FDC precursors to activated FDCs *(Kasajima-Akatsuka and Maeda, 2006)*. Furthermore, the S100 protein is a molecule expressed by activated FDCs, which may explain why CNA.42 yielded a network staining pattern that was widely distributed and anti-S100 gave rise to the staining of individual cells.

The present study describes for the first time in bovine tissue samples the expression of CD208 (DC-LAMP), known as an exclusive marker for mature DCs in T regions (interdigitant DCs) and type II pneumocytes *(de Saint-Vis et al., 1998; Salaun et al., 2004)*. CD208 expression in alveolar surfaces coincides with the previously described expression of this molecule in type II pneumocytes. However, we unexpectedly found CD208-positive cells not only in interfollicular areas but also within lymphoid follicles; given the known exclusive expression of CD208 to DCs in lymphoid organs, these immunopositive cells found within lymphoid follicles might be considered as the previously described germinal center DCs (GCDC) *(Grouard et al., 1996; Goval et al., 2006)*. However, this consideration would be mistaken since GCDC, whose origin is hematopoietic, should express MHCII, a fact that was ruled out by using serial sections immunostained with both

antibodies (Figs 6, 12a,b); CD208-positive cells were located both in MHCII-positive light zones and MHCII-negative dark zones. The typical follicular location, round morphology, and large size suggest that these CD208-positive cells are in fact tingible body macrophages.

Numerous MHCII-positive cells were detected in the thymic medulla, most of them having a stellate shape compatible with that of DCs, which have significant importance in both positive and negative selection during T-cell development *(Ardavin, 1997)*. In this same location, we also observed dendritic-shaped cells immunolabeled against CD208, albeit to a lesser extent. This observation may be explained by the fact that during maturation, DCs acquire a higher capacity to form and accumulate MHCII-peptide complexes, a process that requires a generalized activation of the lysosomal function *(Trombetta et al., 2003)*; the CD208 molecule is included in the lysosomal-associated membrane protein family, which is why these CD208-positive cells in thymic medulla may represent a population of mature DCs, which corresponds to similar descriptions in humans *(Bendriss-Vermare et al., 2001)*.

Langerhans cells (LC) were originally described as immature DCs present in the epidermis, although currently this term has been generalized to include DCs present in all surface stratified-epithelium *(Merad et al., 2008)*. This may support the hypothesis which considers MHCII-positive cells observed in tonsillar epithelium as LC (Fig. 5), as these demonstrated stronger immunopositivity and were more numerous in comparison to those of the epidermis, further highlighting the importance of DCs in the tonsil due to its strategic location in the entrance of numerous airborne pathogens.

It is well established that DCs are present within the dome regions of the intestinal lymphoid tissue *(Niedergang and Kweon, 2005)*, coinciding with the presence of stellated MHCII-positive cells that we found in those intestinal sections examined. However, Peyer's patches follicles barely stained against the MHCII molecule. In ruminants, ileal Peyer's patches are considered to be primary lymphoid organs *(Liebler-Tenorio and Pabst, 2006)*, in which there is no germinal center reaction as observed in jejunal Peyer's patches *(Yasuda et al., 2004)*, which may explain the lack of MHCII expression at this level, similar to that which occurs in the cortex of thymus, another primary lymphoid organ. However, these MHCII-

negative Peyer's patches follicles demonstrated a great quantity of widely distributed CD208-immunopositive cells, supporting the hypothesis that considers these cells to be tingible body macrophages.

Previous studies have determined the phenotypic characteristics of both intestinal and respiratory tract DCs in sheep *(McNeilly et al., 2006; Akesson et al., 2008)* by using antibodies against CD205 and CD1b molecules. ALDC are strong expressors of CD205 *(Gliddon et al., 2004)*, and other tissues have been shown to express this molecule as well *(Parsons et al., 1993; Gliddon et al., 2004)*. Contrary to results obtained by Akesson and collaborators in sheep *(Akesson et al., 2008)*, we did not find cells expressing CD205 in the ileum in this study of Friesian cattle. However, a considerable amount of CD205-immunolabeled cells were detected in the parenchyma and pleura of the lung, which is consistent with other ovine studies *(McNeilly et al., 2006)*. In comparison with other DC markers, very few cells demonstrated immunolabeling against CD1b in the intestine and the lung of sheep *(McNeilly et al., 2006; Akesson et al., 2008)*. Likewise, our results demonstrated that both organs were negative to the expression of CD205 and CD1b. Antibodies for detecting CD205 and CD1b used in this study and in ovine studies from Akesson and McNeilly are bovine specific. Despite this fact, the absence of expression in this study of CD1b in the intestine and the respiratory track and of CD205 in the intestine suggests that the observed species differences between sheep and cattle are due to differences in the technique sensitivity employed; ovine studies were carried out in frozen tissues, whereas this study used tissue samples that had been fixed and embedded in paraffin wax. The latter technique permits an optimal preservation of the cellular morphology and tissue architecture, although it may mask or alter the three-dimensional structure of antigens, which is why antigen retrieval methods are required *(Ramos-Vara, 2005)*.

In summary, despite the existence of other molecules displayed by DCs, the recognition of MHCII expression appears to be one of the most sensitive methods for the detection of any DC of hematopoietic origin in tissue sections. The staining pattern observed with the monoclonal antibody detecting CD208 suggests that the expression of this molecule in bovine lymphoid tissues is restricted not only to interdigitant DCs but also to tingible body macrophages, a finding which has not been previously described. Although further studies will be needed to confirm this

novel finding, this study has demonstrated that CD208 detection allows for the differentiation of mature DCs from all other DCs, a fact of significant interest in the diagnostics and study of infectious diseases. This study further supports the tissue expression of CD1b in dendritic-shaped cells, even though these cells comprised a reduced population out of the total number of the existing DCs and were located in specific sites such as the thymus. Therefore, although global utility is limited, CD1b will provide valuable information on the role of thymic DCs, which is of significance due to the organ's importance in the central immunotolerance. Both CNA.42 and anti-S100 antibodies stained FDCs, but only the latter allowed for the identification of FDCs as isolated cells. Taken together, these results provide a useful general view of the different staining patterns of potential DC markers and will help in future DC studies with pathological tissues.

Acknowledgements

We thank the farm ‘‘Las Rozuelas del Valle’’ (Torrecampo, Spain) for providing the animals.

CHAPTER 2A

Characterization of thymus atrophy in calves with subclinical BVD challenged with BHV-1

Characterization of thymus atrophy in calves with subclinical BVD challenged with BHV-1

Veterinary Microbiology. Under review

Abstract

Since the thymus is a target organ for the bovine viral diarrhea virus (BVDV), our experiment aimed to understand its relationship with the immunosuppressive effect by studying the consequences of a previous infection with BVDV on the thymus of calves challenged with bovine herpesvirus 1.1 (BHV-1). For this purpose, 12 animals were inoculated intranasally with non-cytopathic BVDV-1; 12 days later, 10 of them were coinfected intranasally with BHV-1. These animals were euthanized in batches of two between 0 and 14 dpi with BHV-1. Another 10 calves were inoculated solely with BHV-1 and euthanized in batches of two between 0 and 14 dpi with BHV-1. Thymus samples from these animals were processed for viral detection and histopathological, immunohistochemical, and ultrastructural studies focused on BVDV/BHV-1 antigens, cortex:medulla ratio, apoptosis (TUNEL and caspase-3), collagen deposition, and Factor VIII endothelial detection. Our study revealed the immunohistochemical presence of BVDV antigen in all animals in the BVDV-infected group, unlike BHV-1 detection, which was observed in animals in both infection groups only by molecular techniques. BVDV-preinfected animals showed severe atrophic changes associated with reduced cortex:medulla ratio, higher presence of cortical apoptosis, and increased collagen deposition and vascularization. However, calves solely infected with BHV-1 did not show atrophic changes. These findings could affect not only the numbers of circulating and local mature T cells but also the T cell-mediated immunity, which seems to be impaired during infections with this virus, thus favoring pathogenic effects during secondary infections.

Key words: bovine viral diarrhea virus (BVDV); bovine herpesvirus type 1 (BHV-1); thymus atrophy; apoptosis; immunohistochemistry.

Introduction

Bovine viral diarrhea virus (BVDV), a pestivirus in the family *Flaviviridae* along with classical swine fever virus and Border disease virus, is a major pathogen of cattle, which causes significant economic losses worldwide *(Houe, 1999)*. The clinical manifestations of BVDV infection range from unapparent or mild to the inevitable fatal syndrome of mucosal disease *(Brownlie, 1991)*. Two antigenically distinct genotypes of BVDV exist, types 1 and 2, and according to their *in vitro* effect on bovine epithelial cells, both genotypes can be segregated into noncytopathic and cytopathic biotypes *(Fulton et al., 2000)*. However, it is the type 1 virus of the noncytopathic biotype that usually circulates in cattle populations.

Due to the BVDV predilection for cells of the immune system *(Potgieter, 1995)*, an acute infection with BVDV can lead to immunosuppression that enhances the severity of the disease during mixed infections with other enteric or respiratory pathogens such as Bovine herpesvirus-1.1 (BHV-1) *(Bolin, 2002)*. Due to this immunosuppression, many studies of the pathogenesis of BVD are focused on lymphoid organs. The thymus is a primary lymphoid organ and it has long been considered useless once the immune system is fully developed. However, many studies have demonstrated that the thymus has an important role throughout adult life *(Cunningham et al., 2001)*, with the thymopoiesis being an essential process for the development and maintenance of a robust and healthy immune system. Thereby, due to this role in the adult immune system and to the fact that the thymus is known for being one of the main target organs for BVDV, it is of great interest to characterize the pathological changes that the thymus undergoes, in order to understand the nature of BVDV-associated immunosuppression. Thymic atrophy is one common pathology in a variety of infectious diseases *(Savino, 2006)*, many of which have important immunosuppressive features such as infections with human immunodeficiency virus *(Su et al., 1995)*, porcine reproductive and respiratory syndrome virus *(He et al., 2012)*, classical swine fever virus *(Sanchez-Cordon et al., 2002)*, feline immunodeficiency virus *(Woo et al., 1997)*, or BVDV *(Liebler-Tenorio et al., 2003b)*. The mechanisms driving such changes may vary depending on the pathogen or its virulence, and thus deserve detailed

investigation. Therefore, the objective of this study was to characterize the lesional alterations associated with BVDV-induced thymic atrophy occurring in calves preinfected with BVDV and challenged later with BHV-1, thus contributing to a better understanding of the disease.

Results

At postmortem examination, no remarkable gross lesions were detected in any of the thymus samples, with no differences being observed between both infection groups.

Immunohistochemical and genomic viral detection

Results of the BHV-1 immunohistochemical detection were negative for the thymus samples from all the animals. The BVDV antigen was found in thymus samples from all the animals in the group previously infected with such virus, with a progressive decrease and an occasional increase at 4 dpi being observed during the study period *(Fig. 1a)*. Immunolabeling with the 15c5 monoclonal antibody was observed multifocally located in the cortex and mainly in the surrounding connective tissue, with the medulla being practically negative and thus not quantified *(Fig. 1c)*. Positive staining was observed in cells with a marked pleomorphism, including macrophages, occasional lymphocytes, and many spindle or stellate-shaped cells compatible with reticular epithelial cells and fibroblasts *(Fig. 1c inset)*.

Since the BHV-1 antigen was not detected by immunohistochemistry in any of the thymus samples, further molecular studies were carried out to clarify the presence or absence of this virus. BHV-1 was detected in the thymus via PCR assay from 1 to 14 dpi in the coinfected group and between 2 and 14 dpi in the BHV1 group *(Table 1)*.

Table 1. Results of BHV-1 genomic detection by conventional PCR

	dpi with BHV-1:	0	1	2	4	7	14
BVDV/BHV1 group	Animal 1	-	-	+	+	+	-
	Animal 2	-	+	+	+	+	+
BHV1 group	Animal 1	-	-	+	+	+	+
	Animal 2	-	-	+	-	+	+

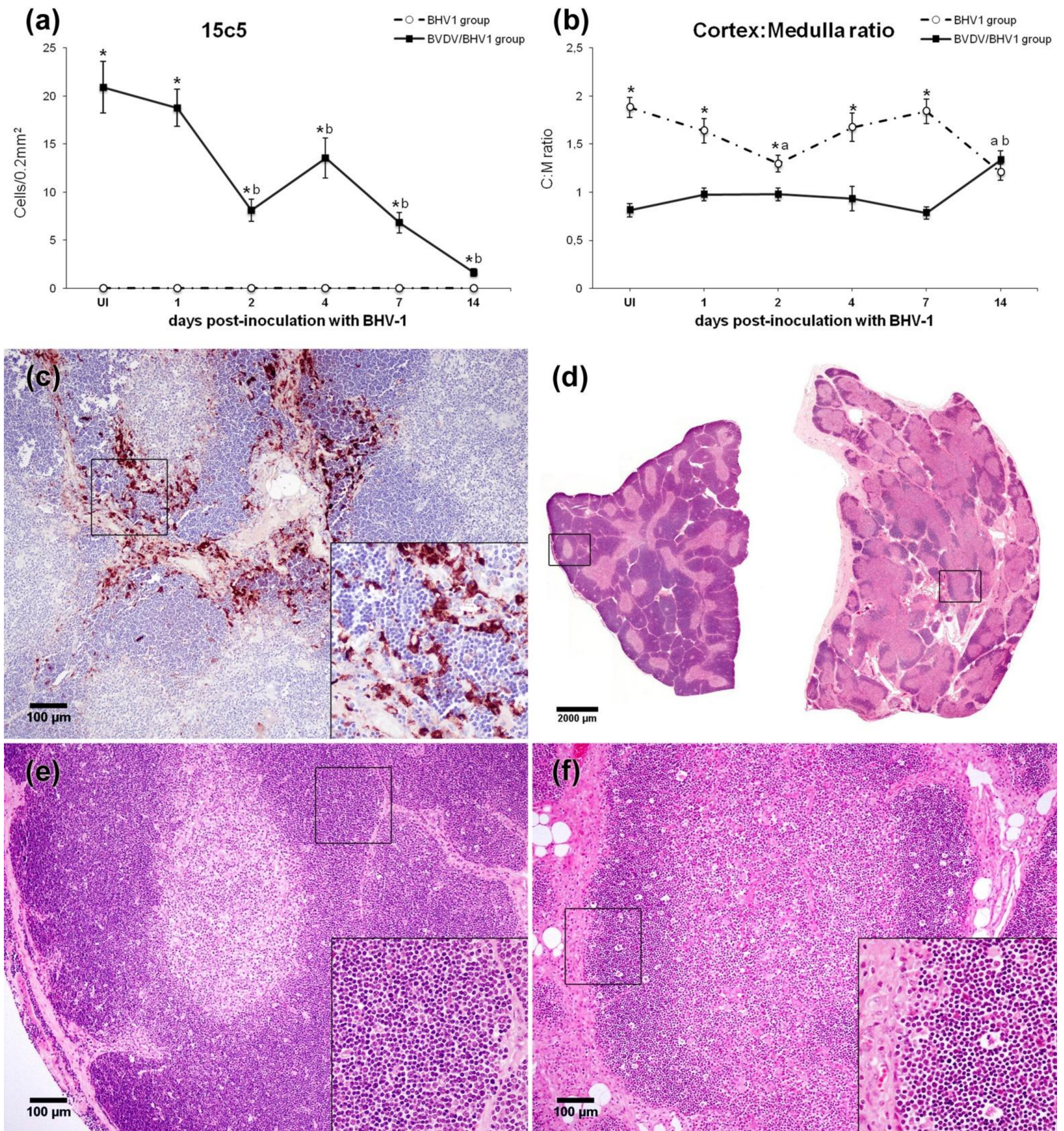

Figure 1. Immunohistochemical detection of BVDV and morphometric assessment of the thymic compartments. **(a)** Counts of BVDV infected cells (mean ± standard error) immunolabeled with the monoclonal antibody 15c5. **(b)** Quantitative assessment (mean ± standard error) of the cortex:medulla ratio. (UI, BHV-1 un-infected: negative controls for the BHV1 group and BVDV infection controls for the BVDV/BHV1 group. *Significant differences ($p<0.05$) between inoculated groups at the same time point, [a,b]Significant differences ($p<0.05$) in the same group at various time points). **(c)** Immunohistochemical detection of BVDV in the thymus of a calf inoculated with BVDV, challenged with BHV-1 12 days later, and euthanized at 1 dpi with BHV-1. Inset: detail of different cell types immunolabeled against BVDV. Notice the immunolabeling located in the cortex and mainly in the surrounding connective tissue, with the medulla

being practically negative. **(d)** Low magnification of thymus sections from calves euthanized at 4 dpi with BHV-1, belonging to the BHV1 group (on the left) and the BVDV/BHV1 group (on the right). Hematoxylin-eosin (HE). **(e,f)** Higher magnifications of the fields framed in black in (d). Notice the lower cortex:medulla ratio and the frequent presence of tingible body macrophages in the animal preinfected with BVDV (on the right).

Morphometric and histopathological findings in the thymus

In general terms, a loss of cortical lymphoid tissue was observed in the animals previously inoculated with BVDV, evidenced by a slimming of the cortex and the frequent presence of large macrophages containing phagocytosed apoptotic bodies (tingible body macrophages), indicative of apoptosis *(Figs. 1d and 1f)*. In addition, a proliferation of an acidophilic fibrous material filling the interlobular spaces and some degree of hyperemia were also observed in this coinfected group in comparison with the BHV1 group. These findings were further investigated by using complementary techniques, as described below.

A generalized reduction in the cortex:medulla ratio was observed in the coinfected group, that reached similar values to the BHV1 group solely at 14 dpi *(Figs. 1b, 1d, 1e and 1f)*. This reduction in the BVDV/BHV1 group was due to both an increase in medulla thickness and a slimming of the cortex, with the exception of the second dpi, whose reduced cortex:medulla ratio was produced only due to a medullar thickening (data not shown). Despite the different changes noticed in this study, no corticomedullary boundary blurring was observed in any of the animals.

To further investigate the different rate of apoptosis observed in the cortex, the presence of DNA fragmentation and the expression of an executioner caspase (caspase-3) were studied. TUNEL staining identified apoptotic cells scattered in the thymic medulla and more densely distributed throughout the cortex *(Figs. 3a and 3b)*. The apoptotic signal was localized mainly in apoptotic bodies within macrophages, but a signal was also seen in free apoptotic bodies. The number of TUNEL-positive cells of the cortex was higher in the BVDV/BHV1 group throughout the study, except for the end of the study (14 dpi) *(Fig. 2a)*. Caspase-3 detection was mainly observed in the nucleus of cortical lymphocyte-like cells *(Figs. 3c and 3d)*. Tingible body macrophages were not considered during the cell counts, because labeling of apoptotic bodies was not constantly detected in these elements. Cortical cell counts remained consistently low in the BVDV-preinfected

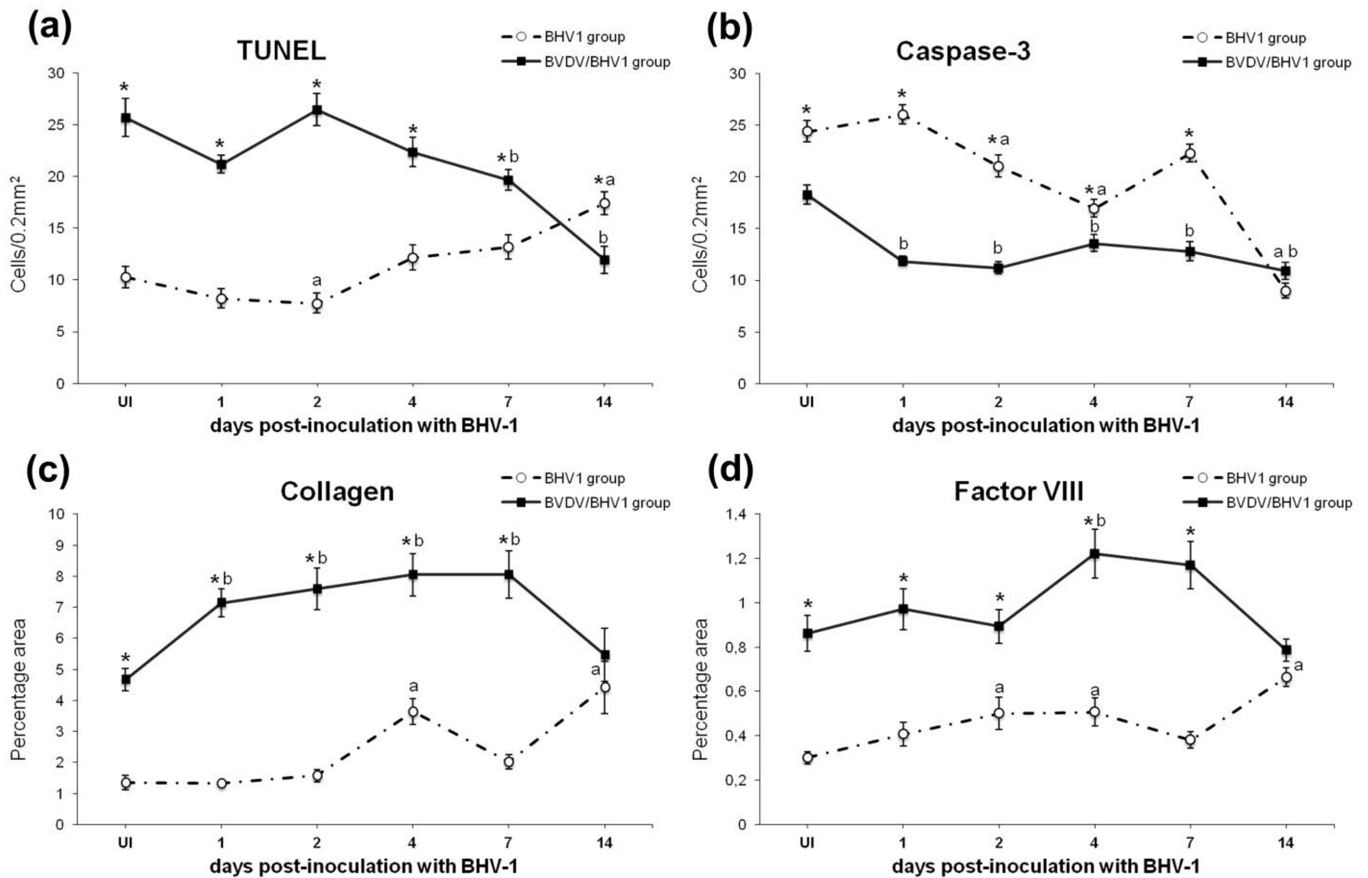

Figure 2. Quantitative assessment (mean ± standard error) of apoptosis, collagen and Factor VIII. **(a)** TUNEL-positive cell counts, **(b)** caspase 3-immunolabeled cell counts, **(c)** percentage area of collagen (Masson's trichrome staining), **(d)** percentage area of Factor VIII-immunolabeled vascular structures. (UI, BHV-1 un-infected: negative controls for the BHV1 group and BVDV infection controls for the BVDV/BHV1 group. *Significant differences ($p<0.05$) between inoculated groups at the same time point, [a,b]Significant differences ($p<0.05$) in the same group at various time points).

group with respect to the BHV1 group *(Fig. 2b)*, although at 14 dpi the BHV1 group decreased to levels similar to those of the BVDV/BHV1 group.

The transmission electron microscopy *(Figs. 3e and 3f)* showed changes consistent with lymphocyte apoptosis more frequently observed in the coinfected group. Apoptotic cells showed condensation and margination of chromatin, as well as fragmentation of lymphocyte nuclei and cytoplasm. Cells with increased size and presence of engulfed apoptotic bodies compatible with tingible body macrophages were also observed. These features were more widely spread in the BVDV/BHV1 group than in the BHV1 group.

Masson's trichrome special stain evidenced the collagen, mainly located surrounding thymic lobules and to a lesser extent infiltrating medullary regions

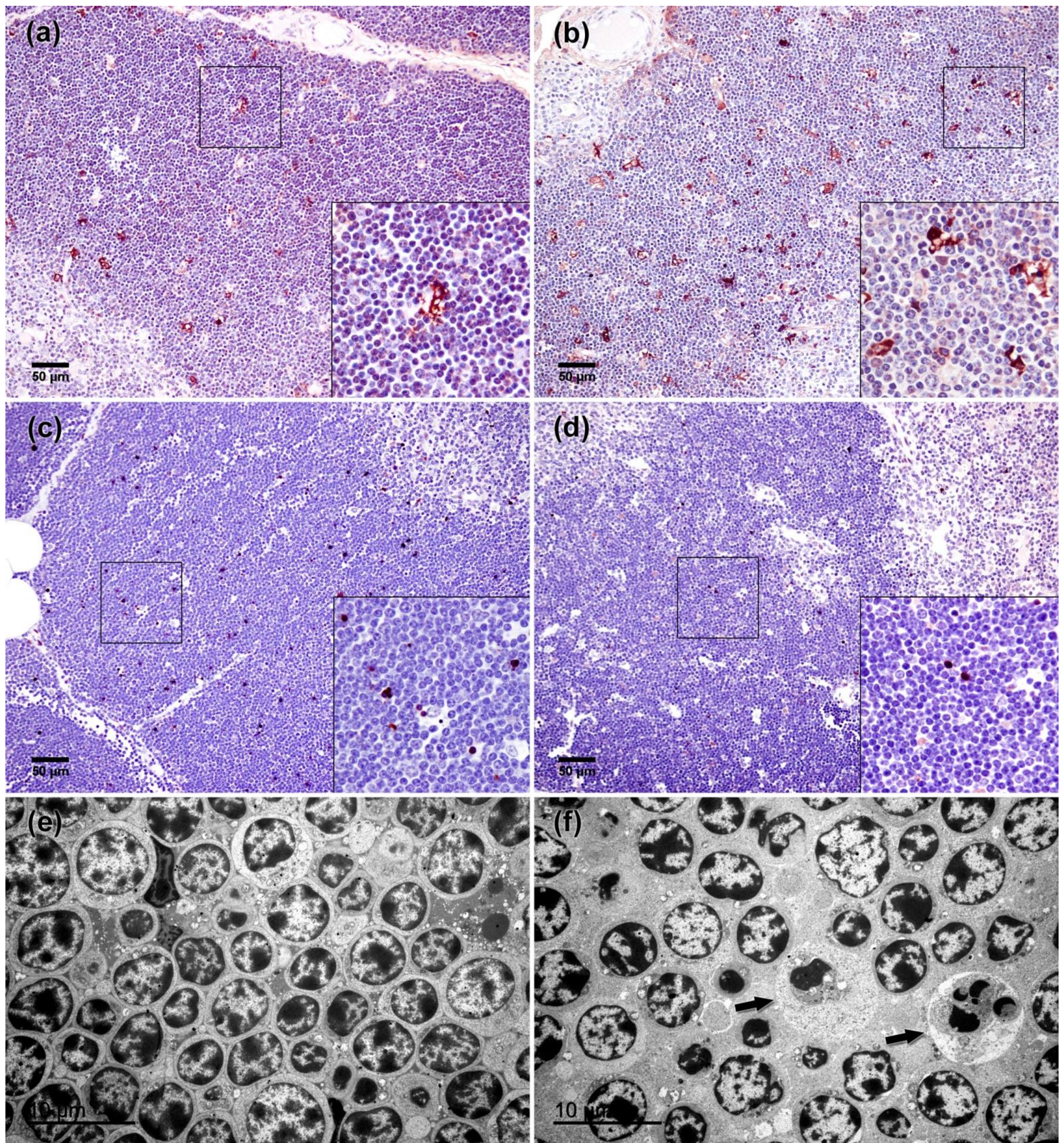

Figure 3. Representative photographs of the apoptosis assessment. **(a,b)** TUNEL staining in animals sacrificed at 2 dpi from the BHV1 group (a) and the BVD/BHV1 group (b). Note the numerous TUNEL-positive cells in BVDV-preinfected calf. **(c,d)** Immunohistochemical detection of Caspase 3-expressing cells in animals euthanized at 1 dpi from the BHV1 group (a) and the BVD/BHV1 group (b). Note the lower amount of immunolabeled cells in the BVD/BHV1 group, possibly due to a more advanced phase of apoptosis where caspase-3 is not expressed anymore. **(e)** Transmission electron microscopy (TEM) photograph of thymocytes from a calf of the BHV1 group euthanized at 4 dpi with BHV-1. Notice the lack of subcellular changes. **(f)** TEM photograph of the thymus from a calf of the BVDV/BHV1 group euthanized at 2 dpi with BHV-1. Notice the presence of nuclear fragmentation resulting in apoptotic bodies (arrows). Insets represent higher magnifications of the fields framed in black.

(Figs. 4a and 4b). The collagen quantification *(Fig. 2c)* revealed a surprisingly higher amount of this substance prior to BHV-1 inoculation in the animals preinfected with BVDV, demonstrating an evident thickening of the interlobular septa. A decrease to values similar to those of the BHV1 group was observed at the end of the study.

The transmission electron microscopy supported the high amount of collagen observed with Masson's trichrome stain *(Figs. 4e and 4f)*. Collagen was identified as nearly 100 nm width fibrils with a characteristic banding pattern repeated every 65 nm and organized into bundles of fibrils (collagen fibers) closely packed of more than 2 μm width. These thick collagen fibers were identified as type I collagen (unlike the thinner fibers of type III collagen, so-called reticular fibers) *(Montes, 1996)*, and were found in different locations intermingled with lymphocytes and other cell types.

Anti-factor VIII labeling of vascular structures showed similar dynamics to those of collagen *(Fig. 2d)*, with a significantly higher vascular proliferation in the coinfected group throughout the study except at 14 dpi, when an increase in the BHV1 group that reached the other group was observed. The vast majority of the blood vessels were observed within the medulla and in the connective tissue surrounding the thymic lobules *(Figs. 4c and 4d)*.

A scarce adipocyte infiltration was observed in this study, with only a very slight increase in some of the BVDV-preinfected animals (data not shown).

Discussion

In this study, thymus lesional changes were evaluated in healthy calves and calves with subclinical BVD both experimentally inoculated with BHV-1, in order to characterize the thymus atrophy associated with BVDV infection. The results show that thymuses of the BVDV-preinfected calves displayed severe atrophic changes that were not observed in the non-BVDV-infected control group.

Several findings highlight the importance of the thymus as a target organ for BVDV. The thymus was elected as the best organ for the purification of a protein that afterwards would be determined as CD46, a cellular receptor for BVDV *(Maurer et al., 2004)*. In the bovine species, the ileal Peyer's patch is a primary

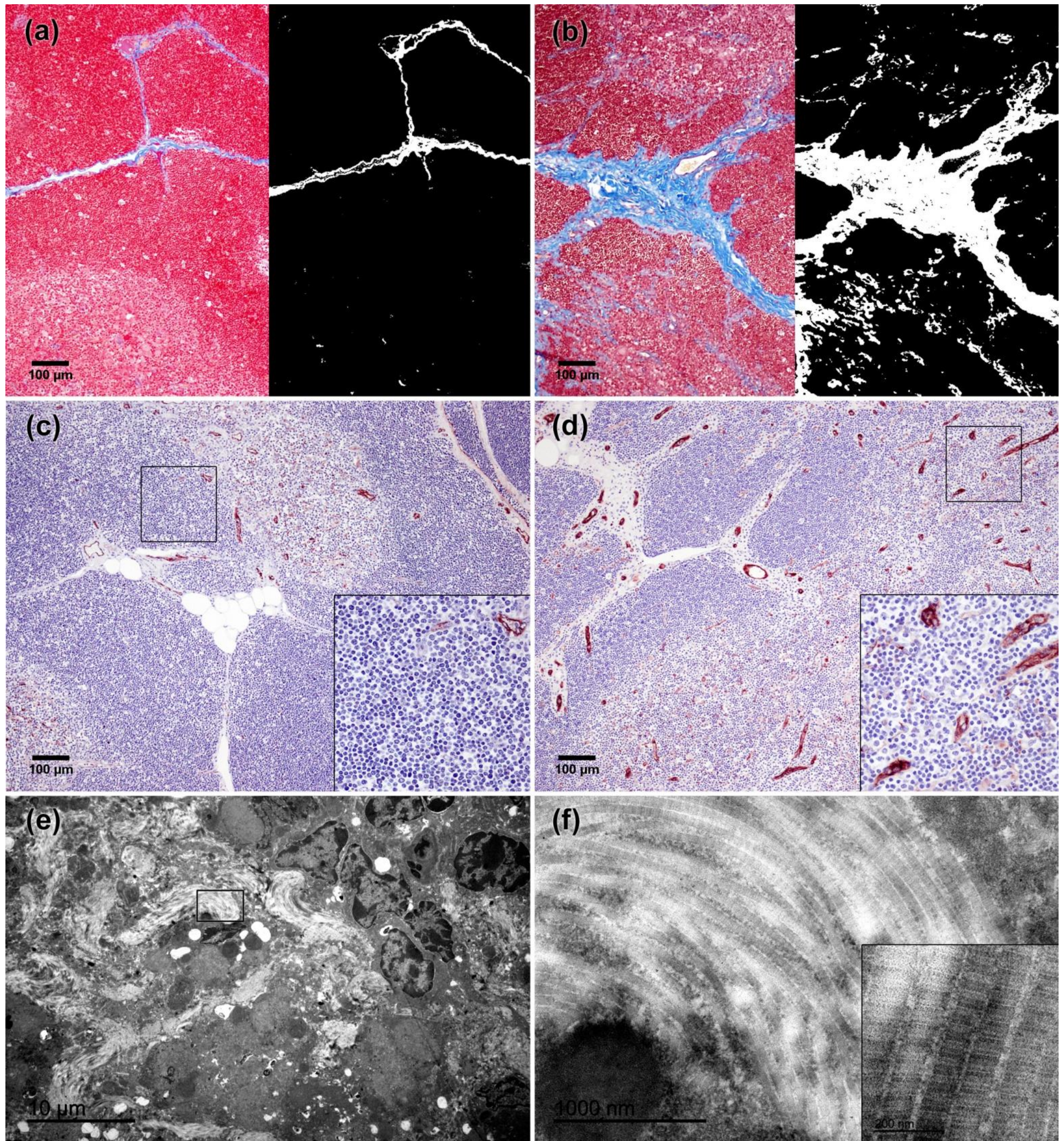

Figure 4. Representative photographs of the collagen and factor VIII assessment. **(a,b)** Masson's trichrome special stain in animals sacrificed at 2 dpi from the BHV1 group (a) and 7 dpi from the BVD/BHV1 group (b). The black and white images on the right represent the images on the left after thresholding for the blue color of collagen. Note the higher presence of collagen in the animal of the BVD/BHV1 group. **(c,d)** Anti-Factor VIII immunostaining of vascular structures in animals sacrificed at 4 dpi from the BHV1 group (c) and the BVD/BHV1 group (d). Notice the greater vascularization in the BVDV-preinfected animal. **(e)** TEM photograph of a calf sacrificed at 4 dpi from the BVD/BHV1 group depicting the intense proliferation of type I collagen. **(f)** Detail of the collagen fiber framed in black in (e), composed of fibrils closely packed with a characteristic banding pattern.

lymphoid organ *(Yasuda et al., 2006)*. Together with the thymus, they constitute the locations where the highest amounts of virus can be found, which may indicate that the main BVDV strategy is to cause immunosuppression by injuring the primary lymphoid organs.

A reduced cortex:medulla ratio was observed in this study in the BVDV-preinfected calves. Thickness measurements of the cortex and medulla revealed that this alteration was due to both a slimming of the cortex and a thickening of the medulla, which may be explained by the generalized cortical depletion due to apoptosis phenomena and a compensatory medullar hyperplasia. Exceptionally, the reduced cortex:medulla ratio observed at 2 dpi was produced solely by the expansion of the medulla, without marked cortical atrophy, which coincides with an important decrease in the counts of BVDV-infected cells. A previous study by Falkenberg and coworkers compared infections of 2-3 week old colostrum-deprived calves with high (HV) and typical virulence (TV) strains of BVDV-2 *(Falkenberg et al., 2014)*. These authors described an apparent cortical atrophy of the thymus in both HV and TV calves, with the greatest degree of atrophy occurring in HV calves compared to TV calves.

In a variety of acute infections accompanied by severe atrophy of the thymus, an intense cortical thymocyte apoptosis has been described *(Savino, 2006)*, especially during infections causing an impairment of the immune system *(Sanchez-Cordon et al., 2002; He et al., 2012)* and for this reason, our study aimed to study apoptosis. The *in situ* identification of nuclear DNA fragmentation (TUNEL assay) revealed greater levels of cortical apoptosis in the BVDV/BHV1 group, coinciding with the morphological changes indicative of apoptosis observed in HE sections and in the ultrastructural study. Curiously, the proportion of BVDV-infected lymphocytes was very low through the experiment. These results agree with previous reports that detected thymic depletion but very low BVDV-infection of lymphocytes by immunohistochemistry *(Raya et al., 2012)*, suggesting indirect mechanisms for the thymocyte apoptosis. The capacity to induce apoptosis has also been reported for BHV-1 *(Hanon et al., 1998; Winkler et al., 1999)*, which may explain the final increase observed in the BHV1 group at the end of the study. However, despite being also infected with BHV-1, the coinfected group did not show this final increase, probably due to the severe loss of cortical tissue.

The caspase-3 detection showed different kinetics, with higher levels in the BHV1 group than in the previously BVDV-infected group. Caspase-3 is a marker for early apoptosis, when DNA fragmentation has not yet taken place. This suggests a hypothesis supporting that caspase-3 levels observed in the BHV1 group correspond to physiological apoptosis that is normally produced in the thymus, with the level of caspase-3 in the BVDV/BHV1 group being lower due to an advanced phase of apoptosis (with DNA fragmentation) where caspase-3 is not expressed anymore, probably due to a degradation of apoptotic bodies by lytic enzymes, as previously suggested *(Dukers et al., 2002)*. An increase in caspase-3 expression induced by BVDV has been observed in other target organs such as ileum *(Pedrera et al., 2009)*, but in this case the period of study corresponded to the first 14 days of infection (acute phase), unlike the later period of our experiment (from 12 dpi with BVDV onwards), when the acute phase of infection had already finished. In fact, at the beginning of our study (0 dpi with HVB-1, 12dpi with BVDV), the coinfected group showed higher counts of cells expressing caspase-3 than those observed subsequently, which could be indicative of the high levels of caspase-3 expression that might have been reached during the first 12 dpi with BVDV. Moreover, previous studies focused on porcine circovirus type 2 have also reported a scarcer immunolabeling of caspase-3 expressing cells associated with intense thymus atrophy *(Resendes et al., 2004)*.

Some controversy regarding the thymic localization of BVDV-infected cells can be found in the literature; previous studies include both the thymic medulla and cortex as sites of BVDV infection *(Ellis et al., 1998; Raya et al., 2012)*. However, in this study, only the cortex and surrounding connective tissue was observed to be susceptible to infection, in agreement with other authors *(Marshall et al., 1996; Liebler-Tenorio et al., 2003a)*. These differences seem to be dependent on the age of the animals, the BVDV strains, and the time of infection, with young animals, high virulent strains, and early periods of infection being the main predisposing factors to thymic medulla infection findings.

In this experiment, no BHV-1-infected cells were observed in the thymus by means of immunohistochemistry (IHC). However, the PCR assay revealed the presence of the virus in this location. It has been previously reported that the IHC detection of BHV-1 is normally restricted to foci of necrosis associated with BHV-1

(Moeller et al., 2013; Risalde et al., 2013), which were not observed in this study. In addition, previous studies have demonstrated that with thymus sections the rate of BHV-1 detection was higher by *in situ* hybridization than by IHC *(Ayers et al., 1989)* making evident the sensitivity differences between molecular and IHC techniques for the detection of BHV-1. Collectively, these data suggest that although BHV-1 can arrive to the thymus, no lesions associated with viral replication sites can be observed in this organ, which is not a target organ for BHV-1, thus making difficult its detection by IHC methods. An absence of lesions attributed to BHV-1 was observed in the thymus of these animals. However, further studies may help to elucidate if the thymus functionality remains unaltered or if on the contrary is disrupted.

This study reveals how local BVDV-infection can exacerbate thymic atrophy, through remodeling extracellular matrix (ECM) with collagen deposition. The intrathymic production of ECM components has been previously described in other infectious diseases such as rabies, syphilis, measles and Chagas diseases *(Savino, 2006)* but this is the first description of increased collagen deposition induced by BVDV infection. The presence of this component coincides to a great extent with the frequent location of BVDV-infected cells in the interlobular connective tissue. A previous study of *Tripanosoma cruzy* infections *(Cotta-de-Almeida et al., 1997)* reported an enhancement of ECM production by the thymic nurse cells (TNC) in response to the release of lymphocytes due to the infection. The TNC, located in the outer cortex, are thymic epithelial cells (TEC) that form multicellular structures together with thymocytes. These results, together with the fact that TEC are targets for the BVDV *(Raya et al., 2014)*, lead us to hypothesize that the TEC might be directly related to the production of ECM when they become infected with BVDV. As we can observe, the lack of lymphocytes due to cortex depletion is substituted not only by a medullary hyperplasia but also by an increased deposition of collagen. Ultrastructural studies identified the bulk of the deposited collagen as type I collagen, normally found in several localizations such as the dermis of the skin, tendons, ligaments and scar tissue *(van Zuijlen et al., 2003; Eroschenko, 2008)*. Hence, this type I collagen deposition may be considered a type of scarring consequent to viral-induced injury, in the context of a healing response.

The higher vascularization observed after Factor VIII endothelial detection in the BVDV/BHV1 group could be considered a viral strategy to facilitate the spreading of infected cells into different organic locations. Given the important role of vascular buds in granulation tissue *(McGavin and Zachary, 2007)*, these vascular changes could also be explained as part of the aforementioned process of healing, which accounts for the apparent recovery observed at the end of the study.

These signs of regeneration at the end of the experiment coincided with previous works where thymic depletion was transient with low virulent strains and followed by recovery *(Liebler-Tenorio et al., 2003b)*. Curiously, these final signs of recovery concurred with the lowest values of BVDV infected cells, highlighting the important contribution of BVDV in producing these changes. However, this signs of morphologic recovery do not necessarily mean a restoration of thymus functionality, which might alter ensuing immune responses. In this respect, further studies focused on the thymic immune cells and their mediators will be required to further investigate thymus functionality.

Conclusion

Collectively, the results obtained in the present study provide a deep characterization of the lesional alterations underlying the BVDV-induced thymic atrophy and shed light on the possible mechanisms that give the thymus an important role in the compromised immune status that favors secondary infections.

Acknowledgements

The authors thank the farm ''Las Rozuelas del Valle'' (Torrecampo, Spain) for providing the animals and especially G. Muñoz for her excellent technical assistance. This work was supported by grants from the Junta de Andalucía-FEDER (project P09-AGR-4671). F. Romero-Palomo was financially supported by a predoctoral grant associated with the aforementioned project.

CHAPTER 2B

Immunopathologic changes in the thymus of calves preinfected with BVDV and challenged with BHV-1

Immunopathologic changes in the thymus of calves preinfected with BVDV and challenged with BHV-1

Abstract

The aim of this work was to investigate the effect of preinfection with bovine viral diarrhea virus (BVDV) on thymus immune cells from calves challenged with bovine herpesvirus 1 (BHV-1), being the thymus in the limelight of our studies due to the elevated tropism of BVDV for this organ. Twelve Friesian calves, aged 8 to 9 months, were inoculated with noncytopathic BVDV-1. Ten of them were subsequently challenged with BHV-1 and euthanized in batches of two at 1, 2, 4, 7, or 14 dpi with BHV-1. The other 2 calves were euthanized prior to the second inoculation and were used as BVDV-infected controls. Another 10 calves were inoculated solely with BHV-1 and euthanized at the same time points. Two calves were not inoculated with any agent and were used as negative controls. Quantitative changes in immune cells were evaluated with immunohistochemical methods to compare coinfected calves and calves challenged only with BHV-1. Results from this study pointed out BVDV as responsible for the thymic lesions observed in the experiment as well as for the majority of immunopathologic changes, including a downregulation of Foxp3 lymphocytes and TGF-β that reverted as BVDV was cleared, and an overexpression of medullary CD8+ T cells. However, despite not inducing evident lesions in the thymus, BHV-1 did seem to prompt also some immune alterations. Collectively, these data contribute to the knowledge on the immunopathologic alterations of the thymus during BVDV infections, and its importance in the development of secondary infections.

Key words: BVDV, BHV-1, thymus, immune response; lymphocytes, antigen-presenting cells, immunohistochemistry.

Introduction

Bovine viral diarrhea virus (BVDV) is an endemic ruminant Pestivirus in populations worldwide *(Ridpath, 2010)*. BVDV has different genotypes and biotypes, but is the ncp genotype 1 the most widely distributed, and is considered a major predisposing factor in the appearance of bovine respiratory disease complex (BRDC) *(Fulton et al., 2002; Srikumaran et al., 2007)*. BVDV shows a preference for lymphoid tissues, reaching its highest concentrations in the tonsils, ileum and thymus *(Bruschke et al., 1998)*. The thymus is a central immune organ essential for development of mature functional T lymphocytes, with an outstanding role not only in young individuals but also after complete development of the immune system *(Cunningham et al., 2001)*. With the aim of recreate the underlying pathologic conditions that exist during the BRDC, we arranged coinfections of calves with two of its major pathogens, BVDV and BHV-1. In the previous chapter *(Chapter 2a)* it has been reported and characterized the thymus atrophy observed in calves preinfected with BVDV. Focusing on the same animals aforementioned, but from an immunopathologic point of view, the purpose of this study was to investigate the effect of preinfection with BVDV on thymus immune cells from calves challenged with BHV-1. Among these immune cells, we studied the main antigen-presenting cells (dendritic cells and macrophages), lymphocyte subpopulations (CD8, γδ T cells, FoxP3+ T cells) as well as the proliferative activity of these cells and the expression of TGF-β, a potent immunoregulatory cytokine. These results are deemed as a step in understanding the impact of BVDV infection on immunosuppression beyond the course of acute disease.

Results

Results of the immunohistochemical detection of **BHV-1** and **BVDV** have been described in Chapter 2a. Briefly, no immunolabeled cells were observed against BHV-1 in none of the thymus samples from the experiment. The BVDV antigen was found in the thymus from all the animals in the group previously infected with such virus, with a progressive decrease being observed during the study period. Immunolabeling with the 15c5 monoclonal antibody was observed

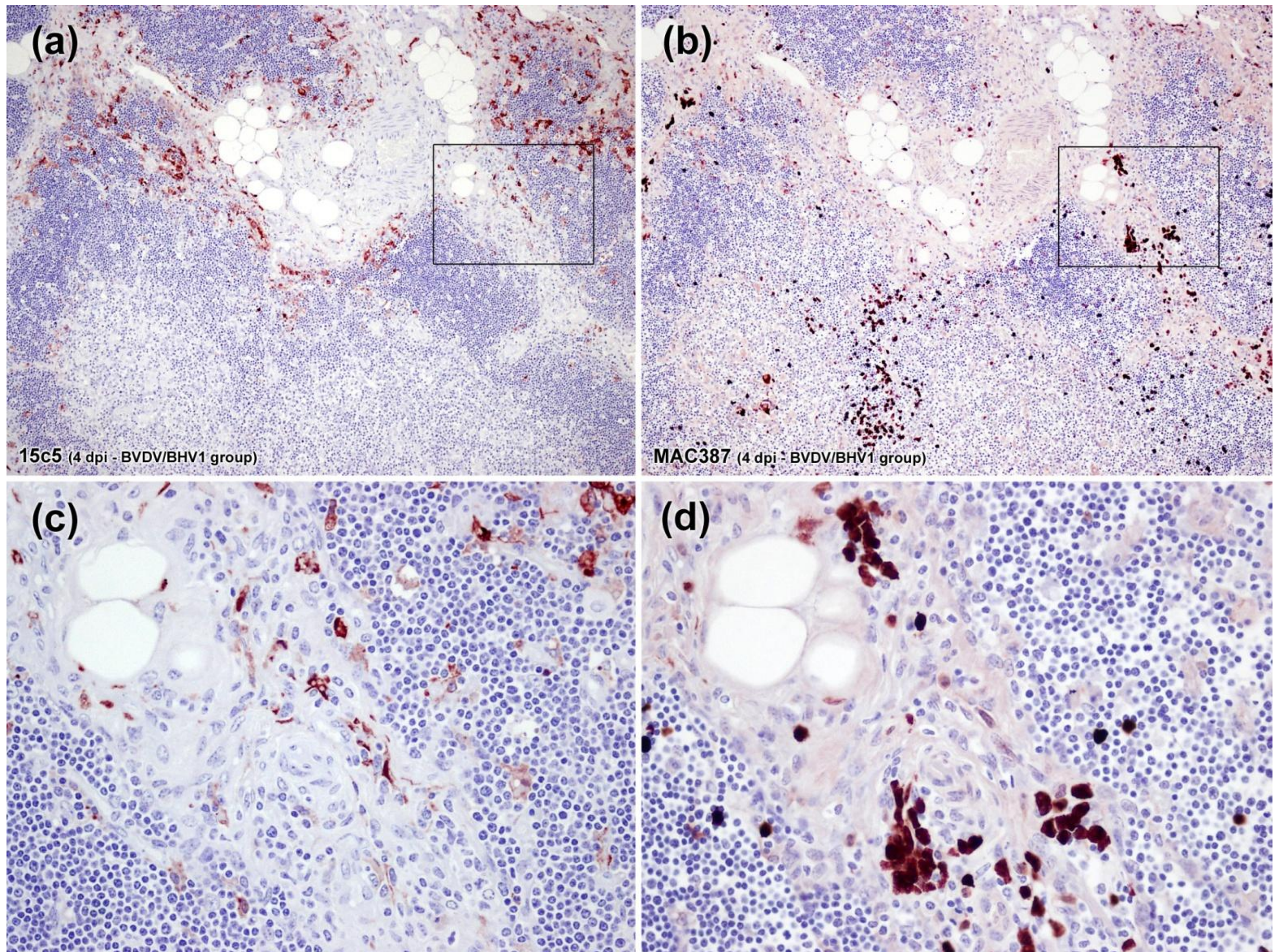

Figure 1. Serial sections of thymus immunostained with 15c5 (a, c) and MAC387 (b, d) antibodies. Positivity to BVDV could be attributed to macrophages only partially, since many other cells with stellate or spindle shape were observed to be positive for BVDV and negative for the macrophage marker. Figures (c) and (d) represent a higher magnification of the fields framed in black in figures (a) and (b). Original magnifications: 10x (a, b), 40x (c, d)

multifocally located in the cortex and mainly in the surrounding connective tissue *(Fig. 1a, Chapter 2a)*, with the medulla being practically negative *(Fig. 1a,c)*. Since the BHV-1 antigen was not detected by immunohistochemistry in any of the thymus samples, further molecular studies were carried out *(described in Chapter 2a)* in order to clarify the presence or absence of this virus. Briefly, BHV-1 was detected in the thymus via PCR assay from 1 to 14 dpi in the coinfected group and between 2 and 14 dpi in the BHV1 group *(Table 1, Chapter 2a (p119))*.

The number of medullar **macrophages** in the coinfected group was lower than the other group, remaining constant throughout the study period *(Fig. 1b and 5a)*. However, in the cortex and mainly in the surrounding connective tissue *(Fig.*

1b,d and 5b), the coinfected group showed prior to BHV-1 infection higher cell counts than the single infected group that was reached subsequently due to a rise in the BHV1 group from 1 dpi onwards.

Ki67 immunostaining revealed a high number of lymphoid cells showing nuclear staining of heterogeneous intensity *(Fig. 2a,b)*. Cell counting of ki67+ cells showed in the medulla a greater proliferative activity in the coinfected group *(Fig. 5c)*, descending progressively until reaching proliferative levels close to those of the BHV1 group from 7 dpi onwards. In the cortex *(Fig. 5d)*, ki67+ cell counts began with lower values in the coinfected group that rose progressively throughout the study with a peak at 4 dpi, and decreasing again from that moment onwards. The BHV group showed constant values in general, that plummeted at the end of the study.

CD1b immunolabeling was restricted to the vast majority of cortical thymocytes along with a fewer amount of cells with dendritic morphology located in the medulla *(Fig. 2c)*. The quantification of these CD1b-positive medullar cells *(Fig. 6a)* revealed highly dynamic kinetics in both groups. Animals in the BHV1 group displayed increasing cell counts during the study period, particularly between 2 and 7 dpi. Likewise, the preinfected group exhibited also an increasing tendency, although much more moderate, and even with a fall at 7 dpi that reached BHV1-preinoculation values.

The expression of **CD208,** known as a marker for mature dendritic cells (DCs), was mainly restricted to cells in the medulla with a marked pleomorphism, and a granular cytoplasmic pattern of immunostaining *(Fig. 2d)*. Immunopositive cells soared progressively in both groups *(Fig. 6b)*, showing similar values during the study period, except for the slightly superior cell counts observed in the coinfected group at 2 and 7 dpi.

The nearly complete staining of the cortex with the anti-**CD8** antibody hampered the quantification of CD8+ cortical lymphocytes *(Fig. 3a,b)*. Immunolabeling in the medulla revealed significant increased numbers in the group preinfected with BVDV *(Fig. 7a)* which decreased to lower values similar to those of the BHV1 group solely by the end of the study (14 dpi), when the presence of BVDV was most reduced.

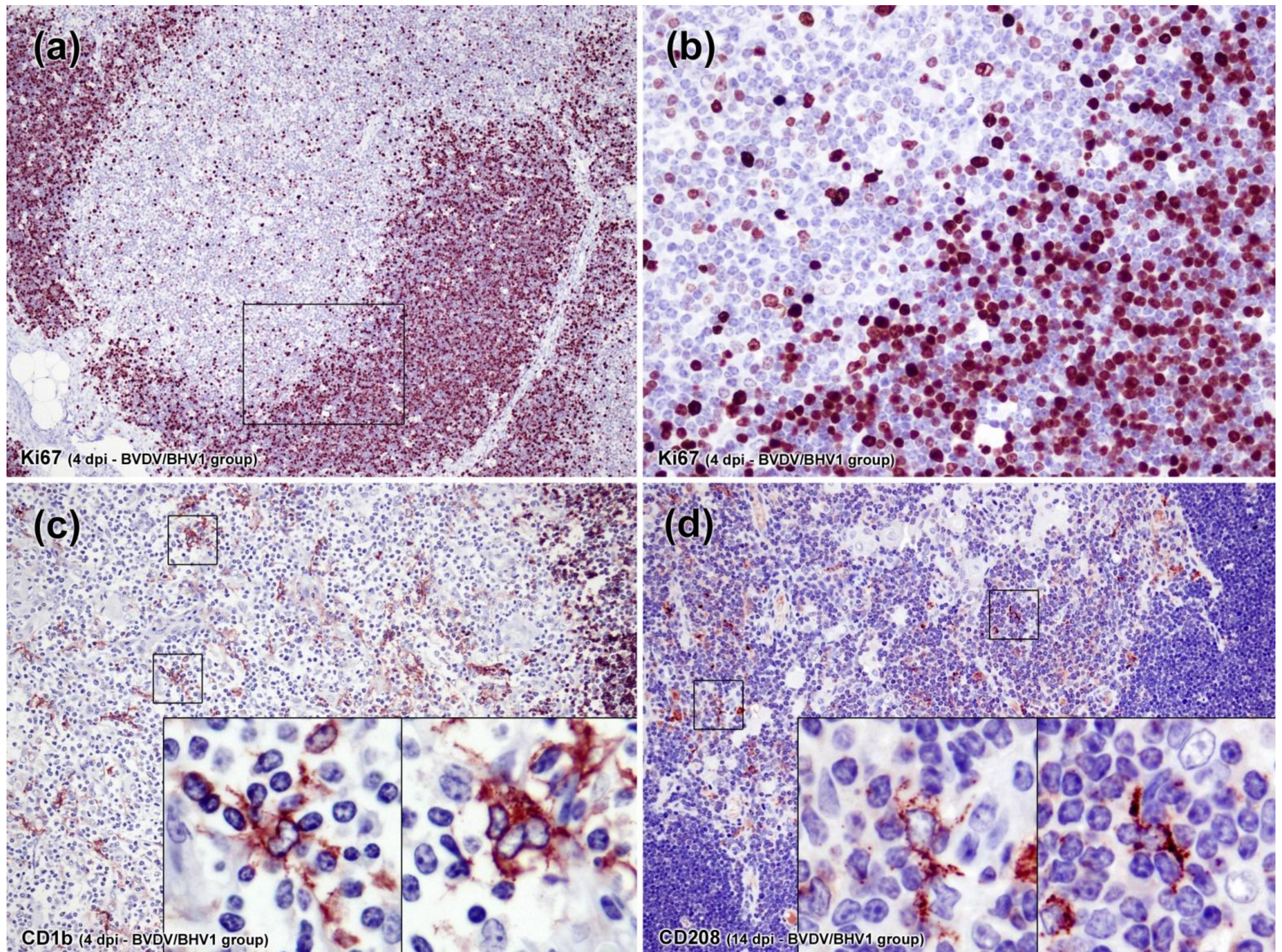

Figure 2. Immunolabeling for Ki67 antigen (a, b), and dendritic cell (DC) markers (c, d). Figure (**b**) represents a higher magnification of the field framed in black in figure (**a**), depicting the nuclear stain pattern of proliferating ki67+ cells, highly abundant in the cortex, and sparser in the medulla. CD1b immunolabeling (**c**) was restricted to the vast majority of cortical thymocytes (right top) along with a fewer amount of cells with dendritic morphology located in the medulla. The expression of mature DC marker CD208 (**d**) was restricted to cells in the medulla with a marked pleomorphism, and a granular cytoplasmic pattern of immunostaining. Insets represent higher magnifications of the fields framed in black. Original magnifications: 10x (a), 40x (b), 20x (c, d).

Immunolabeling of **WC1**+ cells yielded a strong staining pattern restricted to the cytoplasmic membrane, displaying scattered positive cells in the cortex and higher numbers in the medulla *(Fig. 3c,d)*, where these cells could be frequently observed in association with Hassall's corpuscles *(Fig. 3c inset)*. WC1+ γδ T lymphocytes showed similar kinetics in single and dual infections in the medulla *(Fig. 7b)*, with a progressive decrease being observed in the number of this cell type in both groups after BHV1 inoculation. Although cell counts seemed to be higher in the BVDV/BHV1 group, a nadir was observed in these calves at 1 dpi, and

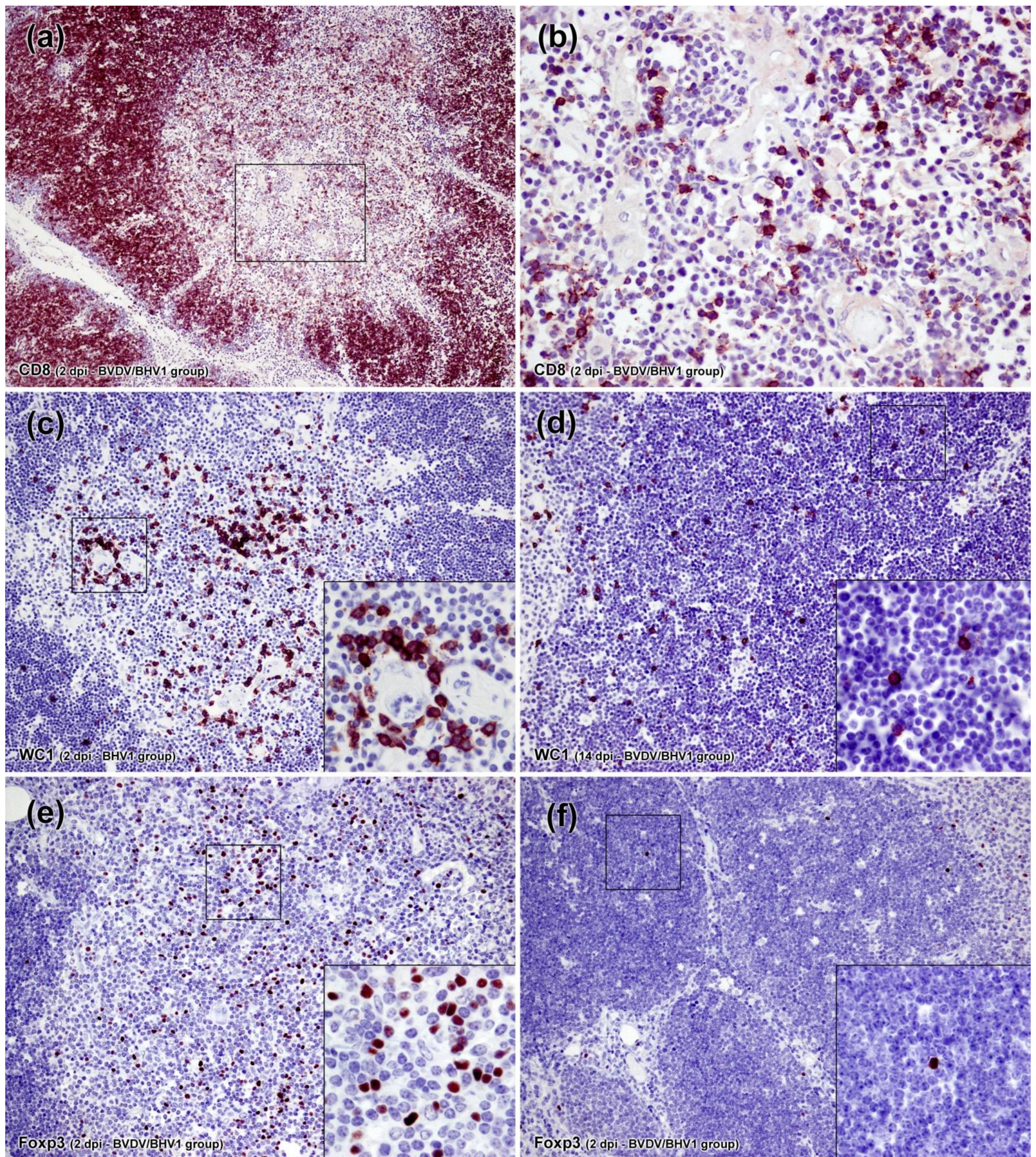

Figure 3. Immunohistochemical detection of lymphocyte subpopulations. Immunolabeling of CD8+ cells (**a**, **b**) displayed a nearly complete staining of cortical thymocytes, and scattered cells in the medulla. Immunolabeling of WC1+ cells yielded a strong staining pattern restricted to the cytoplasmic membrane, displaying higher numbers of positive cells in the medulla (**c**), more scattered in the cortex (**d**). Notice the frequent association of WC1+ cells in association with Hassall's corpuscles ((c) inset). Immunolabeling with the anti-Foxp3 antibody showed an intense nuclear stain of lymphocytes, especially abundant in the medulla (**e**) and rarer in the cortex (**f**). Original magnifications: 10x (a), 40x (b), 20x (c-f)

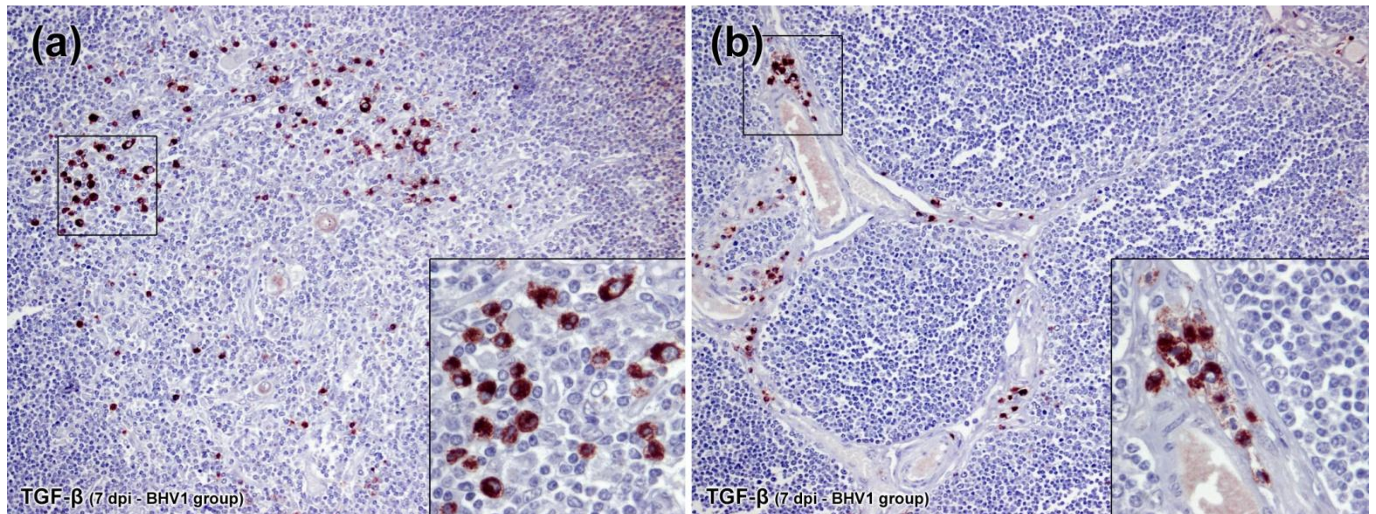

Figure 4. Immunohistochemical detection of TGF-β. Immunolabeled cells display a cytoplasmic granular staining. Positive cells in the cortex (**b**) are referred to cells located in connective tissue surrounding the thymic lobules, were the immunostaining was only observed. Original magnifications: 20x (a, b)

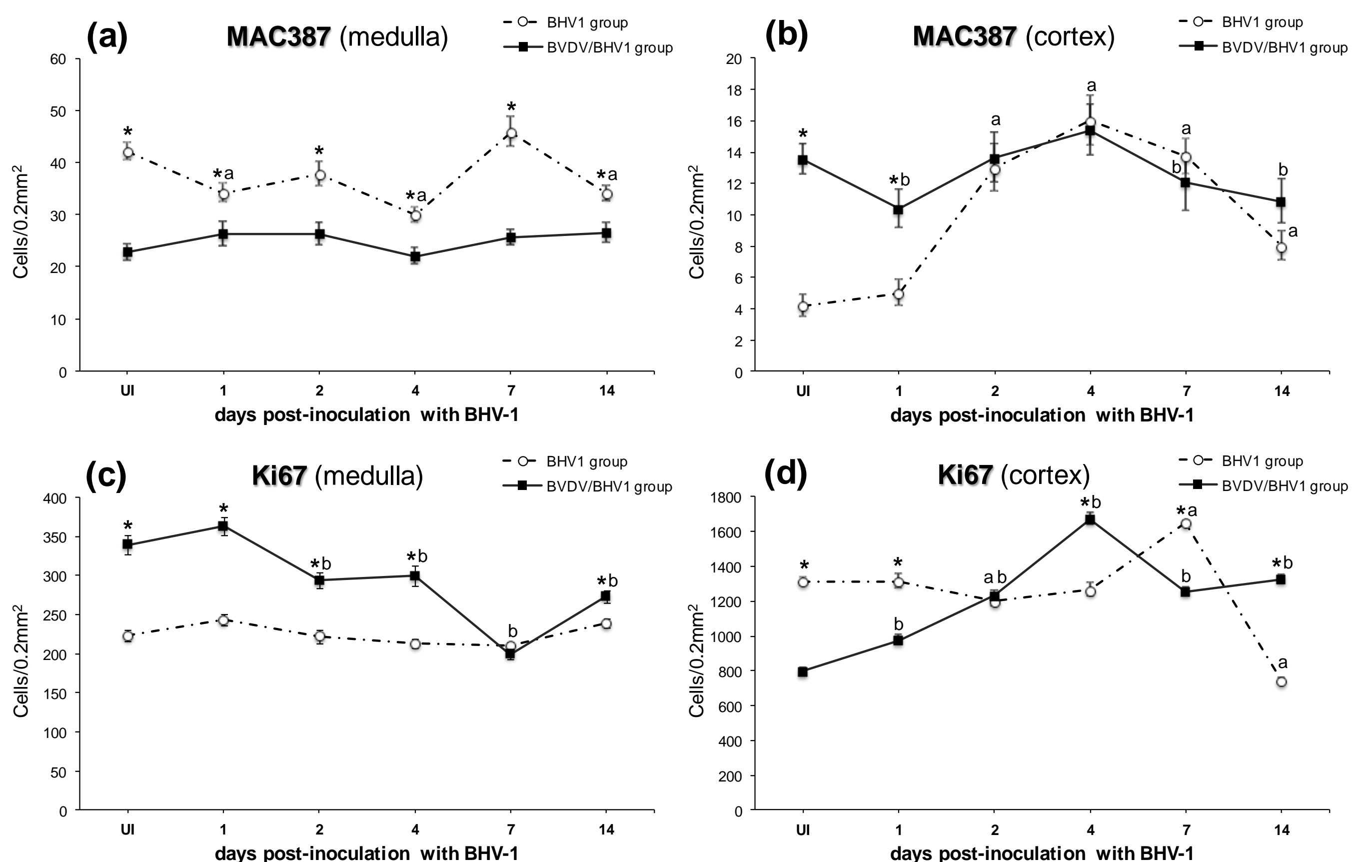

Figure 5. Quantitative assessment (mean ± standard error) of macrophages positive for MAC387 and cells immunolabeled with the proliferation marker ki67 (UI, BHV-1 uninfected: negative controls for the BHV1 group and BVDV infection controls for the BVDV/BHV1 group. *Significant differences ($p<0.05$) between inoculated groups at the same time point, [a,b]Significant differences ($p<0.05$) in the same group at various time points).

the differences between groups were significant only at 4 and 7 dpi. In the cortex, prior to BHV1 infection, animals of the coinfected group showed lower levels of WC1+ lymphocytes *(Fig. 7c)*, but at 4 dpi both groups displayed an important nadir followed by a recovery that was greater in magnitude in the pre-infected group.

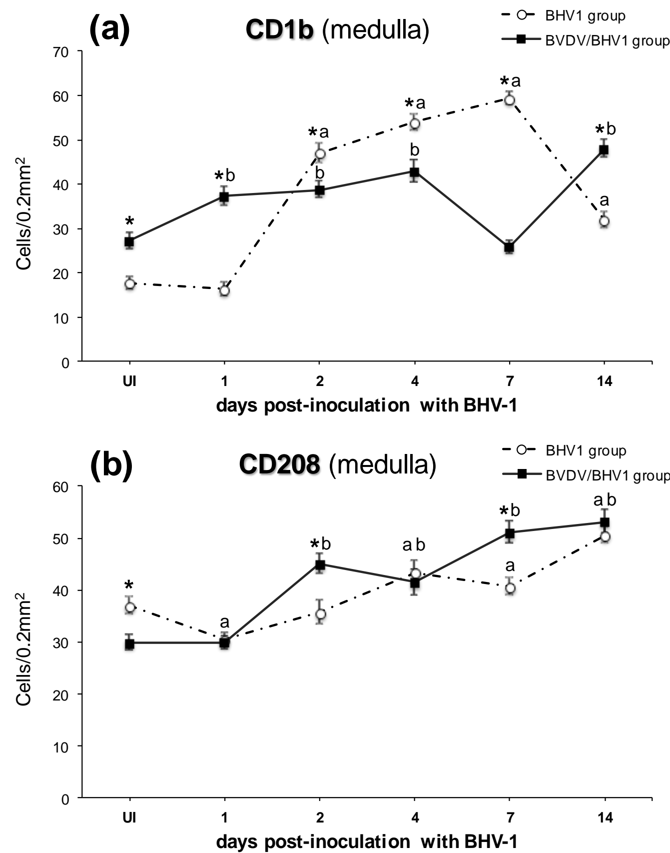

Figure 6. Quantitative assessment (mean ± standard error) of immunopositive cells for dendritic cell (DC) markers CD1b and CD208 (UI, BHV-1 uninfected: negative controls for the BHV1 group and BVDV infection controls for the BVDV/BHV1 group. *Significant differences ($p<0.05$) between inoculated groups at the same time point, [a,b]Significant differences ($p<0.05$) in the same group at various time points).

Immunolabeling with the anti-**Foxp3** antibody showed an intense nuclear stain of lymphocyte subpopulations, especially abundant in the medulla and much more scarce in the cortex *(Fig. 3e,f)*. In general terms, calves infected with BVDV were observed to have a decreased number of FoxP3+ lymphocytes both in the medulla and the cortex *(Fig. 7d,e)*. At 7 and 14 dpi, scattered cortical areas with greater amount of FoxP3-positive lymphocytes were found in the co-infected group, which raised the mean numbers of positive cells, although these differences were not statistically different between both groups.

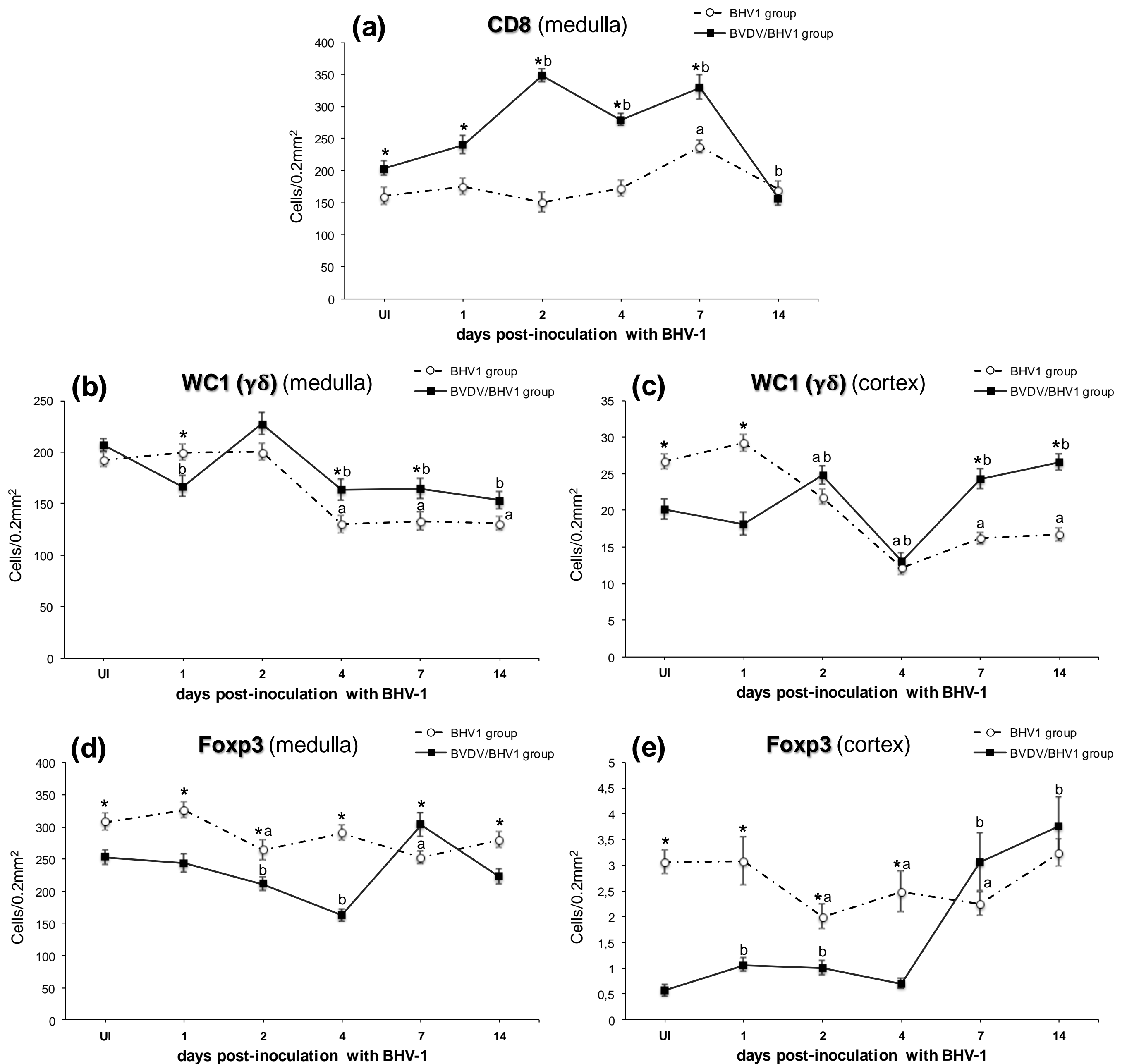

Figure 7. Quantitative assessment (mean ± standard error) of lymphocyte subpopulations (UI, BHV-1 uninfected: negative controls for the BHV1 group and BVDV infection controls for the BVDV/BHV1 group. *Significant differences ($p<0.05$) between inoculated groups at the same time point, [a,b]Significant differences ($p<0.05$) in the same group at various time points).

Immunohistochemical examination of **TGF-β** showed a cytoplasmic granular appearance *(Fig. 4)*. Cell counts in the medulla *(Fig. 8a)* showed lower values in the animals pre-infected with BVDV, except for 2 and 14 dpi, when these levels equaled or overcame the BHV1 group, respectively. Cell counts in the cortex are referred to cells located in connective tissue surrounding the thymic lobules,

were the immunostaining was only observed *(Fig. 4b)*; the number of TGF-β-positive cells of the preinfected animals remained lower than the other group until 2 dpi *(Fig. 8b)*, and from that moment onwards cell counts of the BHV1 group were outnumbered by the BVDV/BHV1 group.

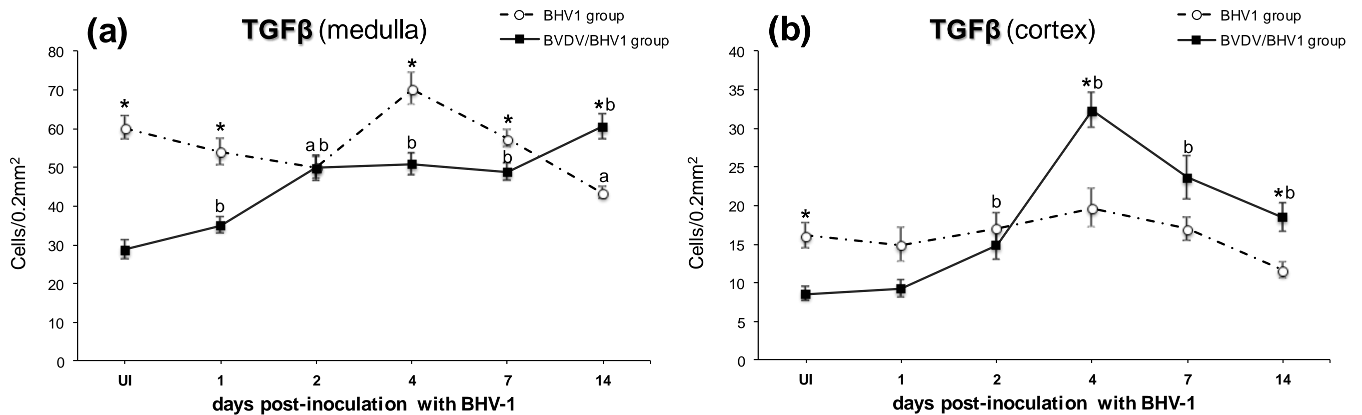

Figure 8. Quantitative assessment (mean ± standard error) of cells expressing TGF-β (UI, BHV-1 uninfected: negative controls for the BHV1 group and BVDV infection controls for the BVDV/BHV1 group. *Significant differences ($p<0.05$) between inoculated groups at the same time point, [a,b]Significant differences ($p<0.05$) in the same group at various time points).

Discussion

The impairment of the immune system arisen during BVDV infection is one major paradigm of the immunology in the modern research of cattle diseases. In this sense, several efforts are being conducted to elucidate the complex mechanisms used by BVDV to evade the host immune response.

Despite being a primary lymphoid organ, evidences supporting the thymus as an active organ throughout the adult life exist *(Bertho et al., 1997; Jamieson et al., 1999; Cunningham et al., 2001)*. This lifelong activity allows the thymus to supply lymphocytes to secondary lymphoid organs when required and to be involved in other aspects of ongoing immune responses such as antibody generation *(AbuAttieh et al., 2012)*. Chronic involution of the thymus in the context of increased longevity is likely to be an important factor in determining the diminished immunocompetence of the elderly, and hence their increased susceptibility to infectious diseases *(Sauce and Appay, 2011)*. In a similar manner, a premature viral-induced thymic atrophy may be directly related to the known

immunosuppressive effects associated with BVDV. As has been aforementioned, the thymus is responsible for supplying lymphocytes to secondary lymphoid organs. These cells that emerge from the thymus are naïve in normal conditions, ie, they have not yet encountered their specific antigen within an immune response. However, since BVDV infects the thymus, lymphocytes facing the virus may fail in recognizing it as a foreign antigen, thus exiting this organ as BVDV-tolerant cells unable to defeat the infection at the periphery.

This experimental design aimed to recreate the underlying pathologic conditions that exist during the BRDC *(Srikumaran et al., 2007)* by means of co-infections with two of its major pathogens, BVDV and BHV1. Previous studies based on this same experimental model revealed different findings, including an earlier depletion of circulating CD8+ lymphocytes and more intense clinical symptoms and lesions in the BVDV-inoculated calves, as well as the observation of BHV1 viremia only in this coinfected group *(Risalde et al., 2011; Molina et al., 2013; Risalde et al., 2013)*. These results, coupled with the evident lesional changes observed in the thymus of the coinfected animals *(Chapter 2a)*, appeal to a potential role for this central immune organ in the BVDV-induced characteristic immunosuppression. Slight signs of morphologic recovery where observed in this organ at the end of the study *(Chapter 2a)*. Nevertheless, since it does not necessarily mean a restoration of the thymus functionality, we aimed to further investigate by focusing on the thymic immune cells and mediators that might alter ensuing immune responses.

A previous study from our group focused on the first 12 dpi with BVDV revealed a transitory decrease of **monocytes** *(Molina et al., 2014)*. After these 12 days of infection with BVDV, circulating monocytes restored to normal values and maintained with no significant changes after BHV-1 inoculation *(Molina et al., 2013)*, coinciding with systemic results from other authors *(Falkenberg et al., 2014)*. However, as regards tissular macrophages, increases were observed in the thymus of calves acutely infected with BVDV *(Raya et al., 2012)*, and in the lungs of calves BVDV-infected from this experiment *(Risalde et al., 2014)*, as well as in the thymus cortex of these calves, as has been pointed out in this study. These results suggest that at systemic levels, BVDV induces transient monocyte depletion possibly associated with a migration to different organs, resulting in an increased

and prolonged presence of tissular macrophages. A migration of macrophages from the medulla to the cortex, were BVDV-infected cells were predominantly detected, may account for the lower counts observed in the medulla of coinfected calves. Despite not being observed in the thymus by IHC, the detection of BHV-1 by PCR in this organ in the BHV1 group from 2 dpi, coupled with the fact that macrophages/monocytes have been shown to be infected by BHV-1 *(Renjifo et al., 1999),* might explain the rise in cortical macrophages in this group from 2 dpi.

By using serial thymus sections immunostained with 15c5 and MAC387 antibodies, positivity to BVDV could be attributed to macrophages only partially, since many other cells with stellate or spindle shape were observed to be positive for BVDV and negative for the macrophage marker *(Fig. 1)*, highlighting the importance of cells like thymic epithelial cells as target for BVDV, as previously described *(Raya et al., 2014)*.

As regards detection of **Ki67** antigen, the higher proliferative activity observed in the medulla of coinfected calves may be associated either with a medullar hyperplasia compensatory to the cortical atrophy described previously for these animals *(Chapter 2a)*, or with a mechanism to fulfill the requirements of T cells in the periphery, since it is in the medulla where the final functional maturation of T lymphocytes takes place *(Chen, 2004)*. Since macrophage cell counts in the medulla are lower in the coinfected animals, then, the higher proliferation observed in these calves may point out particularly to lymphocyte proliferation, coinciding in addition with the superior CD8+ cell counts in the medulla. In the cortex, the level of proliferation of preinfected animals starts with very low values coinciding with the intense lymphoid depletion associated with the great presence of BVDV antigen. These results are in agreement with previous reports on the acute phase of BVD where an inverse correlation between BVDV and proliferation rate is described *(Liebler-Tenorio et al., 2003; Raya et al., 2014)*. As the BVDV is cleared, the rate of proliferation begins to increase in these coinfected animals, remaining constant in the BHV1 group until 4 dpi. In both groups, a retarded decline in the cortical cell proliferation was observed at 7 and 14 dpi (BVDV/BHV1 group) and 14 dpi (BHV1 group). This decline may be accounted for a inhibitory effect of BHV-1 on lymphocyte proliferation, previously

described by Carter and colleagues *(Carter et al., 1989)*, since the PCR study revealed the presence of BHV-1 in the thymus of calves in both groups.

DCs are key elements in the establishment of T-cell immune responses, with an extraordinary potential as antigen-presenting cells (APCs) *(Banchereau and Steinman, 1998; Romero-Palomo et al., 2011)*. Within the thymus, however, DCs do not behave as classical APCs. Thymic DCs are considered the major cell population responsible for the process of immunologic central tolerance, by participating in the deletion of self-reactive developing T cells *(Ardavin, 1997)* and induction of natural regulatory T cells *(Proietto et al., 2008)*. For this reason, we studied this type of cells by immunolabeling against two molecules (CD1b and CD208) previously reported as DC markers in cattle *(Romero-Palomo et al., 2013 - Chapter 1)*.

Unlike other molecules of the CD1 family, **CD1b** is restricted to cortical thymocytes and a dendritic cell subpopulation *(Howard et al., 1993)*. All in all, a progressive increase was observed in both groups along the study period, claiming for the influence of BHV-1 inoculation to this change. However, the magnitude of this increase appeared to be more limited in the preinfected group, suggesting that the presence of BVDV infection might downregulate the proliferation of CD1b+ DCs in response to secondary infections with BHV-1.

CD208, also known as DC-LAMP (dendritic cell–lysosome-associated membrane protein) was found to be specifically expressed in mature DCs located in T zones of lymphoid tissues, known as interdigitant dendritic cells *(de Saint-Vis et al., 1998)*. No remarkable differences between both infected groups were observed in the expression of CD208, although a progressive dual soaring was noted upon BHV-1 inoculation. Immunohistochemical studies revealed the absence of BVDV within the medulla, precisely where DCs are located, and even in the case it was present, it has been previously reported that DCs are not destroyed by BVDV *(Glew et al., 2003)*, what explains the absence of remarkable differences between groups.

Collectively, these data may indicate that the presence of BHV1 in this organ (detected by PCR) may be behind a viral-induced process of DC maturation and proliferation of CD1b+ DCs. A previous study reported that despite the absence of productive infection, DCs were effective APC for BHV-1 antigens when cultured

with T cells *(Renjifo et al., 1999)*, what functionally requires DC maturation, as observed in this study. Many pathogen strategies for immune evasion may be targeted at interfering with DC biology *(Rescigno and Borrow, 2001)*, with a number of steps being proposed at which pathogens may interfere in the DC-T cell interaction outcome. Among these steps, hamper DC maturation does not seem to be the case for BHV-1 in this study. However, it could be the presence of BVDV the reason for the minor proliferation of CD1b+ DCs observed after BHV-1 infection; in this case indirect mechanisms may interfere in the process, since DCs are located in the medulla and BVDV in the cortex.

Given the fact that CD1b is restricted to a subpopulation of conventional DCs *(Mittag et al., 2011; Ruscanu et al., 2013)* and that CD208 expression cannot differentiate between mature conventional or plasmacytoid DC *(Bendriss-Vermare et al., 2001)*, future works should address the study of plasmacytoid DC, cells of great interest during viral infections, due to their potent capacity of IFN-I production *(Reid et al., 2011)*.

The thymus is the organ responsible for the generation and distribution through the vascular system of mature **T lymphocytes** from the medulla to the rest of the organism. Hence, to maintain a stable population, there must be an influx of new T cells replacing the ones that are decaying *(Stromberg and Antia, 2012)*. In this study, we observed an increase of **CD8+** T cells in the thymus medulla of BVDV-infected calves that might take place as a result of a compensatory mechanism of cytotoxic T lymphocyte (CTL) mobilization in response to the intense CD8 systemic depletion observed in the coinfected animals during the acute phase of BVD *(Molina et al., 2014),* which was maintained after BHV-1 inoculation *(Molina et al., 2013)*. Moreover, a greater vascularization was observed in the thymus of preinfected animals in this experiment *(Chapter 2a)*, that may also facilitate the dissemination of CTLs.

Regulatory T cells (Tregs) are T cell subsets that possess immune suppression activities that are essential for maintaining self-tolerance and for controlling pathological immune responses *(Sakaguchi et al., 2009; Peterson, 2012)*. Tregs that arise during the normal process of maturation in the thymus, also known as natural Tregs (nTreg), have a key role in the development of central tolerance. The transcription factor forkhead-box p3 (**Foxp3**) has been long

considered to play a major role in the function and development of humans and mice nTreg cells, representing their most specific marker identified so far *(Hori et al., 2003)*. Previous studies have demonstrated an accurate identification of Foxp3-expressing lymphocytes in cattle by using the same clone (FJK-16s) we used in this study *(Gerner et al., 2010)*. However, additional studies have attributed to **δγ T** cells the role of regulatory T cells in cattle rather than Foxp3+ T cells *(Hoek et al., 2009; Guzman et al., 2014)*. Due to the current **controversies** regarding which cell population acts as Treg in cattle, both markers, Foxp3 and WC1 (recognizing the majority of δγ T cells) were considered in this study.

Regarding **WC1+ δγ** T cells, we observed that medullary δγ thymocytes showed a very distinctive association with Hassall's corpuscles, as previously reported *(Hein and Mackay, 1991)*, suggesting a role for these structures in δγ thymocyte maturation. In this experiment, a progressive decrease in the number of WC1+ δγ T cells was observed in the medulla in both groups of animals, with significantly higher values in the coinfected group only at 4 and 7 dpi. At the systemic level, these animals did not show remarkable differences between groups, except for a peak in preinfected calves at 12 dpi with BVDV (0 dpi with BHV-1) *(Molina et al., 2012; Molina et al., 2014)*. Results from these previous works must be cautiously interpreted, since the monoclonal antibody CACTB6A used in these studies detects only a part of the entire δγ T cells population. These results, in agreement with previous works, do not clearly claim for a major role of δγ T cells in resisting BVDV infection *(Howard et al., 1992)*. Conversely, **Foxp3** expression did show important differences between groups, attaining levels both in medulla and cortex significantly lower in the preinfected group that reached values close to those of the BHV group from 7 dpi onwards, when the expression of BVDV-infected cells showed the lowest values in this organ. Given the fact that Tregs are a subset that generally suppress or downregulate induction and proliferation of effector T cells, the lower levels of Foxp3+ cells observed in the medulla of coinfected animals might be responsible for the higher expression of ki67 detected in these animals in this location, where T cell maturation takes place.

TGF-β is a potent regulatory cytokine produced by a broad variety of cell types and with a wide range of functional properties, including the regulation of T lymphocyte proliferation, differentiation and survival *(Li et al., 2006)*. In this study,

low values of TGF-β were observed in the medulla and cortex of the coinfected animals at the beginning of the study (when the highest amount of BVDV-infected cells was detected). However, these levels of TGF-β underwent subsequently a progressive increase as the BVDV was cleared. It has been described that TGF-β promotes the differentiation of Tregs *(Liu et al., 2008; Murphy et al., 2012)* and has a role as antiapoptotic survival factor for T lymphocytes *(Cerwenka and Swain, 1999; Ouyang et al., 2010)*. Taken together, these data suggest that a possible pathogenic mechanism for BVDV may include the inhibition of TGF-β, thus hampering the differentiation of Foxp3+ Tregs (accounting for the reduced values of Foxp3 until 4 dpi) and favoring the appearance of increased apoptotic lesions, as have been described with the TUNEL technique in the previous chapter. Accordingly, as BVDV is cleared and TGF-β arises, this would result in Foxp3+ proliferation and reduced apoptotic phenomena. This explanation fits with the classical regulatory function attributable to Foxp3+ T cells, but further studies may be required to confirm if the *in vitro* studies considering bovine δγ T cells (instead of Foxp3+ cells) as the actual Tregs in cattle can be extrapolated to natural thymic Tregs.

Conclusions

These findings point out BVDV as responsible for the thymic lesions observed in this experiment as well as for the majority of immunopathologic changes, including a downregulation of Foxp3 lymphocytes and TGF-β that reverted as BVDV was cleared, and an overexpression of medullary CD8+ T cells. However, despite not inducing evident lesions in the thymus, BHV-1 did seem to prompt some immune alterations, as observed in terms of cortical macrophages, dendritic cells or cortical cell proliferation, which may be associated with de immunosuppressive features described also for BHV-1 *(Nandi et al., 2009)*. Collectively, these data contribute to the knowledge on the immunopathologic alterations of the thymus during BVDV infections, and its importance in the development of secondary infections.

CHAPTER 3

In vitro study of the effect of infections with BVDV and BHV-1 on PBMCs

In vitro study of the effect of infections with BVDV and BHV-1 on PBMCs

Abstract

BVDV and BHV-1 infections are spread in cattle populations all over the world causing important economic losses, being considered important predisposing factors in the development of BRDC, as a result of their immunosuppressive features. Since synergic interactions have been observed *in vivo* with both viruses, the aim of this study was to determine the consequences of *in vitro* infections on distinct populations of blood immune cells. For this study, 4 Holstein-Friesian heifers (8-10 months old) were selected from a dairy herd free of BVDV and BHV-1 and each animal was confirmed to be BVDV and BHV-1 antigen and antibody free by using serum neutralization tests, ELISA or PCR. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of these animals and separated in turn into 4 groups of infection: uninfected control cells (CON), cells infected with ncp BVDV-1 (BVDV), cells infected with BHV-1 (BHV) and cells infected with both BVDV and BHV-1 (BVDV+BHV). BVD and BHV-1 viruses were grown and titrated on MDBK cells. PBMCs were infected at 1 m.o.i. and harvested at 18, 24, 48, and 72 hours post infection (hpi) to determine the effect of virus infection by flow cytometry, determining the percentage and/or MFI of expression of different leukocyte differentiation antigens. The main findings of this study included the reduced percentage of monocytes after viral infection, especially pronounced after co-infection with both viruses, which also produces a detrimental effect on CD11b expression in these cells. In addition, monocytes seemed to down-regulate expression of CD80 in response to BVDV infection. These changes in monocytes may be responsible for an undermined innate response to infections and an impaired process of antigen presentation and activation of lymphocytes.

Key words: BVDV, BHV-1, PBMCs, leukocyte populations, flow cytometry.

Introduction

Bovine viral diarrhea virus (BVDV) and bovine herpesvirus 1 (BHV-1) infections are spread in cattle populations all over the world causing important economic losses, not only as the etiological agents of diseases like BVD and infectious bovine rhinotracheitis (IBR), respectively, but also as frequent predisposing factors in the development of bovine respiratory disease complex (BRDC), as a result of their immunosuppressive features *(Srikumaran et al., 2007)*. Though BVDV can infect a wide variety of cell types, there is an apparent predilection for cells of the immune system, including T and B cells, monocytes, macrophages, and dendritic cells *(Sopp et al., 1994; Glew et al., 2003)*. Likewise, although BHV-1 has a special tropism for respiratory epithelial cells, many cell types of the immune system can be infected by this virus *(Hanon et al., 1998; Renjifo et al., 1999; Leite et al., 2005)*

For mounting an effective clearance of intruder pathogens, the participation of both innate and adaptive immune responses is required. Cells like monocytes/macrophages (m-MØs) are key components of innate immunity. These cells migrate to sites of inflammation and exert their abilities to phagocytose pathogens by expressing molecules such as complement receptor 3 (CR3-CD11b/CD18), which regulates leukocyte adhesion and migration and recognizes molecules of invading pathogens acting as a pattern recognition receptor (PRR) *(Solovjov et al., 2005)*. In addition, m-MØs secrete immunomodulatory cytokines, which may contribute to excessive and harmful proinflammatory environments as observed during the BRDC. T and B lymphocytes are the major cells responsible for the development of adaptive immune responses. T cell receptors (TCR) are unable to recognize intact antigens (like B lymphocytes do), and they need the antigen to be processed by an antigen-presenting cell and presented through molecules of the major histocompatibility complex (MHC). The majority of nucleated cells can operate as antigen-presenting cells due to its capacity of presenting intracellular antigens to cytotoxic T lymphocytes (CTL, CD8+) by means of MHC type I

molecules; however, the term "antigen-presenting cell" (APC or professional APC) is commonly referred to those cells that constitutively express MHC type II, including dendritic cells (DC), m-MØs and B cells, which uptake extracellular antigens and present them to helper T cells (Th, CD4+) *(Romero-Palomo et al., 2011)*. This "first signal", antigen recognition by the T cell, must then be followed by secondary signals as a result of the interaction between co-stimulatory molecules like CD80/86 (B7 family) on the APC with their co-receptors on the T cell (like CD28 binding to CD80/86 *(Orabona et al., 2004)*). This secondary signal generated by co-stimulatory molecules is necessary for the correct activation of T lymphocytes *(Johnson and Jenkins, 1992)*. Activation of naive T cells in the presence of co-stimulation through CD28 signaling induces the expression and secretion of IL-2 and the expression of CD25 (α chain of IL-2 receptor (IL-2Rα), responsible for conversion into high-affinity IL-2 receptors). IL-2 binds to the high-affinity IL-2 receptors to promote T-cell growth in an autocrine fashion *(Murphy et al., 2012)*.

Effects of infections with BVDV or BHV-1 on APCs and lymphocytes have been intensively studied separately *(Chase, 2013; Levings and Roth, 2013a; b; Peterhans and Schweizer, 2013)*. The reasons why BRDC is still a problem, despite the use of antibiotics and vaccines, is the complex interactions between the pathogens and the host, and the still incomplete knowledge of the process *(Ellis, 2014)*. Since synergic interactions have been observed *in vivo* with both viruses *(Risalde et al., 2013a)*, the aim of this study was to determine the consequences of *in vitro* infections on distinct populations of blood immune cells.

Results

Both viruses were replicated in epithelial cells and the final viral stocks were titrated, giving values of $10^{4,6}$ $TCID_{50}$/μl for ncp BVDV (7443 strain) and $10^{4.76}$ $TCID_{50}$/μl for BHV-1. Total leukocyte and platelet counts from the animals used in this study had values included within the normal range for the bovine species, with only a slight increase in one animal in the number of monocytes *(Figure 1, Table 1)*.

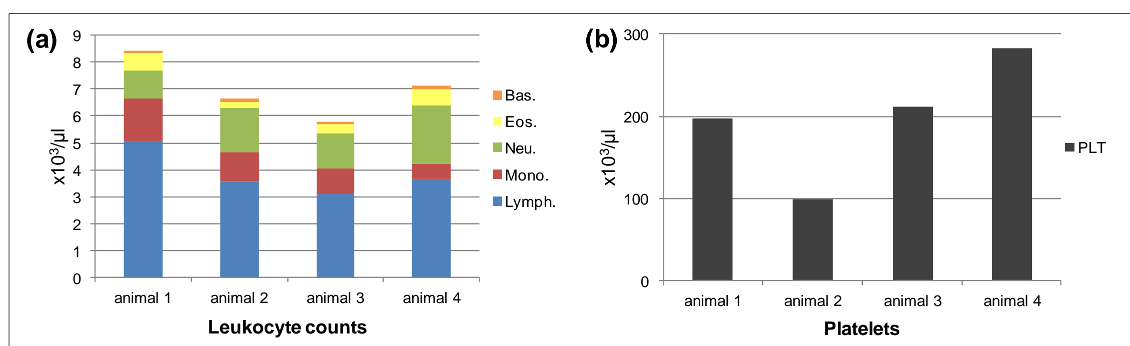

Figure 1. Graphic representation of total leukocyte and platelet counts from the animals used in this study.

Table 1. Values of total leukocyte and platelet counts from the animals used in this study, and normal values of reference.

(n x 10^3/µl)	Animal 1	Animal 2	Animal 3	Animal 4	Normal values[1]
Lymphocytes	5.05	3.59	3.10	3.68	4.5 (2.5-7.5)
Monocytes	1.59	1.08	0.97	0.55	0.4 (0.025-0.84)
Neutrophils	1.06	1.65	1.30	2.17	2 (0.6-4)
Eosinophils	0.62	0.20	0.31	0.59	0.7 (0-2.4)
Basophils	0.10	0.11	0.12	0.15	0.05 (0-0.2)
Total leukocytes	8.42	6.63	5.80	7.14	8 (4-12)
Platelets	197	99	211	283	500 (100-800)

[1] Normal blood values for cattle (Schlam's veterinary hematology. 4th ed. Philadelphia: Lea & Febiger, 1986)

Percentages of CD14+ monocytes revealed remarkable differences among groups of infection, with decreased numbers in all infected groups, but being more pronounced and statistically significant when PBMCs are infected with both viruses compared with control group *(Figure 2a)*. Percentages of expression of CD11b or CD80 within the monocyte population did not show significant differences except for a decreased in BHV and BVDV+BHV groups compared to CON group at 18 hpi *(Figures 2b,c)*. Infected monocytes showed a decreased in the mean fluorescence intensity (MFI) for CD11b, especially pronounced and with significant differences when infected with both viruses (*Figure 2d*). Some significant increments were observed in the CD80 MFI of monocytes associated with the time in culture, although this increasing tendency was observed in all groups of infection *(Figure 2e)*; monocytes of the groups infected with BVDV (alone or along with BHV-1) seemed to show a downregulation of CD80, as observed

when comparing with control and BHV groups, with significant decreases in the co-infected group compared with BHV group.

Table 2. Surface molecule expression by PBMC prior to infection (0 hpi). Average values from four separate experiments (4 animals). MFI: mean fluorescent intensity, geometric mean channel number.

	% (±SD)	**MFI** (±SD)
CD14+	2 (1)	
(CD14) CD11b+	91 (6)	143 (24)
(CD14) CD80+	41 (4)	19 (4)
CD4+	20 (6)	
CD8+	15 (3)	
(CD4) CD25+	12 (5)	11 (2)
(CD8) CD25+	4 (4)	8 (2)
CD28	4 (0)	7 (1)
B-B2	33 (11)	
MHC-I	99 (1)	150 (51)
MHC-II	27 (5)	21 (12)

T lymphocyte did not show differences along the study period neither in the percentages of CD4+ or CD8+ subpopulations *(Figure 3 a,b)*, nor in the percentage of expression of the CD25 activation marker within these subpopulations *(Figure 3 c,d)*. With respect to the CD25 MFI of CD4+ lymphocytes, some significant differences were observed among groups of infection at different time points *(Figure 3e)*, with no changes being observed in the CD8+ subpopulation *(Figure 3f)* except for a slight increment in all groups of infection. No differences among groups of infection were detected in the percentage or MFI of cells expressing CD25 out of the total PBMC population (data not shown).

The T cell activating receptor CD28 did not show significant variations neither in the percentage of cells expressing it nor in their MFI *(Figure 4 a,b)*. No significant changes were observed in the percentage of B lymphocytes *(Figure 4c)*, neither in the MFI for MHC-I or MHC-II positive cells *(Figure 4 d,e)* (nor in their percentages, data not shown).

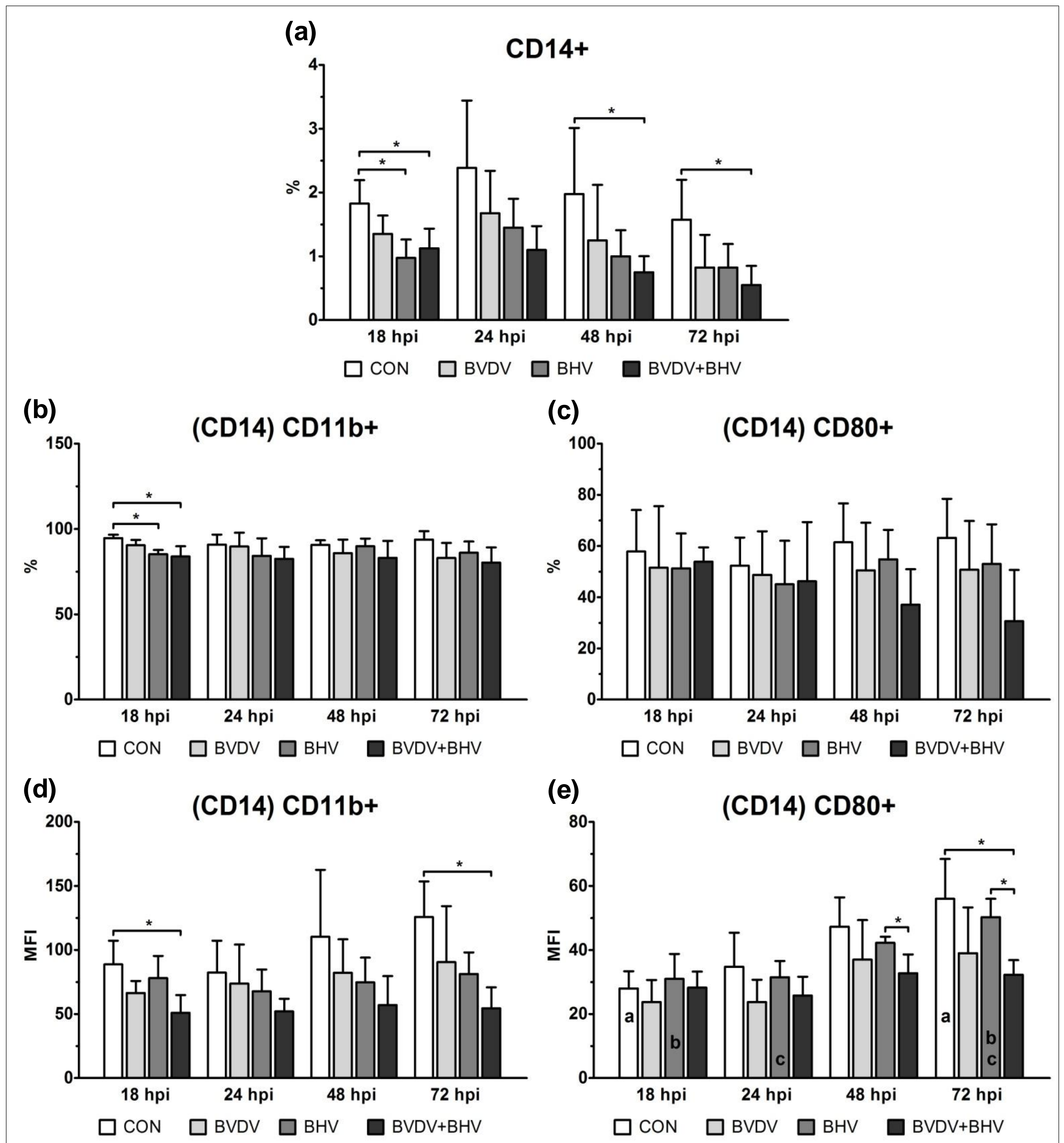

Figure 2. Percentage of monocytes (CD14+) **(a)**, percentage of cells expressing CD11b or CD80 of the monocyte population **(b,c)**, and MFI for CD11b+ or CD80+ cells of the monocyte population **(d,e)** (MFI: mean fluorescent intensity, geometric mean channel number). The values represented in all the graphs are the means (±SD) of four separate experiments (4 animals). (*) Significant differences ($p<0.05$) between different groups of infection at the same time point. (a,b,c...) Significant differences ($p<0.05$) within a group of infection at different time points.

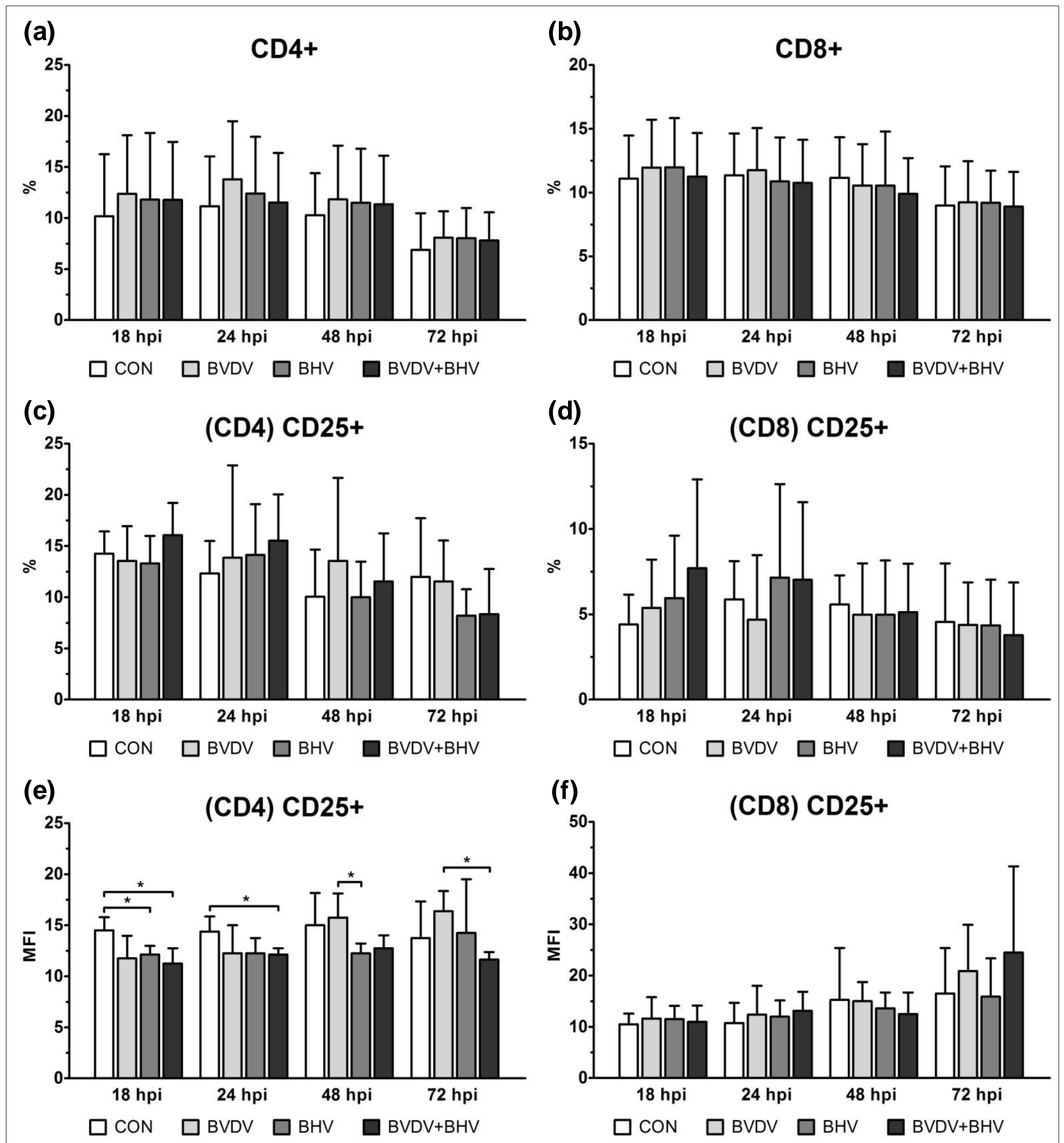

Figure 3. Percentage of CD4+ or CD8+ T lymphocytes **(a,b)**, percentage of cells expressing CD25 of the CD4+ or CD8+ T lymphocyte populations **(c,d)**, and MFI for CD25+ cells of the CD4+ or CD8+ T lymphocyte populations **(e,f)** (MFI: mean fluorescent intensity, geometric mean channel number). The values represented in all the graphs are the means (±SD) of four separate experiments (4 animals). (*) Significant differences ($p<0.05$) between different groups of infection at the same time point. (a,b,c...) Significant differences ($p<0.05$) within a group of infection at different time points.

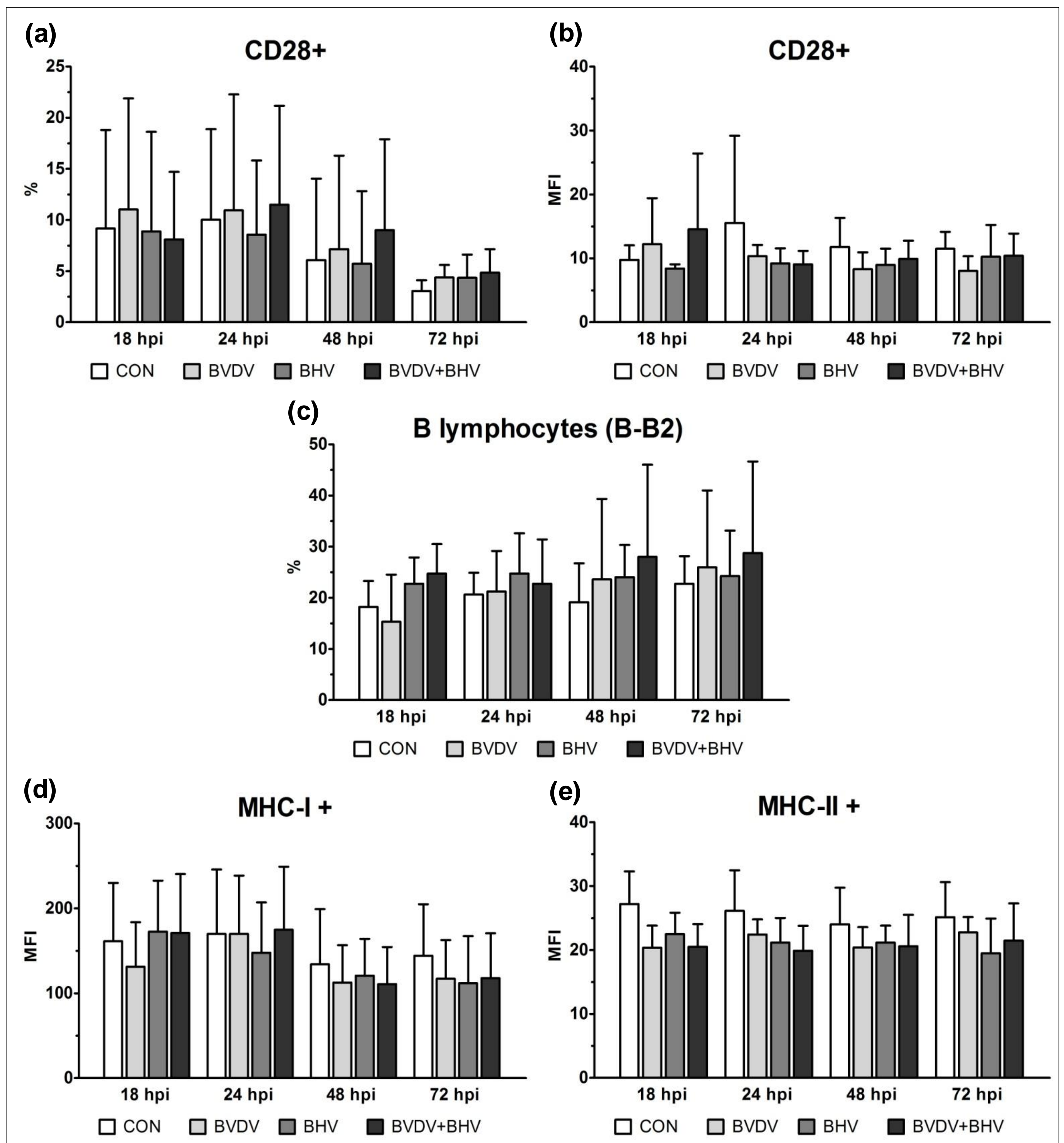

Figure 4. Percentage of cells expressing CD28 and MFI for CD28+ cells **(a,b)**, percentage of B lymphocytes (B-B2+)**(c)**, and MFI for MHC-I or MHC-II positive cells **(d,e)** (MFI: mean fluorescent intensity, geometric mean channel number). The values represented in all the graphs are the means (±SD) of four separate experiments (4 animals). (*) Significant differences ($p<0.05$) between different groups of infection at the same time point. (a,b,c...) Significant differences ($p<0.05$) within a group of infection at different time points.

Discussion

This study analyzes and compares the effect of dual and single infections with ncp BVDV-1 and BHV-1 on different populations of PBMCs obtained from naïve uninfected cattle. The percentages of several constitutive leukocyte differentiation antigens were calculated by flow cytometry, along with the MFI of some of the molecules susceptible to infection-induced regulation.

Values of total leukocyte counts from the animals used in this study were within the normal range, with only one animal showing a slight increase in the number of monocytes; however, the normal reference interval for monocytes shown in Table 1 can be considered even higher according to some studies using a hematology analyzer that gives similar results to those obtained with the hematology analyzer used in this study *(Bauer et al., 2011; Warren et al., 2013)*.

A reduction in the percentage of **monocytes** in the virus-infected groups was reported in this study, especially after co-infection with both viruses, reflecting their marked pathogenicity and tropism for monocytes. In agreement with this observation, monocytes were found to be the leukocyte population with the highest frequency of infection with BVDV *(Sopp et al., 1994)*. Similarly, BHV-1 replicates in purified m-MØs cultures inducing a reduction of monocyte viability *(Hanon et al., 1998; Renjifo et al., 1999)* or without causing apparent cytopathic effects *(Woldehiwet and Rowan, 1990)*. *In vitro* studies revealed that monocytes were killed with cp BVDV but not after ncp infection in purified *(Glew et al., 2003)* or monocyte-enriched cell cultures *(Lambot et al., 1998)*. This absence of cell death after infection with these viruses is based on purified monocyte culture systems, and the detrimental effects that other cells may induce on them are not considered *(Wang and Splitter, 1998)*, as it might be occurring in this study with total PBMC or in *in vivo* experiments *(Molina et al., 2014)*, where a substantial reduction of monocytes is described after infection.

These differences in purified cell cultures have also been observed in the absence of evident changes in the expression of **CD11b** *(Glew et al., 2003)*. However, in this study with PBMCs, along with the reduction in their percentage, monocytes in the infected groups of cells underwent a reduction in the expression of CD11b, especially significant in the dually infected group. Similarly, alveolar

macrophages recovered from BVDV-infected animals showed decreased functionality, including reduction of complement receptor 3 (CR3) expression *(Welsh et al., 1995)*. Given the role of CD11b in leukocyte adhesion, migration and antigen recognition *(Solovjov et al., 2005)*, this reduced expression may be partially responsible for the impairment of the immune response.

Professional APCs express relatively high levels of MHC class I and class II molecules, co-stimulatory molecules induced upon activation, receptors for endo- and phagocytosis, and adhesion molecules *(Murphy et al., 2012)*. To determine if viral strains affect the professional antigen presenting function of monocytes to activate T cells, we assessed **CD80** expression. *In vitro* studies to determine the expression of CD80/86 during infection of bovine monocytes with BVDV have shown variable results, ranging from down-regulation of gene expression *(Lee et al., 2008)* to a lack of effect on the surface expression *(Glew et al., 2003)*. During *in vivo* infections with BVDV, down-regulation of cells expressing CD80/86 *(Archambault et al., 2000)*, and variable levels of mRNA expression of CD80 depending on the organ or viral virulence have also been reported *(Palomares et al., 2014)*. In our study, monocytes of the BVDV-infected groups showed a downregulation in the surface expression of CD80 that may be responsible for an impaired process of antigen presentation and activation of lymphocytes.

To our knowledge, the absence of changes in CD11b and CD80 expression after infection with BHV-1 observed in our study is the first description of the effect of BHV-1 on these leukocyte markers.

We did not observe significant changes in the MFI of **MHC-I** or **MHC-II** positive cells. Studies by Glew *et. al. (2003)* neither observed evidence for MHC down/up-regulatory effect on BVDV-infected monocytes or moDC; however, despite the unaltered expression of co-stimulatory and MHC molecules, these authors reported that monocytes infected with ncp BVDV were compromised in their ability to stimulate allogeneic and memory CD4+ T cells responses. Studies by Lee *et al. (2009)* on purified monocytes reported that ncp BVDV had the strongest inhibitory effect on the MHC class I, MHC class II and MHC-DQ isotype protein expression levels. With regard to BHV-1, previous studies *in vitro* have reported down-regulation of MHC-I and MHC-II after infection *(Nataraj et al., 1997; Hinkley et al., 2000; Koppers-Lalic et al., 2005)*. As we can see in this study, although not

significant, virus-infected groups of cells seem to down-regulate MHC-II expression, suggesting that higher infectious doses might induce more evident changes.

This study revealed an absence of changes in the percentage of **T lymphocytes** despite the viral infections. *In vivo* infections with BHV-1 revealed that CD4+ and CD8+ blood T cells decreased, especially CD4+ T cells, which unlike CD8+ T cells, was correlated with an increase in apoptosis, being CD4+ T cells (but not CD8+) infected with BHV-1 *(Winkler et al., 1999)*. Hanon *et al. (1998)* reported increased levels of apoptosis in T and B lymphocytes and monocytes after *in vitro* infection with BHV-1, although these studies used to infect an m.o.i. as high as 10 PFU per cell, possibly due to the fact that PBMCs are not the principal target cells for BHV-1. *In vitro* studies with homologous strains of BVDV revealed that only the cp biotype, in contrast to the ncp counterpart, induced evident apoptosis in PBMCs after infection *(Lambot et al., 1998)*. On the contrary, *in vivo* BVDV infections showed significant decreases in the number of T lymphocyte subpopulations *(Ellis et al., 1988; Ganheim et al., 2005; Molina et al., 2014)*. The absence of changes in the percentage of T lymphocytes also suggest that these cells do not proliferate in response to antigen presentation of virus, possibly explained by the fact that cells used in this study were obtained from naïve (non-immunized) animals. This absence of pre-immunization might explain the decreased levels or the absence of changes observed in the **CD25** MFI *(Carter et al., 1989; Hou et al., 1998)*, contrasting with CD25 up-regulation observed upon pre-infection or vaccination *(Lan et al., 1996; Endsley et al., 2002; Platt et al., 2006)*.

To our knowledge, there are no previous reports describing the effect of BVDV/BHV-1 infection in **CD28** expression; our studies suggest that none of the viruses we used induce changes in the expression of this molecule, not even up-regulation, as it might be expected in response to a viral stimulation.

Varying effects on circulating **B-lymphocytes** have been reported during *in vivo* infections with BVDV. Ellis *et al. (1988)* found a decrease in the proportion of B-cells, while Archambault *et al. (2000)* and Ganheim *et al. (2005)* observed no effect on the B-cells, possibly due to differences in the viral strains. No changes were observed in the percentage of B-lymphocytes in this study, contrasting with their evident affectation during *in vivo* infections with the same BVDV strain, either

transiently in circulating B-cells *(Molina et al., 2014)* or within lymphoid follicles *(Pedrera et al., 2009b)*. These results support the hypothesis considering that B-lymphocytes may die by indirect mechanisms triggered by other infected cells like macrophages or stromal cells *(Pedrera et al., 2009a; Pedrera et al., 2012)*, which are not present in the present *in vitro* study.

In **summary**, the main findings of this study included the reduced percentage of monocytes after viral infection, especially pronounced after co-infection with both viruses, which also produces a detrimental effect on CD11b expression in these cells, reflecting synergic mechanisms that undermine the response of m-MØs and, in turn, the innate immune response to these viruses. In addition, monocytes seemed to down-regulate expression of CD80 in response to BVDV infection, which may be responsible for an impaired process of antigen presentation and activation of lymphocytes.

Further studies with this experimental design are being carried out to complement this study, focused on late and early markers of apoptosis, cytokine production and viral replication. Additionally this complete set of results from naïve animals will be compared with results from cells of immunized animals. Taken together, this project will help to a better understanding of the possible interaction mechanism lying behind the BRDC and the effectiveness of current vaccine systems.

CONCLUSIONS

1. MHC-II expression is the most sensitive marker for detecting any DC of hematopoietic origin in bovine tissue sections by means of immunohistochemistry, being both anti-S100 and CNA.42 antibodies useful markers for the detection of stromal FDCs. In addition, immunolabeling of CD208 allows the detection of mature DCs in T zones of lymphoid organs, while CD1b identifies DC populations mainly located in the thymus: **CHAPTER 1**

2. Pre-infection of calves with BVDV induces severe atrophic changes in the thymus, an organ of outstanding relevance for the immune system, associated with a delayed persistence of the virus in this organ. This thymus atrophy is characterized by a reduction in the cortex:medulla ratio, and an increase in cortical apoptosis as well as in collagen deposition and vascularization: **CHAPTER 2A**.

3. In calves pre-infected with BVDV and challenged with BHV-1, BVDV gives rise to the majority of immunopathological changes observed in the thymus, including a down-regulation of Foxp3+ T cells and TGF-β that reverts as BVDV is cleared from this organ: **CHAPTER 2B.**

4. *In vitro* infection of PBMCs with BVDV and BHV-1 induces in monocytes (CD14+) a decrease in their percentages and a down-regulation in the expression of CD11b, a molecule of great importance for leukocyte migration and antigen recognition, being the most pronounced reduction observed in the co-infected group. In addition, in response to BVDV infection, monocytes seem to down-regulate the expression of the co-stimulatory molecule CD80, required for an adequate antigen presentation and activation of lymphocytes: **CHAPTER 3.**

SUMMARY

The impairment of the immune system arisen during bovine viral diarrhea virus (BVDV) infections is one major paradigm of the immunology in the modern research of cattle diseases. In this sense, several efforts are being conducted to elucidate the complex mechanisms used by BVDV to evade the host immune response. Although BVDV is not a primary agent in the pathogenesis of bovine respiratory disease complex (BRDC), its suppressive effects on the host immune system can increase the risk of secondary infections, thus enhancing pulmonary colonization by other pathogens. Consequently, the general objective of this work was to contribute to the knowledge of the immunopathogenic strategies by which BVDV infections enhance the susceptibility to secondary infections, as those produced by bovine herpesvirus type 1 (BHV-1).

Due to the important effects that viral pathogens can induce on the immune system, this work was **firstly** focused on a cell type of outstanding relevance for the immune system, the dendritic cells (DC), which are the most effective antigen-presenting cells (APC), and modulates both innate and adaptive immune responses. The absence of a solid literature detailing DC marker candidates for cattle gave rise to the first objective, focused on finding potential DC markers for the bovine species, standardize their immunohistochemical protocol and describe their histological distribution, in order to establish a basis for the study of DCs in diseased tissues (CHAPTER 1). For this purpose, paraffin wax-embedded tissues from 6 healthy Friesian calves were subjected to immunohistochemical studies and the most appropriate protocols were studied for each of the following primary antibodies: MHC-II, CD208, CD1b, CD205, CNA.42 and S100.

Despite labeling other APCs, MHC-II expression was established as the most sensitive marker for detecting any DC of hematopoietic origin in bovine tissue sections. Both CNA.42 and anti-S100 antibodies stained stromal FDCs, but only the latter allowed for the identification of FDCs as isolated cells. Cells stained against

CD205 were scarce, and their location was mainly restricted to non-lymphoid tissues. Immunolabeling of CD208 allowed the detection of mature DCs in T zones of lymphoid organs, while CD1b identified DC populations mainly located in interfollicular areas of some lymph nodes and in the thymus.

Secondly, with the aim of reproducing and studying the pathologic condition developed during the BRDC, by which a primary BVDV infection favors the susceptibility to secondary infections, the following *in vivo* experimental design was proposed. Twelve Friesian calves were inoculated intranasally with noncytopathic BVDV-1 (strain 7443). Twelve days later, when the calves did not show clinical signs of viremia against BVDV, ten of them were subsequently challenged intranasally with BHV-1.1 (strain Iowa) and euthanized in batches of two at 1, 2, 4, 7, or 14 days post-infection (dpi) with BHV-1 (BVDV/BHV1 group). The other 2 calves were euthanized prior to the second inoculation (0 dpi with BHV-1) and were used as BVDV-infected controls for this group. Another 10 calves were inoculated solely with BHV-1 and euthanized in batches of two at the same time points (BHV1 group). Two calves were not inoculated with any agent and were used as negative controls. Several papers with clinical and pathological results from the animals in this experiment have been previously published (enumerated in section “preface”). The thymus was the focus of attention in this work (CHAPTER 2A-B) since it is considered a target organ for BVDV and because it remains as an active organ also throughout the adult life, allowing lymphocyte supply to secondary lymphoid organs when required. For this study, thymus samples from these animals were processed for viral genomic detection and for histopathological, immunohistochemical, and ultrastructural studies focused on BVDV/BHV-1 antigens, cortex:medulla ratio, apoptosis (TUNEL and caspase-3), collagen deposition, and Factor VIII endothelial detection (lesional study - CHAPTER 2A), as well as on thymus immune cells (immunopathological study - CHAPTER 2B). Among these immune cells, we studied the main APCs (DCs and macrophages), lymphocyte subpopulations (CD8, γδ T cells, FoxP3+ T cells) as well as the proliferative activity of these cells and the expression of TGF-β, a potent immunoregulatory cytokine. To study DCs, some of the DC markers described in the first chapter were used (CD208 and CD1b).

Our study revealed the immunohistochemical presence of BVDV antigen in the thymus of all animals in the BVDV pre-infected group, unlike BHV-1 detection, which was observed in the thymus of animals in both infection groups only by molecular techniques. BVDV-preinfected animals showed severe thymic atrophic changes associated with reduced cortex:medulla ratio, higher presence of cortical apoptosis, and increased vascularization and deposition of collagen (ultrastructurally identified as type I-collagen). Calves solely infected with BHV-1 did not show atrophic changes. These findings could affect not only the numbers of circulating and local mature T cells but also the T cell-mediated immunity, which seems to be impaired during infections with BVDV, thus favoring pathogenic effects during secondary infections (CHAPTER 2A).

The immunohistochemical evaluation to compare the quantitative changes in thymus immune cells from coinfected calves and calves challenged only with BHV-1 pointed out BVDV as responsible for the majority of immunopathologic changes, including a downregulation of Foxp3 lymphocytes and TGF-β that reverted as BVDV was cleared, and an overexpression of medullary CD8+ T cells. However, despite not inducing evident lesions in the thymus, BHV-1 did seem to prompt also some immune alterations. Collectively, these data contribute to the knowledge on the immunopathologic alterations of the thymus during BVDV infections, and its importance in the development of secondary infections (CHAPTER 2B).

BVDV is considered as the main predisposing factor for the occurrence of BRDC through an alteration of the immune response, thus favoring the colonization of the respiratory system by other pathogens. BHV-1 is one of these pathogens, which is the etiological agent of infectious bovine rhinotracheitis (IBR), and, in turn, has also some detrimental effects on the immune system. Results from our *in vivo* experimental model revealed a synergic action of the pathogenic mechanisms between both viruses. Therefore, the **last** objective was to shed light on these mechanisms by establishing an additional experimental model with *in vitro* infections with ncp BVDV-1 (strain 7443) and BHV-1.1 (strain Iowa) on distinct populations of blood immune cells (CHAPTER 3). For this study, 4 Friesian heifers were selected from a dairy herd free of BVDV and BHV-1 and each animal

was confirmed to be BVDV and BHV-1 antigen and antibody free by using serum neutralization tests, ELISA or PCR. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of these animals and separated in turn into 4 groups of infection: uninfected control cells (CON), cells infected with BVDV-1 (BVDV), cells infected with BHV-1 (BHV) and cells infected with both BVDV and BHV-1 (BVDV+BHV). BVD and BHV-1 viruses were grown and titrated on MDBK cells. PBMCs were infected at a multiplicity of infection (m.o.i.) of 1 $TCID_{50}$ per cell and harvested at 18, 24, 48, and 72 hours post infection (hpi) to determine the effect of virus infection by flow cytometry, determining the percentage and/or mean fluorescence intensity (MFI) of expression of different leukocyte differentiation antigens (CD14, CD11b, CD80, CD4, CD8, CD25, CD28, MHC-I, MHC-II, and B-B2 (B-cells)).

After *in vitro* infection of PBMCs, BVDV and BHV-1 induced in monocytes (CD14+) a decrease in their percentages and a down-regulation in the expression of CD11b, a molecule of great importance for leukocyte migration and antigen recognition, being the most pronounced reduction observed in the co-infected group, reflecting synergic mechanisms that undermine the response of monocytes and, in turn, the innate immune response to these viruses. In addition, in response to BVDV infection, monocytes seemed to down-regulate the expression of the co-stimulatory molecule CD80, which may be responsible for an impaired process of antigen presentation and activation of lymphocytes (CHAPTER 3).

Further studies with this experimental design are being carried out to complement this study, focused on late and early markers of apoptosis, cytokine production and viral replication. Additionally this complete set of results from naïve animals will be compared with results from cells of immunized animals. Taken together, this project will help to a better understanding of the possible interaction mechanism lying behind the BRDC and the effectiveness of current vaccine systems.

RESUMEN

El deterioro del sistema inmunológico que surge durante las infecciones con el virus de la diarrea vírica bovina (VDVB) es uno de los principales paradigmas de la inmunología en la investigación actual de las enfermedades del vacuno. En este sentido, se están llevando a cabo numerosos esfuerzos para dilucidar los complejos mecanismos utilizados por el VDVB para evadir la respuesta inmune del hospedador. Aunque el VDVB no es un agente primario en la patogénesis del complejo respiratorio bovino (CRB), sus efectos supresores sobre el sistema inmune del hospedador pueden aumentar el riesgo de infecciones secundarias, potenciando así la colonización pulmonar por otros patógenos. En consecuencia, el objetivo general de este trabajo fue contribuir al conocimiento de las estrategias inmunopatógenas mediante las cuales, infecciones con el VDVB aumentan la susceptibilidad a infecciones secundarias, como las producidos por el herpesvirus bovino tipo 1 (HVB-1).

Debido a los importantes efectos que los patógenos virales pueden inducir sobre el sistema inmune, este trabajo se centró en **primer** lugar en un tipo de células de excepcional relevancia para el sistema inmune, las células dendríticas (CDs), que son las células presentadoras de antígeno (CPA) más eficaces y modulan las respuestas inmunitarias tanto innatas como adaptativas. La ausencia de una literatura sólida que detalle candidatos marcadores de CDs para bovino dio lugar al primer objetivo, centrado en encontrar marcadores potenciales de CDs para la especie bovina, estandarizar su protocolo inmunohistoquímico y describir su distribución histológica, con el fin de establecer una base para el estudio de las CDs en tejidos patológicos (CAPÍTULO 1). Para este propósito, tejidos incluidos en parafina de 6 terneros frisones sanos fueron sometidos a técnicas inmunohistoquímicas y se estudiaron los protocolos más apropiados para cada uno de los siguientes anticuerpos primarios: MHC-II, CD208, CD1b, CD205, CNA.42 y S100.

A pesar de marcar otras CPA, la expresión de MHC-II se estableció como el marcador más sensible para detectar cualquier CD de origen hematopoyético en secciones de tejido bovino. Ambos anticuerpos CNA.42 y anti-S100 tiñeron células dendríticas foliculares (CDF) estromales, pero sólo este último permitió la identificación de CDF como células aisladas. Las células teñidas frente a CD205 fueron escasas, y su ubicación se limitó principalmente a tejidos no linfoides. El inmunomarcaje de CD208 permitió la detección de CDs maduras en zonas T de órganos linfoides, mientras que CD1b identificó poblaciones de CDs localizadas principalmente en áreas interfoliculares de algunos nódulos linfáticos y en el timo.

En **segundo** lugar, con el objetivo de reproducir y estudiar el estado patológico que tiene lugar durante el CRB, por el cual una infección primaria favorece la susceptibilidad a infecciones secundarias, se propuso el siguiente modelo experimental *in vivo*. Doce terneros frisones fueron inoculados por vía intranasal con VDVB-1 no citopático (cepa 7443). Doce días más tarde, cuando los terneros no mostraban signos clínicos de viremia frente al VDVB, diez de ellos fueron infectados intranasalmente con HVB-1.1 (cepa Iowa) y sacrificados en lotes de dos a los 1, 2, 4, 7, o 14 días post-infección (dpi) con HVB-1 (grupo VDVB/HVB1). Los otros 2 terneros fueron sacrificados antes de la segunda inoculación (0 dpi con HVB-1) y se utilizaron como controles infectados con VDVB para este grupo. Otros 10 terneros fueron inoculados únicamente con HVB-1 y sacrificados en lotes de dos a los mismos tiempos (grupo HVB-1). Dos terneros no se inocularon con ningún agente y se utilizaron como controles negativos. Varios artículos con resultados sobre clínica y patología de estos animales han sido anteriormente publicados (enumerados en la sección "prefacio"). El timo fue el foco de atención en este trabajo (CAPÍTULO 2A-B) ya que se considera un órgano diana para el VDVB y porque continua como un órgano activo también durante la vida adulta, permitiendo el aporte de linfocitos a órganos linfoides secundarios cuando es necesario. Para realizar este estudio, se procesaron muestras de timo de los animales anteriormente citados para la detección de genoma vírico y para estudios histopatológicos, inmunohistoquímicos y ultraestructurales, centrados en antígenos de VDVB/HVB-1, ratio corteza:médula, apoptosis (TUNEL y caspasa-3), depósito de colágeno, y detección de Factor VIII endotelial (estudio lesional -

CAPÍTULO 2A), así como en las células inmunes del timo (estudio inmunopatológico - CAPÍTULO 2B). Entre estas células inmunes, se estudiaron los principales CPA (CDs y macrófagos), subpoblaciones de linfocitos (CD8, células T γδ, células T FoxP3+), así como la actividad proliferativa de estas células y la expresión de TGF-β, una potente citoquina inmunorreguladora. Para estudiar las CDs, se utilizaron algunos de los marcadores de CDs descritos en el primer capítulo (CD208 y CD1b).

Nuestro estudio reveló mediante inmunohistoquímica la presencia del antígeno de VDVB en el timo de todos los animales del grupo preinfectado con VDVB, a diferencia de la detección de HVB-1, que se observó en el timo de los animales infectados en ambos grupos sólo mediante técnicas moleculares. Los animales preinfectados con VDVB mostraron cambios atróficos severos en el timo, caracterizados por un reducido ratio corteza:médula, una mayor presencia de fenómenos de apoptosis cortical y un incremento en la vascularización y el depósito de colágeno (ultraestructuralmente identificado como colágeno tipo I). Los terneros únicamente infectadas con HVB-1 no mostraron cambios atróficos en el timo. Estos hallazgos podrían afectar no sólo a la cantidad de células T maduras circulantes y locales sino también a la inmunidad mediada por células T, que parece verse afectada durante las infecciones con el VDVB, favoreciendo así los efectos patógenos durante infecciones secundarias (CAPÍTULO 2A).

La evaluación inmunohistoquímica para comparar los cambios cuantitativos en las células inmunes del timo de los terneros coinfectados y de los infectados solamente con HVB-1, apuntaron al VDVB como responsable de la mayoría de los cambios inmunopatológicos, incluyendo una disminución de linfocitos Foxp3 y de expresión de TGF-β que revirtieron a medida que el VDVB se iba eliminando del organismo, y un incremento de linfocitos T CD8+ medulares. Sin embargo, a pesar de no inducir lesiones evidentes en el timo, el HVB-1 pareció provocar también algunas alteraciones inmunes. En conjunto, estos datos contribuyen al conocimiento sobre las alteraciones inmunopatológicas que sufre el timo durante infecciones con VDVB, y su importancia en el desarrollo de infecciones secundarias (CAPÍTULO 2B).

El VDVB se considera como el principal factor predisponente en la aparición del CRB a través de una alteración de la respuesta inmune, lo que favorece la

colonización del aparato respiratorio por otros patógenos. El HVB-1 es uno de estos patógenos, el cual es el agente etiológico de la rinotraqueítis infecciosa bovina (IBR), y a su vez, tiene también algunos efectos perjudiciales sobre el sistema inmunológico. Resultados de nuestro modelo experimental *in vivo* revelaron una acción sinérgica entre los mecanismos patogénicos de ambos virus. Por lo tanto, el **último** objetivo fue profundizar en el conocimiento sobre estos mecanismos mediante el establecimiento de un modelo experimental adicional de infecciones *in vitro* con VDVB-1 no citopático (cepa 7443) y HVB-1.1 (cepa Iowa) en distintas poblaciones de células inmunes sanguíneas (CAPÍTULO 3). Para este estudio, se seleccionaron 4 vacas frisonas de una explotación lechera libre de VDVB y HVB-1 y cada animal fue confirmado como libre de antígenos y anticuerpos de VDVB y HVB-1 mediante el uso de pruebas de seroneutralización vírica, ELISA o PCR. Se aislaron células mononucleares de sangre periférica (PBMCs) de la sangre de estos animales y se separaron a su vez en 4 grupos de infección: células control no infectadas (CON), células infectadas con VDVB-1 (VDVB), células infectadas con HVB-1 (HVB) y células infectadas con VDVB y con HVB-1 (VDVB+HVB). Los virus VDVB y HVB-1 se hicieron previamente replicar y se titularon en una línea celular establecida de células epiteliales de riñón bovino (MDBK). Los PBMC fueron infectados a una multiplicidad de infección (m.o.i.) de 1 $TCID_{50}$ por célula y se recogieron a las 18, 24, 48, y 72 horas post- infección (hpi) para analizar el efecto de la infección viral mediante citometría de flujo, determinando el porcentaje y/o la intensidad de fluorescencia media (MFI) de la expresión de diferentes antígenos de diferenciación leucocitaria (CD14, CD11b, CD80, CD4, CD8, CD25, CD28, MHC-I, MHC-II, y B-B2 (células B)).

Tras la infección *in vitro* de PBMCs, el VDVB y el HVB-1 indujeron en los monocitos (CD14+) una disminución en sus porcentajes y una reducción en la expresión de CD11b, una molécula de gran importancia para la migración de leucocitos y el reconocimiento de antígenos, observándose la reducción más pronunciada en el grupo de células co-infectadas, lo que evidencia la presencia de mecanismos sinérgicos que perjudican la respuesta de los monocitos y, a su vez, la respuesta inmune innata a estos virus. Además, en respuesta a la infección con el VDVB, los monocitos parecían mostrar una reducida expresión de la molécula

coestimuladora CD80, que puede ser responsable de una alteración en los procesos de presentación antigénica y activación linfocitaria (CAPÍTULO 3).

Estudios adicionales sobre este último diseño experimental se están llevando a cabo para complementar este estudio, centrados en marcadores tempranos y tardíos de apoptosis, producción de citoquinas y replicación vírica. Además, este conjunto completo de resultados con animales indemnes a ambos virus se comparará con resultados procedentes de células de animales inmunizados. En conjunto, este proyecto ayudará a una mejor comprensión de los posibles mecanismos de interacción que subyacen tras el CRB así como de la eficacia de los actuales sistemas vacunales.

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ANNEX

I. Supplemental material

Figure S1. Immunohistochemical detection of CD1b+ cells in lymph nodes and dermis. (CHAPTER 1)

Figure S2. Positive-infection tissue controls for the immunohistochemical detection of BHV-1. (CHAPTER 2A)

Figure S3. Low magnification of thymus sections. Hematoxylin-eosin (CHAPTER 2A)

Figure S4. Representative figure of BVDV-induced thymus atrophy. (CHAPTER 2A)

Figure S5. Transmission electron microscopy photographs showing structures compatible with BHV-1 particles. (CHAPTER 2A)

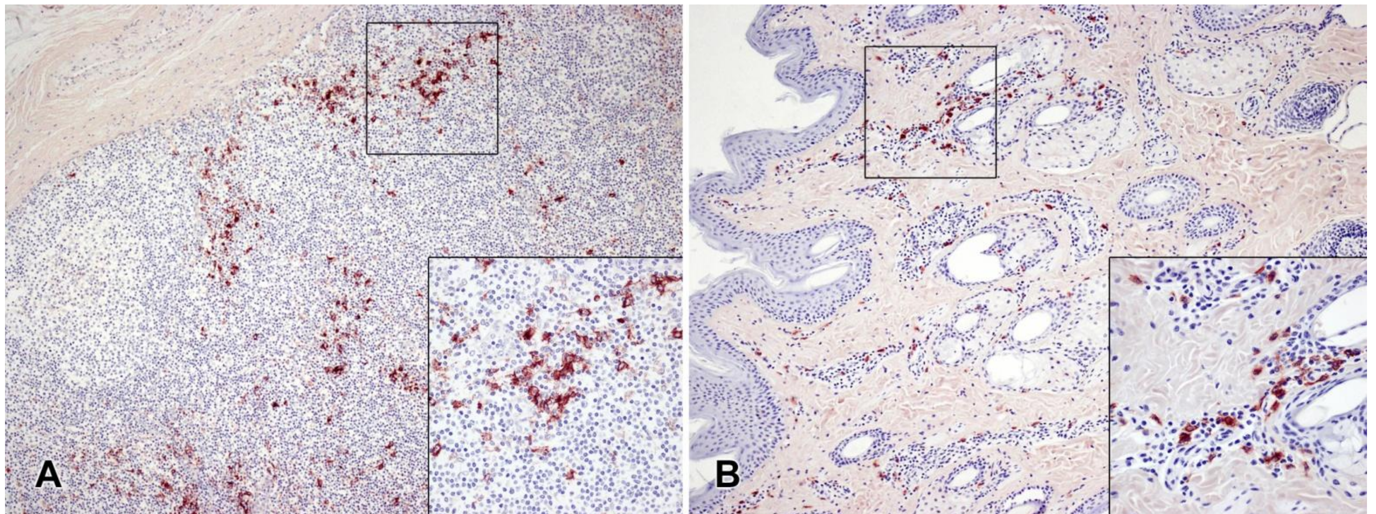

Figure S1. Immunohistochemical detection of CD1b+ cells in the interfollicular areas of some lymph nodes (A), and in the dermis (B). (CHAPTER 1)

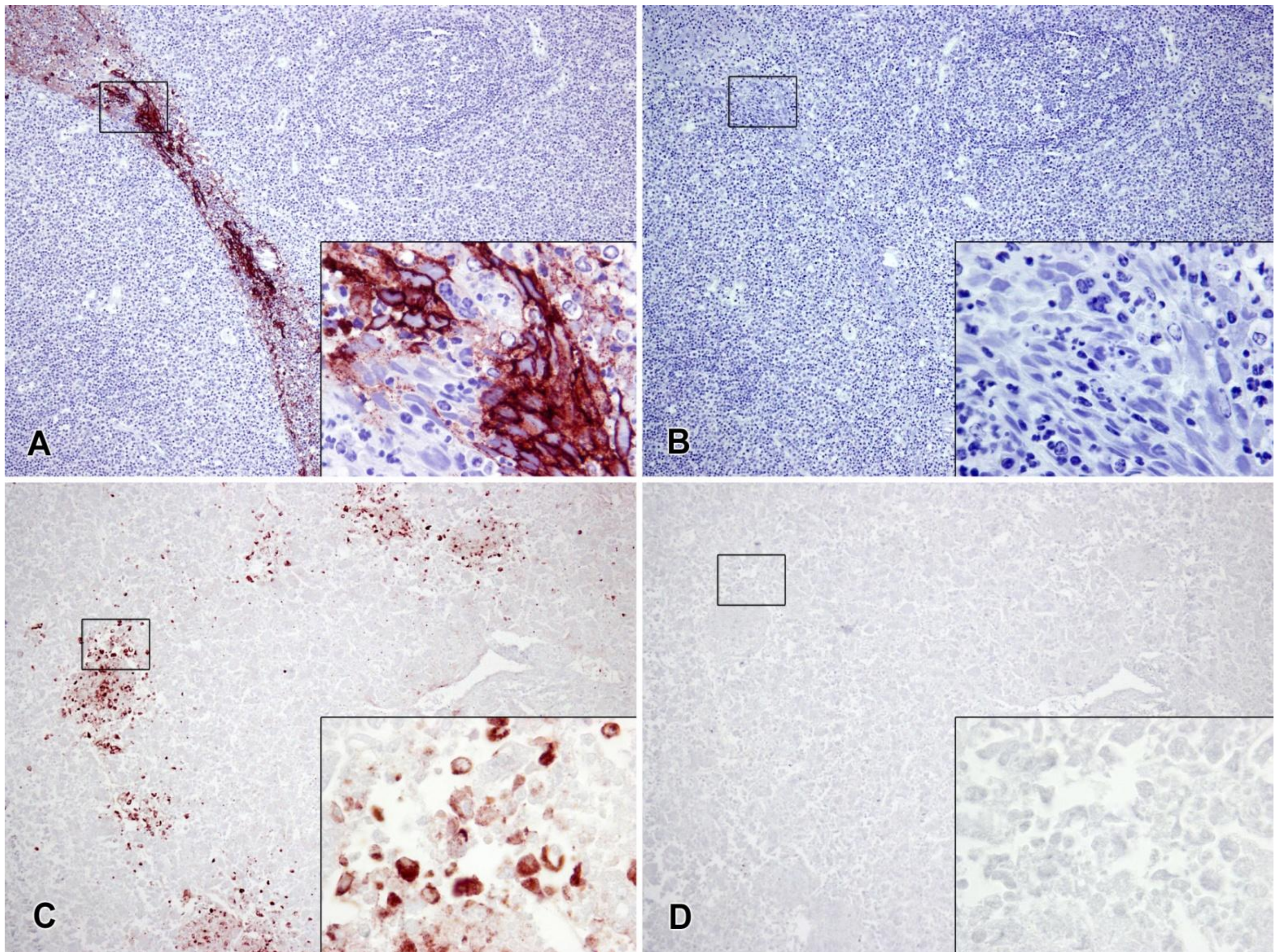

Figure S2. Positive-infection tissue controls for the immunohistochemical detection of BHV-1. Palatine tonsil from a calf euthanized at 4 dpi with BHV-1 (BVDV/BHV1 group) immunolabeled with F2 monoclonal antibody (**A**) and isotype-matched negative control (**B**). Liver from an aborted fetus that was positive for BHV-1 immunolabeled with F2 monoclonal antibody (**C**) and isotype-matched negative control (**D**). Insets represent higher magnifications of the fields framed in black. (CHAPTER 2A)

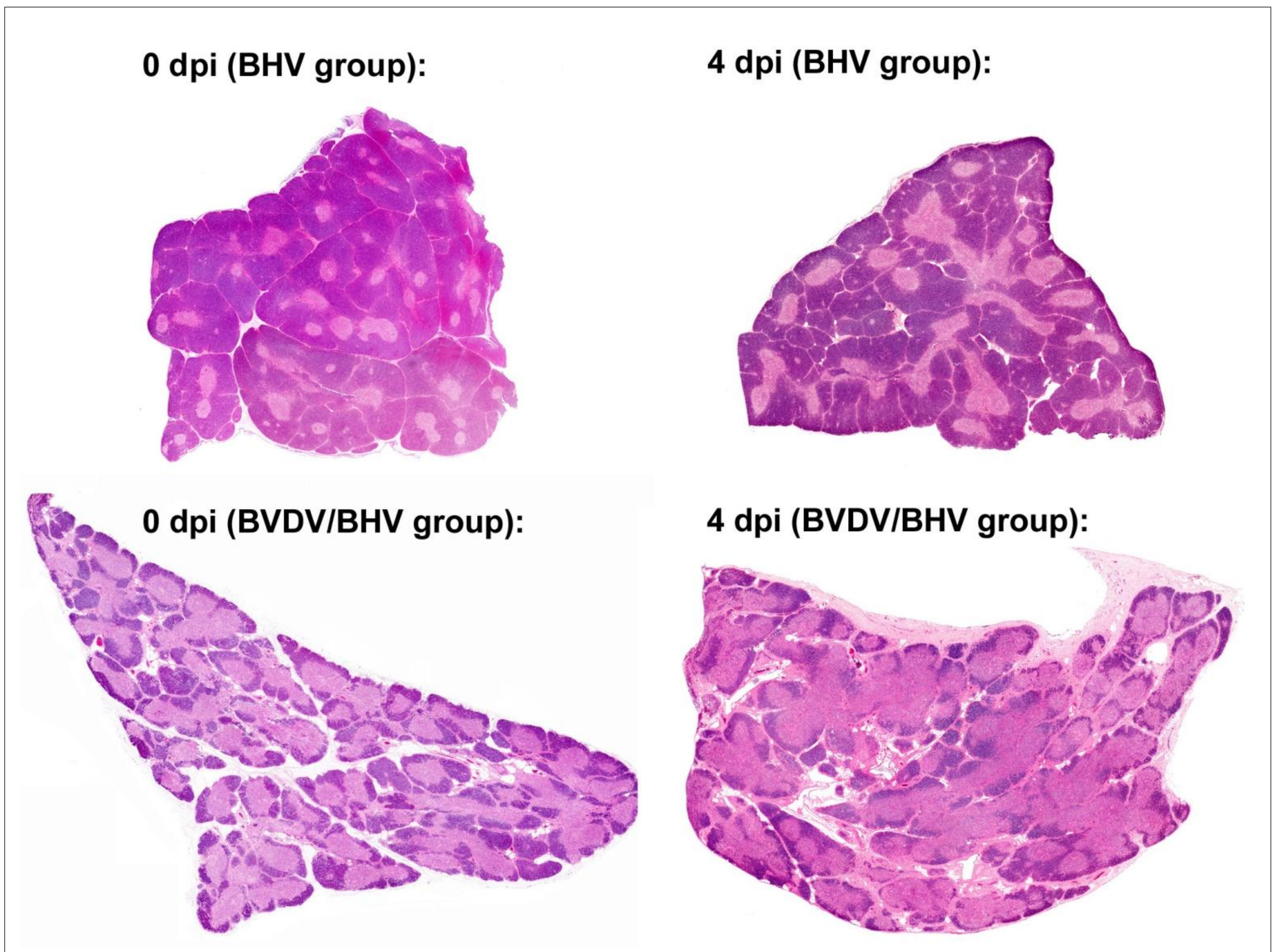

Figure S3. Low magnification of thymus sections. Hematoxylin-eosin (CHAPTER 2A)

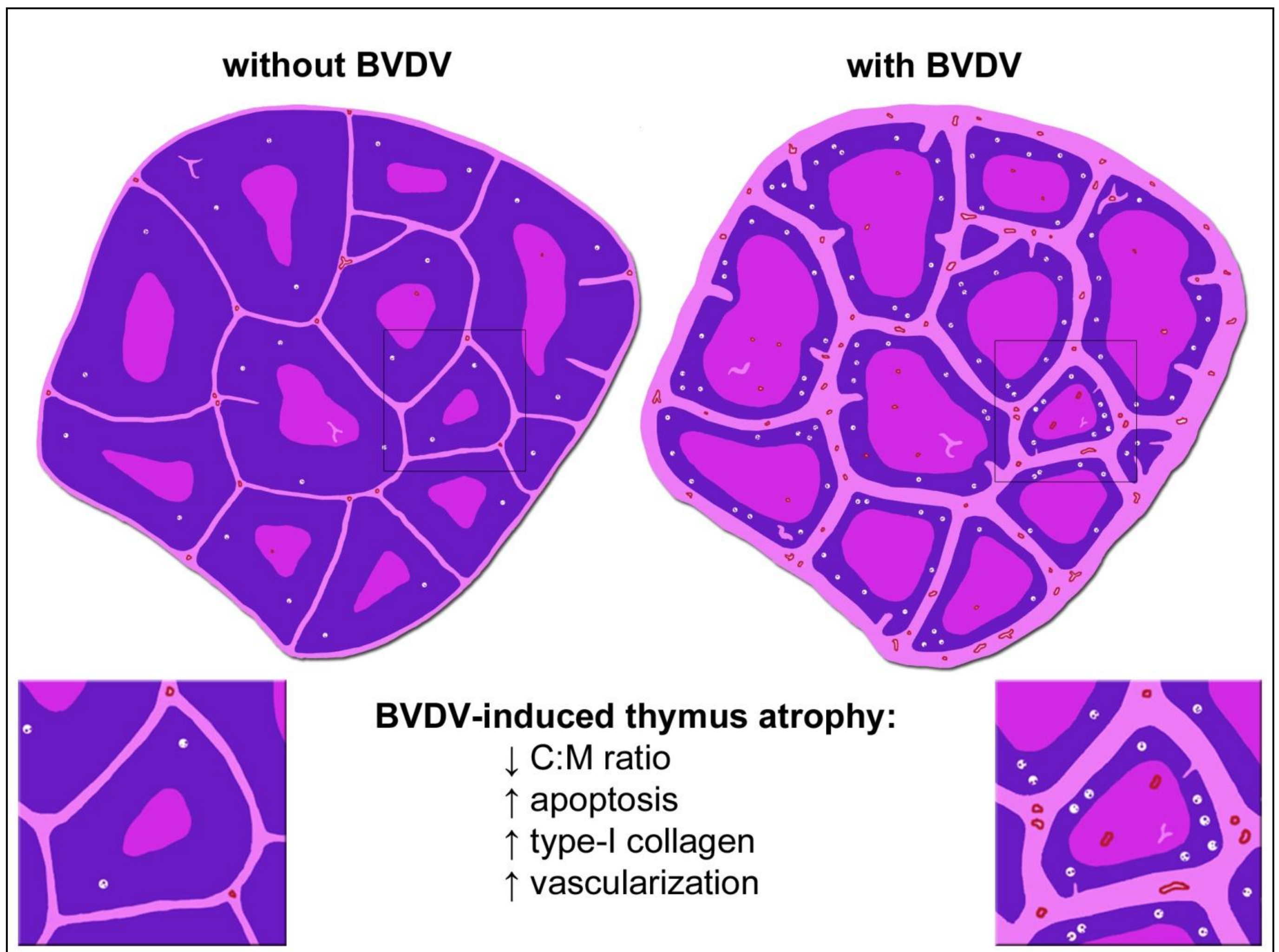

Figure S4. Representative figure of BVDV-induced thymus atrophy, characterized by a decreased C:M ratio, a high grade of apoptosis, and an increased type-I collagen deposition and vascularization. (CHAPTER 2A)

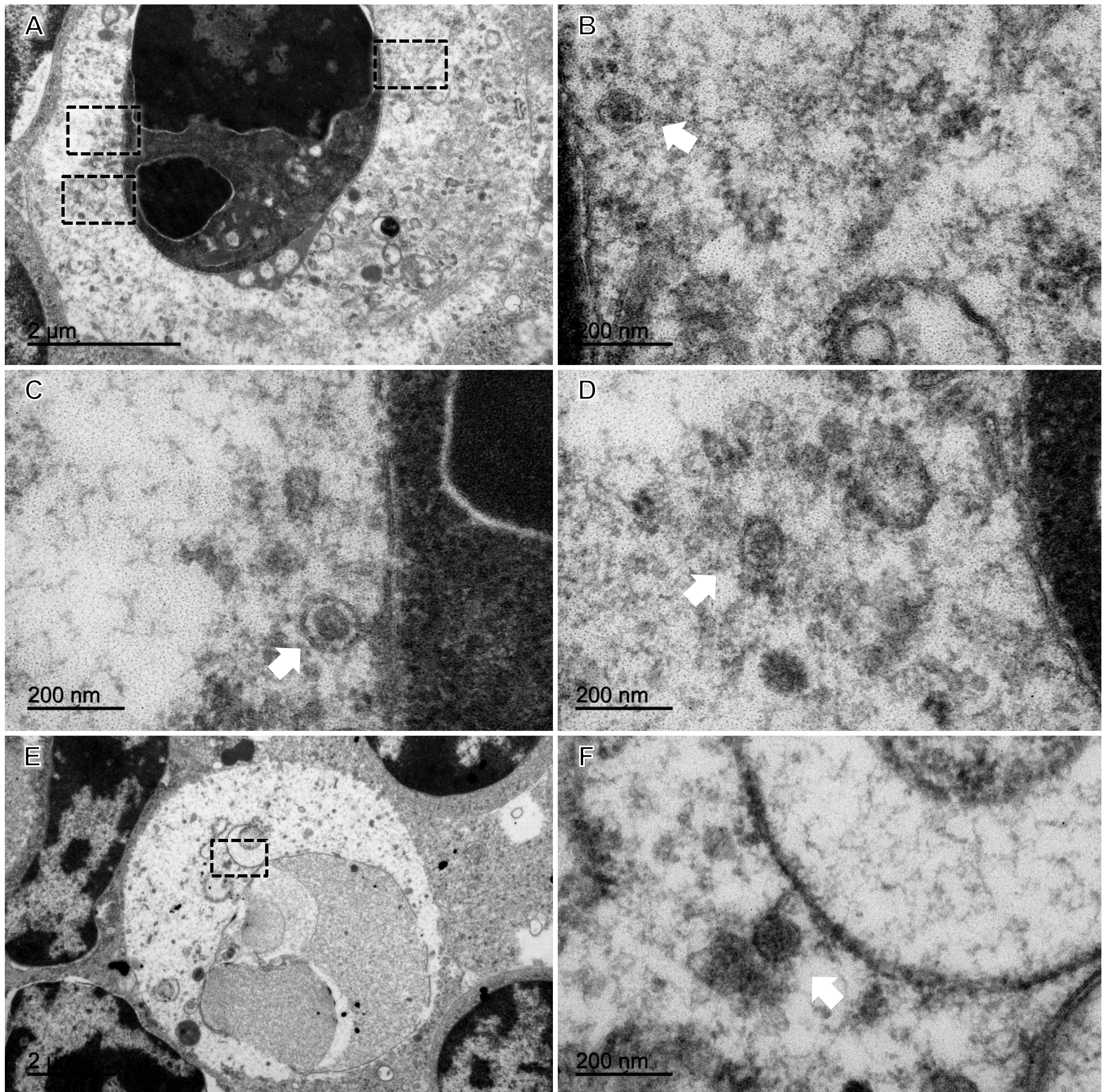

Figure S5. Transmission electron microscopy photographs showing the presence of round structures composed by an outer envelope and an inner electrodense core, compatible with BHV-1 particles, located in the cytoplasm of tingible body macrophages of a calf euthanized at 14 dpi with BHV-1. Figures B, C and D correspond to higher magnifications of the field outlined in A. Figure F corresponds to a higher magnification of the field outlined in E. (CHAPTER 2A)

ii. Scientific contributions

PUBLICATIONS

Romero-Palomo F, Risalde MA, Molina V, Lauzi S, Bautista MJ and Gomez-Villamandos JC (**2014**). Characterization of thymus atrophy in calves with subclinical BVD challenged with BHV-1. *Veterinary Microbiology*: under review.
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Romero-Palomo F, Risalde MA, Molina V, Sanchez-Cordon PJ, Pedrera M, and Gomez-Villamandos JC (**2013**). Immunohistochemical detection of dendritic cell markers in cattle. *Veterinary Pathology*: 50 (6), 1099-1108.
Área: Ciencias Veterinarias. Índice de impacto: 2.038. Posición: 15 de 129.

Romero-Palomo F, Sanchez-Cordon PJ, Risalde MA, Pedrera M, Molina V, Ruiz-Villamayor E, and Gomez-Villamandos JC (**2011**). Funciones y clasificación de las células dendríticas. *Anales de la Real Academia de Ciencias Veterinarias de Andalucía Oriental*: 24 (1), 167-192.
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COMMUNICATIONS AT MEETINGS

Romero-Palomo F, Risalde MA, Bautista MJ, Molina V, Sánchez-Cordón PJ, Gómez-Villamandos JC (**2014**). Immunohistochemical, morphometric and histopathological study of the thymus of calves with subclinical BVD and healthy calves, both challenged with BHV-1. In: *Second Joint European Congress of the European Society of Veterinary Pathology, European Society of Toxicologic Pathology and European College of Veterinary Pathologists (ESVP/ESTP/ECVP) - Cutting Edge Pathology* (Berlin, Germany). Type of participation: Oral presentation. **Granted with an Oral Presentation Award**

Romero-Palomo F, Risalde MA, Raya AI, Molina V, Sánchez-Cordón PJ, Bautista MJ, Gómez-Villamandos JC (**2013**). Immunohistochemical study of the thymus of calves infected with bovine viral diarrhoea virus type 1. In: *10th International Veterinary Immunology Symposium* (Milan, Italy). Type of of participation: Poster communication.

Romero-Palomo F, Risalde MA, Molina V, Sánchez-Cordón PJ, Pedrera M, Gómez-Villamandos JC (**2013**). Estudio inmunológico del timo de terneros con DVB subclínica y de terneros sanos, ambos infectados con HVB-1.1. En: *XXV Reunión de la Sociedad Española de Anatomía Patológica Veterinaria* (Toledo (España). Tipo de participación: Comunicación oral.

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Romero-Palomo F, Risalde MA, Molina V, Sánchez-Cordón PJ, Pedrera M, Gómez-Villamandos JC (**2012**). Immunohistochemical detection of dendritic cell markers in cattle paraffin wax-embedded tissues. In: *(Joint Meeting) 30th Meeting of the European Society of Veterinary Pathology and European College of Veterinary pathologists. 24th Annual Meeting of the Spanish Society of Veterinary Pathology* (Leon, SPAIN). Type of participation: Poster communication.

Romero-Palomo F (**2012**). Estudio *in vivo* e *in vitro* del papel de las células dendríticas en la respuesta inmune frente al virus de la Diarrea Vírica Bovina. En: *I Congreso Científico de Investigadores en Formación en Agroalimentación. II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba* (Córdoba, España). Tipo de participación: Comunicación poster.

iii. Published article: →

Veterinary Pathology
50(6) 1099-1108
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DOI: 10.1177/0300985813482951
vet.sagepub.com

Immunohistochemical Detection of Dendritic Cell Markers in Cattle

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Abstract

Dendritic cells (DCs) are "professional" antigen-presenting cells with a critical role in the regulation of innate and adaptive immune responses and thus have been considered of great interest in the study of a variety of infectious diseases. The objective of this investigation was to characterize the in vivo distribution of DCs in bovine tissues by using potential DC markers to establish a basis for the study of DCs in diseased tissues. Markers evaluated included MHCII, CD208, CD1b, CD205, CNA.42, and S100 protein, the latter 2 being expressed by follicular dendritic cells whose origin and role are different from the rest of hematopoietic DCs. Paraffin wax–embedded tissues from 6 healthy Friesian calves were subjected to the avidin-biotin-peroxidase method, and the most appropriate fixatives, dilutions, and antigen retrieval pretreatments were studied for each of the primary antibodies. The most significant results included the localization of CD208-positive cells not only in the T zone of lymphoid organs but also within lymphoid follicles; CD1b-positive cells were mainly found in thymus and interfollicular areas of some lymph nodes; cells stained with anti–CD205 antibody were scarce, and their location was mainly in nonlymphoid tissues; and CNA.42- and S100 protein–positive cells localized in primary lymphoid follicles and light zones of germinal centers, although showing differences in the staining pattern. Furthermore, MHCII was established as one of the most sensitive markers for any DC of hematopoietic origin. These results increase our understanding of DC immunolabeling and will help in future DC studies of both healthy and diseased tissues.

Keywords

bovine, CD208, CD205, CD1b, dendritic cells, follicular dendritic cells, immunohistochemistry, MHCII

Dendritic cells (DCs) are "professional" antigen-presenting cells (APCs) and are well known for their unique ability to present processed antigens to naive T lymphocytes.[20] In addition to their critical role in the regulation of the adaptive immune response, DCs serve as sentinels, recognizing the presence of invading pathogens and secreting proinflammatory cytokines involved in host defense and thereby linking both the innate and adaptive immunity.[35] These cells have a hematopoietic origin and express the major histocompatibility complex class II (MHCII) molecule. This is contrasted with the so-called follicular dendritic cells (FDCs) present in lymphoid follicles, which have a stromal origin, do not express MHCII, and present intact antigens to B cells in the form of surface immune complexes, being related to B-cell homeostasis.[39,41]

Different authors have studied the role of DCs in various cattle diseases by means of in vitro infections of different subtypes of DCs, including monocyte-derived DCs[9,21,37] and afferent lymph DCs (veiled cells or ALDCs).[14,15] To our knowledge, this report is the first that describes in vivo distribution of DCs in bovine tissues on the basis of their differential expression of potential dendritic cell markers such as MHCII, CD208, CD1b, CD205, CNA.42, and S100 protein.

The MHCII molecule is expressed by professional APCs, which includes all subtypes of DCs as well as macrophages and B lymphocytes. APCs are responsible for T helper cell activation by means of a synapse established between the antigen-loaded MHCII molecule and the T-cell receptor. CD208, also known as DC-LAMP (dendritic cell–lysosome-associated membrane protein) was found to be specifically expressed in mature DCs located in T zones of lymphoid tissues, known as interdigitant dendritic cells.[6] This molecule is not present in any other cell type, with the exception of type II pneumocytes,[38] demonstrating the potential usefulness of this marker in diagnostics of DC-related pathology. CD1 molecules are a family of cell surface–associated glycoproteins now recognized as having a role in the presentation of lipid antigens to certain subpopulations of T cells, as MHC molecules do with protein antigens.[32] This family comprises several members, but

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not all are present in cattle. CD1b has been described as the most important CD molecule in this species.[36,42] The majority of CD1 molecules are strongly expressed in cortical thymocytes as well as in certain other cell types, to include DCs.[16] CD205, also known as DEC-205, is a C-type lectin that belongs to the same family of the macrophage mannose receptor and is primarily expressed on certain DCs and epithelial cells, acting as an endocytic receptor, and is involved in the capture of antigens from extracellular spaces and transferal to a specialized antigen-processing compartment.[8,18] In addition, bovine CD205 has been previously described as the WC6 antigen, showing a strong expression on ALDCs.[10] In this study, we used a monoclonal antibody (clone CNA.42) for the labeling of FDCs[34] and a polyclonal antibody for the detection of S100 protein, which is expressed in a wide variety of cell types, including FDCs.[17,23]

Because of the complicated interactions between the distinct cells associated with both normal and pathologic mechanisms of action of the immune system, it is of critical interest to examine DCs not only in cell culture but also in vivo. This study aims both to standardize the immunohistochemical method for the detection of different potential DC markers but also to elucidate the histological distribution of these markers, some of which are commonly used in many in vitro studies, thus helping to expand the understanding of the various roles of DCs in different cattle diseases.

Materials and Methods

Six Friesian healthy male calves, aged 8 to 9 months, were obtained from farms free of tuberculosis, brucellosis, bovine leukosis virus, bovine viral diarrhea, and infectious bovine rhinotracheitis and were housed in the Animal Experimental Center of Cordoba University (Spain). Only those animals clinically healthy and with blood parameters within the normal range were included in the study. Parasitic coprological analyses were negative in all animals. Animals were sedated with xylazine (Rompun 2% solution; Bayer Healthcare, Kiel, Germany) and euthanized by overdose with thiopental-sodium (Thiovet; Vet Limited, Leyland, Lancashire, UK). This work was carried out in accordance with the code of Practice for Housing and Care of Animals used in Scientific Procedures, approved by the European Economic Community Union in 1986 (86/609/EEC) amended by the directive 2003/65/EC.

No significant macroscopic pathologic lesions were observed at necropsy. Samples from liver, intestine, and lung were subjected to microbiological routine cultures by using standard procedures, and no bacteria were isolated beyond the normal intestinal microflora. Tissue samples (0.5–1 cm thick) from a wide range of organs (Table 1) were immersed in 3 different fixatives: 10% neutral buffered formalin for 24 hours, Bouin's solution for 8 hours, and zinc salts fixative for 24 hours. Samples were processed by routine methods and embedded in paraffin wax. Sections (4 μm) were stained with hematoxylin and eosin (HE) and examined microscopically.

Two monoclonal antibodies (CD1b and CD205) directed against bovine antigens were used in this study. Anti–S100 antibody has been raised against S100 protein isolated from cow brain, and CNA.42 monoclonal antibody cross-reacts with FDCs in different species, including cattle.[34] Studies by Ababou et al[1] and Grüneberg et al[13] have demonstrated that the clone TAL.1B5 (anti–human HLA-DR α chain) also binds to an intracellular epitope of the BoLA-DR α chain. Given the close phylogenetic relationship between cattle and sheep, an anti–mouse CD208 antibody that cross-reacts with sheep[38] was used in this study (Table 2).

The avidin-biotin-peroxidase complex method was used.[30,31] Tissue sections (3 μm) were dewaxed and rehydrated using graded ethanol, and endogenous peroxidase activity was exhausted by incubation of the sections with H_2O_2 3% in methanol for 45 minutes at room temperature. Tissue sections were subjected to one of the following antigen retrieval pretreatments for the stated antibodies (Table 3): no pretreatment, when no antigen retrieval methods were performed; Tween 20 (Merck, Munich, Germany) 0.1% in 0.01M phosphate-buffered saline (PBS), pH 7.2 (10 minutes at room temperature); protease type XIV (Sigma-Aldrich Chemie, Steinheim, Germany) 0.1% in PBS (7 minutes at room temperature); and microwave heat–induced epitope retrieval (HIER) in 0.01M citrate buffer at different pH levels (3.2, 6, and 9), in addition to different time points from the onset of boiling. After pretreatment, sections were given three 5-minute rinses in PBS. For the primary antibodies MHCII, CNA.42, CD1b, and CD205, tissue sections were covered with 1% normal horse serum (Pierce-Endogen, Woburn, MA) in 0.05M Tris-buffered saline (TBS), pH 7.6, for 30 minutes at room temperature and incubated with the primary monoclonal antibodies at 4°C overnight. For the primary antibodies against CD208 and S100 protein, 1% normal rabbit serum and 20% normal goat serum, respectively, replaced normal horse serum. Details of the commercial primary antibodies tested in this study are summarized in Table 2. After primary incubation, slides were washed in PBS (3 times for 5 minutes each) and then incubated with the secondary antibodies for 30 minutes at room temperature. Biotinylated horse anti–mouse IgG secondary antibody (Pierce-Endogen) diluted 1:200 in TBS containing normal horse serum 1% was used for the primary antibodies MHCII, CNA.42, CD1b, and CD205. Biotinylated rabbit anti–rat Igs secondary antibody (Dako, Glostrup, Denmark) diluted 1:100 in TBS containing normal rabbit serum 1% was used for the primary antibody against CD208. Biotinylated goat anti–rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS containing normal goat serum 1.5% was used for the polyclonal primary antibody anti-S100. After 3 further 5-minute washes in PBS, samples were incubated with the avidin-biotin-peroxidase complex (Vectastain ABC Kit Elites; Vector Laboratories) for 1 hour at room temperature in the dark. All tissue sections were finally rinsed in TBS, and labeling was "visualized" by application of a chromogen solution (NovaRED Substrate Kit; Vector Laboratories). Slides were counterstained with Mayer's hematoxylin. Mouse or rat nonimmune sera were used in place of specific monoclonal primary antibodies as negative controls.

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Table 1. Distribution of Cells Immunolabeled With Dendritic Cell Markers in Different Organs.

	Cell Markers					
	MHCII	CD208	CD1b	CD205	CNA42[a]	S100[a]
Lymphoid tissues						
Palatine tonsil						
LF	++++	+++	–	–	+	+
IFA	+++	++	–	+		
Epithelium	++	–	–	–		
Thymus						
Cortex	–	–	++++	–		
Medulla	++++	++	++	–		
Spleen						
LF	++	+++	–	+	++	++
RSP	+++	–	–	–		
Lymph nodes[b]						
LF	++	+++	–	–	++	++
IFA	+++	++	++[c]	–		
Medulla	++	–	–	+		
Respiratory tract						
Lung						
Alveolar septa	++	+++	–	++		
BALT	++	++	–	+	+	+
Trachea						
Epithelium	–	–	–	–		
Lamina propria	++	–	–	+		
Digestive and tegumentary system						
Liver	+	–	–	++		
Distal ileum						
Lamina propria	+++	–	–	–		
PP follicles	–	+++	–	–	+	+
PP dome regions	++	++	–	–	++	+
Ileocecal valve						
Lamina propria	+++	–	–	–		
LF	++	+++	–	–	+	+
IFA	+++	++	–	–		
Haired skin						
Epidermis	+	–	–	–		
Dermis	+	–	+	–		

Results expressed as number of immunolabeled cells per area of 0.2 mm^2: – (none), + (0–5), ++ (0–20), +++ (20–60), and ++++ (>60). BALT, bronchus-associated lymphoid tissue; IFA, interfollicular areas; LF, lymphoid follicles; MHCII, major histocompatibility complex class II molecule; PP, Peyer's patches; RSP, red splenic pulp.
[a]CNA.42 and anti–S100 antibodies were used for the detection of follicular dendritic cells, and thus only cells stained within lymphoid follicles were assessed in this table. Intensity and quantity of cells stained with these antibodies were assessed as + (slight) or ++ (high).
[b]Lymph nodes included in this study were retropharyngeal, submandibular, mediastinal, ileocecal, and superficial inguinal lymph nodes.
[c]Positivity to CD1b antibody was mainly observed in superficial inguinal lymph nodes and submandibular lymph nodes.

A semiquantitative assessment of the immunolabeled cells was performed by 2 experienced observers in 25 fields of 0.2 mm^2 randomly chosen. Results were expressed as number of immunolabeled cells per area of 0.2 mm^2: – (none), + (0–5), ++ (0–20), +++ (20–60), and ++++ (>60). Intensity and quantity of cells stained with CNA.42 and anti–S100 antibodies were assessed only within lymphoid follicles as + (slight) or ++ (high) (Table 1).

Results

Tissues used in this study were assessed by a board veterinary pathologist who certified the absence of histopathological lesions. Signs of inflammation indicative of infectious or toxic agents were not observed in any of the animals included in the study.

Optimization of the Immunohistochemical Method

Despite using other fixatives such as Bouin's solution or zinc salts fixative, the best results were obtained with buffered formalin. Tissue expression of the MHCII molecule was observed with each of the different antibody concentrations as well as with the different unmasking pretreatments, although the higher dilution (1:100) and the unmasking method with citrate at pH 3.2 during 6 minutes at subboiling temperature were

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Table 2. Primary Antibodies Used in the Present Study.

Primary Antibody	Specificity	Cellular Expression	Isotype/Form	Clone	Source
Mouse anti–human HLA-DR	MHCII	DCs, B cells, Mø	IgG1 κ, Supernatant	TAL.1B5	Dako
Rat anti–mouse CD208	DC-LAMP (CD208)	Mature DCs, PnIIs	IgG2a, Purified	1010E1	Dendritics
Mouse anti–bovine CD1w2	CD1b	DCs, cortical thymocytes	IgG2a, Supernatant	CC20	Serotec
Mouse anti–bovine CD205	DEC-205 (CD205)	DCs, some B/T cells, some epithelial cells	IgG2b, Purified	CC98	Serotec
Mouse anti–human FDC	FDCs	FDCs	IgM κ, Supernatant	CNA.42	Dako
Polyclonal rabbit anti-S100	S100 protein	Wide (including FDCs)	Purified	Polyclonal	Dako

DC, dendritic cell; DC-LAMP, dendritic cell–lysosomal-associated membrane protein; FDC, follicular dendritic cell; MHCII, major histocompatibility complex class II molecule; Mø, macrophages; PnII, type II pneumocytes.

Table 3. Immunoreactivity Produced by Various Primary Antibodies on Calf Tissues Fixed in Formalin Solution and Subjected to Various Antigen-Retrieval Pretreatments.

Antibody dilutions		Pretreatments					
					Citrate Microwave		
		None	Tween 20	Protease	pH 3.2	pH 6	pH 9
MHCII	1:10	++	++	++	++	++	++
	1:50	++	++	++	++	++	++
	1:100	++	++	++	+++[a]	++	++
CD208	1:10	–	–	–	Bs	Bs	Bs
	1:50	–	–	–	Bs	Bs	Bs
	1:100	–	–	–	+	++[a]	Bs
CD1b	1:10	–	+	+++[a]	–	–	–
	1:50	–	–	+	–	–	–
	1:100	–	–	+	–	–	–
CD205	1:10	Bs	++[a]	+	–	–	–
	1:50	–	–	–	–	–	–
	1:100	–	–	–	–	–	–
CNA.42	1:10	–	–	–	+	+	++
	1:50	–	–	–	–	+	+++[a]
	1:100	–	–	–	–	–	–
S100	1:10	Bs	Bs	–	Bs	++	–
	1:50	++	++	–	++	++	++
	1:400	++	++	–	+++[a]	++	+

–, none; +, slight positivity and light background; ++, positive reaction and light background; +++, positive reaction without background; Bs, positive reaction but intense background staining. MHCII, major histocompatibility complex class II molecule.
[a]Elected dilution and pretreatment for each antibody.

considered the most effective (Table 3). Immunolabeling against CD208 was possible with the higher dilution (1:100), although only when using the HIER methods at pH 6 during 20 minutes at subboiling temperature. This technique appeared to be the most appropriate, since staining using pH 3.2 was too weak, and pH 9 gave rise to intense background staining. The most suitable antigen retrieval method for the CD1b molecule was enzymatic digestion with protease but required the highest concentration of anti–CD1b antibody (1:10) to obtain the best results. In the case of anti–CD205 antibody, the only dilution that yielded positive results was the most concentrated (1:10), with the HIER methods being ineffective and the detergent permeabilization with Tween 20 being considered the most appropriate. For the detection of FDCs with CNA.42 monoclonal antibody, use of the HIER methodology was necessary, with the best results obtained at pH 9 during 30 minutes at subboiling temperature and with a 1:50 antibody dilution. S100 protein expression was observed with all of the unmasking methods except for enzymatic digestion, although the best results were obtained with citrate at pH 3.2 during 6 minutes at subboiling temperature and with a 1:400 antibody dilution.

Quantification and Location of Immunolabeled Cells

Immunolabeled cells were counted for each animal, with minor differences among animals being observed and included within the stated intervals (Table 1).

MHCII followed by CD208 appeared as the primary molecules expressed in the different tissues, both in intensity of labeling and quantity of labeled cells. All lymphoid organs displayed a high amount of MHCII-positive cells. These variably sized cells were typically stellate or polygonal and had

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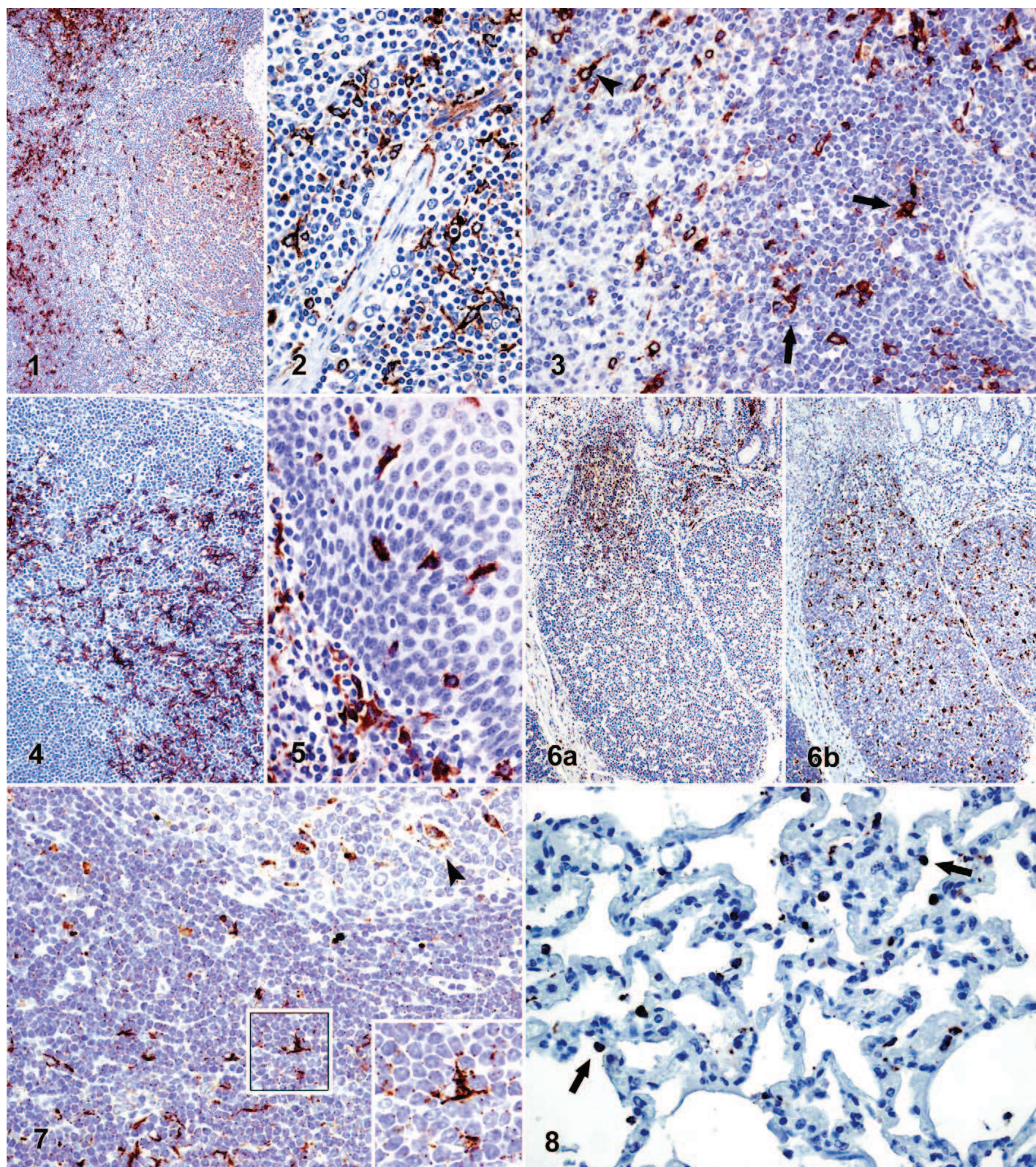

Figure 1. Lymph node; cattle No. 3. Immunostaining against major histocompatibility complex class II (MHCII) demonstrated strong labeling intensity in interfollicular areas and also stained cells within lymphoid follicles. Immunohistochemistry (IHC) with the avidin-biotin-peroxidase complex (ABC) method counterstained with hematoxylin. **Figure 2.** Lymph node; cattle No. 2. MHCII immunostaining of medulla, where many positive cells with dendritic morphology can be appreciated. IHC (ABC method) counterstained with hematoxylin. **Figure 3.** Spleen; cattle No. 1. Cells with dendritic morphology and that stained against MHCII can be observed in both the red splenic pulp (arrowhead) and the periarteriolar lymphoid sheaths (arrows). IHC (ABC method) counterstained with hematoxylin. **Figure 4.** Thymus; cattle No. 4. There is strong labeling against the MHCII molecule in the thymic medulla, which includes a population of dendritic-shaped cells. IHC (ABC method) counterstained with hematoxylin. **Figure 5.** Palatine tonsil; cattle No. 2. The presence of MHCII-positive cells with dendritic morphology in the stratified

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homogeneous cytoplasmic staining. Immunopositive cells in palatine tonsil and lymph nodes could be clearly differentiated from one another in the interfollicular areas (Fig. 1). A general faint staining was observed in the majority of lymphoid follicles, and in some of these lymphoid follicles, more strongly immunostained cells were noted and mainly located in the lymphoid follicle light zones (Figs. 1, 12a). Immunolabeled cells were also observed in the medulla of lymph nodes, although to a lesser degree in comparison with the cortex. The cells identified within the medullary regions had a very characteristic dendritic morphology (Fig. 2). Immunostaining observed in the spleen was evident in red splenic pulp and periarteriolar lymphoid sheaths, displaying many immunopositive dendritic-shaped cells (Fig. 3), in contrast to lymphoid follicle staining being weaker if more diffuse. In the thymus, immunolabeling against MHCII was restricted to the medulla, where strong immunoreactivity was observed, including many cells with a dendritic morphology (Fig. 4). Rare immunopositive cells were observed in the pulmonary alveolar septa and periportal areas of the liver. Positive cells were also noted within the tonsillar epithelium and, to a lesser degree, in the epidermis and dermis of the skin, frequently showing dendritic morphology (mainly in tonsillar crypts epithelium) (Fig. 5). MHCII-positive cells in the distal ileum were located in the lamina propria as well as within the dome region of the Peyer's patches but not within Peyer's patches follicles (Fig. 6a), in contrast to the immunopositivity observed in the ileocecal valve lymphoid follicles.

Staining against CD208 (DC-LAMP) was cytoplasmic and had a dark granular appearance. Positive cells were mainly stellate or polygonal and were widely distributed in lymphoid tissues. A slight generalized staining was observed as tiny dark dots that could be appreciated on the cell surfaces (Fig. 7, inset). This was more evident as the pH of the HIER method was increased. Immunopositive cells were located in the interfollicular areas and, to a greater extent, within the lymphoid follicles (Figs. 7, 12b), where the cells were homogeneously distributed, with no differences observed between the dark and light zones of the germinal center, unlike the differences noted with anti–MHCII and anti–FDC antibodies. In general, not all the lymphoid follicles of the same lymph node presented the same quantity of immunopositive cells, and the medulla of lymph nodes was observed to be free of CD208-positive cells. Immunostained cells against CD208 had a round morphology and were located on the alveolar surfaces in the lung (Fig. 8). A moderate amount of immunolabeled cells that were predominantly stellate was identified in the periarteriolar lymphoid sheaths of the spleen and within the thymic medulla. In addition, numerous large round immunopositive cells were detected within the ileum Peyer's patches and the lymphoid follicles of the ileocecal valve (Fig. 6b).

Very few organs were noted to have CD1b-immunopositive cells. Cortical thymocytes were uniformly immunopositive along with some cells with a dendritic morphology located in the thymic medulla (Fig. 9). Most lymph nodes were immunonegative for anti–CD1b antibody, with the exception of the submandibular and superficial inguinal lymph nodes and, to a lesser extent, the mediastinal lymph nodes, where numerous dendritic-shaped immunolabeled cells were identified in the interfollicular areas. Some scattered immunopositive cells were also demonstrated in the dermis of the skin.

Immunolabeling against CD205 demonstrated a granular appearance, located in the cytoplasm of cells with either round or spindle morphology. In general, few immunolabeled cells were observed by using this antibody, and these were located in uncommon regions such as in tissue adjacent to tonsil mucous glands or to large blood vessels (data not shown), in trabeculae of lymphoid organs, or in the connective tissue of hepatic portal spaces, central veins, and Glisson's capsule. It was difficult to find any immunopositive cells within the interfollicular areas or the lymphoid follicles (Fig. 10). A greater number of immunolabeled cells were identified in the lung, located in the alveolar septa, the pulmonary pleura, or surrounding bronchioles and arteries (Fig. 11).

Use of the CNA.42 antibody yielded a staining pattern in the form of a network distributed among lymphocytes of the primary lymphoid follicles, as well as the light zones and mantle zones of germinal centers (Fig. 12c). Curiously, the lymphoid follicles of palatine tonsil, Peyer's patches, and ileocecal valve demonstrated only a scarce staining intensity, unlike the stronger labeling intensity noted in the lymphoid follicles of the examined lymph nodes and the spleen. Expression of S100, also observed in the primary lymphoid follicles and the light zones of the germinal centers, showed organic differences similar to those observed with CNA.42, although staining against S100 was located in both the cell cytoplasm and the nucleus (Fig. 12d). Both antibodies (CNA.42 and anti-S100) yielded immunostaining of cell types other than cells located within the lymphoid follicles, which are not included in the aims of this study.

Discussion

It is well known that DCs are a heterogeneous cell population categorized according to their location, functionality, and cell marker expression. The present study has focused on the comparison and characterization of the location of dendritic cells of

Figure 5. (continued). epithelium, compatible with Langerhans cells, is demonstrated. IHC (ABC method) counterstained with hematoxylin. **Figure 6.** Serial sections of distal ileum; cattle No. 1. (a) Cells staining against the MHCII molecule are restricted to Peyer's patches domes and the lamina propria. (b) CD208-positive cells are widely distributed in Peyer's patches domes and follicles. IHC (ABC method) counterstained with hematoxylin. **Figure 7.** Lymph node; cattle No. 1. Cells immunopositive for CD208, having a dendritic cell morphology compatible with interdigitant dendritic cells, are demonstrated (inset). Positive cells are also observed within the lymphoid follicles, having the appearance of tingible body macrophages (arrowhead). IHC (ABC method) counterstained with hematoxylin. **Figure 8.** Lung; cattle No. 6. CD208 immunolabeled cells are found among the surface cells of the alveoli, compatible with type II pneumocytes (arrows). IHC (ABC method) counterstained with hematoxylin.

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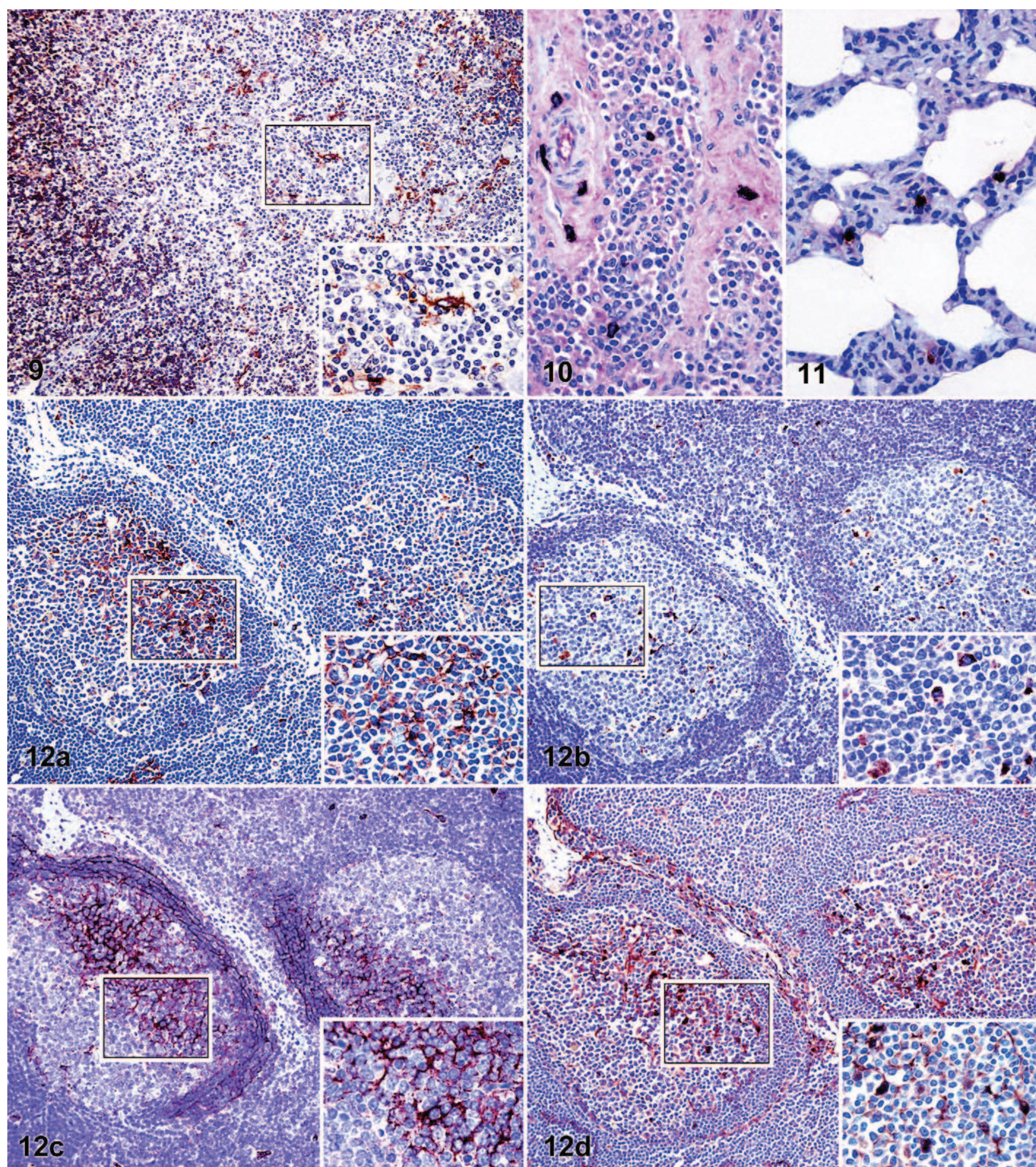

Figure 9. Thymus; cattle No. 3. Anti-CD1b immunolabeling of cortex thymocytes and some dendritic-shaped cells of the medulla are demonstrated (inset). Immunohistochemistry (IHC) with the avidin-biotin-peroxidase complex (ABC) method counterstained with hematoxylin. **Figure 10.** Lymph node; cattle No. 1. CD205-positive cells in the medulla, located inside trabeculae, surround a blood vessel and rarely are noted in lymphatic sinuses. IHC (ABC method) counterstained with hematoxylin. **Figure 11.** Lung; cattle No. 3. Cells immunostained against CD205 with round morphology are located inside the alveolar septae. IHC (ABC method) counterstained with hematoxylin. **Figure 12.** Lymph node; cattle No. 4. Serial sections showing the staining pattern within lymphoid follicles with different antibodies; insets represent a higher magnification of the field framed in black. (a) Anti–major histocompatibility complex class II (MHCII) immunolabeling of the lymphoid follicle light zone. (b) Anti-CD208 immunolabeling of cells compatible with tingible body macrophages all over the lymphoid follicles. (c) CNA.42 antibody staining forming a cytoplasmic pattern restricted to the light zone. (d) Anti–S100 staining of cells located in the light zone of germinal centers showing the dual cytoplasmic and nuclear staining pattern. IHC (ABC method) counterstained with hematoxylin.

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Friesian calves based on their expression for different cellular markers. This was accomplished by using fixed and paraffin wax–embedded tissues, which preserve an optimal cell morphology, in comparison with frozen tissues, in which there can be a significant loss of morphology, making the identification of a precise location of the antigen-expressing cells within the tissue much more difficult.[2,5,33] Furthermore, frozen tissue handling may involve a higher degree of difficulty to produce cryostat sections of consistent quality.

The detection of the MHCII molecule is frequently conducted in tissue sections, although the vast majority of these studies are based on frozen tissue sections and typically for purposes other than detecting DCs.[24,25] The novel information presented by this study employed the MHCII molecule to describe the type of immunolabeled cells within a wide variety of organs, thereby providing a standardization of the immunohistochemical methodology, as well as taking advantage of the fact that DCs are strong expressors of this molecule and that fixed paraffin-embedded tissues preserve the characteristic morphology of these cells, allowing for a mapping of their localization within select organs. To our knowledge, this is the first report of the use of this list of antibodies in the detection of DCs in bovine paraffin-embedded tissues.

This study revealed that of the antibodies examined, the MHCII antibody yielded the greatest quantity of labeled cells in the different tissues analyzed. This is due to the expression of the molecule not only in DCs but also in macrophages and certain populations of B cells, as well as being inducible in endothelial cells. Despite this variety of positive cells, use of this antibody also permits the detection of all DC populations, unlike other markers that detect only certain subpopulations. A striking finding in the results obtained with the anti–MHCII antibody was the marked staining polarization in most of the germinal centers of secondary lymphoid organs, which coincided to a great extent with the immunolabeling observed with the use of CNA.42 and anti–S100 antibodies, which was restricted solely to the light zone (and adjacent mantle zone in the case of CNA.42) of the germinal centers. This observation was possible by using serial sections of the tissue immunostained with the antibodies of interest (Fig. 12a,c,d). It is known that FDCs do not internalize or present processed protein antigens in the context of MHCII molecules,[41] a fact that would establish as incompatible the coincidence in location of FDCs in light zones with the strong expression of MHCII. However, it has been shown that this cell type can acquire MHCII molecules not expressed by FDCs themselves,[7] which may explain why a strong immunostaining against MHCII in light zones can be observed, where FDCs are located. Human studies have demonstrated the CNA.42 antibody as an immunomarker for FDCs for each level of maturation, ranging from FDC precursors to activated FDCs.[19] Furthermore, the S100 protein is a molecule expressed by activated FDCs, which may explain why CNA.42 yielded a network staining pattern that was widely distributed and anti-S100 gave rise to the staining of individual cells.

The present study describes for the first time in bovine tissue samples the expression of CD208 (DC-LAMP), known as an exclusive marker for mature DCs in T regions (interdigitant DCs) and type II pneumocytes.[6,38] CD208 expression in alveolar surfaces coincides with the previously described expression of this molecule in type II pneumocytes. However, we unexpectedly found CD208-positive cells not only in interfollicular areas but also within lymphoid follicles; given the known exclusive expression of CD208 to DCs in lymphoid organs, these immunopositive cells found within lymphoid follicles might be considered the previously described germinal center DCs (GCDCs).[11,12] However, this consideration would be mistaken since GCDCs, whose origin is hematopoietic, should express MHCII, a fact that was ruled out by using serial sections immunostained with both antibodies (Figs. 6, 12a,b); CD208-positive cells were located both in MHCII-positive light zones and MHCII-negative dark zones. The typical follicular location, round morphology, and large size suggest that these CD208-positive cells are in fact tingible body macrophages.

Numerous MHCII-positive cells were detected in the thymic medulla, most of them having a stellate shape compatible with that of DCs, which have significant importance in both positive and negative selection during T-cell development.[3] In this same location, we also observed dendritic-shaped cells immunolabeled against CD208, albeit to a lesser extent. This observation may be explained by the fact that during maturation, DCs acquire a higher capacity to form and accumulate MHCII-peptide complexes, a process that requires a generalized activation of the lysosomal function;[40] the CD208 molecule is included in the lysosomal-associated membrane protein family, which is why these CD208-positive cells in thymic medulla may represent a population of mature DCs, which corresponds to similar descriptions in humans.[4]

Langerhans cells (LCs) were originally described as immature DCs present in the epidermis, although currently this term has been generalized to include DCs present in all surface-stratified epithelium.[27] This may support the hypothesis that considers MHCII-positive cells observed in tonsillar epithelium as LCs (Fig. 5), as these demonstrated stronger immunopositivity and were more numerous in comparison to those of the epidermis, further highlighting the importance of DCs in the tonsil due to its strategic location in the entrance of numerous airborne pathogens.

It is well established that DCs are present within the dome regions of the intestinal lymphoid tissue,[28] coinciding with the presence of stellated MHCII-positive cells that we found in those intestinal sections examined. However, Peyer's patches follicles barely stained against the MHCII molecule. In ruminants, ileal Peyer's patches are considered primary lymphoid organs,[22] in which there is no germinal center reaction as observed in jejunal Peyer's patches,[43] which may explain the lack of MHCII expression at this level, similar to that which occurs in the cortex of thymus, another primary lymphoid organ. However, these MHCII-negative Peyer's patches follicles demonstrated a great quantity of widely distributed CD208-immunopositive cells, supporting the hypothesis that considers these cells to be tingible body macrophages.

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Previous studies have determined the phenotypic characteristics of both intestinal and respiratory tract DCs in sheep[2,26] by using antibodies against CD205 and CD1b molecules. ALDCs are strong expressors of CD205,[10] and other tissues have been shown to express this molecule as well.[10,29] Contrary to results obtained by Akesson and collaborators[2] in sheep, we did not find cells expressing CD205 in the ileum in this study of Friesian cattle. However, a considerable amount of CD205-immunolabeled cells were detected in the parenchyma and pleura of the lung, which is consistent with other ovine studies.[26] In comparison with other DC markers, very few cells demonstrated immunolabeling against CD1b in the intestine and the lung of sheep. [2,26] Likewise, our results demonstrated that both organs were negative to the expression of CD205 and CD1b. Antibodies for detecting CD205 and CD1b used in this study and in ovine studies from Akesson et al[2] and McNeilly et al[26] are bovine specific. Despite this fact, the absence of expression in this study of CD1b in the intestine and the respiratory track and of CD205 in the intestine suggests that the observed species differences between sheep and cattle are due to differences in the technique sensitivity employed; ovine studies have been carried out in frozen tissues, whereas this study used tissue samples that had been fixed and embedded in paraffin wax. The latter technique permits an optimal preservation of the cellular morphology and tissue architecture, although it may mask or alter the 3-dimensional structure of antigens, which is why antigen retrieval methods are required.[33]

In summary, despite the existence of other molecules displayed by DCs, the recognition of MHCII expression appears to be one of the most sensitive methods for the detection of any DC of hematopoietic origin in tissue sections. The staining pattern observed with the monoclonal antibody detecting CD208 suggests that the expression of this molecule in bovine lymphoid tissues is restricted not only to interdigitant DCs but also to tingible body macrophages, a finding that has not been previously described. Although further studies will be needed to confirm this novel finding, this study has demonstrated that CD208 detection allows for the differentiation of mature DCs from all other DCs, a fact of significant interest in the diagnostics and study of infectious diseases. This study further supports the tissue expression of CD1b in dendritic-shaped cells, even though these cells comprised a reduced population out of the total number of the existing DCs and were located in specific sites such as the thymus. Therefore, although global utility is limited, CD1b will provide valuable information on the role of thymic DCs, which is of significance due to the organ's importance in the central immunotolerance. Both CNA.42 and anti–S100 antibodies stained FDCs, but only the latter allowed for the identification of FDCs as isolated cells. Taken together, these results provide a useful general view of the different staining patterns of potential DC markers and will help in future DC studies with pathologic tissues.

Acknowledgements

We thank the farm "Las Rozuelas del Valle" (Torrecampo, Spain) for providing the animals.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by a grant from the Andalusian Regional Government-ERDF (P09-AGR-4671). F. Romero-Palomo was supported by a predoctoral grant associated with project P09-AGR-4671.

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