UNIVERSITÀ DEGLI STUDI DI PARMA

Dottorato di ricerca in Scienze Medico-Veterinarie

Ciclo XXVIII

ENDOMETRIAL RESPONSE TOWARD BOVINE HERPESVIRUS 4 INFECTION

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"Non est quod timeas ne operam perdideris, si tibi didicisti".

(L. A. Seneca, Epistulae morales ad Lucilium)

The research reported in this PhD thesis was published in a international scientific journal:

- **Interferon gamma-mediated BoHV-4 replication restriction in bovine endometrial stromal cells is host IDO1 gene expression independent and BoHV-4 IE2 gene expression dependent.**

  Jacca S, Franceschi V, Agosti M, Cavirani S, Mistretta F, Donofrio G. Biol Reprod. 2014 Nov;91(5):112. doi: 10.1095/biolreprod.114.123000. Epub 2014 Oct. (Jacca, Franceschi et al. 2014)

Three months of my PhD course (May 26/August 31 2014) were spent at Swansea University (Wales), under the supervision of Prof. I.M. Sheldon. During this period, I acquired the ability to generate and manipulate primary endometrial cells (stromal cells, epithelial cells and macrophages) and to work with explants, in order to study the interaction between bovine endometrium and viruses, in term of cytokines and interleukins production.

Abstract

Metritis and endometritis are the main responsible of great economical loss inside dairy herds, especially in post partum period. While the first one involves all the uterus and it is especially characterized by the presence of systemic symptoms, the second one consists of the inflammation of the only endometrium, with the presence of purulent discharge, destruction of epithelial surface, vascular congestion, stromal edema and accumulation of lymphocytes and plasma cells. These pathologies can cause ovarian dysfunction, leading to infertility or reduction of conception rate, but also abortion and decreasing in milk production. Although they are usually related to bacterial infection, which can overcomes inside uterus directly during parturition, the role of some viruses in the development of uterine diseases was recently investigated and the relationship between Bovine Herpesvirus type 4 and the onset of metritis and endometritis was demonstrated. Bovine Herpesvirus type 4 (BoHV-4) is a gamma-herpesvirus and its genome is constituted by a linear double stranded DNA molecule with a B-type structure, characterized by the presence of a long unique genome region (LUR) flanked by poly-repetitive DNA (prDNA) elements. It was isolated both from healthy and from cattle with diseases, such as ocular and respiratory diseases, but especially from cows with metritis, endometritis, vaginitis or abortion. Usually, the role played by the virus in this kind of pathologies is associated to the co-presence with other pathogens, which could be other viruses, such as the Bovine Viral Diarrhea virus (BVDV) in the case of abortion, as well as bacteria. Generally, The initial defense of the bovine endometrium against microbes is dependent on the innate immune system and the activation of some specific cellular receptors determines the synthesis and production of several pro-inflammatory cytokines and chemokines, which can modulate BoHV-4 replication. BoHV-4 genome possesses two major Immediate Early (IE) RNAs, among which ORF50/IE2 is the most important and its product, called Rta/ORF50, is well conserved among Herpesviruses. It is responsible of the direct trans-

activation of many cellular or viral genes and it can be modulated by different extracellular stimuli too.

Previously, it was demonstrated how TNF-α, produced by stromal cells and macrophages inside endometrium, as a consequence of bacterial infection, is able to enhance BoHV-4 replication through the activation of NFkB pathway, directly acting on Immediate Early 2 gene (IE2) promoter activation.

For these reasons, it was interesting to investigate how BoHV-4 replication could be decreased. In this work, the interaction between Bovine Herpesvirus 4 (BoHV-4) infected bovine endometrial stromal cells (BESCs) and interferon gamma (IFN-γ) was investigated and the capability of this molecule to restrict BoHV-4 replication in a IDO1 independent and IE2 dependent manner was demonstrated. Furthermore, the presence of a potential responsive elements (RE) interacting with inhibitory transcription factors induced by IFN-γ inside BoHV-4 IE2 gene promoter confirmed this hypothesis. Basing on these data, we could suppose the relationship between IFN-γ axis activation and the reduced BoHV-4 replication, paving the way to new efficient treatment and prevention of uterine diseases.

Furthermore, as the right mechanism by which BoHV-4 infects the endometrial substrate is not still understood, it was of interest to deeply investigate the interaction between the virus and the endometrial layer, analyzing the differences showed by BoHV-4 infected and uninfected bovine endometrial stromal cells, in terms of gene expression patterns. Basing on preliminary data obtained through RNA sequencing analysis (RNAseq), it was noticed that several genes are up-regulated after BoHV-4 infection and that, among these genes, Metalloproteinase 1 is one of the most relevant, because of its possible implication in the development of bovine endometritis. Subsequent analysis elicited by western-blot and real time techniques, were able to corroborate these results, underlying the efficiency of a new experimental approach, based on RNAseq technology, for the study of the onset of pathologies.

Riassunto

Metriti ed endometriti sono le patologie maggiormente responsabili delle perdite economiche negli allevamenti bovini da latte, specialmente nel periodo successivo al parto. Mentre le metriti coinvolgono e si sviluppano in tutto l'utero e sono caratterizzate dalla presenza di sintomi sistemici, le endometriti consistono in una infiammazione che riguarda il solo endometrio, con la presenza di perdite purulente, distruzione della superficie epiteliale, congestione vascolare, edema stromale ed accumulo di linfociti e plasmacellule. Queste patologie, inoltre, possono causare, disfunzione ovarica, con conseguente infertilità e riduzione sia dell'efficienza riproduttiva della vacca sia della produzione stessa di latte. Nonostante queste malattie siano, nella maggior parte dei casi, correlate all'instaurarsi di infezioni batteriche, che possono subentrare nell'utero direttamente durante il parto, il ruolo di alcuni virus nello sviluppo di queste patologie è stato recentemente approfondito e la correlazione tra l' Herpesvirus Bovino 4 e l'insorgere di metriti ed endometriti è stata dimostrata. L' Herpesvirus Bovino 4 (BoHV-4) è un gamma-herpesvirus ed il suo genoma è costituito da una molecola lineare di DNA a doppio filamento con una struttura genomica di tipo B, caratterizzata dalla presenza di un'unica lunga sequenza centrale (LUR) fiancheggiata da multiple sequenze poli-ripetute (prDNA). BoHV-4 è stato isolato sia da animali sani sia da animali con differenti patologie, tra cui malattie oculari e respiratorie, ma soprattutto da casi di metriti, endometriti, vaginiti o aborti. Generalmente, il ruolo svolto dal virus in questo tipo di patologie è associato alla compresenza di altri tipi di patogeni, che possono essere virus, come nel caso del Virus Della Diarrea Virale Bovina (BVDV), o più frequentemente batteri. Usualmente, l'iniziale difesa dell'endometrio bovino nei confronti dei microbi si fonda sul sistema immunitario innato e l'attivazione di specifici recettori cellulari determina la sintesi e la produzione di citochine e chemochine pro infiammatorie, che possono essere in grado di modulare la replicazione di BoHV-4. Il genoma di BoHV-4 possiede due principali trascritti

per i geni Immediate Early (IE), trai quali ORF50/IE2 è il più importante ed il suo prodotto, Rta/ORF50, è fortemente conservato tra tutti gli Herpesvirus. Esso è responsabile della diretta trans-attivazione di numerosi geni virali e cellulari e può essere modulato da differenti stimoli extracellulari. Precedentemente è stato dimostrato come il TNF-α, prodotto dalle cellule stromali e dai macrofagi all'interno dell'endometrio, in conseguenza ad infezione batterica, sia in grado di aumentare la replicazione di BoHV-4 attraverso l'attivazione del pathway di NFkB e direttamente agendo sul promotore di IE2.

Per queste ragioni, è risultato di forte interesse investigare quali potessero essere, invece, i fattori limitanti la replicazione di BoHV-4. In questo lavoro è stata studiata la relazione tra cellule endometriali stromali bovine infettate con l'Herpesvirus Bovino 4 e l'interferon gamma (IFN-γ) ed è stata dimostrata la capacità di questa molecola di restringere la replicazione di BoHV-4 in maniera IDO1 indipendente ed IE2 dipendente. Inoltre, la presenza di alcuni elementi in grado di interagire con l' IFN-γ, all'interno del promotore di IE2 di BoHV-4, ha confermato questa ipotesi. Basandoci su questi dati, abbiamo potuto supporre l'esistenza di uno stretto vincolo tra l'attivazione dell'asse dell'interferon gamma e la ridotta replicazione di BoHV-4, andando a porre le basi per una nuova efficiente cura e prevenzione per le patologie uterine. Poiché il meccanismo corretto attraverso il quale BoHV-4 infetta l'endometrio bovino non è ancora ben compreso, è stato interessante approfondire in maniera più accurata l'interazione presente tra il virus ed il substrato endometriale, analizzando le differenze esistenti tra cellule infettate e non, in termini di espressione genica. Basandoci su dati preliminari ottenuti attraverso analisi con RNA sequencing (RNAseq), abbiamo visto come numerosi geni risultino over-espressi in seguito ad infezione con BoHV-4 e come, tra questi, la Metalloproteasi 1 sia uno dei più interessanti, a causa delle sue possibili implicazioni nello sviluppo delle patologie dell'endometrio uterino bovino. Successive analisi, effettuate tramite westernblotting e real time PCR, sono state in grado di confermare tale dato, sottolineando

l’efficacia di un nuovo approccio sperimentale, basato sul RNAseq, per lo studio dell’insorgenza delle patologie.

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Appendix: List of papers published during 3 years of the PhD course in "Medical and Veterinary Science" (2013-2015).

1 CHAPTER 1

Introduction

1.1 *Herpesvirales*

The International Committee on Taxonomy of Viruses recently changed the criteria for Herpesvirus classification. In fact, the initial *Herpesviridae* family is now noticed as a new order, the *Herpesvirales*, which includes 3 families, 3 subfamilies, 17 genera and 90 species (Davison 2010). Inside this order, there are about 120 viruses, sharing some peculiar structural and biological characteristics and causing diseases both in animals and humans. The name “Herpes” derived from the Greek word “herpein” ("to creep"), associated to a typical trait of these viruses, which is their ability to establish a latent and recurring infection in their host. *Herpesvirales* are double stranded DNA viruses, with a well-defined structural organization, composed by a DNA molecule, core, capsid, tegument and envelope. Every single viral particle is called virion; it is spherical and it has a diameter varying from 150 to 200 nm.

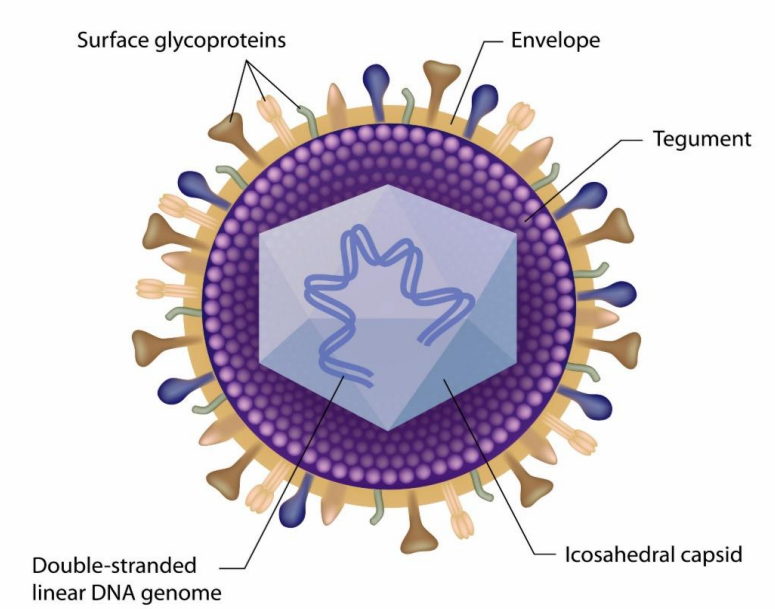

FIGURE 1: Schematic representation of the typical structure of an Herpesvirus (http://www.alilamedicalmedia.com/-/galleries/images-only/microbiology-images/-/medias/f0071360-09ab-11e3-974a-db7884278797-herpes-simplex-virus).

The DNA molecule, composed by a single, linear, double stranded DNA, is included inside a preformed capsid, filled by liquid crystalline array, to constitute the core. The mature capsid is icosahedric, of about 100 nm in diameter, and it is composed by 162 prismatic capsomeres, among which there are 12 pentavalent and 150 hexavalent ones (Davison 2010). The constitution of the capsid structure starts from the assembly of capsomeres around a pro-capsid scaffold through a co-condensation process. This is the reason why all the subunits are connected by weakly bonds (Davison A.J. a. C. 1997). Capsid is immersed inside a poorly-defined proteinaceous matrix, known as tegument (Davison 2010). Historically described as an amorphous structure, it was demonstrated that tegument plays an important role during virion assembly and egress of herpes viral particles (Guo, Shen et al. 2010). Virion is surrounded by an external envelope, which is similar to a membrane because of its lipid bilayer, containing a number of different integral viral glycoproteins, which are the main important determinants of viral pathogenicity (Spear 1985). In fact, glycoproteins mediate viral entry, they spread inside cells and they influence tissue tropism and host range. As consequence, viral membrane glycoproteins are also responsible for the stimulation of host protective immune responses (Spear 1985). Herpes viral genome is composed by linear ds DNA of about 125-240 kb in size (Whitley. 1996), depending on the virus, and from 32 to 75% in G+C content while the number of Open Reading Frame (ORFs) contained ranges from about 70 to more than 200. All Herpesviruses share a common DNA structure based on the presence of long regions and repeated sequences. The number and the position of these sequences varies according to the subfamily, genus and species. Five different genomic structures were identified by Roizman at all. (Roizman 2001): the first one (A) is characterized by a unique region flanked by two direct terminal repeated sequences, which can be short as 30 bp, like in the case of Murine Herpesvirus type 1 (MuHV-1), or larger than 10 kb, as in the case of Human Herpesvirus type 6 (HHV-6); the second one (B), which characterizes rhadinoviruses, presents

variable and multiple flanked repeated sequences; the third one (C) is composed by a unique long and unique short region divided and flanked by multiple direct repeated sequences; the fourth one (D) differs from the previous one as the long unique region and the short unique one possess inverted repeats; finally, in the fifth one (E) the terminal repetitions are also repeated in inverted orientation internally.

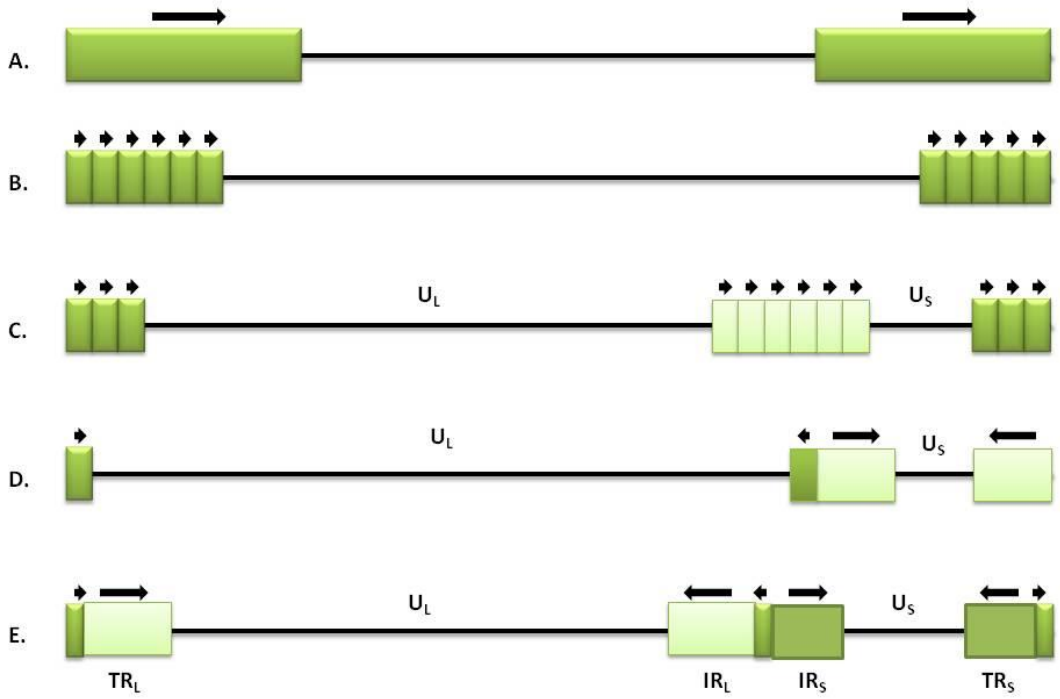

FIGURE 2: Diagrams representing different genome organizations in Herpesvirus. Unique regions are represented by lines, repeated regions by rectangles. Rectangle of different colors stand for repetition of different sequences. The arrows indicate the orientation of the repetitions. U_L: Unique long; U_S: Unique short; TR_L: Terminal Repeat long; TR_S: Terminal Repeat short; IR_L: Internal Repeat long; IR_S: Internal Repeat short. Adapted from (Faquet C.M 2005).

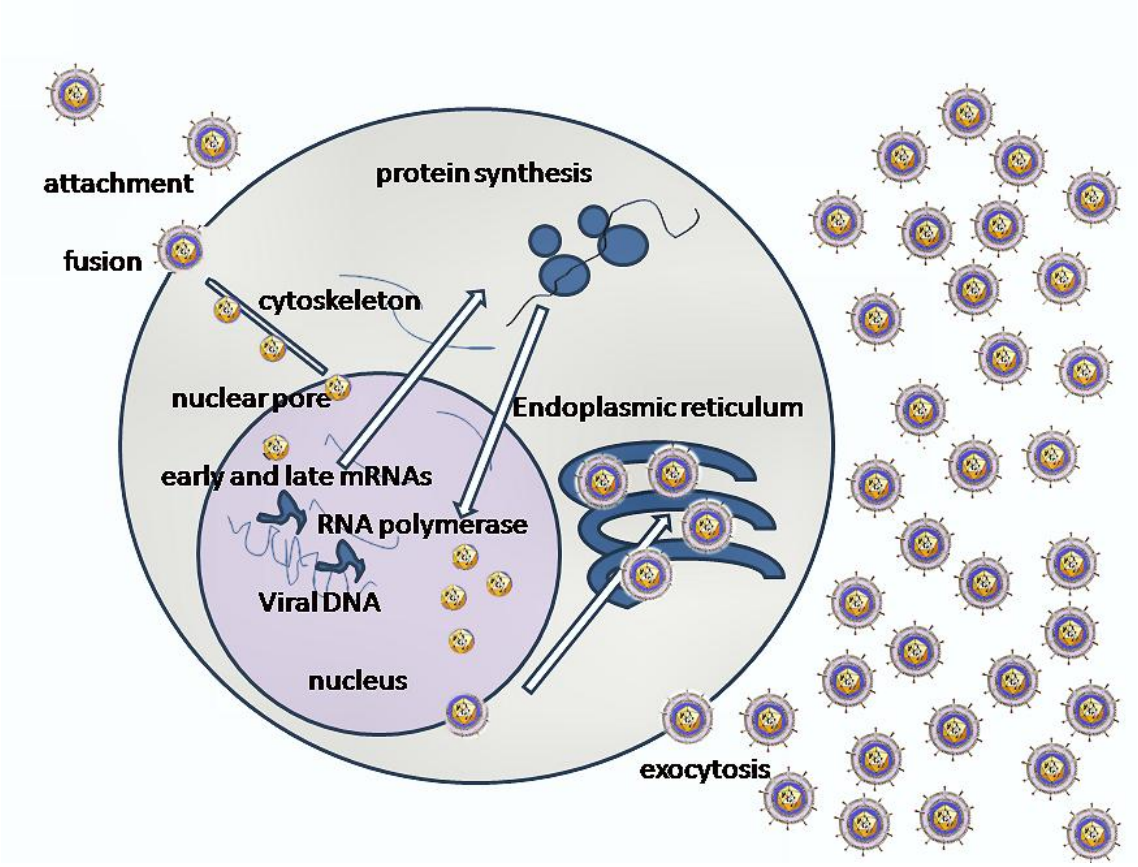

FIGURE 3: Schematic representation of replication mechanism adopted by Herpesviruses (http://www.wikidoc.org/index.php/Herpes_simplex_virus).

Adsorption and penetration involve the interaction of multiple viral envelope proteins with cell surface receptors and it is possible by fusion of the envelope with the plasma membrane. The nucleocapsid is transported to the region of a nuclear pore by unknown mechanisms, while tegument proteins, many of whose functions are unknown, interfere with cellular metabolism. In permissive cells, viral entry is followed by transcriptional cascade activation. Immediate early (IE) genes, which differ among subfamilies, regulate gene expression by transcriptional and post-transcriptional mechanisms. Usually, IE genes encode for DNA replication complex and for a variety of enzymes and proteins involved in modifying host cell metabolism, while the structural proteins of the virus are encoded by late genes. Generally, transcription is directed by host RNA polymerase II. Viral DNA synthesis is supposed to occur from one or

more origins of replication probably through a rolling circle mechanism. Newly synthesized DNA is packaged from the concatamers into pre-formed immature capsids within the nucleus by processes that involve several viral proteins. Nucleocapsids are observed budding through the inner nuclear membrane into the perinuclear space and the resulting enveloped virions may then be transported by exocytosis to the cell surface. The expression of the Immediate Early genes (IE) in infected cell supports the viral lytic cycle and it can be induced by some external stimuli or cell differentiation, causing the passage from the circular episomal state of the input genome to the productive cycle. Herpesviruses are widely spread in nature and their natural hosts are vertebrates. Despite natural and occasional transfers to other species in nature are possible, only one Herpesvirus infecting invertebrates is known, the Ostrid Herpesvirus-1 (OsHV-1) (Roizman 2001). Typical lesions characterizing these viruses are localized vesicular eruptions on epithelial surface, which sometimes can cause damages to the respiratory, digestive and genital mucosal tracts; tissue damage can also produce localized giants cell proliferation in glandular epithelium, necrosis of liver or lymphoid tissues; in particular, neural lesions can lead to diffuse meningo-encephalitis. Furthermore, infection of the foetus is streaky connected to foetal death, abortion or in new borne diseases. Herpesviruses are usually divided in three distinct subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*) according to some of their characteristics, such as biological properties, host range, multiplication rate, cytopathology and latency sites. These families are:

1.1.1 *Alphaherpesvirinae*

This subfamily is characterized by short replication cycle, wide host range, latency in sensorial ganglions and lysis of the infected cells. Examples are Human Herpesvirus-1 (HHV-1, also

known as Herpes Simplex virus 1), Human Herpesvirus-3 (HHV-3, also known as Varicella-zoster virus, VZV), Bovine Herpesvirus-1 (BoHV-1) and Caprine Herpesvirus-1 (CpHV-1).

1.1.2 *Betaherpesvirinae*

This subfamily presents a slow replication cycle, limited host range and latency in monocytes, secretory glands, kidney and other tissues. Among these viruses, there is Human Herpesvirus-5 (HHV-5, or Human Cytomegalovirus, HCMV), which has a high prevalence and it causes severe and sometimes fatal diseases in immunologically immature or compromised patients. HHV-5 is mentioned as the mammalian DNA virus having the largest genome, with its 235 kb double-stranded DNA molecule.

1.1.3 *Gammaherpesvirinae*

These viruses have a limited host range and latency in lymphocytes or in lymphoid tissues. Many of them are not able to replicate in cultured cells (Ackermann 2006). They are divided into the lymphocryptovirus (γ1 Herpesviruses) and rhadinovirus (γ2 Herpesviruses) genera. Epstein-Barr virus (EBV, agent of infectious mononucleosis and Burkitt's lymphoma) is one of the main exponent of lymphocryptoviruses, while rhadinoviruses include a great number of viruses really interesting for medicine and research, such as Saimiriine Herpesvirus-2 (SaHV-2) in monkeys, Equid Herpesvirus 2 (EHV-2) in equines, Murid Herpesvirus-4 (MuHV-4 or Murine gammaherpesvirus-68, MuHV-68) in mice, Bovine Herpesvirus-4 (BoHV-4) in cattle and Human Herpesvirus-8 (HHV-8 or Kaposi' Sarcoma associated Herpesvirus, KSHV) in man. Some other rhadinoviruses have been identified in ruminants: Alcelaphine Herpesvirus 1 (AlHV-1), Ovine Herpesvirus 2 (OvHV-2), Bovine Lymphotrophe Herpesvirus (BLHV) and Caprine Herpesvirus 2 (CpHV-2).

1.2 Bovine Herpesvirus 4 (BoHV-4)

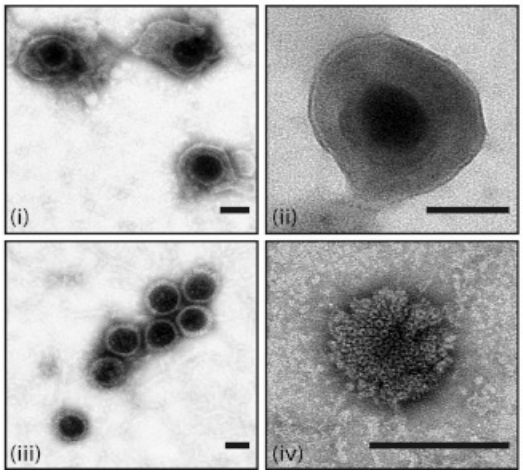

FIGURE 4: BoHV-4 particles as appear at electronic microscope, adapted from (Lete, Palmeira et al. 2012).

Bovine Herpesvirus type 4 (BoHV-4) is a gamma-herpesvirus and it is greatly studied because of its economic importance in dairy cattle. Anciently, it was referred to us as "bovid" as well as all viruses isolated from members of *Bovidae*: cattle, sheep and goats. Naturally, there are several Bovid Herpesviruses (BHV), which were characterized on the basis of the serology and restriction-enzyme analysis of DNA and which were isolated both from healthy animals and from animals with clinical signs of disease.

Virus	*Natural host*	*Clinical signs*
BHV-1	Cattle	pustular vulvovaginitis, bovine rhinotracheitis, abortion, respiratory disease, conjunctivitis, meningoencephalitis
BHV-2	Cattle	Bovine herpesmammillitis, pseudo-lumpy skin disease
BHV-3	Wildebeest	Malignant catarrhal fever
BHV-4	Cattle	Unapparent infection, abortion, metritis, mammary pustular dermatitis, respiratory and enteric infection
BHV-5	Sheep	Pulmonary adenomatotis
BHV-6	Goat	Latent infections, enteritis

TABLE 1: Classification of bovine Herpesviruses, adapted from (Ludwig 1982)

BoHV-4 was firstly isolated in 1963 by Bartha et al. (prototype strain Movar 33-63) (Bartha, Juhasz et al. 1966), in calves showing respiratory and conjunctival signs. At the beginning, in a lot of places worldwide this virus was isolated, giving it different names, such "orphan Herpesvirus", "Movar-type Herpesvirus", "Bovid cytomegalovirus" and "Bovid Herpesvirus-4". To overcome this confusion, Bartha gave to all these isolates, sharing equal characteristics, the name "Bovine Herpesvirus-4" (BHV-4) (Bartha, Fadol et al. 1987). The international committee of viral Taxonomy decided then to adopt the official name of BoHV-4 in 2000 (Fauquet 2005). BoHV-4 was classified among *Herpesviridae* family because of its morphological characteristics visualized to the microscope. It was included primarily among *betaherpesvirinae* because of its similarity to cytomegalovirus, for example the presence of inclusion body inside cytoplasm and nucleus of infected cells as well as its slow replication cycle (Storz, Ehlers et al. 1984). After several studies on BoHV-4 genomic structure and DNA sequence, it was classified as a member of *gammaherpesvirinae.* In fact, BoHV-4 genome is constituted by a B-type structure (Roizman 2001), characterized by the presence of a long unique genome region (LUR) flanked by poly-repetitive DNA (prDNA) elements. Some of its genes are homologous to that of other Herpesviruses such as HHV-8 and SaHV-2 (Bublot, Lomonte et al. 1992; Goltz, Broll et al. 1994) and its genome contains also a typical thymidine kinase gene (Kit, Kit et al. 1986). Later, it was specifically defined as a gamma-2 genus, also called rhadinovirus (Kit, Kit et al. 1986). Among these viruses there are also Alcelaphine Herpesvirus 1 (AlHV-1), Ovine Herpesvirus 2 (OvHV-2), Bovine lymphotrophe Herpesvirus (BLHV) and Caprine Herpesvirus 2 (CpHV-2); BoHV-4 seems not to be phylogenetically related to neither of them (McGeoch, Gatherer et al. 2005). Nowadays, more than 40 BoHV-4 strains have been isolated in the world and they can be grouped in the European strain (or Movar 33-63 like strain), the American strain and the African Buffalo strain (Dewals, Thirion et al. 2006).

1.2.1 BoHV-4 genome

Both the V-test European strain (Palmeira, Machiels et al. 2011) and the North American one (strain 66-p-347) (Broll, Buhk et al. 1999; Zimmermann, Broll et al. 2001) of BoHV-4 genome are now completely sequenced. BoHV-4 has a type 2 genomic organization. It has a linear double stranded DNA of about 144 kb, constituted by a central long unique region of ~110 kb and with a high G+C content flanked by direct polyrepetitive DNA (prDNA). The number of tandem repeats inside genomic termini can be variable according to the strain, 1450 to 2850 bp, but the total amount usually is constant (Ehlers, Buhk et al. 1985).

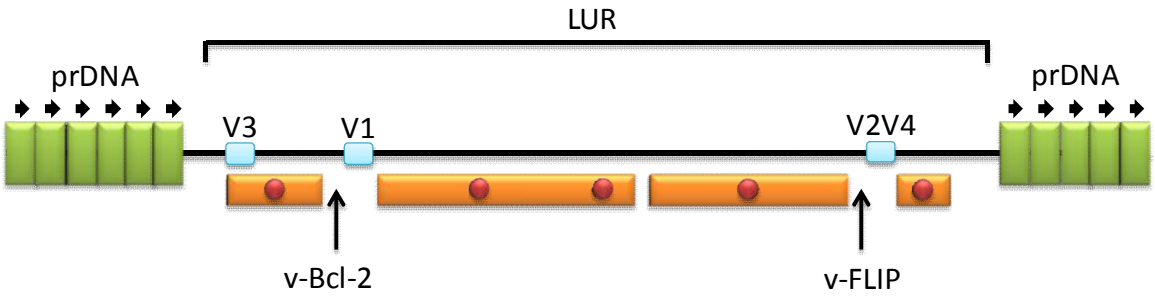

FIGURE 5: BoHV-4 genomic organization (the orange bars represent the 5 conserved blocks within the LUR; V1-V4 the variable regions between strains) (Moran, Perez et al. 2015).

These repetitive regions do not contain any typical coding sequences (Chang and Van Santen 1992) and their importance can be showed during viral replication; in fact they play an important role in the cleavage of concatamers produced after rolling cycle replication and during viral incapsidation. *Pac1* and *pac2* are the two sites, identified in all Herpesviruses, indispensable for cleavage and packaging (Ehlers, Buhk et al. 1985; Broll, Buhk et al. 1999). P*ac-1* consists in a T-A rich region located near the left end of the region and *pac-2* is in a T motif near the right end. Some gammaherpesvirus use these prDNA also during latency, as an anchorage point for viral episomes (Fejer, Medveczky et al. 2003); They could contain an *oriP* latency-associated origin of replication too, as in the case of SaHV-2 (Collins, Medveczky et al. 2002). The BoHV-4 LUR of both 66-p-347 strain and V-test one show 99.1% of identity, but also

a large variability at the genome level (Palmeira, Machiels et al.). The first one has a 108.873 bp LUR, with a 41.4% G+C content, as determined through shotgun analysis by Zimmermann et al, in 2001; the second one, consists of a 108.241 bp LUR with an average G+C content of 41.21%. Genes of gamma-herpesviruses are usually arranged in conserved blocks, whose number and organization vary according to the virus. BoHV-4 has five conserved blocks (1 to 5 in 5'-3' direction), and ORFs inside them generally have same organization and orientation (Bublot, Van Bressem et al. 1990; Bublot, Lomonte et al. 1992). Blocks 1, 2 and 4 include genes "core", conserved in all *Herpesviridae* and probably deriving from a common ancestor, involved in viral capsid formation, in viral replication, DNA incapsidation and capsid release from the nucleus. ORFs in blocks 3 and 5 are proper of gammaherpesvirus. There are some regions, called "inter-block", which are able to encode genes typical for each species. Two regions of multiple direct repeats have been identified inside the LUR, called R1 and R2. R1 consists of several complete and incomplete direct repeats of 23, 25 and 65 bp in length; its length and sequence differ between the strains; R2 consists of two different repeat stretches, R2a and R2b. R2a consists of 28 perfect and 3 imperfect direct repeats of 22-23 bp and R2b contains several different repeats from 8 to 68 bp in length and one inverted repeat with an hairpin-loop predicted structure too. Downstream of the G+C rich R2a stretch, in the R2b hairpin-region, partially overlapping with the gene Bo11 and Bo12, it was identified, a putative *Ori Lyt*, matching with the position of other Herpesviruses. The analysis of BoHV-4 sequence revealed the presence of at least 79 ORFs, 62 homologous to that of other rhadinovirus while 17 are unique for BoHV-4 (Fig.6). ORFs, which are typical and unique of BoHV-4, are named from Bo1 to Bo17, according to their position in the genome from 5' to 3' end, except for Bo5, which has a corresponding gene in SaHV-2. Furthermore, Bo9 and Bo10 have only a position and organization with a great homology to SaHV-2. BoHV-4 has some peculiar genomic traits which makes it different from all the other Herpesviruses. For example, BoHV-4 has no genes

coding for cytokines, cytokines receptor (as in the case of SaHV-2, HHV-8 and EHV-2), interleukine receptors (while SaHV-2, HHV-8, MuHV-68 and EHV-2 have), chemokines or viral macrophage inflammatory protein (as in the case of HHV-8) (Lomonte, Bublot et al. 1995).

BoHV-4 lacks also of genes coding for cycline D, complement regulatory protein, dehydrofolate reductase and thymidilate synthetase, which are present both in SaHV-2 and in HHV-8. Although BoHV-4 is able to encode for proteins involved in cell cycle and survival, such as v-Bcl-2 (ORF 16) and V-FLIP (ORF 17), which are able to prevent apoptosis, nowadays there is no evidence of a relationship between the virus and some lympho-proliferative disorders or transforming capability, usually associated to other gamma-herpesviruses such as HHV-8 or MuHV-68. However, it was demonstrated that BoHV-4 can induce apoptosis at a late stage of infection in permissive cells (Sciortino, Perri et al. 2000).

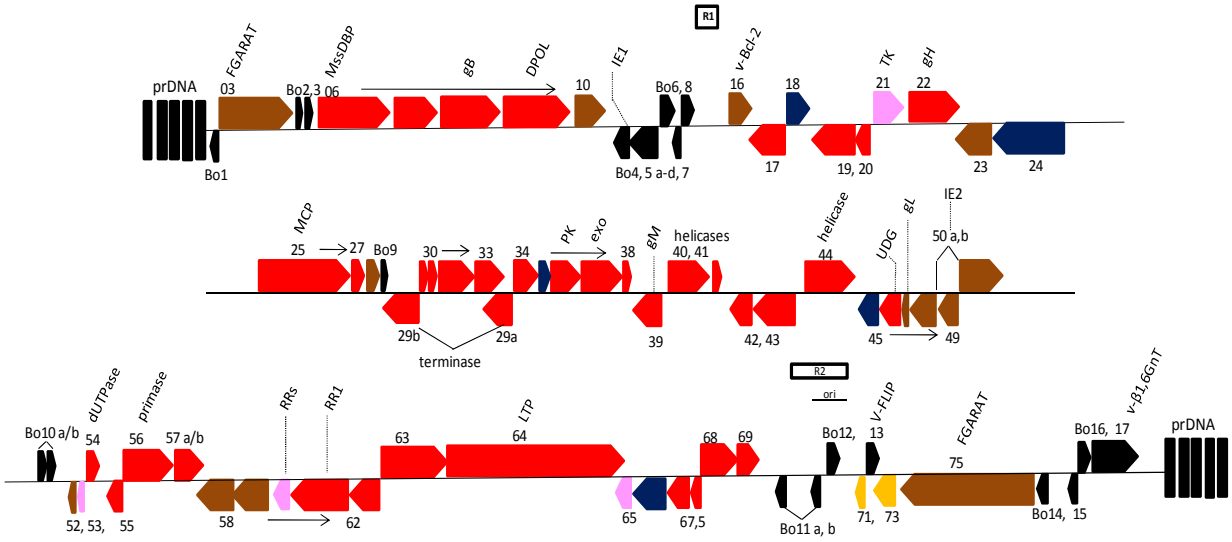

FIGURE 6: BoHV-4 genomic organization, not to scale, adapted from (Zimmermann, Broll et al. 2001). ORFs common to all Herpesviruses are represented in red, in blue those one common to β and γ; in pink those to α and γ; in brown those peculiar to γ herpesviruses. ORFs of γ2 herpesviruses are in yellow; the gene unique for BoHV-4 are in black.

ORFs Bo4 and the spliced Bo5 are part of the Major Immediate Early I transcript (IE1), as described by Van santen; it was demonstrated that this gene, although it is one of the firstly transcribed after infection, is not essential for viral replication and that the removal of this gene can affect virus replication in terms of viral rescue of 1 log respect to the parental virus (Franceschi, Capocefalo et al. 2015). ORF Bo8 overlaps partially with the 1.7kb late gene, described by Bermudez-Cruz et al (Bermudez-Cruz, Zhang et al. 1997). The L1.7 sequence is unique and no homologous sequences have been found among other rhadinoviruses. Interestingly, L1.7 mRNA does not contain long open reading frames (ORFs) coding for functional proteins; in fact, the central portion of this gene is constituted by some repeat arrays, whose varies among BoHV-4 isolates (Capocefalo, Mangia et al. 2013).

Bo10 encodes a non-essential viral envelope protein, gp180, which regulates viral tropism, also interacting with glycosaminoglycans (GAGs). Two different Bo10 mutant viruses were constituted and it was demonstrated that gp180 is dispensable for establishment and maintenance of latency *in vivo*, but it drastically reduced the susceptibility of BoHV-4 to neutralization by immune serum on various cell line (Machiels, Lete et al.); in fact, gp180 appears to be highly O-glycosylated, and removing O-linked glycans from virions sensitized them to neutralization (Machiels, Lete et al. 2011); gp180 seemed also to hide partially several epitopes on gB, gH and gL, providing a O-glycan shield able to hide these epitopes (Machiels, Lete et al.). Machielis et al. demonstrated also that BoHV-4 Bo11 is able to produce an alternative splicing of its Bo10 gene, in order to have distinct viral populations that behave differently basing on the originating cell. It means that the amount of gp180 produced can be higher or lower according to the cell where the virus replicated and that this molecular switch promotes on the one hand its dissemination into the organism, on the other hand, its transmission between hosts (Machiels, Stevenson et al. 2013). Bo17 encodes a viral β-1,6-N-acetylglucosaminyltransferase (β-1,6GnT), that is 81,1% homologous with the human one,

involved in cellular differentiation and in immunological processes. Interestingly, BoHV-4 is the only virus in which this gene has been identified (Vanderplasschen, Markine-Goriaynoff et al. 2000). Not only Bo5, Bo10 and Bo11 are subjected to splicing, but also ORF29, 50 and 57: ORF29 encodes for a terminase and it is present and spliced in all Herpesviruses sequenced until now; ORF50 product is a putative trans-activator, coded by the spliced IE2 gene; the intron of the IE2 transcript contains the complete ORF49, which encodes for a 299 aa protein, conserved among other Herpesviruses (van Santen 1991; van Santen 1993); finally, ORF57 is responsible of the production of a putative post-transcriptional trans-activator. As well as the other Herpesviruses, BoHV-4 has a thymidine-kinase (TK) gene, which is not essential for its replication and for this reason it is usually chosen as a target gene for the insertion of foreign DNA in BoHV-4 vector system (Donofrio, Sartori et al. 2008). TK gene is involved in pyrimidine metabolism, in particular in the salvage pathway of pyrimidine biosynthesis (Lomonte, Bublot et al. 1992). This gene is encoded by ORF21 and it is trans-activated by IE2 gene product (Zhang and van Santen 1995). This protein was not detected among virion proteins while, in the other Herpesviruses, TK is expressed as a tegument protein (Lete, Palmeira et al. 2012).

1.2.2 BoHV-4 tegument and envelope protein composition

BoHV-4 has an icosahedric capsid, constituted by 150 hexamers and 12 pentamers; the nucleocapsid is of about 90-100 nm while the total virion particle is of about 200 nm. The nucleocapsid is immersed inside the tegument and it is surrounded by an envelope containing different viral glycoproteins involved in attachment, penetration, budding and diffusion of the viral particle.

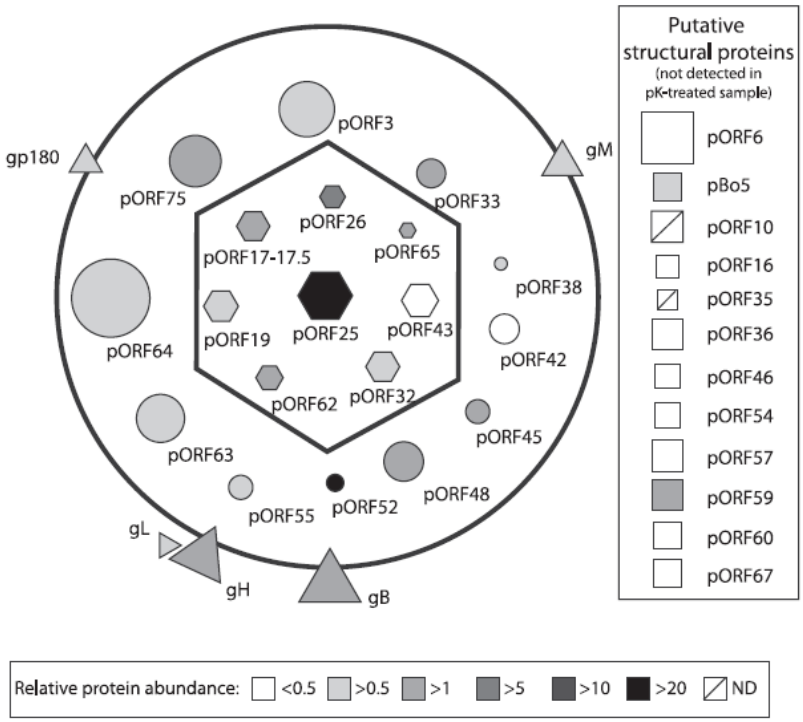

FIGURE 7: Schematic representation of the protein composition of mature extracellular BoHV-4 virions. (Lete, Palmeira et al. 2012).

BoHV-4 tegument is predicted to contain about 15 proteins. Among these, there is ORF52 product, which is one of the mostly abundant, encoding for a small protein of about 20 kDa (Lete, Palmeira et al. 2012). Basing on structural analysis of this protein, it works as a dimer and the N-terminal α-helix is involved in the interaction with other components. For example, ORF52 protein interacts with pORF33, pORF75, gM and gN in Human Kaposi's Sarcoma Virus (KSHV) (Lete, Palmeira et al. 2012). Other tegument protein are produced by ORF6, Bo5, ORF10, ORF16, ORF36, ORF46, ORF54, ORF57, ORF59 and ORF60 (Lete, Palmeira et al. 2012). Among the envelope proteins, there are 5 glycoproteins which are extremely conserved in all Herpesviruses: these are gB, gH, gL, gM and gN. Specifically, BoHV-4 possesses 29 glycosylated polypeptides in the envelope (Dubuisson, Thiry et al. 1989), four of which are mostly studied. The main important protein complex in BoHV-4 envelope is constituted by gp6/gp10/gp17

complex (150kDa/120kDa/51kDa); gp10-gp17 are bounded by disulfides bonds and gp6 by non-covalent bonds. The heterodimer gp10-gp17 derives from the proteolytic cleavage of BoHV-4 gB gene product;it is conserved in all Herpesviruses and it is essential for host cell penetration (Dubuisson, Thiry et al. 1989; Goltz, Broll et al. 1994). BoHV-4 gB protein is essential for viral life and replication and it cannot be replaced either with an homologous protein from other gamma-herpesviruses or with an heterologous protein from other Herpesviruses (Franceschi, Capocefalo et al. 2013). Another complex is characterized by gp11/VP24 (120kDa/16,5kDa), in which glycosylated gp11 is bound to not glycosylated VP24 through a non-covalent bond, and six neutralizing epitopes were identified in this complex (Dubuisson, Thiry et al. 1989). The third major glycoprotein of BoHV-4 envelope is gp8 (135kDa), involved in heparin-like molecules contacting at the cell surface. This late protein is poorly glycosylated and its secreted form can interferes with the immune humoral response of the host (Dubuisson, Koromyslov et al. 1992); a lot of studies have demonstrated that the protein identified as gp8 in reality is another form of gp6/gp10/gp17 protein. The last major protein characterized is gp1 (>300kDa), a strictly late protein as described by Dubuisson et al. (Dubuisson, Bublot et al. 1991). Other BoHV-4 envelope proteins are the homologous of gH and gL proteins; in particular, gp110, corresponding to the gH glycoprotein and gp31-35 or gp45-65, corresponding to gL protein. gH is important for fusion of viral envelope with host membrane; gL role was usually associated to gH glycoprotein because it was demonstrated that BoHV-4 lacking gL showed altered gH glycosylation and incorporation, although it remain infectious (Lete, Machiels et al. 2012). BoHV-4 lacking gL glycoprotein showed also poor growth associated with an entry deficit impaired endocytosis (Lete, Machiels et al. 2012).

Finally, there is gp21 (26-27kDa), described in the sera of infected animals. gM and gN are not greatly studied in gamma-herpesviruses, and their function has never been determined. BoHV-4 does not present gD, which is the major immunogen of BoHV-1, or gE, which is used as a

marker in vaccine against BoHV-1. It was also demonstrated that Bovine Herpesvirus 4 detects some proteins from the host to constitute virion components. Among these proteins actin, cofilin-1 and annexin-2 are particularly abundant. In particular, annexin-2 is associated to the capsid of BoHV-4 as well as other viral species (Lete, Palmeira et al. 2012).

1.2.3 BoHV-4 *in vitro* infection

1.2.3.1 *In vitro* host range

BoHV-4 is able to replicate both in primary and in immortalized bovine cells coming from different kinds of tissue such as kidney, tests, lungs, skin, mammary gland, nasal turbinate, endothelial cells, B-T lymphocytes, macrophage-derived cells, histiocytes, thyroid, embryo tracheal primary cells, fetal bovine bone marrow, in lymphosarcoma calf thymus; also in different kidney's continuous lines (MDBK for Madin Darby Bovine Kidney, GBK for Georgia Bovine kidney and BEK for Bovine Embryonic cells) or lung's derived (EBL for embryonic bovine lung). BoHV-4 can establish persistent infection in bovine macrophage cells (BoMac) (Donofrio and van Santen 2001). It was demonstrated that BoHV-4 can infect and replicate also in cell lines deriving from different origins, among which there are buffalo, sheep, goat, swine, cat, dog, rabbit, mink, turkey, ferret, horse, chicken rat, mouse, hamster (Bartha, Juhasz et al. 1966; Egyed 2000; Markine-Goriaynoff 2003), but also some human cell line (Egyed and Bartha 1998; Donofrio, Cavirani et al. 2000; Gillet, Minner et al. 2004). The ability of BoHV-4 to infect human cells was considered of particular interest because of the potential use of BoHV-4 as a viral vector for therapeutic and vaccine purposes or oncolytic and gene therapy; in fact, it was demonstrated the oncolytic capacity of BoHV-4 in the treatment of glioma in mice and rats (Redaelli, Franceschi et al. 2012). The fact that BoHV-4 possesses a broad host range, which is not common among other gamma-herpesviruses, is probably related to its interaction with host cell heparan-like structures as a first contact (Vanderplasschen, Bublot et al. 1993), which

are usually present on the surface of the majority of vertebrate's cells. This observation could lead to the hypothesis that a cross-species transmission can be possible among animals, even humans. To date there are no reported cases of human infection with BoHV-4. Scientists demonstrated that human serum neutralizes efficiently BoHV-4 through activation of complement by natural antibodies raised against host cell derived epitopes expressed on the virion surface (Machiels, Gillet et al. 2007).

1.2.3.2 Viral replication *in vitro*

BoHV-4 replication cycle is strictly associated to S phase of the cell cycle (Vanderplasschen, Goltz et al. 1995); it has a slow growth (Storz, Ehlers et al. 1984), which could be due to the slow rate of thymidine kinase induction in infected cells (Kit, Kit et al. 1986). Kinetics of extra and intracellular viral particles produced show that BoHV-4 is a cells-associated virus (Storz, Ehlers et al. 1984). Usually, Cytopathic Effect starts to be visible 48-72 hours after cell infection and it is characterized by the comparison of rounded cells, around the monolayer, without the formation of canonical plaques.

BoHV-4 genes are usually divided in three different kinetic classes according to the time of expression. They are chronologically expressed as immediate early (IE or alpha), early (E or beta) and late (L or gamma) proteins. Immediate early proteins are firstly expressed after release of the viral genome from the capsid into the nucleus. E protein expression occurs after synthesis of the IE protein, while L protein expression is the last one to be executed.

BoHV-4 interacts with heparan sulfate-like structures of host cellular surface through its gB envelope protein; probably, there are some other protein interactions able to make stronger this bond, but it is not well clarified until now. Immediately after the attachment, the envelope fuses with the plasma membrane, and the envelope proteins as well as the nucleocapsid are released in the cytoplasm and transported to the nucleus through the interaction with microtubules.

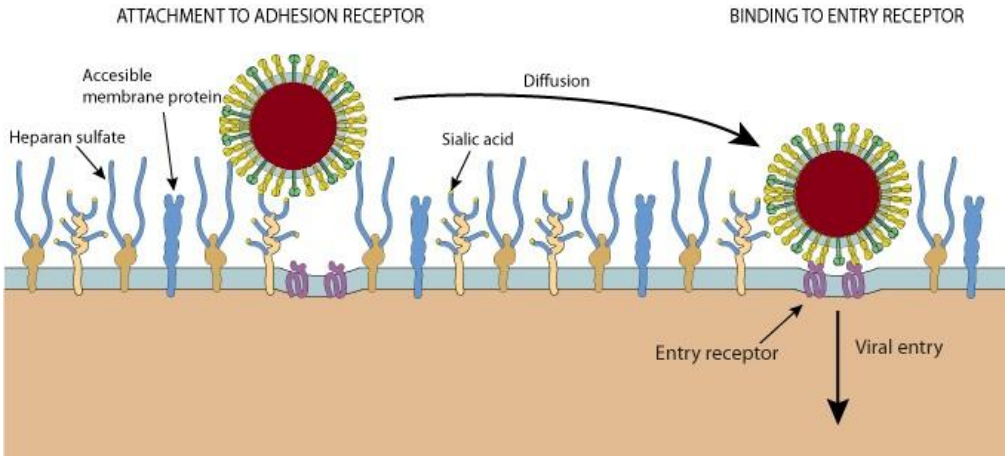

FIGURE 8: Schematic representation of viral attachment and mechanism of viral entry into the cell (http://viralzone.expasy.org/all_by_protein/956.html).

Viral DNA is released into the nucleus passing through a nuclear pore. After DNA circularization, some proteins of the envelope interact with the host cell transcriptional machinery to induce the IE genes transcription by the host RNA polymerase II. IE1 and IE2 proteins are the two major immediately early proteins of BoHV-4; they migrate to the nucleus where they block the expression of IE gene and activate the one of E and L genes. E genes are expressed at the major level from 4 to 8 hours after the infection; they are involved in the metabolism of nucleotides and in DNA replication. Between late genes (γ), we distinguish γ1 (partial late), whose expression is accelerated by DNA synthesis, and γ2 (real late). γ proteins are usually constituted by structural protein, which are useful for the assembly of new forming capsids. Replication of viral DNA starts from the Ori (Origin of replication) through a rolling circle mechanism, producing concatamers subsequently cleaved and packaged inside new capsids. The new-formed capsids are then transported to the periphery of the nucleus. The passage through at first the endoplasmic reticulum and then the vesicles of Golgi, provides probably the precursor or the final viral envelope proteins, according to a process which is not

still clear. BoHV-4 can establish both latent and lytic infection, which is usually initiated on rare occasions. BoHV-4 possesses three main transactivating proteins, transcribed from immediate early genes, which are able to initiate the lytic cycle. These proteins are ORF50 (Rta/IE2 protein), ORF57 (Mta/IE1) and Bo4/5. Promoter activation by these proteins has also a strong effect on DNA synthesis from the *Ori* of lytic replication; as a result, virions are generated and released from the productively infected cells (Ackermann 2006). The typical inclusion bodies appear in the cytoplasm from 48 to 72 hours after the infection and are composed by viral particles at different stages of maturity, with capsids empty or full, enveloped or not. On the contrary, the absence of replication generally characterizes the latency. There is not a clearly pattern of gene expression required for establishment, maintenance, and reactivation of latency. Usually, the beginning of a latent infection is in common with lytic one but, when the viral DNA arrives to the nucleus, it does not start the classical gene transcription cascade through the expression of alpha and beta genes. During BoHV-4 latency, the viral genome persists in the nucleus as an episome, a circularized form of viral DNA, which is distributed to the new formed cells thanks to the protein LANA (latency associated nuclear antigen, coded by ORF73). LANA binds the cellular chromatin with its N-terminal and it interacts with different sequences in prDNA with its C-terminal. In this way it starts the viral replication and the anchorage of episome to cellular chromosomes is also permitted. BoHV-4 ORF73 deletion does not affect the virus replication *in vitro*, but it affects virus persistence *in vitro* and it prevents latent infection *in vivo* in a rabbit model (Thirion, Machiels et al. 2010). It was demonstrated that in BoMac cells persistently infected with a recombinant BoHV-4 carrying the neomycin-resistance gene and drug selected with geneticin, the viral DNA can exist as an episome or it can be integrated in the cellular DNA, at random sites, in this way confirming that persistent BoHV-4 infection is compatible with cell survival and replication (Donofrio and van Santen 2001). The capability to establish a lytic cycle is really important to understand the kind of

infection, which can characterize a cell line. In fact, a cell is usually defined “susceptible” to the infection when it possesses on its surface the receptor required for virus attachment and entry; a cell can become also “permissive” to the infection when it is able to support a complete productive infection cycle. There are some cases characterized by non-productive infections: the latent infection, which can be usually reactivated from different exogenous stimuli in a productive infection; the abortive infection, in which the viral replication is irreversible blocked, evolving to cellular death or to a totally unapparent infection. It was observed that over-expression of Immediate Early protein 2 (IE2) was able to induce virus replication in non-permissive cell lines (Donofrio, Cavirani et al. 2004).

1.2.4 *In vivo* BoHV-4 infection

1.2.4.1 *In vivo* host range

Although the bovine specie is considered the natural host of BoHV-4, it was observed that BoHV-4 has been isolated in several animals. For example, in African buffalo (*Syncerus caffer*) it was observed a prevalence higher than in cattle, and this information can mean that, maybe, African buffalo was the original host specie for BoHV-4, and that other species, including domestic cattle, acquired the virus more recently (Dewals, Thirion et al. 2006). BoHV-4 was also been isolated in the American bison (*Bison bison*), zebus (*Bos indicus*) (Moreno-Lopez, Goltz et al. 1989), sheep and goat. Some felines are also susceptible to BoHV-4 infection: lions (Egyed, Kluge et al. 1997) or cats suffering from urolithiasis (Fabricant, King et al. 1971), causing severe pathologies, varying from conjunctivitis to pneumonia, often with a lethal prognosis (Egyed, Kluge et al. 1997). BoHV-4 strain was isolated from the kidney of an apparently healthy monkey (*Aotus trivirgatus* (Bublot, Dubuisson et al. 1991). In monkeys this virus is neither pathogen not oncogenic, and a high prevalence has been demonstrated, such as 42%. An Herpesvirus related to BoHV-4 could circulate between exotic animals such as Asian

elephants (*Elephas maximus*), which presented antibodies against BoHV-4 (Metzler, Ossent et al. 1990) and in black rhinoceros (*Diceros bicornis*), in which a DNA polymerase strictly related to that of BoHV-4 was identified. Some experimental animals can be infected by BoHV-4, such as the rabbit, which is considered a good model to study BoHV-4 biology (Egyed, Kluge et al. 1997; Egyed 2000; Lin, Jiang et al. 2000; Egyed and Baska 2003). In fact rabbit can support BoHV-4 replication and, eventually, persistence. The site of viral infection is the lymphoid tissue, but the virus was isolated also from conjunctival swabs and spleen or, during latency, in bone marrow, lung, kidney, salivary glands and liver with a low titer (Osorio, Reed et al. 1982).

1.2.4.2 *In vivo* pathologies

BoHV-4 was isolated both from healthy and from cattle with diseases. BoHV-4 infection is firstly associated with ocular and respiratory diseases; in fact, it was isolated from animals with keratoconjuncivitis and respiratory illness, with nasal discharge, cough, dyspnoea and pulmonary lesions (Bartha, Juhasz et al. 1966; Mohanty, Hammond et al. 1971). The development of genital diseases, such as metritis and endometritis, vaginitis or abortion, is also related to BoHV-4 infections. The role played by the virus in relation to this kind of pathologies is usually associated to the co-presence with other pathogens, which could be other viruses, such as the Bovine Viral Diarrhea virus in the case of abortion (Reed, Langpap et al. 1979), as well as bacteria, in the case of post-partum metritis and endometritis. BoHV-4 was also detected, as a secondary pathogen, in mastitis cases, as well as in cellular fraction of milk (Donofrio, Cavirani et al. 2000), in skin lesions , such as ulcerative mammilitis or mammary pustular dermatitis, enteric diseases and some other pathologies, for example neoplastic diseases, as lymphosarcoma, ocular carcinoma, T cell lymphoma and some others (Kaminjolo, Mugera et al. 1972; Anson, Benfield et al. 1982; Toho 1985). It was observed that the experimental inoculation of the virus in cattle or experimental animals does not produce clinical signs in each case, so that its pathogenicity is

still unclear. For example, intranasal and intratracheal inoculation of BoHV-4 is able to reproduce almost all the respiratory symptoms, leading sometimes to cattle death (Mohanty, Lillie et al. 1972) even if, in some cases, *Pasteurella multocida* was also isolated from the same experiment. Inoculation of virus isolated from a metritis case into the fetus causes unequivocally to its death, about at 3 or 4 month of gestation (ParK and Kendrick 1973); intranasal and intra-mammary inoculation reproduces subclinical mastitis (Wellenberg, Bruschke et al. 2002), while intradermal inoculation into the udder produces vesicular lesions (Osorio and Reed 1983); experimental infection can induce a febrile response if a BoHV-4 isolated from a mammary pustular dermatitis is used but enteric symptoms were never reproduced in cattle (Eugster 1978/1979).

1.2.4.3 *In vivo* transmission and replication

BoHV-4 transmission probably occurs by direct contact with the respiratory route, inspiring air or swallowing virus aerosol, or with the genital or the alimentary tracts. In fact, BoHV-4 is usually detected or from upper respiratory tracts or from genital tracts, even if genital transmission is not yet totally understood. It was demonstrated also that the vertical transmission to the foetus is possible (Deim, Szeredi et al. 2007). From the apparently healthy animals the virus is continuously detected from the blood leukocytes, spleen and lymph nodes for months (Osorio, Reed et al. 1982).

In general, BoHV-4 replicates firstly in the entry site, for example the oronasal route, and then it disseminates through mononuclear blood cells, which usually are monocite/macrophage lineage cells; in fact, endothelial cells are sensitive to BoHV-4 infection and probably the virus spreads from the mononuclear blood cells to the endothelial cells, opening the route to infect tissues and organs. The virus replicates also in epithelial cells of the intestines, larynx, trachea and bronchioles, causing slight catarrhal symptoms and fever, so that the disease is usually a result cell destruction and it can cause also an organ specific, lytic infection.

The presence of viral DNA in blood of infected animal is detectable from 10 to 30 days after the infection (Egyed, Berencsi et al. 1999) and viremia, not always detectable, can re-appear at different times. Usually, conjunctiva, upper respiratory tract mucosa, and genital mucosa are considered as primary and secondary multiplication sites, causing ocular, nasal and vaginal excretions.

Rabbits experimentally infected with BoHV-4 show an active replication of the virus in the spleen as well as in macrophages, which can be the site of both acute and latent infections; identification of BoHV-4 in non-T, non-B cells located in the marginal zone of the spleen of persistently infected cattle and rabbits has implied that cells of the monocytes/macrophage lineage are one site of persistency for BoHV-4 infection (Osorio, Reed et al. 1982; Osorio, Rock et al. 1985).

For these reasons, although different kinds of tissues were proposed as a probably site for BoHV-4 latency, it was established that splenic mononuclear cells (macrophages) could be the main site for viral persistence (Lopez, Galeota et al. 1996). It was observed that animals euthanized more than one year after the experimental infection possessed viral genome specifically in lymph nodes and medulla, spinal cord and trigeminal ganglion, supporting the hypothesis that even the nervous system could be a latency site (Asano, Inoshima et al. 2003). Lymphoid organs and mononuclear blood cells were suggested as a site of viral latency also in cattle. External stimuli, natural as the parturition, or experimental as dexamethasone treatment, can reactivate BoHV-4 from latency.

1.2.5 Immune response to BoHV-4, prophylaxis and diagnosis

BoHV-4 is associated to a really weak immune response, which can result from the minimum exposure of the virus to the immune system because of its cell association and its capacity to induce latency. After BoHV-4 infection in cattle, high titre of anti-BoHV-4 antibodies can be identified through indirect immuno-fluorescence test (IFI) (Storz, Ehlers et al. 1984; Castrucci,

Frigeri et al. 1987; Castrucci, Frigeri et al. 1987), but a weak or null production of serum neutralizing antibodies can be observed. When a response is detectable, serum neutralizing antibodies (SNA) appeared from 22 to 34 days after the first infection. Mohanty et al. reported the absence of antibodies in BoHV-4 infected calves but, when challenged with the virus 8 weeks after the primary infection, the surviving calves were found to be immune to the virus (Mohanty, Lillie et al. 1972).

Nowadays, no vaccines are developed against BoHV-4 infection. As it was observed a variation in the pathogenic potential of several isolates of BoHV-4 (Castrucci, Frigeri et al. 1987; Castrucci, Frigeri et al. 1987), it could be possible to generate a vaccine based on the use of non-pathogenic isolate. However, the control of BoHV-4 infection is now only provided by hygienic measures or by the identification and physical separation of latently infected animals from the serum-negative ones. The serum-positive cows, which are in the post-partum period, have to be controlled in a particular way, because they can excrete a lot of virus, for long period in the uterine exudates (Thiry 2000).

Not many tests are available for BoHV-4 detection; usually, the isolation of the virus from tissues or explants is successfully adopted and viral replication can be measured by conventional cell culture cultivation. Complement fixation, dot-immuno-blotting assay, virus neutralization, immuno-fluorescence (IFAT) and ELISA tests have been used for serological studies (Naeem and Goyal 1990); an high serum-conversion was also observed after primary infection as well as re-infection or viral reactivation. Later, during the infection, also the complement-dependent neutralization can be successfully used, even if the virus elicits a poor neutralizing antibody response. This kind of test was demonstrated to show a particular efficiency in animal immunized with a recombinant BoHV-4, deleted in Bo10 gene, responsible for hiding the serum neutralizing epitopes of gB and gH/gL (Machiels, Lete et al.). BoHV-4 can be detected also by specific PCR, for example with primers designed over BoHV-4 gB sequence

(Boerner, Weigelt et al. 1999) or thymidine kinase gene (Egyed, Ballagi-Pordany et al. 1996; Egyed and Bartha 1998; Wellenberg, Verstraten et al. 2001). Other techniques include *in situ* hybridization or restriction enzyme analysis, which is able to distinguish different BoHV-4 isolates (Ludwig 1982).

1.2.6 BoHV-4 as a vector

Several characteristics made BoHV-4 an ideal viral vector: (1) its ability to establish a persistent infection, both in the natural host, the cattle, and in the experimental host, the rabbit, maintaining the viral genome as an episome, (2) the lack of oncogenicity, (3) the possibility to delete even the 30% of the viral genome preserving the viral replication and so, (4) the high capacity to include exogenous DNA, (5) the fact that it is completely sequenced, (6) the possibility to manipulate its genome as a Bacterial artificial chromosome (BAC), (7) no correlation with diseases, with the exception for uterine diseases in cattle as secondary pathogen, and (8) broad host range both *in vitro* and *in vivo*.

The possibility to manipulate BoHV-4 as a BAC made possible the generation of different recombinant viruses, expressing exogenous antigenic proteins, both for vaccinal and for therapeutic purposes. For example, the efficiency of BoHV-4 for the treatment of glioma was demonstrated. Starting from the evaluation of the *in vitro* susceptibility of rat glioma F98 cell line to BoHV-4 infection, the virus was then injected into the rat brain, observing as glioma cells were induced to a non-apoptotic death, without the presence of any clinical signs. For all these reasons, BoHV-4 could be considered a good vector for glioma therapy (Redaelli, Cavaggioni et al. 2008). In fact, it was observed also that a recombinant BoHV-4, expressing an enhanced green fluorescent protein (EGFP,) was able to infect only glioma cells, being safe for the nervous system (Redaelli, Mucignat-Caretta et al. 2010). Taking advantages from these information, a new recombinant BoHV-4, expressing the thymidine kinase gene of HSV-1 (TK-HSV-1), was used to be injected into the rat glioma in combination with the pro-drug

ganciclovir, which is usually converted in a toxic compound by the thymidine kinase gene product. It was observed an high reduction of glioma, with no consequences for rat (Redaelli and Denaro 2011).

The capability of BoHV-4 to be used as a viral vector for vaccine purposes was efficiently tested in mice (Franceschi, Capocefalo et al. 2011) as well as other animals, such as rabbits, swine, goats, sheep (Donofrio, Franceschi et al. 2009), and chicken (Donofrio, Manarolla et al. 2008).

For example, recombinant bovine Herpesvirus 4 (BoHV-4) vectors, expressing different Monkeypox virus (MPXV) glycoproteins, A29L, M1R and B6R, were investigated in terms of protection from a lethal MPXV challenge in STAT1 knockout mice. BoHV-4-A-CMV-A29LgD106ΔTK, BoHV-4-A-EF1α-M1RgD106ΔTK and BoHV-4-A-EF1α-B6RgD106ΔTK were successfully constructed by recombineering, and their capacity to express their transgene was demonstrated. A challenge study was performed and all three recombinant viruses appeared safe, without weight-loss or obvious adverse events. It was also demonstrated that BoHV-4-A-EF1α-M1RgD106ΔTK alone or in combination with BoHV-4-A-CMV-A29LgD106ΔTK and BoHV-4-A-EF1α-B6RgD106ΔTK, was able to protect, 100% alone and 80% in combination, STAT1(-/-) mice against mortality and morbidity (Franceschi, Parker et al. 2015).

1.2.7 Cloning of BoHV-4 as a Bacterial Artificial Chromosome (BAC)

BoHV-4 was cloned as a BAC for the first time in 2005 by Gillet *et al.* (Gillet, Daix et al. 2005). BoHV-4 BAC cassette is about 10 kb and it includes an origin of replication as well as some genes, which are essential for BAC replication, such as *redF* and *repE*, and to control the replication rate, such as *parA* and *parB*. Inside the BAC cassette, there is also an antibiotic resistance marker, which is a chloramphenicol resistance, to make possible the metabolic selection. It is also flanked by 500-1000 bp sequences, homologous to the target sequence where the BAC vector is inserted, which are Bo2 and Bo3 inter-genic regions of BoHV-4 genome. The insertion of the EGFP (Enhanced Green Fluorescent Protein) marker is useful and

necessary to isolate BAC-containing recombinant virus and to easily visualize the infected cells by fluorescence microscopy analysis. In order to have the possibility to reconstitute the wild type genome of the virus, the BAC cassette was flanked with two *loxP* sites, exploiting the Cre-loxP system. The BAC cassette, flanked by viral sequences, is usually transfected in cells, resulting in the insertion of the BAC cassette in viral genomes by homologous recombination. During the replication cycle, BoHV-4 forms some circular intermediates which can be extracted from cells and used to transform *E.coli*; in this way, a shuttle vector containing the viral genome cloned as a BAC is constituted, and subsequently it can be manipulated in bacteria cells. The so purified BAC clone can be transfected in eukaryotic permissive cells BEK fincre (Bovine embryo kidney cells) expressing the *cre* recombinase (Gillet, Daix et al. 2005) to reconstitute the recombinant virus, thanks to the presence of two *loxP* sites into the BAC cassette.

A phage-mediated recombination systems was used in *E.coli* through the λ phage Red function. *Exo* and *Beta* are two genes able to generate recombinants in the complete absence of RecA, through a process called single strand annealing. Two homologous DNA sequences receive a double strand break, provided by *Exo,* which can degrade the 5'ended strands exposing 3' overhangs, and these breakings can be bound by *Beta*, which is able to anneal the two strands and generate recombinants. The third gene involved in recombination is *Gam,* which encodes a RecBCD exonuclease inhibitor and protect linear DNA-targeting cassette from degradation by RecBCD. These three genes can be expressed from a stably integrated defective λ prophage, where *exo, bet and gam* are controlled by the strong phage promoter *pL*, under stringent control of the temperature-sensitive repressor, *cI857*. In this system *exo, bet and gam* are not expressed when the bacteria are kept at 32°C; on the contrary, the exposition at 42°C for 15 minutes rapidly induces at high level of these genes and homologous recombination is

efficiently performed. Genetic engineering with phage-encoded genes has been named as "Recombineering", a conventional term used to describe homology-dependent, recombination-mediated, genetic engineering (Court, Sawitzke et al. 2002; Warming, Costantino et al. 2005).

Warming et al., described the development of a novel Galk-based selection system for BACs manipulation in *E.coli* SW102 bacteria cells. The *E.coli* galactose operon consists of 4 genes, GalE, GalT, Galk and GalM, necessary for the use of galactose as the unique carbon source. The Galk gene, codes for galactokinase, an enzyme able to convert the galactose in galactose-1-phosphate; it is also able to catalyze the phosphorylation of a galactose analog, 2-deoxy-galactose (DOG) in a toxic compound. The *E.coli* SW102 strain is $Galk^-$ and it is unable to use galactose as carbon source; leading to positive clone selection in galactose enriched minimal medium and the negative one in medium enriched with DOG (Warming, Costantino et al. 2005). Donofrio et al., added another selection step, flanking the Galk cassette with the gene which confers resistance to Kanamicine; the addiction of a negative selection in kanamicine reduces the number of background colonies growing during the selection step (Donofrio, Franceschi et al. 2009).

For BoHV-4 expression system, It was decided to insert KanaGalk expression cassette inside Timidine Kinase gene (TK) because it was demonstrated that this gene is not essential for viral replication and that its deletion does not consistently affect viral behavior *in vitro* (Donofrio, Cavirani et al. 2002). Recently, it was observed also that an highly attenuated viral vector could be generated by disrupting the late gene encoding the 1.7 kb polyadenylated RNA (L1.7). In fact, BoHV-4-A-KanaGalKΔL1.7 showed attenuation in terms of competence to reconstitute infectious viruses, viral replication, and plaque size when compared to the wild type as well as other recombinant viruses deleted in TK gene. It was tested also the capability of BoHV-4-A-KanaGalKΔL1.7 to deliver and express a heterologous antigen, for example the vesicular

stomatitis virus glycoprotein (VSVg) expression cassette, replacing the KanaGalK cassette, generating BoHV-4-A-EF1αVSVgΔL1.7. BoHV-4-A-EF1αVSVgΔL1.7 infected cells robustly expressed VSVg, thus confirming that the replication deficiency resulting from L1.7 disruption did not prevent heterologous gene delivery and expression (Capocefalo, Mangia et al. 2013).

1.2.8 BoHV-4 IE2/Rta

Two major Immediate Early (IE) RNAs were identified from BoVH-4 genome, during its lytic infection in MDBK (Madin Darby bovine kidney) cells. The first one is a spliced 1.7 kb RNA, transcribed from right to left on BoHV-4 restriction map. The nucleotide sequence of the DNA encoding the major IE early RNA contains 60 % A+T and 40 % G+C nucleotides. It does not contain multiple repeat motifs, which are characteristic of the major IE promoter regions, and it encodes for a 284 aa protein of about 33KDa, called IE1. IE1 is a putative DNA binding protein and it could be involved in zinc finger structures formation near its N-terminal, because of its similarity to HSV-1 IE110 protein. The other IE RNA, transcribed from left to right on BoHV-4 genome, is of about 1.8 Kb, and it encodes a 61 KDa protein that shows amino acid homology with EBV trans-activating protein R, Rta (van Santen 1991; Chang and Van Santen 1992) and also with SaHV-2 Rta. BoHV-4 IE2 gene product, called Rta/Orf50, is encoded by open reading frame 50 (ORF 50) and it is well conserved among Herpesviruses. The gamma-Herpesviruses ORF50 transcripts have similar genomic location, amino acid sequence and splicing pattern and they have also a typical architecture, essentially constituted of two exons, separated from an intron, that contains ORF49 gene, located in the opposite orientation. Its promoter is probably located inside the Rta/ORF. After the splicing, it results of a single major Rta transcript, but differential splicing can also occur (Damania 2004; Damania, Jeong et al. 2004) (Fig. 3).

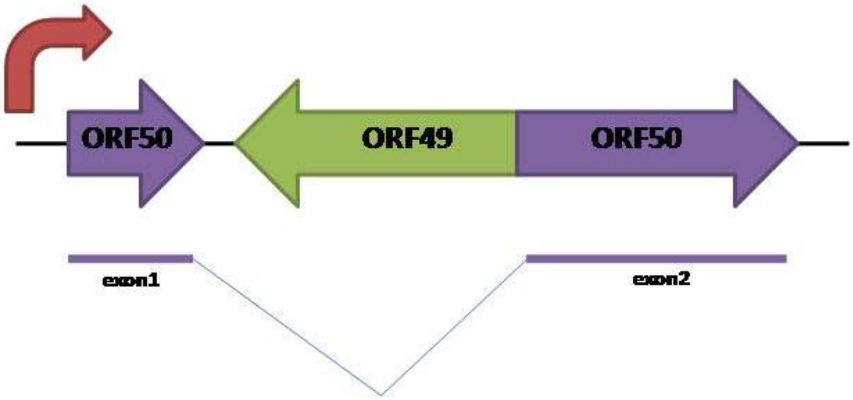

FIGURE 9: Organization of Bovine-Herpesviruses-4 ORF50.

Transactivation studies showed that the BoHV-4 IE2 gene product specifically trans-activates the promoter-regulatory regions of the thymidine kinase gene (Zhang and van Santen 1995). The exact mechanisms whereby Rta/Orf50 activates expression are still not clear, but two possibilities can be supported by the existing data: (1) Rta can directly trans-activate by binding the target gene promoters through specific RRE (Rta Responsive Element) target sequences to stimulate transcription or (2) by interacting with cellular factors and or other viral factors to enhance transcription indirectly.

Donofrio et al., were able to activate the lytic replication of the virus in a non-permissive cell line, RD4 (human rhabdomyosarcoma cells), by over-expression of BoHV-4 IE2 gene, provided in trans by a plasmid vector, suggesting that even BoHV-4 Rta was the master switch protein involved in viral replication, not only during reactivation of persistently infected permissive cells, but also in *de novo* infection of non permissive cells (Donofrio, Cavirani et al. 2004). Furthermore, the high tropism of BoHV-4 for the endometrial stromal and epithelial cells is probably due to the existence of an endometrial-specific TATA box in IE2 promoter. Some extracellular stimuli belonging to intrauterine microenvironment can stimulate the BoHV-4 IE2 gene promoter in a concentration dependent manner and it has been documented challenging bovine endometrial stromal cells (BESC) with Lipopolysaccharide (LPS), which caused the

activation of IE2 promoter in BESC (Bovine Endometrial Stromal Cells) cells. IE2 promoter is involved also in an increased IL-8 mRNA expression in endometrium, which causes the recruitment of granulocytes, such as macrophages and neutrophils, in the site of infection. These experimental data had led to the identification of an ORF50/Rta responsive element also on IL-8 promoter (Donofrio, Capocefalo et al. 2010). These observations may represent an endometrial defence mechanism against viral infection or a virulence mechanism by which viral replication stimulates a chemokine to attract more susceptible host cells to the endometrium.

1.3 Postpartum uterine diseases

Bos Taurus, especially dairy cattle, are particularly subjective to uterine infections, especially after calving. Parturition is an high risk period for mothers of all species, included cows. In fact, during gestation, animal immunity is suppressed and this immunosuppressive mechanism associated with pregnancy persists in the endometrium also after parturition, predisposing the development of uterine diseases. Cows support an high risk of physical damage during the birth process, because of the possibility to failure the releasing of the placenta and because they have a consistent possibility to contract microbial infections. For example, mammary gland infections, during late gestation, can cause premature parturition or compromise fetal or calf health (Sheldon, Williams et al. 2008). However, the greatest impact on health and productivity is associated with microbial contamination of the uterine lumen during parturition because of the dilatation of the cervix (Sheldon 2004; Sheldon and Dobson 2004), which facilitates the entry of environmental bacteria inside the uterus, causing infection in 90% of cows (Griffin, Hartigan et al. 1974; Sheldon, Noakes et al. 2002; Sheldon, Noakes et al. 2002; Sheldon, Lewis et al. 2006). The development of postpartum pathologies, such as metritis and endometritis, is responsible of many physical and physiological changes regarding bovine reproductive tract: uterine tissue inflammation and disruption as well as uterine regression are evident signs of pathology progression. Sometimes these conditions can cause embryonic survival disorder or abortion and they can alter hormone secretion. In fact, animals with postpartum diseases are characterized by changes in the activation of the hypothalamic-pituitary axis with resulting variations in the secretion of estradiol, progesterone and LH (Luteinizing Hormone). As a consequence of this, dairy cattle affected from pelvic inflammatory diseases have deficit of ovarian function connected with lower capacity to ovulate because of the slower postpartum ovarian follicular growth and related reduction in the conception rate (Sheldon, Cronin et al. 2009). All these events are strictly connected with

infertility, reduction of pregnancy and milk production. This is the reason why bovine uterine diseases are responsible of a great economical loss in farms and it is really important to understand the etiology of these pathologies for preventive and therapeutic purposes. The development of uterine infection depends on the balance between different variables, such as animal immunological system, the number and pathogenicity of microbes and the uterine environment. According to the symptoms and the clinical signs showed by cows, it is possible to distinguish between metritis, endometritis and subclinical endometritis. Usually, 25–40% of animals have clinical metritis in the first 2 weeks after calving, and the disease persists in more than 20% of animals as clinical endometritis (Sheldon, Williams et al. 2008). Metritis is generally related to enlarged uterus, purulent uterine discharge with a fetid odor and a panel of systemic signs such as increased temperature, limited milk yield, depression and lack of appetite (Sheldon, Cronin et al. 2009); It was proposed that cows with abnormally enlarged uterus and a purulent uterine discharge without any systemic signs of ill-health were classified as having grade 1 metritis; cows with additional signs of systemic illness, such as decreased milk yield and fever more than 39.5 °C, are classified as having grade 2 clinical metritis; finally, animals with inappetance, cold extremities and depression are classified as grade 3 metritis. On the contrary, endometritis should be defined by inflammation of endometrium and of underlying glandular tissues and it is also associated with the presence of purulent uterine discharge or mucopurulent (approximately 50% pus, 50% mucus) discharge but without systemic signs (Sheldon, Lewis et al. 2006). Animals with endometritis show destruction of epithelial surface, vascular congestion, stromal edema and accumulation of lymphocytes and plasma cells (Bondurant 1999). In the absence of this kind of symptoms, animals are classified as having subclinical endometritis. The role of subclinical uterine disease is less well characterized but it is an emerging problem. About 50% of cows shows the presence of neutrophils in the uterine lumen or endometrium, and inflammation of the tissues until 60

days after parturition, which reduces conception rates (Kasimanickam, Duffield et al. 2004). The high incidence of postpartum uterine inflammation in reproductive genital tract of dairy cattle, approximately 40% for metritis, 20% for endometritis (Donofrio, Capocefalo et al. 2010) and about 30% for chronic uterine inflammation without clinical signs (Sheldon, Cronin et al. 2009), is the reason why bovine uterine diseases represent a major economic problem in farms. Usually, postpartum uterine lumen is able to support the growth of a variety of aerobic and anaerobic bacteria, many of which are contaminants of uterine lumen and are normally eliminated by a range of uterine defense mechanisms. In case of development of pathology, the most common bacteria associated with uterine disease are *Escherichia coli* (37%), *Arcanobacterium pyogenes* (49%) (Sheldon, Williams et al. 2008), *Fusobacterium necrophorum* and *Prevotella*, *F.Nucleatum* (Sheldon, Cronin et al. 2009). Particularly, *A. pyogenes, F. necrophorum* and *Prevotella* species have been demonstrated to enhance the probability and the severity of uterine disease (Sheldon, Williams et al. 2008).

While the involvement of bacteria in uterine diseases and the process of immune response are well understood, the role of viruses in the development of metritis and endometritis is relatively investigated also because viral serology or isolation is less convenient to perform. However, the relationship between Bovine Herpesvirus type 4 (BoHV-4) and uterine inflammatory diseases as well as chronic infertility of dairy cattle (Czaplicki and Thiry 1998) was demonstrated. Bovine Herpesvirus type 4 was isolated for the first time from a metritis case in U.S.A. in 1973 (Parks 1973; Parks and Kendrick 1973) but it was subsequently found in different geographic areas of the world such as Spain (Monge 2006), Serbia (Nikolin, Donofrio et al. 2007), Italy (Castrucci, Frigeri et al. 1986) and India (Mehrotra 1986). BoHV-4 is tropic for endometrial stromal cell (Donofrio, Herath et al. 2007) and, like other Herpesviruses, it is able to establish persistent infection in macrophages (Donofrio and van Santen 2001). It has been hypothesized a possible vicious circle between bacteria and BoHV-4 in the onset of bovine

postpartum uterine diseases, since it has been observed a correlation between increased viral replication and the production of cellular mediators of inflammation, such as PGE2 (Donofrio, Ravanetti et al. 2008), produced by endometrial cells in response to bacterial infection. It was also demonstrated that LPS, largely produced by *E.Coli*, not only increases PGE2 production but it directly promotes BoHV-4 replication (Donofrio, Ravanetti et al. 2008). A lot of cellular mediators are usually produced during inflammation process, among which there are interleukin 1 (IL-1 β and α), Interleukin 6 (IL-6), Interleukin 8 (IL-8), prostaglandins, Tumor Necrosis Factor alpha (TNF-α), Type I and II Interferons; the study of the interaction between the production of these immunological molecular effectors, secreted by endometrium in the case of metritis or endometritis after bacterial infection, and Bovine Herpesvirus 4 replication, could be of great interest.

1.4 Innate immunity inside Endometrium

A range of functional and immunological barriers are usually adopted by cows to avoid microbial infection inside uterus. At first, vulva, vestibule and cervix are able to limit bacterial penetration through the presence of mucus and circular or longitudinal layers of uterine musculature which stop the ascent of bacteria along the reproductive tract (Bondurant 1999). When physical barriers are overcome, the uterine defense is permitted by the influx of neutrophils from the bloodstream into the endometrium and uterine lumen together with other cellular components, which include lymphocytes, eosinophils, mast cells and macrophages (Singh, Murray et al. 2008).

All the endometrium is characterized by a first layer of polarized epithelial cells, which are strictly connected by tight junctions and which are covered by a physical barrier of mucus; under these cells, there is the stromal layer, which is characterized by the presence of tissue macrophages with infiltrating immune cells such as dendritic cells.

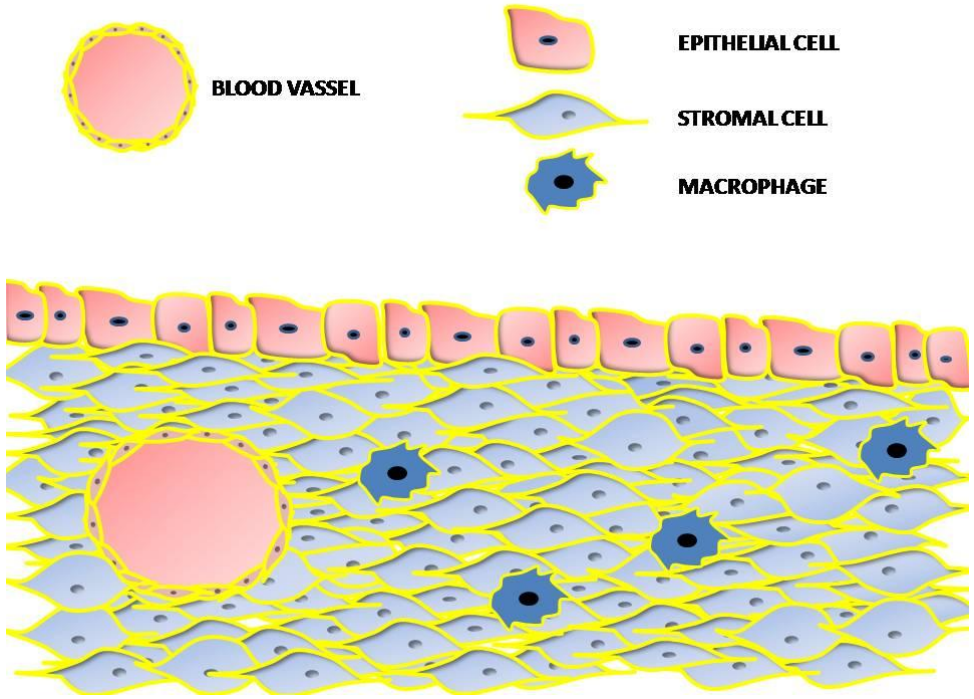

FIGURE 10: Schematic representation of the structure of bovine endometrium.

Epithelial cells are the first ones to be in contact with exogenous microbes, and for this reason they play an important role in the immune response. Both epithelial and stromal cells express Pattern Recognition Receptors (PRR), which are able to recognize some Pathogen Associated Molecular Patterns (PAMPS), causing cellular activation and the production of a lot of molecules involved in inflammation and uterine immune response. Among these cellular mediators there are acute phase proteins, which are produced in the liver in response to pro-inflammatory cytokines, increased during the first few weeks of postpartum period in cattle (Sheldon, Noakes et al. 2001), interleukins and chemokines. Toll-like receptors (TLRs) represent one of the main important group of receptors involved in the innate immunity inside uterus. Whole endometrium of a normal non-pregnant cow expresses from TLRs 1 to 10; in particular, epithelial cells express TLRs 1 to 7 and 9, while stromal cells express TLRs 1 to 4, 6, 7, 9 and 10 (Sheldon, Cronin et al. 2009) (Jacca, Franceschi et al. 2013). TLR1, TLR2, and TLR6 recognize bacterial lipids, TLR3, TLR7, TLR8 and TLR9 are all exclusively located in intracellular compartments, such as endosomes, and are involved in nucleic acid recognition following virus internalization and lysing (Thompson and Locarnini 2007). Specifically, the TLR3 ligand is double-stranded (ds) RNA, which can be generated from single-stranded (ss) RNA viruses as a replication intermediate during infection (Thompson and Locarnini 2007) (Seth, Sun et al. 2006). TLR7 and TLR9 are confined into the endoplasmic reticulum until ligand stimulation, when they traffic to the endosomes. Both TLR7 and TLR8 respond to ssRNA; endosomes contain also TLR8, which is up-regulated after infection and expressed in various tissues, especially monocytes. The TLR9 ligand is unmethylated CpG DNA motifs, which is typical of bacterial and viral genomes, whereas most CpG in the mammalian genome is methylated (Kawai and Akira 2010). TLR5 binds flagellin, while lipopolysaccharide (LPS), produced from Gram-negative bacteria, such as *E. coli,* is recognized by TLR4 in complex with CD14 and LY96 (MD2). The binding between TLRs and microbial targets causes intracellular TLRs

homodimerization and conformational changes necessary for the recruitment of adaptor molecules. Two main different pathways can be activated after TLRs binding: the first one is the MyD88-dependent pathway, which is essential for all TLRs except for TLR3 and which involves the recruitment of NFκB and AP-1 transcription activators, able to promote pro-inflammatory cytokine production; the second one is the TRIF-dependent pathway, dispensable only for TLR3 and TLR4. It causes the activation at first of TRIF and then of both IRF3 and NFκB, resulting in the release especially of type I Interferons (IFNs) (Kawai and Akira 2010), which have antiviral, immuno-modulatory and anti-proliferative functions (Presti, Pollock et al. 1998), also promoting the transcription of interferon stimulated genes responsible of viral replication inhibition (Seth, Sun et al. 2006). Another component of the innate immune response against bacteria inside uterus is characterized by the production of antimicrobial peptides (AMPs), which are an ancient component of the immune system, in particular of mucosal immunity (Selsted and Ouellette 2005). Bovine uterine tissue expresses lingual antimicrobial peptide (LAP), tracheal antimicrobial peptide (TAP), bovine neutrophil β-defensins (BNBD4, DEFB5) and bovine β-defensins (BBD19, BBD123 and BBD124) (Cormican, Meade et al. 2008). Furthermore, Mucin 1 (MUC1) is an epithelial cell glycosylated transmembrane protein, which may also have a role in microbial defense of the endometrium in mammals (Sheldon, Cronin et al. 2009) (Kasimanickam, Kasimanickam et al. 2014) (Brayman, Thathiah et al. 2004) and its expression is increased by LPS production (Davies, Meade et al. 2008).

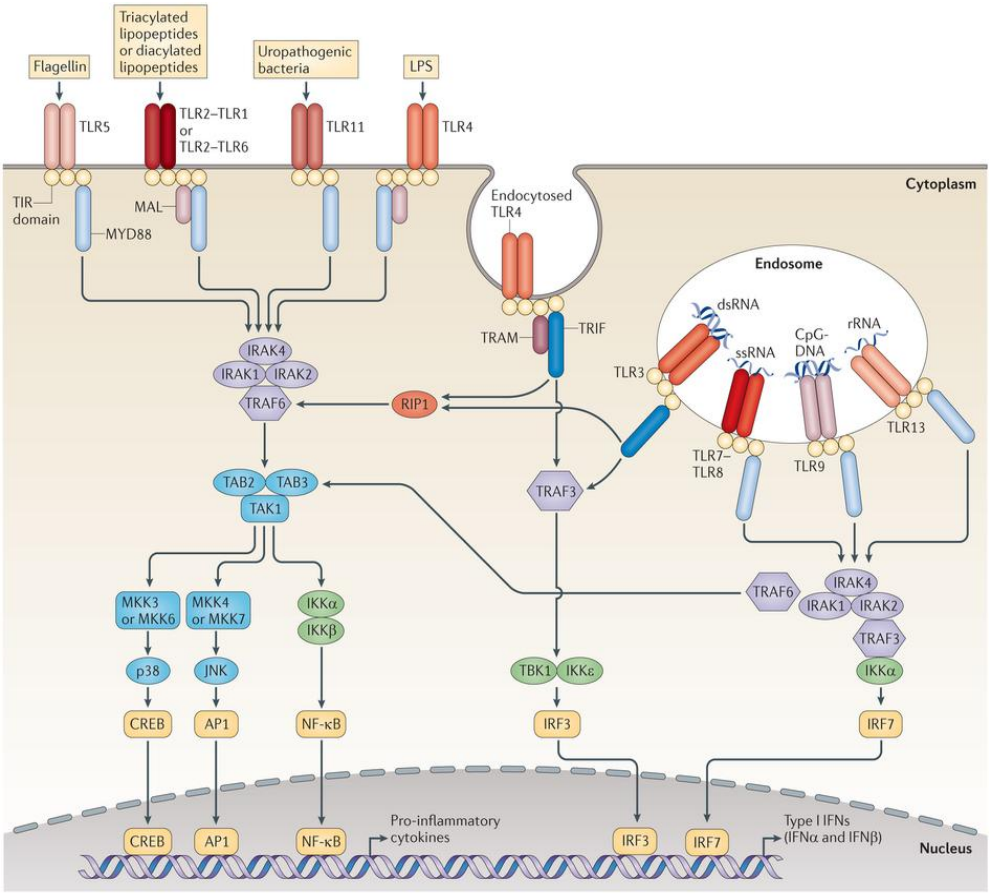

FIGURE 11: Mammalian TLR signaling pathway (O'Neill, Golenbock et al. 2013).

The delicate balance between bacterial or viral infection and immune response from the cows establishes the positive or the negative faith of the endometrial environment and the consequent development of pathologies, such as metritis and endometritis, rather than health.

1.5 Interferons and immunity

The name "interferon" (IFN) derives from the particular ability of some substances to "interfere" with viral replication *in vitro* (Zhang, Boisson-Dupuis et al. 2008). The activity of IFNs was originally described as a soluble factor produced by cells treated with inactivated, non-replicating viruses that blocked subsequent infection with live virus (Isaacs and Lindenmann 1957). Firstly, they were classified according to the secreting cell type but subsequently they were divided into type I and type II interferons, in relation to receptor specificity and sequence homology. Type I interferons include IFN-α (different subtypes, according to the species), IFN-β, IFN-ω and IFN-τ (reported only in ruminants (Bazer, Spencer et al. 1997). They share a significant structure homology and they all bind to a common heterodimeric receptor, named IFNAR, comprising IFNAR1 and IFNAR2. These receptors are able to activate cellular signaling through the classical Janus Kinase (JAK) signal transducer and activator of transcription (STAT) pathway, which is responsible of the activation of hundreds IFN induced genes (ISGs) with different antiviral response (Kang, Cheong et al. 2014). ISGs are involved in direct inhibition of protein translation, mRNAs degradation, induction of apoptosis inside infected cells and macrophages, increasing of antigen presentation on cell surface, limiting viral propagation *in vivo* (Kang, Cheong et al. 2014). The phosphorylation of tyrosine residues in STATs allows the translocation into the nucleus of STAT complexes (Platanias 2005) and the following transcription activation (Wen, Zhong et al. 1995), thanks to the binding with some specific sequences inside gene promoters, referred to us as IREs (IFN-stimulated responsive elements). Although type I interferons are secreted from a lot of cell types , hematopoietic cells are the major producers of IFN-α and IFN-ω while the main source of IFN-β are macrophage, after stimulation (Schroder, Hertzog et al. 2004). IFN-α and IFN-β are extremely important for a lot of biological functions, especially in terms of immuno-modulatory properties and defense against intracellular infections. In fact, they are able to

regulate natural killer (NK) cells and cytotoxic T cells (CTLs) as well as to facilitate cross-presentation of viral antigens from DCs to $CD8^+$T cells (Stetson and Medzhitov 2006). Many studies demonstrated also how type I IFNs are necessary for differentiation and function of effector $CD8^+$T cells too (Stetson and Medzhitov 2006). This kind of response is guaranteed by the production of several chemokines, able to recruit NK cells and CTLs cells to the site of infection, such as IL-15, which allows the proliferation and the maintenance of NK and memory $CD8^+$T (Stetson and Medzhitov 2006). The crucial role of type I IFNs in the development of an "antiviral state" was demonstrated for a variety of viruses, among which there are also some *Herpesviruses*. In particular, it was proved the IFN-β production from fibroblast and macrophages in response to Murine Gammaherpesvirus 68 infection (Kang, Cheong et al. 2014).

On the other side, Interferon gamma (IFN-γ) is the only type II interferon. It is particularly characterized by a unique amino acid sequence and by the binding to a specific receptor. IFN-γ receptors consist of two trans-membrane chains, IFN-γR1 and IFN-γR2; although both of them are required for the activation, IFN-γR2 is generally referred as the limiting factor in IFN-γ responsiveness because IFN-γR1 chain is usually in surplus (Schroder, Hertzog et al. 2004). Furthermore, IFN-γR2 is constitutively expressed but its expression level may be strongly regulated on the base of cellular differentiation and activation (Schroder, Hertzog et al. 2004). Both IFN-γRs lack intrinsic kinase/phosphatase activity and so they are associated with signaling machinery in order to consent signal transduction (Schroder, Hertzog et al. 2004); IFN-γR1 is connected to Janus tyrosine kinase (Jak)1 while IFN-γR2 to Janus tyrosine kinase (Jak)2. IFN-γ signal cascade not only involves the Jak-Stat pathway but also the recruitment and activation of several other proteins, members of the Janus family of kinases (Jaks 1,3 and Tyk2) and the Stats (Stats 1,6, including Stat5a and Stat5b), in order to strictly control the transcription of target genes via specific response elements. The phosphorylation of tyrosine

residues in STATs allow the translocation into the nucleus and the consequently binding with IFN-γ activated sites (GAS). Firstly, it was supposed that only $CD8^+$ cytotoxic lymphocytes and NK cells could produce IFN-γ, but later there was the evidence that also B cells, NKT cells and professional antigen-presenting cells (APCs) were able to secrete it (Schroder, Hertzog et al. 2004).

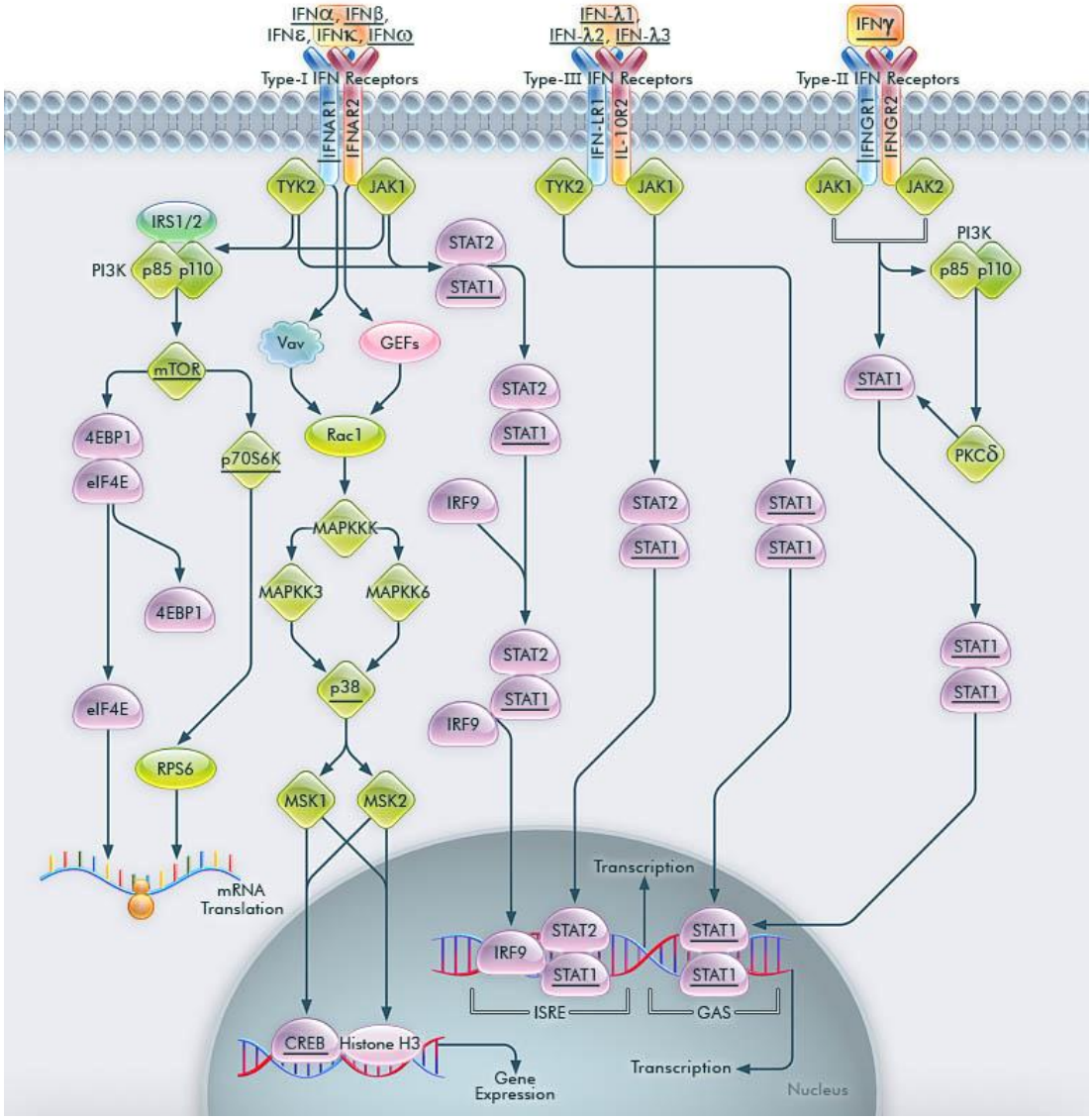

FIGURE 12: Interferon Pathway (http://www.ebioscience.com/resources/pathways/interferon-pathway.htm).

IFN-γ production is regulated by several cytokines mostly secreted by APCs cells, such as IL-12 and IL-18, which work like a bridge between infection and IFN-γ production in the innate immune response (Golab, Zagozdzon et al. 2000). In fact, pathogen recognition by macrophages allow IL-12 and other chemokines secretion with consequent recruitment in the site of inflammation of NK cells, responsible of IFN-γ production (Schroder, Hertzog et al. 2004). On the contrary, IFN-γ negative regulation is based on IL-4, IL-10, TGF-β and glucocorticoids production (Sen 2001). IFN-γ is able regulate both innate and adaptive immune response against viruses. In fact, it coordinates the immune response and the establishment of the antiviral state for long term control (Schroder, Hertzog et al. 2004). It stimulates class I antigen presentation pathway because it increases the numbers of different peptides presented to cell surface in the context of MHC I presentation, allowing a better intracellular response against pathogens as well as a stronger cytotoxic T cell activity and cell-mediated immunity (Schroder, Hertzog et al. 2004). IFN-γ is also responsible of MHC II over-expression, promoting peptide-specific activation of $CD4^+$T cells, in cells constitutively expressing MHC II receptor, such as B cells, DCs cells and cells of monocyte/macrophage lineage, but also to induce its expression in cells were MHC II is not usually produced, such as non professional APCs (Billiau, Heremans et al. 1988). IFN-γ promote the immune response to a Th1 phenotype, enhancing cell-mediated immunity, through the activation of NK cells, cytotoxic immunity, through the interaction between APCs cells and T cells, and macrophages activation (Boehm, Klamp et al. 1997). The antiviral state promoted by IFN-γ is characterized also by the induction of key antiviral enzymes, among which there are NADPH-dependent phagocyte oxidase (NADPH oxidase), responsible of reactive oxygen species (ROS) production, or some others involved in NO production, lysosomal microbe destruction and tryptophan depletion (Decker, Stockinger et al. 2002). The induction of indoleamine 2, 3 dioxygenase, an enzyme catalyzing the passage from tryptophan to N-formylkyneurine, mediated by IFN-γ, is responsible of

tryptophan depletion (Hassanain, Chon et al. 1993) and this mechanism was demonstrated to be relevant for many antimicrobical and antiparasitic immune activation (Pfefferkorn 1984). Macrophages are another important target of IFN-γ activity because their capacity to respond to LPS ligand is greatly improved after IFN-γ priming and because IFN-γ is able to induce TLR4 expression on the cell surface and to inhibit its LPS mediated down-regulation (Bosisio, Polentarutti et al. 2002). All these aspects explain why IFNs are well regulated at multiple levels: in fact, dysfunction in their production can cause severe biological and functional dysregulation, sometimes leading to development of diseases (Boehm, Klamp et al. 1997; Schroder, Hertzog et al. 2004). In fact, IFN-$\gamma^{-/-}$ or IFN-γR1 $^{-/-}$ mice show deficiencies in natural resistance and response against bacterial, parasitic and viral infection (Schroder, Hertzog et al. 2004). Furthermore, human disorders of IFN-γ responses, caused by IFN-γR1 and IFN-γR2 mutations, confer predisposition to mycobacterial diseases (Newport, Huxley et al. 1996).

1.6 Role of Matrix metalloproteinases inside endometrium

Matrix metalloproteinases (MMPs) are a family of more than 20 different Zinc ion (Zn+) dependent endopeptidases, which are characterized by the ability to degrade various components of extracellular matrix (ECM), such as collagen, elastin, fibronectin and laminin (Mastroianni and Liuzzi 2007). MPPs were firstly described in 1962 by Jerome Gross and Charles Lapiere, who observed the enzymatic activity of MMP-1 on collagen triple helix during the metamorphosis process in tadpole tail (Newby, Pauschinger et al. 2006). Later, MPPs were identified in vertebrates, in invertebrate and plants and they were purified from human skin too (Stricklin, Bauer et al. 1977). Structurally, MMPs are conserved multi-domain enzymes, essentially characterized by the presence of a catalytic domain, the N-terminal pre-domain and the pro-domain (Mastroianni and Liuzzi 2007). The catalytic domain is high conserved and it determines the substrate specificity. It contains a zinc ion, linked to three conserved hystidine residues inside the active site, which is essential for the proteolytic activity. The catalytic domain is connected to the C-terminal one thanks to a flexible hinge, of about 75 amino acids, without a determinable structure, called ”linker domain”. The C-terminal domain is a hemopexin like domain because of its similarity to the serum protein hemopexin, also responsible of the binding with tissue inhibitors of metalloproteinases. The pro-domain contains a cysteine residue, which interacts with the zinc inside the active site preventing the binding and the cleavage of the substrate, keeping the enzyme in an inactive form. In the majority of the MMPs, the cysteine residue is inside the conserved sequence PRCGxPD and it is connected to Zn^{2+} ion through its thiol group. The dissociation of the Cysteine residue (Cys) from the zinc ion determines a “switch” that leads to the enzyme activation, which is commonly called “cysteine switch” (Van Wart and Birkedal-Hansen 1990). The pre-domain is a signal peptide that activates secretion and it is clipped off when new synthesized MMPs travel to the cell surface.

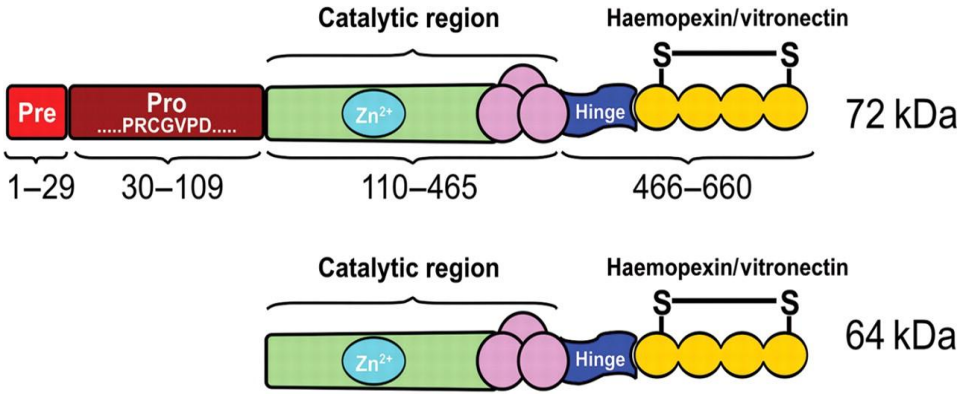

FIGURE 13: General structure of metalloproteinase (Kandasamy, Chow et al. 2010).

Generally, MMPs are grouped basing on their localization inside cells (secreted or trans-membrane MMPs) and their specific substrate (collagenases, gelatinases, stromelysins and membrane-type MMPs). MMPs play an important role not only in remodeling and degrading ECM, but also in immune response activation mechanism, modulating chemokines and cytokines activity (Elkington PT 2005). For example, MMP-1, 2, 3, 7, 9 and 12 can release active TNFα from the membrane associated precursor in a similar way to that of TNFα converting enzyme; MMPs can cleave IL-1β to an active or inactive state; MMP-9 induces the fragmentation of CXCL8 (IL-8) increasing its potentiality about ten times (Van den Steen, Proost et al. 2000). MMPs are also able to promote a variety of cell processes, among which there are cell proliferation and migration, tissue repair, differentiation, angiogenesis, apoptosis, organogenesis and cancer progression (Chang and Werb 2001). Since they play an important role in many physiological and cellular behaviors, it could be observed that a right balance between MMPs activity and MPPs inhibitors production can determine the development of a pathogenic or healthy condition in the organism. For this reason, MMPs are subjected to different levels of regulation. They can be regulated at the transcriptional level, in response to the production of exogenous signals: among these, there are chemokines and cytokines, which are able to induce or repress their expression, such as TNF-α or IFN-γ (Lockwood, Basar et al.

2014), growth factors, LPS (Mastroianni and Liuzzi 2007) or some other compounds (Struewing, Durham et al. 2009). Post-transcriptional regulation is based on the production of an inactive enzyme (zymogen), requiring chemical or catalytic changes to be activated. The last level of regulation is governed by the interaction between the c-terminal hemopexin like domain of MMPs and tissue inhibitors of matrix metalloproteinases, among which there are both chemical and natural compounds, such as TIMPs (Tissue inhibitors of matrix metalloproteinases) The dysregulation of TIMPs expression and the consequent unbalance between MMPs and TIMPs activity can cause excessive matrix degradation, development of pathogenesis and diseases. Among the pathological conditions associated with the overexpression of MMPs there are cancer progression (Chang and Werb 2001), some degenerating diseases, such as Alzheimer diseases (overexpression of MMP-2 and MMP-9) (Hernandez-Guillamon, Mawhirt et al. 2015), cirrhosis, arthritis, rheumatoid arthritis (overexpression of MMP-1 and MMP-3) (Zhang, Chen et al. 2015) (Zhang, Chen et al. 2015), pathogenesis of Aortic Aneurysm or degradation of the structural proteins of the aortic wall (Rabkin 2014). It was demonstrated also the correlation between dysregulation of MMPs and different infectious diseases (Elkington PT 2005). In fact, certain MMPs are preferentially up-regulated by infections. Among the viral and bacterial pathogens that can cause metalloproteinases over production there are Human immunodeficiency Virus (HIV), Human T lymphotrophic Virus (HTLV-1), Hepatitis B virus as well as *Helicobacter pylori* and *Mycobacterium tuberculosis* (Elkington PT 2005) (Mastroianni and Liuzzi 2007). In the particular context of mammalian endometrium, (Swangchan-Uthai, Lavender et al. 2012) MMPs were demonstrated to be involved both during menstruation (Izumi, Koga et al. 2015) and after parturition (Wathes, Cheng et al. 2011). In fact, they contribute to the restoration of the extracellular matrix, but also promote re-epithelialization. Among the main MMPs produced inside endometrium, there are MMP-1, MMP-2, MMP-3 and MMP-9 (Wathes, Cheng

et al. 2011). It was demonstrated MMP-1 overexpression during menstruation, playing an important role in the breakdown of endometrium (Wathes, Cheng et al. 2011), and during uterine involution after parturition, which was especially observed in rats (Manase, Endo et al. 2006). MMP-1, 2, 3, 9 and 13 are the mostly ones expressed inside bovine endometrium during late gestation (Kizaki, Ushizawa et al. 2008) and MMP-1 and MMP-13 are specifically down-regulated immediately in post-partum uterus. Furthermore, MMP-1 over-expression was observed inside bovine endometrium during pro-inflammatory processes, such as metritis or endometritis, as a consequence of bacterial or viral infections (Swangchan-Uthai, Lavender et al. 2012) and MMP-1 and MMP-13 increased production, also related to cows with severe negative energy balance status (NEB), is induced by LPS. For these reasons, cows with NEB and uterine infections can delay the mechanisms of endometrial repair in postpartum period because of alteration of MMPs activity.

2 CHAPTER 2

Aim of work

Bovine uterine diseases are able to cause a strong impact on health costs of dairy herds, because they are responsible of reduced reproductive efficiency of cows as well as the loss of pregnancy and decrease of milk production. In order to prevent these pathologies and to idealize a new therapy, it could be important to understand their etiology and mechanism of pathogenesis. Usually, the development of bovine uterine diseases is associated to bacterial infection but, recently, the involvement of viruses was also demonstrated and, among these viruses, Bovine Herpesvirus type 4 is the most prevalent (Parks and Kendrick 1973). It was proved the existence of a vicious cycle between bacterial infection and viral replication inside endometrium. Prostaglandin E and LPS, produced by bacteria, as well as *E. Coli* itself, can induce endometrial tissue damage and inflammation leading to development of bacterial endometritis, through the trans-activation of BoHV-4 Immediate Early 2 gene promoter (Donofrio, Ravanetti et al. 2008). Furthermore, the activation of some cellular receptors, belonging to the innate immunity, generally results in the synthesis and production of several pro-inflammatory cytokines and chemokines, which are able to modulate BoHV-4 replication. Among these cytokines, TNF-α was demonstrated to enhance BoHV-4 replication. Its activity was associated with BoHV-4 Immediate Early 2 (IE2) gene promoter trans-activation, mediated by nuclear factor KB (NFkB), which is directly induced by TNF-α production (Jacca, Franceschi et al. 2013). These data focalize the attention on the important role that pro-inflammatory molecules can play on BoHV-4 replication and on the development of chronic uterine diseases.

Aim of this project was to deepen the interaction between bovine endometrium and BoHV-4 infection and replication. Specifically, this work investigates on cellular and molecular mediators which can be involved in viral enhanced or restricted replication.

Firstly, it was noticed how IFN-γ secreted by macrophages but especially by T lymphocytes and NK cells, is able to counteract BoHV-4 replication end to prevent viral spreading inside

endometrium. It was observed that the activity of IFN-γ is directly exercised on BoHV-4 Immediate Early Gene 2 (IE2) promoter; this activation determines the induction of ORF50/Rta expression and the consequently reduction of BoHV-4 lytic replication. Therefore, it was hypothesized that IFN-γ is able to avoid the shift between an acute infection to a chronic one and that deficit on the activation of IFN-γ axis can represents the reason why some cow are predisposed to the development of endometritis compared to others (Jacca, Franceschi et al. 2014).

Secondly, as the right correlation between BoHV-4 infection and uterine pathology is still not well characterized, it was of great interest to analyze the response of BoHV-4 infected endometrial cells in terms of gene expression profile, in order to identify the genes directly up-regulated or down-regulated by the virus. Preliminary data, obtained with RNA sequencing Technology (RNAseq), highlighted how BoHV-4 is able to induce the expression of Matrix Metalloproteinase 1. To verify this supposition, MMP-1 over-expression, as a consequence of BoHV-4 infection, was analyzed through qReal Time PCR and Western Blotting techniques and its probable involvement in the development of bovine uterine diseases was investigated.

3 CHAPTER 3

Experimental section

3.1 *Interferon gamma-mediated BoHV-4 replication restriction in bovine endometrial stromal cells is host IDO1 gene expression independent and BoHV-4 IE2 gene expression dependent.*

3.1.1 Abstract

Metritis and endometritis are responsible of great economical loss inside dairy herds, especially in post-partum period, because of the reduced reproductive efficiency and pregnancy as well as the decrease of milk production. Although the etiology of the majority of bovine uterine diseases is usually related to bacterial infection, the correlation between viruses and endometrial pathologies was demonstrated and, among these viruses, Bovine Herpesvirus type 4 is the most prevalent. For these reasons, it could be of great interest to understand the way by which viruses are involved, for preventive or therapeutic purposes. The initial defense of the mammalian endometrium against microbes is dependent on innate immune system and the activation of cellular receptors results in the synthesis and production of several pro-inflammatory cytokines and chemokines, which can modulate BoHV-4 replication. Previously, it was demonstrated how TNF-α, produced by stromal cells and macrophages inside endometrium, is able to enhance BoHV-4 replication through the activation of NFkB pathway, directly acting on Immediate Early 2 gene (IE2) promoter activation (Jacca, Franceschi et al. 2013). Similarly, it could be interesting to investigate how BoHV-4 replication can be decreased. In this work, the interaction between Bovine Herpesvirus 4 (BoHV-4) infected bovine endometrial stromal cells (BESCs) and interferon gamma (IFN-γ) was investigated. Firstly, a pure population of bovine endometrial stromal cells (BESCs), free of CD45 positive cells and proved for their mesenchymal derivation through a vimentin positive and cytokeratin negative staining, was developed and the presence of IFN-γ receptors (IFN-γR) 1 and 2 on the cells but not of IFN-γ ligand was certified. The functionality of IFNGR 1 and 2 was tested through a transfection assay with a new reporter construct, made by cloning the bovine indoleamine 2, 3-dioxygenase 1 (IDO1) promoter in front of the luciferase reporter gene, activated by exogenous IFN-γ treatment. Further, It was noticed how IFN-γ treated or

constitutively expressing IFN-γ BESCs, strongly restricted BoHV-4 replication and consequent cytopathic effect (CPE) in time and dose dependent manner.

As IDO1 expression is usually related with IFN-γ treatment and anti-microbial response, it was important to check if the restriction of BoHV-4 replication was due to the activation of IFN-γ/IDO pattern or to something different. It was observed that IDO1 inhibitors and IDO1 constitutive expression were not able to counteract IFN-γ effect on BoHV-4 infected BESCs, while BoHV-4 Immediate Early (IE2) gene expression was transcriptionally decreased by IFN-γ axis activation. The presence of a potential responsive elements (RE), interacting with inhibitory transcription factors induced by IFN-γ, inside BoHV-4 IE2 gene promoter, confirmed this hypothesis. Based on these data, we can therefore say that IFN-γ mediated BoHV-4 restriction in BESCs cells is IDO1 independent and IE2 dependent and that the study of the relationship between IFN-γ axis activation and reduced BoHV-4 replication could pave the way to new efficient treatment and prevention of uterine diseases.

3.1.2 Introduction

The reduction of reproductive efficiency inside dairy herd greatly increases the cost of health management because of the loss of pregnancies as well as the decrease of milk production. Bovine uterine diseases are one of the main causes of infertility in dairy herds and, for these reasons, they have a great impact on their economic output. Therefore, it is very important to understand the etiology and the pathogenesis of these diseases for their correct prevention and treatment. Bovine uterine infections are commonly developed during parturition and they are associated with the presence of different bacteria species such as *Escherichia coli* and *Arcanombacteium pyogenes*, which are the most prevalent, followed by a range of anaerobic bacteria, including *Prevotella spp, Fusobacteium necrophorum* and *Fusobacterim nucleatetum* (Bonnett, Miller et al. 1991; Sheldon, Noakes et al. 2002). Usually, the epithelial layer of the uterus is considered the primary site of infection for the bacteria coming from the environment and able to infect vagina during parturition (Sheldon, Cronin et al. 2009). On the contrary, the role of viruses in the development of bovine uterine diseases is not commonly investigated. Nevertheless, the association between Bovine Herpesvirus 4 (BoHV-4) and post-partum diseases in cattle, especially as a secondary pathogen along with different bacteria species, was demonstrated. This kind of viral infection, coming through the systemic route in chronically infected animals, involves the stromal layer of the uterus as a primary site of infection. A complex co-infection model for BoHV-4 as a cofactor for the development of bovine post-partum metritis was hypothesized. Usually, during parturition, infection of the uterus can take place from environmental bacteria but, in normoergic animals, the infection can be clear within 3 weeks (Sheldon, Cronin et al. 2009) (Sheldon and Dobson 2003). In BoHV-4 persistently infected animals, the inflamed uterus attracts BoHV-4 persistently infected macrophages (Donofrio, Ravanetti et al. 2008) from the periphery. Furthermore, inflammatory molecules produced by inflamed endometrium and proliferating bacteria, such as

Prostaglandin E2 (PGE2) and Lipopolysaccaride (LPS), can induce BoHV-4 replication in persistently infected macrophages and consequently stromal cells can become infected with new replicating virus. Tumor Necrosis Factor alpha Receptor 1 binds Tumor Necrosis Factor alpha produced by macrophages induced by LPS (Dumitru, Ceci et al. 2000) on the surface of BoHV-4 infected endometrial stromal cells, thus inducing the over-expression of IE2 BoHV-4 gene and enhancing BoHV-4 replication (van Santen 1993; Jacca, Franceschi et al. 2013). The IE2 gene product, ORF50/Rta (Replication and Transcription Activator) is also able to induce Interleukin 8 (IL-8) production (Donofrio, Capocefalo et al. 2010), thus shifting inflammation from a transitory and acute state to a chronic one (Chastant-Maillard 2013). Although the association between BoHV-4 and bovine post-partum metritis, reproductive disorders and infertility was often certified (Frazier, Baldwin et al. 2002) (Monge, Elvira et al. 2006) (Nikolin, Donofrio et al. 2007), it is not still understood which are the factors, inside BoHV-4 persistently infected animals, responsible of the outcome of infection, decreeing between the development of disease and healthiness. Among the molecules probably involved in anti-viral response in infected animals, it was observed that interferon gamma (IFN-γ) is up-regulated in cases of BoHV-4 infection (Godfroid, Czaplicki et al. 1996). Furthermore, a link between natural intracellular parasite infection with *Neospora Caninum* and IFN-γ production in pregnant dairy cattle, with consequent protection against abortion, was previously noticed (Lopez-Gatius, Almeria et al. 2007). IFN-γ is considered to be mainly produced by T lymphocytes and Natural Killer (NK) cells and it is able to enhance innate immunity via induction of antimicrobial factors or degradative pathways in other immune cells, such as macrophages. BoHV-4 can establish persistent infections in cattle, particularly in macrophages (Donofrio and van Santen 2001) and it has a remarkable tropism for those same uterine locations and stromal layer (Donofrio, Herath et al. 2007) where T lymphocytes as well as NK cells can be mainly found (Oliveira, Mansourri-Attia et al. 2013). For these reasons, in the present paper a relationship between

IFN-γ axis activation and BoHV-4 replication in bovine endometrial stromal cells was underlined, highlighting new possibilities for prevention and therapy of uterine diseases.

3.1.3 Materials and methods

Endometrial cell isolation and primary cultures

Bovine uteri from post-pubertal non-pregnant BoHV-4 serum negative animals with no evidence of genital disease were collected at a local abattoir immediately after slaughter and kept on ice until further processing in the laboratory. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the ovarian morphology. Stage I includes interval between ovulation and the time when epithelium grows over the rupture point, thus forming the apex of a new corpus luteum and marking the beginning of stage II. During stage II a corpus luteum is fully formed with vasculature visible around its periphery. When the corpus luteum is dissected, the apex is red or brown, while the remainder is orange or yellow. Stage III begins when the red or brown colour disappears leaving the entire corpus luteum bright orange or yellow. Late in this stage, vasculature is visible over the apex of the corpus luteum. During stage IV, the ovary usually contains at least one large follicle and a regressed corpus luteum with no vasculature visible on its surface. Genital tracts with an ovarian Stage I corpus luteum were selected for endometrial cell isolation and culture and only the horn ipsilateral to the corpus luteum was used. The intercaruncular areas of the endometrium from 3 post-pubertal non-pregnant BoHV-4 serum negative animals were cut into strips and placed into serum-free RPMI-1640 (Sigma) supplemented with 100 IU/ml of penicillin (Sigma), 100 µg/ml of streptomycin (Sigma) and 2.5 µg/ml of Amphotericin B (Sigma), working under sterile conditions. The strips were then chopped into $1mm^3$ pieces and placed into Hank's balanced salt solution (HBSS; Sigma). Tissue was digested in 25 ml sterile filtered digestive solution, which has made by dissolving 50 mg trypsin III (Roche), 50 mg collagenase II (Sigma), 100 mg bovine serum albumin (BSA; Sigma) and 10 mg DNase I (Sigma) in 100 ml HBSS without phenol red. Following a 1.5 h incubation in a shaking water bath at 37 °C, the cell suspension was filtered through a 40 µm mesh (Fisher)

to remove undigested material and the filtrate was resuspended in washing medium, comprising of phenol-red free HBSS containing 10% FBS (Sigma) and 3 μg/ml trypsin inhibitor (Sigma). The suspension was centrifuged at 100 x g for 10 min and, following two further washes in washing medium, the cells resuspended in RPMI-1640 containing 10% FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin (sigma) and 2.5 μg/ml of Amphotericin B (Sigma). The cells were plated at a density of 1 x 10^5 cells in 2 ml per well using 24-well plates (Nunch). To obtain separate stromal and epithelial cell population, the cell suspension was removed 18 h after plating, which allowed selective attachment of the bovine endometrial stromal cells (BESCs). The purity of stromal and epithelial cells was verified by function assays and by histology and the absence of immune cells confirmed by RT-PCR for the CD45 pan-leukocyte marker. The culture media was changed every 48 h until the cells reached confluence. All cultures were maintained at 37 °C with 5% CO_2 in air, in a humidified incubator.

Reagents

Recombinant full length mature Bovine Interferon gamma (IFN-γ) protein was purchased from Abcam, L-Tryptophan and 1-Methyl-L-tryptophan (1-MT) from Sigma.

Cell lines

Human Embryo Kidney cells 293T (HEK 293T, ATCC), Madin Derby Bovine Kidney cells (MDBK, ATCC: CCL-22) and Bovine Embryo Kidney cells (BEK from Dr. Ferrari, Istituto Zooprofilattico Sperimentale, Brescia, Italy) were cultured with complete medium Dulbecco modified essential medium (E-MEM, Sigma) containing 10% FBS, 2mM of L-glutamine (Sigma), 100 IU/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma) and 2.5 μg/ml of Amphotericin B (Sigma) and incubated at 37 °C, 5% CO2 in a humidified incubator.

Transient Transfection Luciferase reporter Assay

Confluent BESCs in 24-well plates were co-transfected with reporter or/and effector plasmids using LTX transfection reagent (Invitrogen) prepared in D-MEM without serum and antibiotics

and left on the cells for 6 hours at 37 °C with 5 % CO2 in air, in a humified incubator. The transfection mixture was replaced with complete medium (RPMI-1640, 10%FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin and 2.5 μg/ml of Amphotericin B) and treated or untreated with IFN-γ. Luciferase reporter assays were performed using a Dual Luciferase Reporter Assay System Kit (Promega). Following transfection and treatment, cells were washed with PBS and lysed with 100 μl of lysis passive buffer by freeze-thawing at 80°C. Then 20 μl of the cell lysate was added to 50 μl of luciferase assay reagent (LAR) and Luciferase activity was determined with a Victor^3 Multilabel Counter (PerkinElmer), according to the manufacturer's instructions.

Viruses and viral replication

Confluent monolayers of BEK or MDBK cells were infected at multiplicity of infection (M.O.I.) of 0.5 P.F.U./cell with BoHV-4-EGFPΔTK (Donofrio, Franceschi et al. 2013) and BoHV-4-A-LucΔTK (Franceschi, Stellari et al. 2014). Infected cells were maintained in minimal essential medium (E-MEM; Sigma) with 2% FBS for 2h. Subsequently the medium was replaced with fresh EMEM (Sigma) containing 10% fetal bovine serum (FBS, Sigma), 2mM of I-glutamine (Sigma), 100 IU/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma) and 2.5 μg/ml of Amphotericin B (Sigma). When approximately 90% of the cell monolayer exhibited CPE (about 72 hours post infection), the virus was prepared by freezing and thawing cells three times and pelleting virions through 30% sucrose, as described previously. Virus pellets were resuspended in cold MEM without FBS. T.C.I.D.$_{50}$ (50% Tissue Culture Infective Dose) were determined with MDBK cells by limiting dilution. For viral replication, the supernatants of infected cultures were harvested after 48 and 72 hours and the amount of infectious virus was determined by limiting dilution on BEK cells.

Generation of stably transfected BESCs

The electroporation of a sub confluent 75-cm^2 flask of BESCs was carried out under the following conditions: Equibio apparatus; 300 V, 25 IF, 240 V, 1050 IF, and 481 R; Opty-Pulse) with 10 μg of pbIFN-γ or pbIDO1P-Luc DNA in D-MEM high at 10% of FBS. Afterwards, the cells were placed inside of a new 75 cm^2 flask in the presence of E-MEM (Sigma) containing 10% FBS (Sigma), 2mM of I-glutamine (Sigma), 100 IU/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma) and 2.5 μg/ml of Amphotericin B (Sigma). 24 hours after electroporation, cells were selected with 500 μg/ml of G418 (Sigma) until visible colonies appeared on the surface of the flask. Ten G418 resistant cell colony were encircled with a small cloning cylinder (Corning), harvested, plated in a 24 well plate and growth till they reached confluence. The clones were selected based on their ability to secrete IFN-γ as measured by ELISA (Duo Set Bovine IFN-γ, R &D System). Although all clones secreted IFN-γ, only 3 of them were kept for further expansion and analysis.

Western blotting

Protein cell extracts were obtained from 25-cm^2 confluent flasks of BESCs adding 100 μl of cell extraction buffer (50 mM Tris-HCl, 150 mM NaCl and 1% NP-40, pH 8). Cell extracts containing 50 mg of total protein were electrophoreses through 10% SDS-PAGE and transferred to nylon membranes by electroblotting. Membranes were incubated firstly with mouse anti-IDO (Indoleamine 2,3-Dioxygenase) monoclonal antibody (clone 10.1, cat 05-840, Millipore), diluted 1:15.000, and then with an horseradish peroxidase-labeled anti-mouse immunoglobulin antibody (Sigma), diluted 1:10.000, and visualized by enhanced chemiluminescence (ECL kit, Pierce).

Cell immunostaining

$2.5x10^5$ BESCs at the 2-3th passage were seeded in a 6-well plate and incubated at 37°C with 5% CO_2 in air in humidified incubator. When BESCs were sub-confluent, the culture medium was removed and the cells were fixed with acetone/methanol solution (1:1 proportion) for 20 minutes at RT (Room Temperature). After two quick washes with PBS the fixed cells were blocked for 1 hour at RT with 10% FBS diluted in PBS + 1% BSA for 1 hour at RT; a quick wash with PBS was then done before adding the primary antibody, diluted 1:200 in PBS + 1% BSA, and the cells were incubated for 1 hour at RT. The antibody was then removed and the cells washed extensively with PBS three times for 3 minutes each. Cells were incubated with the secondary antibody, diluted 1:500 in PBS + 1% BSA, for 1 hour at RT in the dark and after that washed for three times with PBS. As a counterstaining staining, 4'6-Diamidino-2-Phenylindole, Dilactate (DAPI) (Invitrogen) was added to the cells and incubated for 10 minutes in the dark; after a final PBS washing, cells were observed at the microscope. As primary antibodies, anti-alpha-vimentin mouse monoclonal antibody (sc-32332, Santa Cruz Biotecnology Inc.), diluted 1:200 and anti –cytokeratin 14 (CK14) rabbit polyclonal antibody (KS-B17.2, Sigma), diluted 1:200, were used, while as secondary antibodies a goat anti -mouse IgG AlexaFluor 488 conjugated (A11029, Life Tech.) and a goat anti-rabbit AlexaFluor 594 conjugated (A11037, Life Tech.) were utilized. These antibodies were previously validated for bovine specimens (Martignani, Eirew et al. 2010).

***In vivo* Bioluminescence Imaging (BLI)**

A 24-well plate was prepared with $5x10^4$ BESCs cells/well and subsequently incubated at 37°C with 5% CO_2 in air in a humidified incubator. When BESCs were sub-confluent, the culture medium was removed and the cells were treated with different concentrations of IFN-γ (50, 10, 2, 0.4, 0.08, 0.0016 ng/ml and untreated) and infected with 1 M.O.I. of BoHV-4-A-LucΔTK (Franceschi, Stellari et al. 2014) in the presence of MEM with 2% of FBS for 2 hours. The

medium was then removed and replaced with fresh MEM containing 10% FBS. *In vivo* imaging was performed 48 hours after the infection using an IVIS imaging system (Caliper Life Sciences, Alameda, CA). Photons emitted were quantified using Living Image software (Caliper Life Sciences, Alameda, CA). After the *in vivo* analysis, the cells were fixed with 1xPBS + 10% of paraformaldheyde for 15 min at room temperature. After washing with tap water the cells were colored with crystal violet (2% crystal violet and 2% formaldehyde) for at least 5 minutes at RT, then the monolayers were washed again in tap water and completely dried.

Reverse transcription

Reverse transcription PCR for BoHV-4 IE2 was performed from total RNA isolated by Trizol (Invitrogen) infected BESCs, treated with 50 ng/ml of IFN-γ or untreated; GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as normalizing control. 3 μg RNA were reverse transcribed using Ready to Go, T-Primed first–Strand Kit (Amersham Bioscience) according to the manufacturer's instructions. PCR amplification was carried out in a final volume of 50 μl containing 10 mM Tris-hydrochloride pH 8.3, 0.2 mM deoxynucleotide triphosphates, 2.5 mM MgCl2, 50 mM KCl, 5% Di Methyl Sulfoxide (DMSO) and 0.25 μM of each primer. The primers used for the amplification of IE2 and GAPDH are listed in the table 2. One hundred nanogram of cDNA samples were amplified over 30 cycles, each cycle consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and chain elongation with 1U of Taq DNA Polymerase (Fermentas) at 72 °C for 30 sec. The so generated 386 and 103 bp (IE2 and GAPDH) amplicons were then checked in 2.5% agarose gel and visualized after ethidium bromide staining in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-Acetate, 1mM EDTA). Reverse transcription PCRs for bovine IFN-γ, IFN-γR1 and IFN-γR2 were performed from total RNA isolated by Trizol (Invitrogen) from primary BESCs; 3 μg RNA were reverse transcribed as described above. The PCR amplification was carried out using GAPDH as internal control and with the same reaction conditions were used to amplify BoHV-4 IE2. The primers

used for the amplification are listed in the table 2. The 518, 300 and 227 bp amplicons were then checked in 2% agarose gel.

Reverse transcription PCRs for bovine indoleamine 2,3-dioxygenase 1 (IDO1) and inducible nitric oxide synthase (iNoS) were performed from total RNA isolated by Trizol (Invitrogen) from primary BESCs treated with different concentration of IFN-γ or untreated, from BESCs self-producing IFN-γ (BESCs/INF-γ) and compared to mock transfected or transduced BESCs (BESCs/Mock). Three micrograms RNA were reverse transcribed as described above and the PCR conditions used were denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min and chain elongation with 1U of Taq DNA polymerase (Fermentas) at 72 °C for 40 sec, for 30 cycles. GAPDH was used as internal control and the amplicons of 139, 372 and 103 bp, respectively for IDO, iNoS and GAPDH, were checked in 2% agarose gel.

Reverse transcription PCRs for bovine IDO1 ORF was performed from total RNA isolated by Trizol (Invitrogen) from primary BESCs treated with IFN-γ and 3 μg RNA were reverse transcribed as described above. The PCR conditions used were denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min and chain elongation with 1U of Pfu DNA Polymerase (Fermentas) at 72 °C for 90 sec, for 35 cycles. The primers used were Nhe-bIDO1 ORF sense and HindIII-bIDO1 ORF antisense (table 2). GAPDH was used as an internal control and the amplicons of 1244 and 103 bp, respectively for IDO1 ORF and GAPDH, were checked in 2 % agarose gel and the specificity of the PCR product was determined by sequencing.

Genomic DNA from Primary BESCs and MDBK was also used to successfully amplify IDO promoter (~1,6 kb), using the primer bIDOprom sense and anti-sense (table 2). Genomic DNA was extracted with classical procedure: cells were lysed overnight in Proteinase K Buffer containing 10mM Tris-HCl, pH 7.5, 1mM EDTA, 0.5 % sodium dodecyl sulfate (SDS) and 100 μg of proteinase k per ml at 37 °C. Nucleic acids were extracted with phenol-chloroform and precipitated with ethanol. One microgram of DNA samples was amplified over 35 cycles, each

cycle consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and chain elongation with 1U of Pfu DNA polymerase (Fermentas) at 72 °C for 2 min. The specificity of the PCR product was determined by sequencing. cDNA was also obtained from Peripheral Blood Mononuclear Cells (PBMC) stimulated with LPS; IFN-γ amplification was carried over for 35 cycles, with an initial denaturation step of 5 min at 94 °C, then 1 min of denaturation at 94°C, 1 min of primer annealing at 55°C and 1 min of primer extension at 72°C. Using the primer pair bIFN-γ sense and anti-sense (table 2) an amplicon of 518 bp was obtained and checked by sequencing.

PRIMER NAME		SEQUENCE 5' -> 3'	PRODUCT SIZE (bp)
bIDO	sense	CGA ATA TAC TTG TCT GGT TGG	139
bIDO	anti-sense	GGA GAA CAT CAA AGC ACTG	
biNOS	sense	TAG AGG AAC ATC TGG CCA GG	372
biNOS	anti-sense	TGG CAG GGT CCC CTC TGA TG	
bIFN–γR-1	sense	ATG GCT CTCCTC TTT TTC CTC GTC C	300
bIFN–γR-1	anti-sense	TTT AAC TCT ACC CCA GAC AGA ACT TGA TGG	
bIFN–γR-2	sense	TGG GAG CCG GTG TCC CTG	227
bIFN–γR-2	anti-sense	GAA ACG AGG CCC CCG AGC T	
bIFN-γ	sense	CCC GCT AGC CCA CCA TGA AAT ATA CAA GTC ATT TC	518
bIFN-γ	anti-sense	CCC CTC GAG TTA CGT TGA TGC TCT CCG GCC	
bIDOprom	sense	ACG CGT ATC GAT ATG GCA ACT CTA TTT TTA GTT TTC TGA GGA	1626
bIDOprom	anti-sense	TAAATTTAGAGCTCAGAACGTCAGTTGAGACAAGGAGGAGACCG	
GAPDH	sense	GGC CTG AAC CAC GAG AAG TAT AA	103
GAPDH	anti-sense	CCC TCC ACG ATG CCA AAG T	
IE2	sense	ACA AAC ACA CAG ACC AGT CA	386
IE2	anti-sense	GTT TCA CAA CAG ATT GAG CA	
KpnI-IE2P	sense	AAA CCC GGT ACC GCC AGT GCC AAG CTT TTT AAG	1130
KpnI-IE2PΔ225	sense	AAA CCC GGT ACC ACT CCC TCG CTT TGT TGT GAC	905
KpnI-IE2PΔ450	sense	AAA CCC GGT ACC GTC CAA GAC AAG GTC AGA TAG	680
KpnI-IE2PΔ675	sense	AAA CCC GGT ACC ACA ATC AGA AAA CAT ATC CTC	455
KpnI-IE2PΔ900	sense	AAA CCC GGT ACC TCT GAC ATT TCC CCT TTT TGC	230
NheI-IE2	anti-sense	GGG AAC TAG CTA GCC TGT TGT TCT GCT CCC TTT TA	
NheI-bIDO1ORF	sense	CCC GCT AGC CCA CC ATG GCA GGT GAC ATG TCA TCT CCT	1250
HindIII-bIDO1ORF	anti-sense	CCC AAG CTT CCC GGG TTA TTC TTC CTT CAA CAG GGA ATT	

TABLE 2: List of primers used in this work

qReal time PCR

Total RNA was extracted from BoHV-4 EGFPΔTK infected BESCs, treated with 50 ng/ml of IFN-γ or untreated and reverse transcribed as described above. IE2 gene expression was measured by real-time PCR using the StepOne Real-Time PCR System (Applied Biosystem) and Maxima Syber Green qPCR Master Mix (Fermentas; ROX solution provided) starting with 50 ng of reverse transcribed total RNA. GAPDH expression was used as internal control using the primers listed in table 2 (GAPDH sense and anti-sense). IE2 primers were chosen on the base of IE2 exon I and exon II sequences matching (Table 2, IE2 sense and IE2 antisense). PCR parameters were the following: an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95 °C for 1 min, 55 °C for 30 sec and 72 °C for 30 sec, with a final extension at 72 °C for 5 min.

Total RNA was also extracted from BESCs treated with different concentration of IFN-γ or untreated BESCs self-producing IFN-γ (BESCs/ IFN-γ) and compared to mock transfected or transduced BESCs (BESCs/Mock) and reverse transcribed as described above. IDO gene expression was measured by real-time PCR using the StepOne Real-Time PCR System (Applied Biosystem) and Maxima Syber Green qPCR Master Mix (Fermentas); ROX solution provided) starting with 50 ng of reverse-transcribed total RNA. GAPDH expression was used as an internal control and amplified using the primers listed in the table 2 (GAPDH sense and GAPDH anti-sense). PCR parameters were the following: an initial denaturation at 95°C for 10 min, followed by 40 cycles of 94 °C for 20 sec, 56 °C for 30 sec and 72 °C for 30 sec, with a final extension at 72 °C for 5 min.

Constructs generation

pbIFNG was generated by subcloning the 518 bp amplicon, cut with NheI and XhoI, in the MCS (multicloning site) of pEGFP-C1 (Clontech), cut with the same enzymes. pbIFN-γ construct functionally was tested by Duo Set ELISA for bovine IFN-γ detection (R&D system), in cell

supernatant pf pbIFN-γ transiently transfected HEK 293T cells. The 1.6 kb IDO Promoter was amplified by PCR from bovine genomic DNA, cut with MluI and XhoI and inserted in pGL3 basic (Promega), opened with the same restriction enzymes, to generate pbIDOP-Luc. pbIDO1 was obtained after the PCR amplification of IDO cDNA (1244 bp), the amplicon was cut with NheI/HindIII and inserted in pEGFP-C1, cut with NheI/HindIII. The full promoter of BoHV-4IE2 gene was subcloned in front of the reporter gene Luciferase to generate pIE2prom-Luc (Donofrio, Ravanetti et al. 2008). pIE2promΔ225, pIE2promΔ450, pIE2promΔ675 and pIE2promΔ900 deleted constructs were subsequently obtained from pIE2prom-Luc. pIE2promΔ225 construct was obtained by subcloning a 905 KpnI/NheI amplicon, amplified by PCR from pIE2prom-Luc DNA with the primer pair KpnI- IE2Δ225 sense and NheI-IE2P anti-sense (listed in table 2), in KpnI/NheI pGL3 Basic vector (Promega); pIE2promΔ450 construct was generated by subcloning a 680 bp KpnI/NheI amplicon, amplified by PCR from pIE2prom-Luc DNA with the primer pair KpnI IE2Δ450 sense and NheI-IE2P anti-sense (listed in table 2), in KpnI/NheI pGL3 Basic vector (Promega); pIE2promΔ675 construct was generated by subcloning a 455 bp KpnI/NheI amplicon, amplified by PCR from pIE2prom-Luc DNA with the primer pair KpnI IE2Δ675 sense and NheI-IE2P anti-sense (listed in table 2), in KpnI/NheI pGL3 Basic vector (Promega); similarly, pIE2promΔ900 construct was obtained by subcloning a 230 bp KpnI/NheI amplicon, amplified by PCR from pIE2prom-Luc DNA with the primer pair KpnI IE2Δ900 sense and NheI-IE2P anti-sense (listed in table 2), in KpnI/NheI pGL3 Basic vector (Promega).

Statistical methods

For luciferase assay in figure 17, signal intensity was expressed as folds of induction for each treatment over the luciferase construct transfected vehicle-treated (Untreated control =1). For luciferase assay in figure 40, signal intensity was expressed as percentage of luciferase activity for each treatment over that of the untreated control or that of cells transfected only with

pIE2P-Luc (=100%). For luciferase assay in figure 41, data are reported as percentage of luciferase activity by normalizing the signal for each deleted construct to that obtained with transfected, but IFN-γ untreated BESCs (=100%). All luciferase assay experiments were repeated three times, with 4 replicates at each time point and identical results were obtained. Results are reported as means ± SD and were considered significant when $P \leq 0.05$ (*). For all viral titre, the data are the means ± standard errors of triplicate measurements and each infection was repeated 8 times ($P > 0.05$ for all time point as measured by Student's t test or ANOVA). The quantitative real time PCR data presented are the means ± standard errors of triplicate measurements ($P \leq 0.05$) for all points as measured by Student t test.

3.1.4 Results

BESCs express functional IFGR1 and 2

To better understand the interaction of potential pathogens or specific ligands, such as cytokines or growth factors, with the endometrial substrate, a pure population of cells without leukocytes contamination, was obtained from pathogen free animals and used for these experiments. The absence of immune cells in the BESCs culture, has been verified through RT-PCR for the CD45 pan-leukocyte marker (data not shown); cell stromal nature was tested by immune staining for vimentin (stromal marker) (Fig. 14).

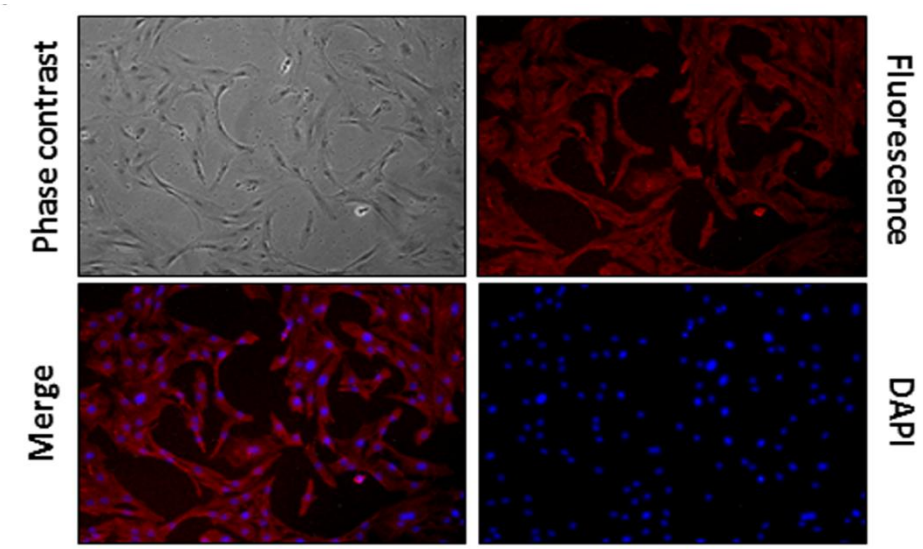

FIGURE 14: Phase contrast and fluorescent images of BESCs expressing the stromal marker, vimentin (red rodamine staining). Nuclei were visualized by counterstaining with DAPI (4',6-diamino-2-phenylindole) and they were merged with red fluorescent image (merge).

It has been shown that BoHV-4 has a particular tropism for bovine endometrium (Donofrio, Herath et al. 2007) and that its replication can be modulated by several cytokines belonging to innate immunity (Donofrio, Herath et al. 2007; Donofrio, Franceschi et al. 2008; Donofrio, Capocefalo et al. 2010; Jacca, Franceschi et al. 2013). On the other side, these molecules are also able to regulate BoHV-4 gene expression cascade inside infected cells (Jacca, Franceschi et

al. 2013). Since IFN-γ is an important bridge molecule between innate and adaptive immunity (Schoenborn and Wilson 2007), it was of great interest to investigate if BESCs express and/or respond to this cytokine. As shown in Fig. 15, Interferon Gamma Receptor 1 (IFN-γR1) and 2 (IFN-γR2) transcripts are well expressed while IFN-γ ligand was not detected (data not shown).

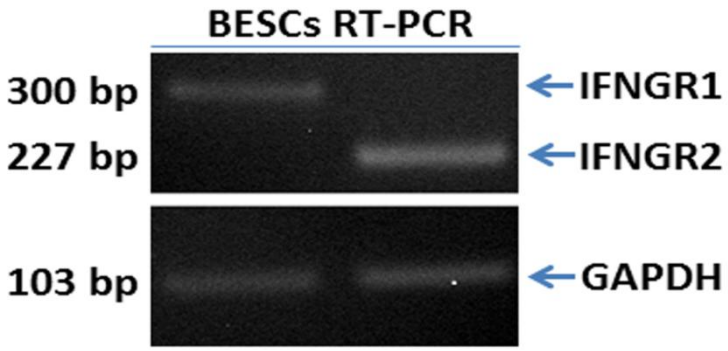

FIGURE 15: Expression of IFN-γR1 and 2 in BESCs. mRNA was isolated and the resulting cDNA was analyzed by PCR for the presence of IFN-γR1 and 2 gene transcripts, using the primer pairs described in Table 2. GAPDH expression was used as internal control and amplified with primer pairs described in Table 2.

To verify BESCs IFN-γRs functionality, a bovine IFN-γ responsive gene promoter, bovine indoleamine 2,3-dioxygenase 1 (IDO1), was initially cloned from bovine genomic DNA (Fig. 16). For this purpose, IDO1 gene promoter, which is mapped on Bos Taurus chromosome 27, was amplified using two primer spanning this locus (see Table 2). Then, the resulting 1.6 kb amplicon generated by PCR, was verified through restriction enzyme analysis and subsequently by sequencing. Amplicon sequence was identical to that present in the data bank (data not shown).

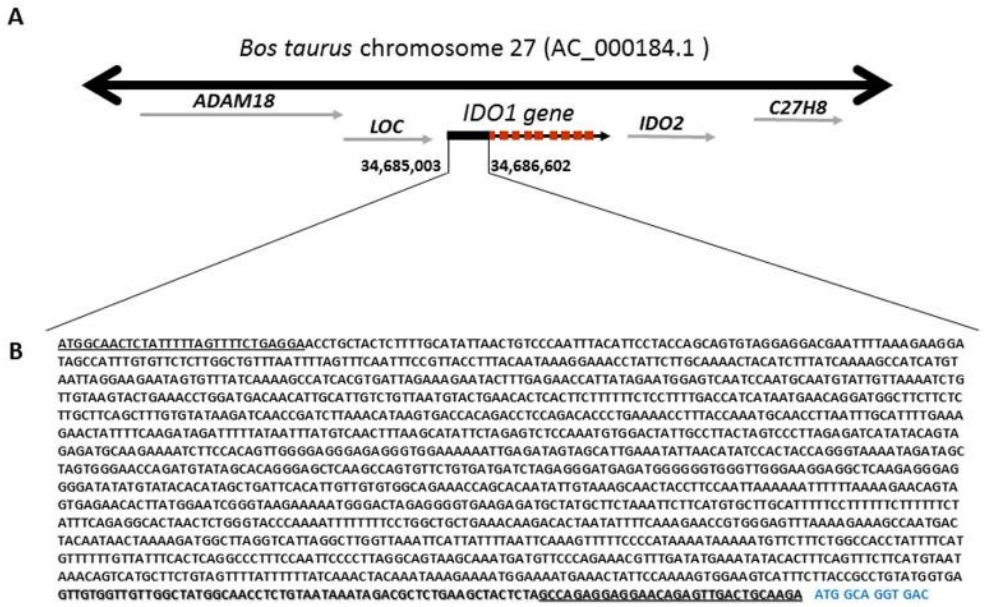

FIGURE 16: IDO1 gene promoter cloning. A) Bos taurus chromosome 27 diagram (not to scale) with the IDO1 gene promoter followed by exons annotated by numbers and surrounding genes [metallopeptidase domain 18 (ADAM18), disintegrin and metalloproteinase domain-containing protein 5-like (LOC), indoleamine 2,3-dioxygenase 2 (IDO2) and chromosome 27 open reading frame homologous to human chromosome 8 open reading frame 4 (C27H8). **B) IDO1 gene promoter sequence** comprising the putative TATA box, the transcriptional start site (+1), the untranslated region (UTR) highlighted with bold characters, part of the coding sequence belonging to the first exon and primers (underlined) employed to clone the 1600 bp IDO1 gene promoter.

To assess quantitatively the transcriptional level of IDO1 promoter activation, BESCs were transiently transfected with a luciferase reporter construct (pbIDO1P-luc), obtained by sub-cloning the 1.6 Kb IDO1 promoter in front of a luciferase reporter gene. It has been observed that, 24 hours after transfection, BESCs were well responsive to different doses (0.005, 0.05, 0.5, 5 and 50 ng/ml) of exogenous IFN-γ (Fig. 17).

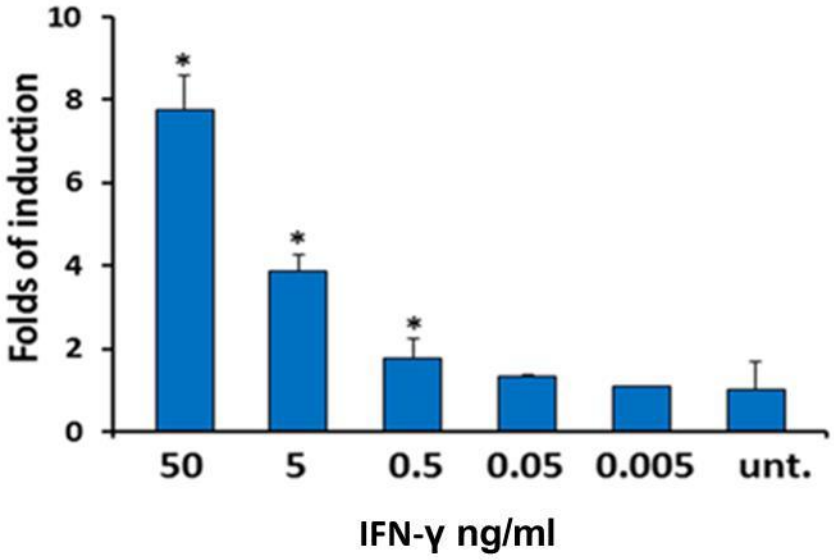

FIGURE 17: Dose dependent IDO1 response of IFN-γ treated BESCs respect to the untreated control. Results are reported as mean ± SD and were considered significant when P≤0.05 (*).

IFN-γ strongly restricts BoHV-4 replication in BESCs

As previously it was demonstrated how TNF-α is able to modulate and to enhance BoHV-4 replication in bovine endometrial stromal cells (Jacca, Franceschi et al. 2013), it was of interest to investigate if some other molecules are able to repress viral growth. Since BESCs are responsive toward exogenous IFN-γ, it has been investigated if the IFN-γ axis activation could have an impact on the viral replication. For this purpose, BESCs were pretreated with 50 ng/ml of IFN-γ and, after 4 hours, they were infected with 0.1 M.O.I. of BoHV-4-EGFPΔTK, a recombinant BoHV-4 expressing EGFP to easily monitor the progression of the infection (Donofrio, Cavirani et al. 2002). It was observed that in IFN-γ treated BESCs, BoHV-4 replication was highly compromised, respect to untreated control; moreover, both EGFP expression and cytopathic effect (CPE) caused by the virus replication appeared only in IFN-γ untreated BESCs at 72 hours post infection (P.I.) and the difference in viral titer was considerable at 48 and 72h P.I. (Fig. 18-19).

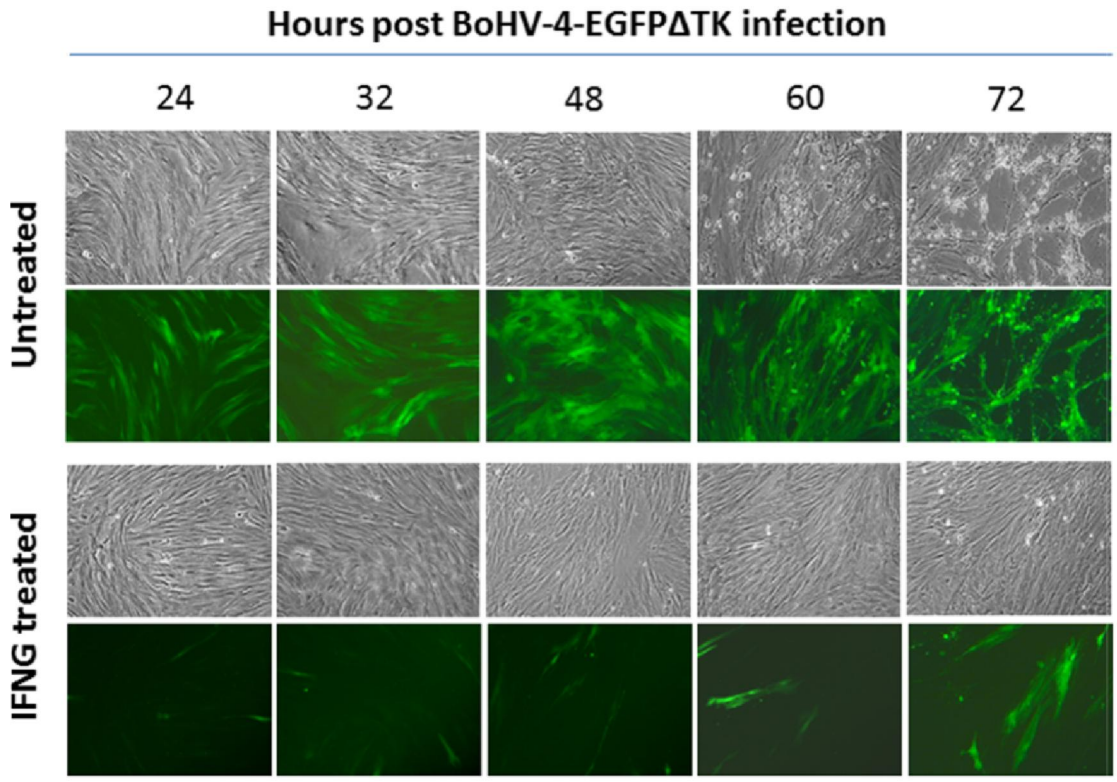

FIGURE 18: Restriction of BoHV-4 replication in BESCs by IFN-γ. Phase contrast and fluorescence representative images (magnification 10x) of IFN-γ treated and untreated BESCs at different time (24, 32, 48, 60 and 72 hours) post BoHV-4-EGFPΔTK infection.

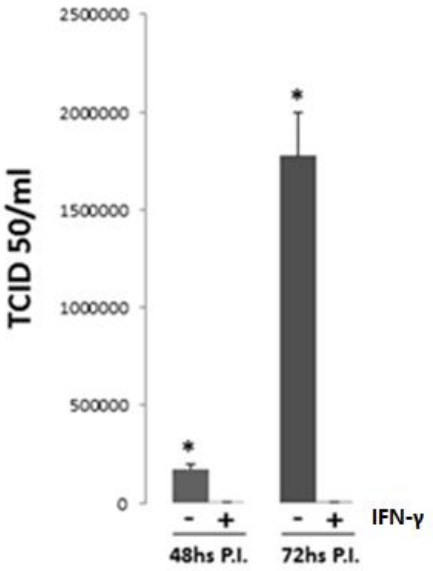

FIGURE 19: BoHV-4EGFPΔTK titer at 48 and 72 hours post infection/treatment of BESCs and expressed as T.C.I.D.$_{50}$/ml. Results are reported as mean ± SD and were considered significant when P≤0.05(*).

BESCs simultaneously infected and treated with IFN-γ showed similar results to that obtained with the pretreatment, although IFN-γ effect was less intense as observable by increase of EGFP expression at the first stage of infection (Fig. 20).

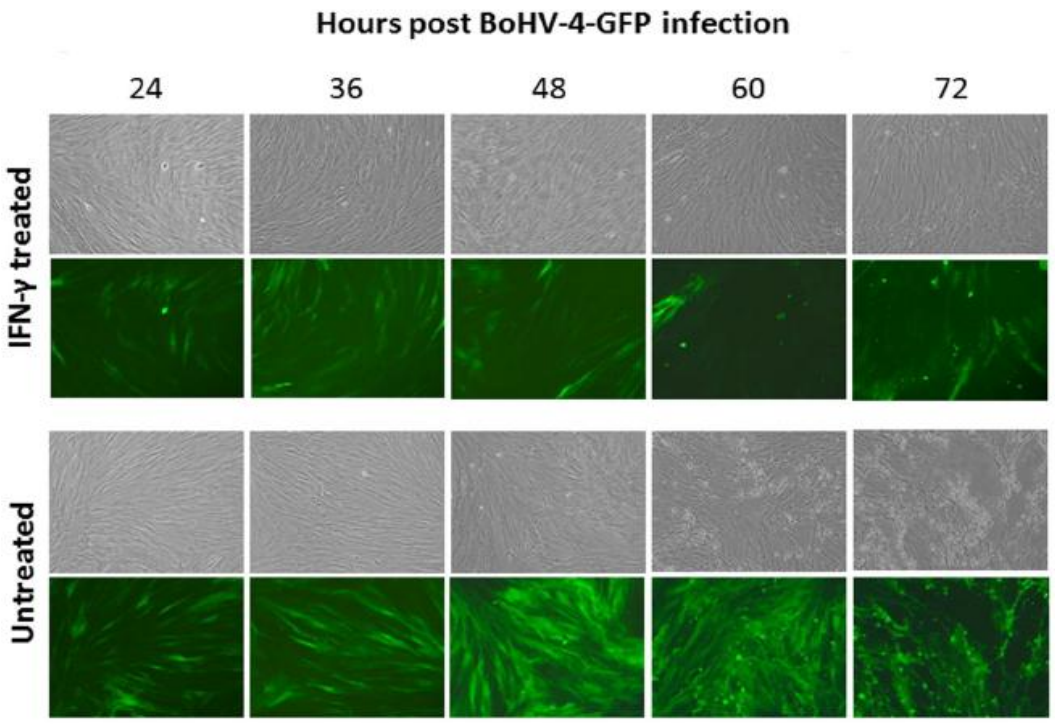

FIGURE 20: Restriction of BoHV-4 replication in BESCs by IFN-γ. Phase contrast and fluorescence representative images (magnification 10x) of IFN-γ treated and untreated BESCs at different time (24, 32, 48, 60 and 72 hours) post BoHV-4-EGFPΔTK infection.

To better understand and quantify the effect of IFN-γ on BoHV-4 restricted replication, BESCs were treated with different doses of IFN-γ (50, 10, 2, 0.4, 0.08, 0.0016 ng/ml and untreated) and infected with 1 M.O.I. of BoHV-4-LucΔTK (Franceschi, Stellari et al. 2014), in order to be monitored through *in vivo* bioluminescence imaging (BLI) analysis. Both bioluminescence signal and CPE (Fig. 21) increased with the decrease of IFN-γ concentration.

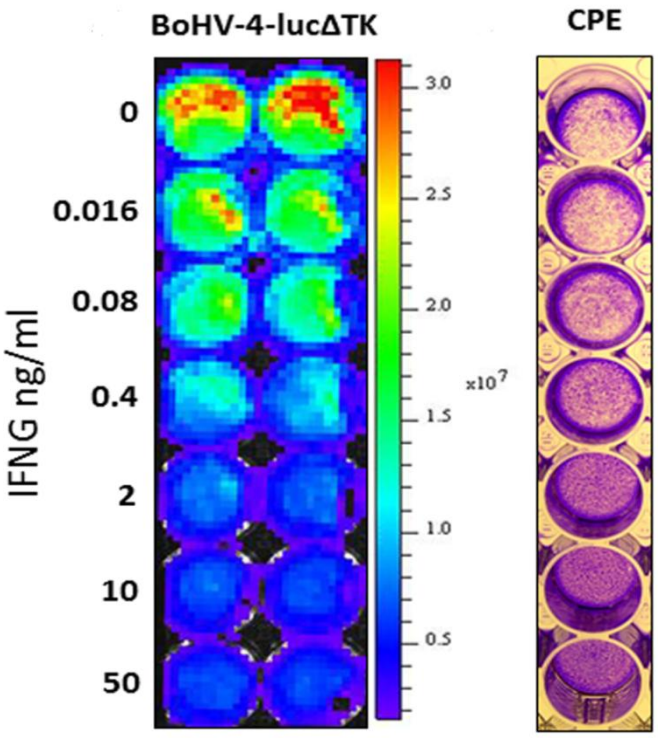

FIGURE 21: On the left representative BLI image of BoHV-4-A-CMVlucΔTK infected and treated with different doses of IFN-γ. Photons emissions were quantified by BLI and expressed as photons/second(S)/cm^2. On the right cristal violet staining of BoHV-4-A induced CPE in BESCs treated with different amounts (50, 10, 2, 0.4, 0.08, 0.0016 ng/ml and untreated) of IFN-γ.

Stably transfected BESCs expressing IFN-γ are resistant to BoHV-4 replication

To confirm these data, about the capability of IFN-γ to restrict BoHV-4 replication, a cell line of BESCs self-producing IFN-γ was prepared in order to see if BESCs are able to induce an anti BoHV-4 state to themselves (autocrine) or to the near-by cells (paracrine) (Fig. 22).

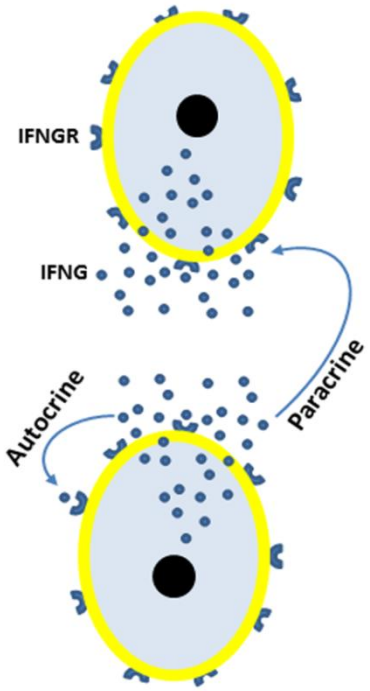

FIGURE 22: Schematic diagram of BESCs self-producing IFN-γ and paracrinally/autocrinally activating IFN-γRs to induce an anti BoHV-4 state.

BESCs cells were stably transfected with the plasmid vector pbIFN-γ, expressing bovine IFN-γ, to generate BESCs/IFN-γ, or with the empty vector, to generate a negative control, BESCs/Mock. Subsequently, BESCs/IFN-γ and BESCs/Mock were infected with BoHV-4-EGFPΔTK, and viral infection and replication was observed only in BESCs/Mock (as shown in figure 23).

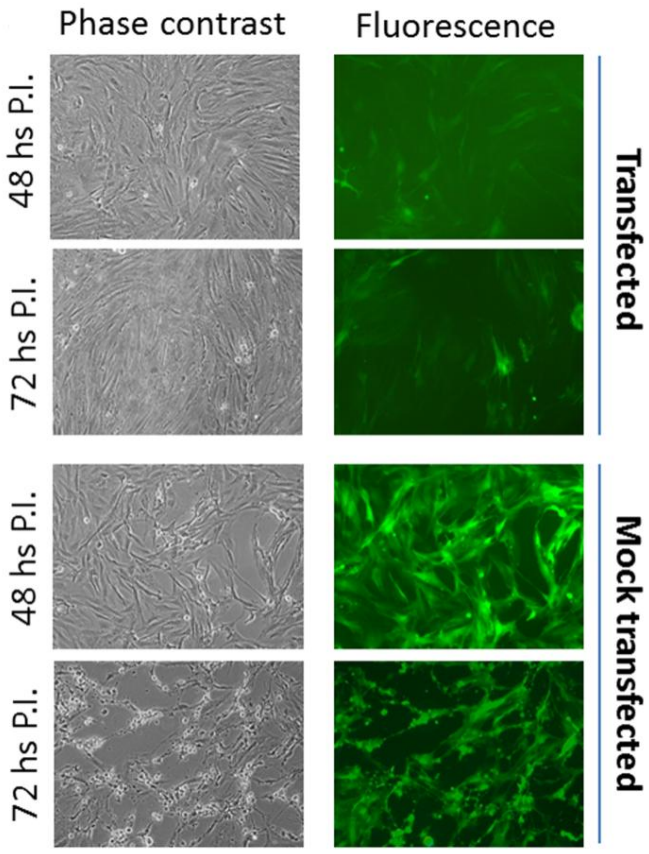

FIGURE 23: Phase contrast and fluorescence representative images (magnification 10x) of BESCs/IFN-γ (transfected) and BESCs/Mock (Mock-transfected) at different times (24 and 48 hours) post BoHV-4-EGFPΔTK infection.

Actually, the titers measured at 48 and 72 hours post BoHV-4-EGFPΔTK were much higher in BESCs/Mock than in BESCs/IFN-γ (Fig. 24).

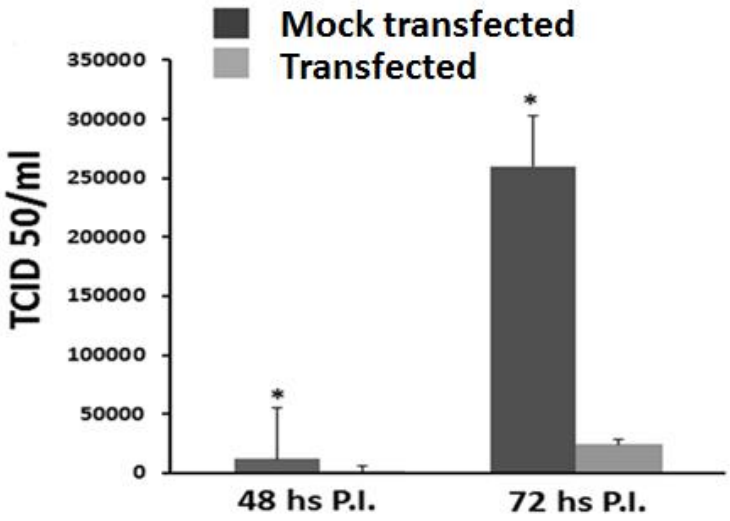

FIGURE 24: BoHV-4EGFPΔTK titer at 48 and 72 hours post infection, expressed as T.C.I.D.$_{50}$/ml. Results are reported as mean ± SD and were considered significant when P≤0.05 (*).

Since BESCs is a primary culture, drug selection could be potentially deleterious to the physiology of the cells; for these reasons, the same experiment was also performed by a transiently transfection with pbIFN-γ trough electroporation and, in addition to this, cells were transduced with the lentiviral vector expressing IFN-γ; in both cases identical results were obtained (data not shown).

IFN-γ treatment tightly induces IDO expression in BESCs

Referring to previous data, concerning the IDO1 gene promoter activation in response to IFN-γ treatment in BESCs, revealed by a luciferase reporter assay, it was of interest to investigate this response on the endogenous gene. BESCs were treated with different doses of IFN-γ (0.5, 5 and 50 ng/ml) for 12 hours and IDO1 mRNA was semi-quantified trough classical reverse transcription PCR (Fig. 25), quantitative real time PCR (figure 26) and trough western immuno-blotting IDO1 protein product (Fig. 27).

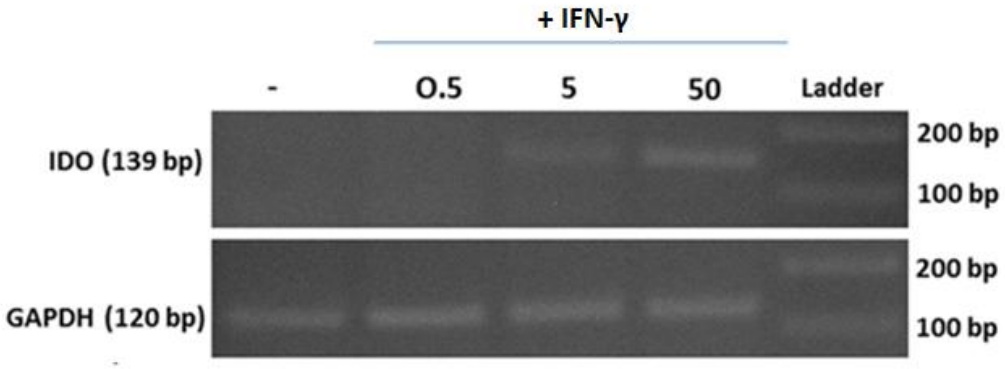

FIGURE 25: Semi quantitative reverse transcription PCR of BESCs treated with different concentrations of IFN-γ or untreated (-). GAPDH was used as a positive control of reaction.

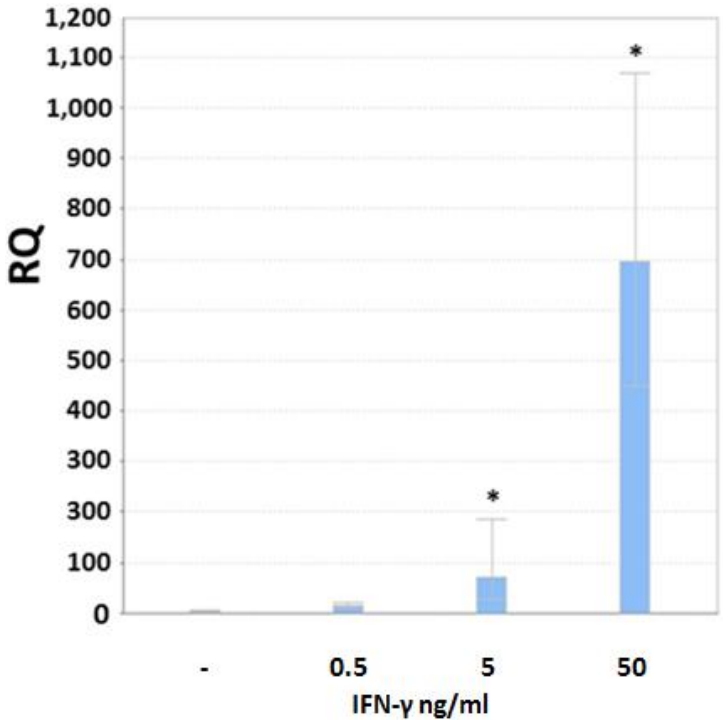

FIGURE 26: Quantitative real time PCR of BESCs treated with different concentrations of IFN-γ or untreated (-). Results are reported as mean ± SD and were considered significant when P ≤ 0.05 (*).

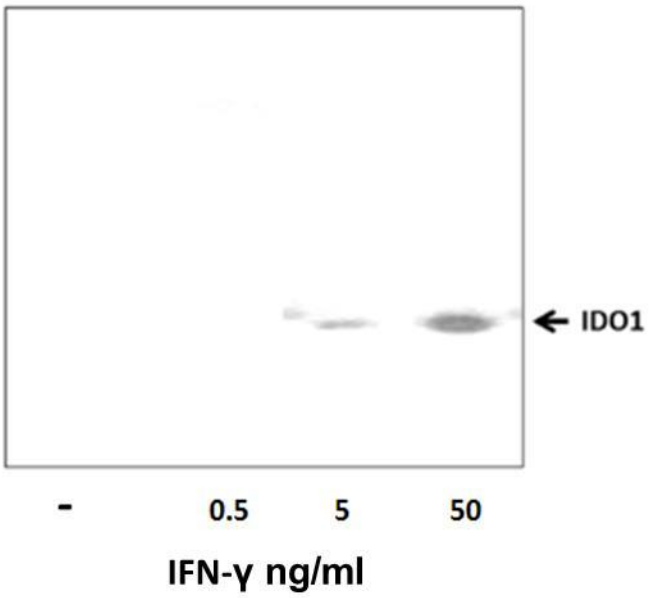

FIGURE27: Western immune-blotting of BESCs treated with different concentrations of IFN-γ or untreated (-).

In all cases, IDO1 transcription and translation increased with IFN-γ concentration. It was also observed that, when BESCs self-producing IFN-γ were compared to Mock transfected or transduced BESCs, similar results to those obtained treating BESCs with IFN-γ were obtained. In particular, this experiment has highlighted that IDO1 mRNA (Fig. 28-29) and IDO1 protein (Fig. 30) were up regulated in BESCs self-producing IFN-γ if compared with Mock transfected or transduced.

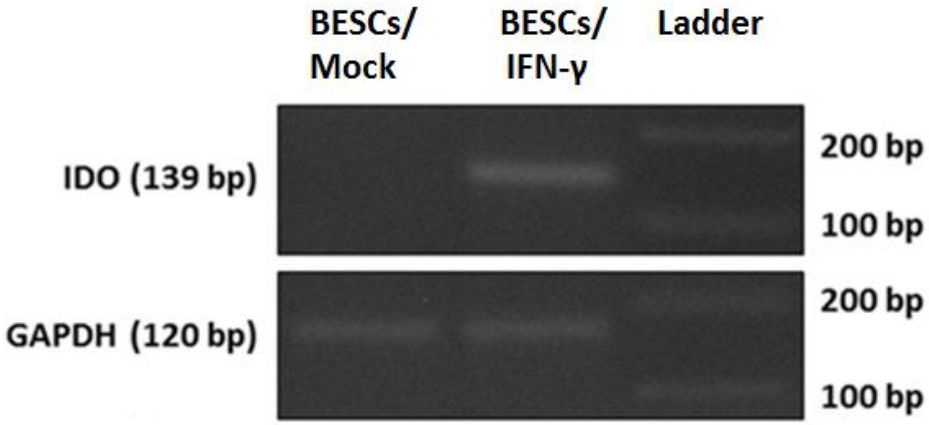

FIGURE 28: Semi quantitative reverse transcription PCR. GAPDH was used as a positive control of reaction.

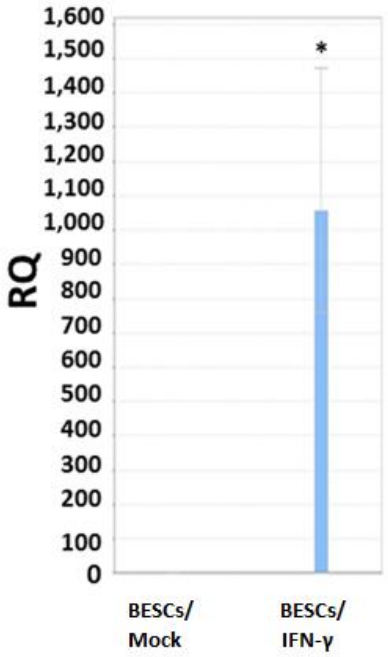

FIGURA 29: Quantitative real time PCR. The results are reported as mean ± SD and were considered significant when P≤0.05 (*)

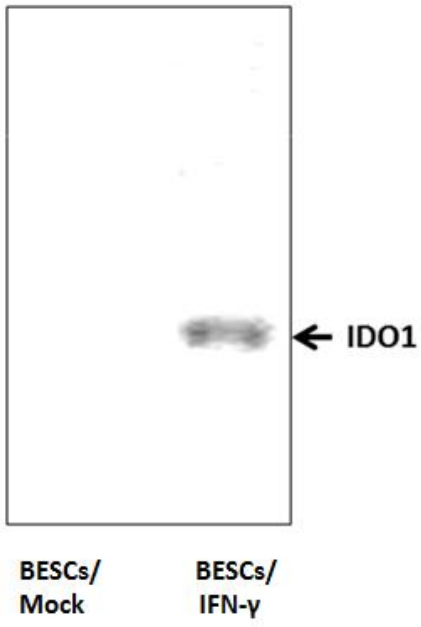

FIGURE 30: Western immunoblotting of BESCs self-producing IFN-γ (BESCs/IFN-γ) compared to mock transfected or transduced BESCs (BESCs/Mock).

It was also investigated the presence of inducible nitric oxide synthase (iNOS) gene transcript, one of the IFN-γ inducible gene product, which has both bacteriostatic and virostatic properties (Adler, Frech et al. 1995), but it was not detected (data not shown).

IFN-γ mediated BoHV-4 replication restriction in BESCs is IDO1 independent

IDO1 is a rate-limiting enzyme in the depletion of tryptophan, an essential amino acid within a highly conserved metabolic pathway, which is implicated in the replication of some pathogenic microorganisms (Zelante, Fallarino et al. 2009). Because of its antimicrobial properties, it was hypothesized a possible relationship between IDO1 expression and IFN-γ mediated BoHV-4 replication restriction. To verify this supposition, BESCs were treated with IFN-γ in association with or without 1-methyl-tryptophan [(1-MT); IDO1 specific inhibitor], as well as in the presence or absence of an excess of tryptophan to contrast its depletion, and subsequently infected with 0.1 M.O.I. of BoHV-4EGFPΔTK. 72 hours after treatment/infection, BESCs were microscopically monitored for the appearance of the CPE and the supernatant viral titer was analyzed. Surprisingly, it has been observed that, both 1-MT and extra tryptophan supplementation could not relieve IFN-γ mediated BoHV-4 replication restriction in BESCs, either in terms of viral titer or CPE (Fig. 31).

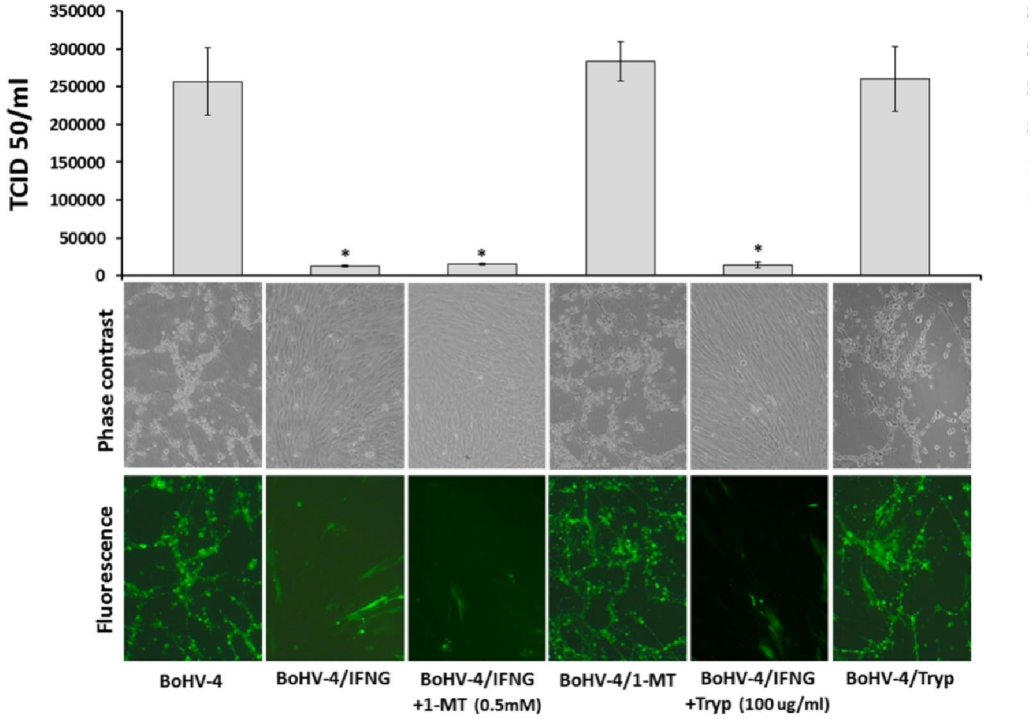

FIGURE 171: BoHV-4EGFPΔTK titer in the BESCs supernatant at 72 hours after different combinations of infection/treatment and expressed as T.C.I.D.$_{50}$/ml. Results are reported as mean ± SD and were considered significant when P≤0.05 (*)

Therefore, no difference were observed in BoHV-4 infected BESCs constitutively expressing IDO1 (BESCs/IDO1) and the control un-expressing IDO1 (BESCs/Mock), in terms of viral titer or CPE (Fig. 32).

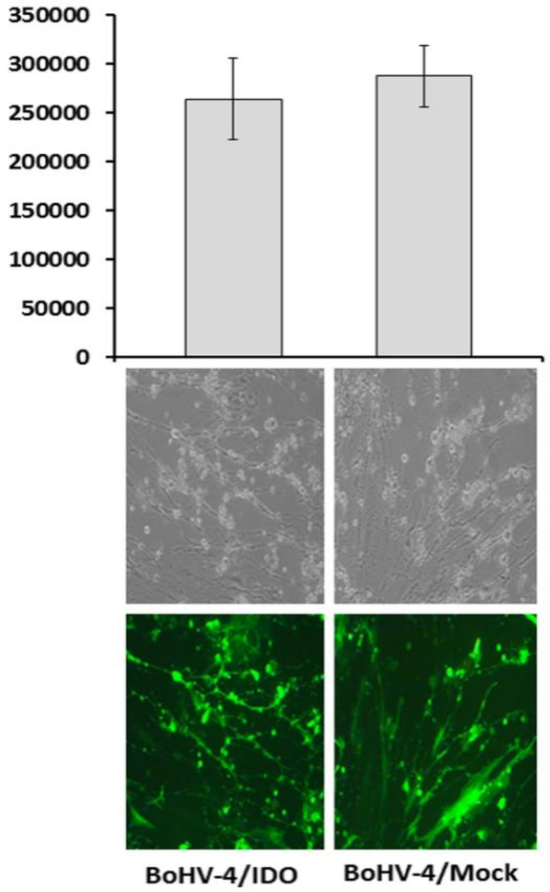

FIGURE 32: BoHV-4EGFPΔTK titer in the supernatant of BESCs constitutively expressing IDO1 at 72 hours post infection and compared with BESCs mock transfected. Viral titers are expressed as T.C.I.D.$_{50}$/ml. Along with the viral titer the equivalent phase contrast and fluorescence representative images (magnification 10x) of infected/treated BESCs are shown.

IFN-γ mediated BoHV-4 replication restriction in bovine endometrial stromal cells is IE2 dependent

The BoHV-4 gene expression program is controlled by the essential immediate-early lytic switch gene *50,* the only gene needed to initiate BoHV-4 lytic replication during *de novo* infection (Franceschi, Capocefalo et al. 2011). This gene encodes *Rta* protein (replication and transcription activator), able to transactivate both viral and cellular genes, directly linking on Rta-responsive elements inside target promoters or through an indirect route which involves an interaction with unknown cellular transcription factors. Although gene *50* promoter regulation is poorly characterized, the possible correlation between IFN-γ axis activation,

BoHV-4 gene *50* expression and BoHV-4 lytic replication was investigated. BESCs were treated with IFN-γ (50 ng/ml) and infected with BoHV-4 (0.1 M.O.I.). 12 hours after infection, IE2 expression was compared to that of BoHV-4 infected but untreated BESCs through quantitative real time PCR (Fig. 33) and classical reverse transcription PCR (Fig. 34).

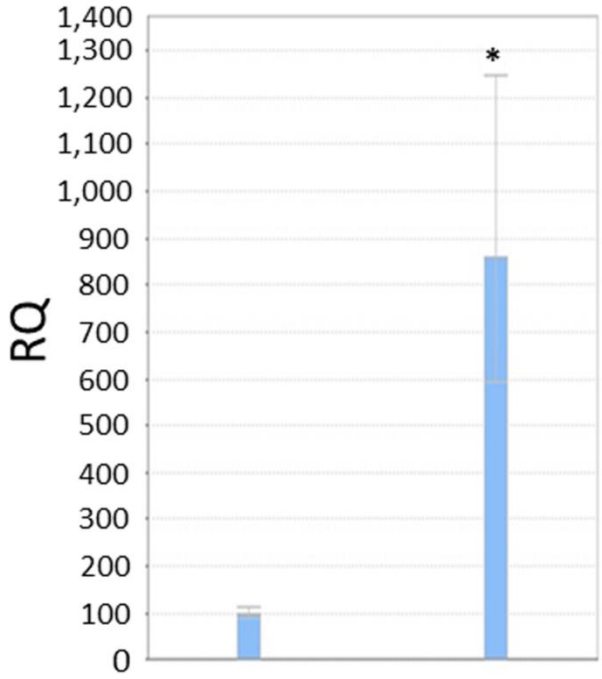

FIGURE 33: Quantitative real time PCR of BoHV-4-EGFPΔTK infected BESCs, treated with 50ng/ml of IFN-γ or untreated (-).

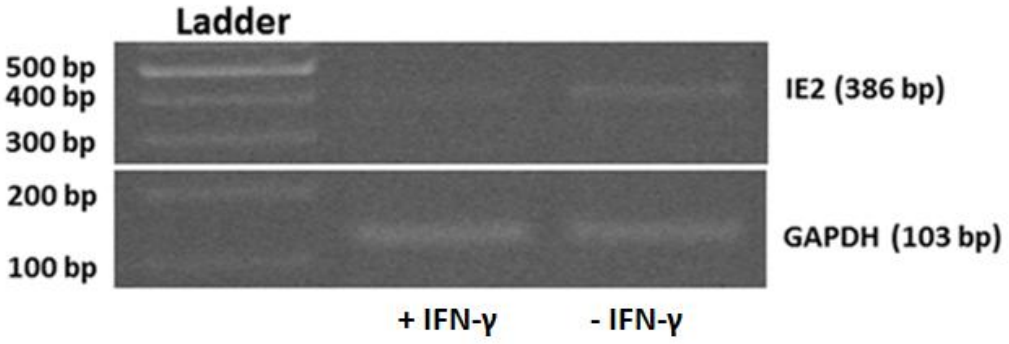

FIGURE 34: Semi quantitative reverse transcription PCR of BoHV-4-EGFPΔTK infected BESCs, treated with 50ng/ml of IFN-γ or untreated (-). Bovine GAPDH was employed as an internal control. Amplicon molecular size is present beside each amplicon band.

As IE2 expression resulted strongly reduced in IFN-γ treated cells respect to untreated control, it was of interest to understand how IE2 promoter activation could be involved in this mechanism. For these reasons, BESCs were transfected with pIE2P-Luc, a reporter construct expressing luciferase under the control of the IE2 promoter, and treated with different doses of IFN-γ (50, 5, 0.5 ng/ml and untreated control). It has been observed that luciferase activity was strongly reduced in IFN-γ treated BESCs respect to untreated control (Fig. 35). The results were confirmed co-transfecting BESCs with pIE2P-Luc in association with pbIFN-γ effector construct; luciferase activity was heavily reduced in co-transfected cells respect to the control cells transfected with pIE2P-Luc and pEGFP-C1 (a commercial plasmid expressing Green Fluorescent Protein under the control of human cytomegalovirus Immediate Early gene promoter) (Fig. 35).

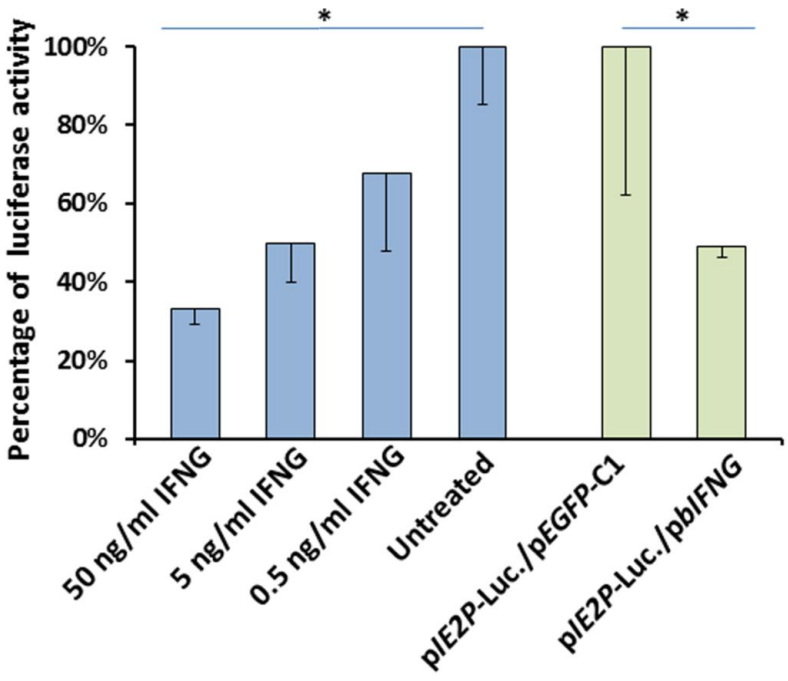

FIGURE 35: On the left, dose dependent pIE2P-Luc response of IFN-γ treated BESCs respect to the untreated control. On the right inhibition of theIE2gene promoter by IFN-γ ORF product. BESCs were cotransfected with the target plasmid (pIE2P-Luc) and the effector plasmid (pbIFN-γ), whereas the control was established co-transfecting pIE2P-Luc with pEGFP-C1, so that the total amount of DNA cotransfected remained the same. Results are reported as mean ± SD and were considered significant when P≤0.05 (*).

IFN-γ mediated BoHV-4 IE2 transcription down regulation resides within IE2 promoter gene

It was interesting to know which were the IE2 promoter regions responsive to a possible transcriptional inhibitor induced in IFN-γ treated BESCs. For this purpose, starting from the BoHV-4 IE2 gene promoter characterized by 1146 bp, a series of 4 deleted fragments (Δ225, Δ451, Δ675, Δ900) were amplified through classical reverse transcription PCR and subsequently cloned within the pGL3-Basic vector reporter plasmid. These fragments in the resulting plasmids shared the same 3' end, with 5' beginning at bases -905 (Δ225), -680 (Δ451), -455 (Δ675), -230 (Δ900) respectively. Afterwards plasmids were transfected into BESCs which in turn were treated or untreated with 50 ng/ml of IFN-γ for 12 hours. Post IFN-γ treatment, luciferase assay showed a reduction of IFN-γ response for deletions between -680 and -455 in IE2 promoter (Fig. 36).

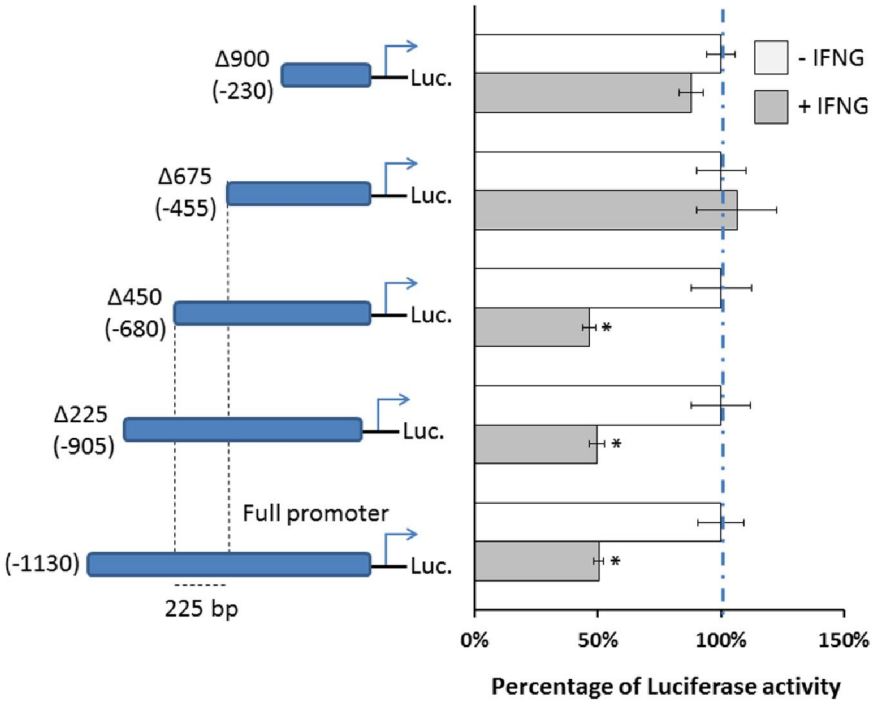

FIGURE 36: IE2 promoter gene analysis. Deletion constructs were designed with numbering relative to transcriptional start site (+1) and the resulting deletion at their 3'end. They correspond to:pIE2P-luc (-1130, undeleted full promoter), pIE2PΔ225-luc (-905), pIE2PΔ450-luc (-680), pIE2PΔ675-luc (-455) and pIE2PΔ900-luc (-230). 225 bp indicates the region comprised between -680 and -455. Results are reported as mean± SD and were considered significant when P≤0.05 (*).

It has been observed that Δ900 promoter maintained the level of luciferase expression comparable to the un-stimulated full promoter. From these data, there seems to be a responsive element as a target site for unknown transcriptional inhibitors inside the IE2 gene promoter between -680 and -455 positions.

3.1.5 Discussion

During post partum period, a lot of dairy cattle are prone to uterine infection and disease, which represent the main responsible of the reduced reproductive efficiency of a dairy herd, causing a significant increase of its health management. Among the bovine uterine pathologies, metritis, endometritis and subclinical endometritis are the most prevalent and they are usually defined according to some characteristic clinical signs, such as uterine horn diameter or consistence and odor of the vaginal mucus (Sheldon and Noakes 1998). Infertility is often associated with uterine disease, because of the damages of the endometrial tissue and the failure of ovarian cyclic activity (Sheldon, Williams et al. 2008). The etiology of uterine diseases is principally ascribed to bacterial infection. Generally, a lot of aerobic and anaerobic bacteria are able to penetrate inside bovine uterine lumen during parturition; among these, there are some pathogens, such as *Arcanobacterium pyogenes, Fusobacterium necrophorum*, *Prevotella species* and especially *Escherichia coli,* which seems to pave the way for subsequent infections by other bacteria or viruses (Donofrio, Ravanetti et al. 2008; Sheldon, Cronin et al. 2009). The role played by viruses on the development of uterine diseases is not well investigated, maybe because of viral serology or isolation is more difficult to perform; however, the relationship between bovine uterine pathologies and Bovine Herpesvirus type 4 (BoHV-4) infection was demonstrated. BoHV-4 is a *Gammaherpesvirus* and it was firstly isolated in a metritis case in 1973 (Parks and Kendrick 1973). Like other *Herpesviruses*, BoHV-4 is able to establish a persistent infection both in its natural host and in the experimental one (rabbit) (Osorio, Reed et al. 1982; Castrucci, Frigeri et al. 1987). In persistently infected animals, BoHV-4 DNA was found in different tissues, through PCR analysis (Egyed and Bartha 1998), in situ hybridization (Lopez, Galeota et al. 1996) or by the recovery of the virus after explants culture (Osorio, Reed et al. 1982; Castrucci, Frigeri et al. 1987). In addition to this, it was demonstrated that BoHV-4 is able to establish a persistent infection in

monocyte/macrophage lineage (Donofrio and van Santen 2001). This data are corroborated by its recovery in non T, non B cells situated in the marginal zone of the spleen of persistently infected natural host and rabbit experimental host (Osorio, Reed et al. 1982; Lopez, Galeota et al. 1996). Probably, the ability of BoHV-4 to persist inside macrophages allows it to move from the periphery and to reach the stromal layer, for which it has a particular tropism (Donofrio, Herath et al. 2007) and where it can actively replicate. BoHV-4 is usually ascribed as a secondary pathogen in the onset of bovine uterine diseases (Donofrio, Cavirani et al. 2005) and the development of a pathological condition is strictly related to the simultaneous presence of environmental contaminant bacteria, among which *E. Coli* is the most prevalent. A vicious cycle between bacterial infection and BoHV-4 replication was supposed (Donofrio, Ravanetti et al. 2008). Inflammatory molecules produced by endometrium and proliferating bacteria, such as LPS and Prostaglandin E2 (PGE2), strongly enhance BoHV-4 replication, through the direct up-regulation of IE2 gene promoter (Donofrio, Ravanetti et al. 2008), both in stromal cells and in persistently infected macrophages. Recently, it was also demonstrated that TNF-α, produced by LPS-induced macrophages, is able to bind TNF-αRs on the surface of BoHV-4-infected endometrial stromal cells, inducing BoHV-4 IE2 gene expression and enhancing BoHV-4 replication (Jacca, Franceschi et al. 2013). IE2 gene product ORF50/Rta induces not only BoHV-4 replication but also IL-8 production, thus probably allowing the shift between a transitory and acute status of inflammation (metritis) to a chronic one (endometritis). Searching for a BoHV-4 replication limiting factor, IFN-γ was considered the best candidate for different reasons: firstly, it was always recognized as an antiviral molecule, even if it was never tested on BoHV-4 infection before; secondly, an increasing of IFN-γ level was observed in BoHV-4 infected animals (Godfroid, Czaplicki et al. 1996) and it was proved to be protective against many intracellular pathogens too, such as *Chlamydia* (Ibana, Belland et al. 2011), *mycobacteria* (Desvignes and Ernst 2009) and *Neospora caninum* (Innes, Panton et al. 1995).

To verify this supposition, a pure population of BESCs cells, without leukocytes contamination, was used for this work and the expression of IFNGR1 and 2 was demonstrated on stromal cell surface, whereas ligand transcript or protein could not be detected. Generally, IFN-γ is supposed to be produced by CD8, CD4 Th1 effector T cells, B cells, professional antigen presenting cells (APCs) of the adaptive immune system and natural killer (NK) cells, and natural killer T (NKT) cells, which play an important role in innate immune response (Gough, Levy et al. 2008). All these cellular types are effectively located inside bovine endometrial stromal layer (Leung, Derecka et al. 2000; Oliveira, Mansourri-Attia et al. 2013), highlighting a possible paracrine interaction between IFN-γ locally produced with IFN-γRs exposed on the surface of BESCs. Since IFN-γ is considered a strong gene transcription inducer and IDO1 is one of the most interferon gamma stimulated genes (ISGs) (Taylor and Feng 1991), the functionality of IFN-γRs was evaluated through a luciferase assay, performed with a luciferase reporter construct based on IDO1 gene promoter, generated by cloning, for the first time, the bovine IDO1 gene promoter from bovine genomic DNA. As expected, it properly responded to IFN-γ treatment, in a dose relate manner. Since previous studies have shown that IFN-γ is fundamental to contrast chronic murine γ-herpesvirus 68 infection (MHV68) in mice (Tibbetts, van Dyk et al. 2002; Steed, Barton et al. 2006), it was of interest to investigate if this cytokine could have the same effect on BoHV-4 lytic replication on bovine endometrial stromal cells. Infection of BESCs revealed that IFN-γ strongly restricts BoHV-4 replication respect to the untreated control. This result was obtained by different methods, both pre-treating or post treating BESCs with IFN-γ and establishing BESCs self-expressing IFN-γ. Although the role of this cytokine has been shown to be essential during BoHV-4 infection in BESCs, the molecular mechanism by which this occurs is unknown. As IDO1 is one of the most important genes activated by IFN-γ, its potential involvement in IFN-γ restricted BoHV-4 replication was investigated. IDO1 enzyme is a single chain oxidoreductase catalyzing the first, rate-limiting

step of tryptophan degradation in the biosynthesis of the central metabolic regulator nicotinamide adenine nucleotide (NAD) along the kynurenine pathway. In mammals, the IFN-γ mediated induction of IDO1 expression was discovered to be crucial in the contest of antimicrobial activity, depleting tryptophan essential for the replication of some pathogens, such as bacteria and viruses (Adams, Besken et al. 2004; Adams, Besken et al. 2004). It was also found to be a potent immune-regulatory molecule in pregnancy, long-term immune responses, chronic infection and tumors (Mellor 2005; Zelante, Fallarino et al. 2009). For these reasons, IDO1 was hypothesized to be responsible of the IFN-γ mediated restriction of BoHV-4 replication in BESCs. In order to verify this supposition, BESCs were treated with IFN-γ, in the presence of IDO1 specific inhibitor and subsequently infected with BoHV-4. However, IDO1 inhibitors could not be able to abolish BoHV-4 replication restriction in IFN-γ treated BESCs as well as IDO1 constitutively expressed BESCs could not overturn IFN-γ effect on BoHV-4 infected BESCs. Based on these data, it was supposed the possible involvement of Immediate Early gene 2 (IE2) as a probable protagonist of this mechanism of inhibition.

Immediate early (IE) genes, as the name suggest, are the first genes immediately transcribed after infection and they do not require prior viral protein synthesis for their expression but only host cell transcription factors, already present at the moment of infection (Staudt and Dittmer 2007). BoHV-4 IE2 protein (homologous to Epstein-Barr virus replication and transcription activator, Rta, and thus designed as BoHV-4 Rta) is encoded by open reading frame 50 (ORF 50) and it is well conserved among all *Herpesviruses* (Zimmermann, Broll et al. 2001). BoHV-4 Rta expression plays an essential role in initiating viral replication, both during reactivation of latently infected non permissive cells and during *de novo* infection of permissive cells (Donofrio, Cavirani et al. 2004). On the basis of these data, it was interesting to evaluate if IFN-γ activity on BoHV-4 replication could be related to IE2 gene. As expected, IE2 expression was heavily reduced in IFN-γ treated BoHV-4 infected cells, when compared

with the untreated control. To understand if the IE2 promoter was effectively involved in this effect, BESCs were transfected with a reporter construct expressing the luciferase under the control of IE2 gene promoter and subsequently treated with IFN-γ. BoHV-4 IE2 gene promoter activity resulted strongly down-regulated in a dose-dependent manner. To identify which sites of IE2 promoter were involved in IFN-γ mediate IE2 inhibition, four deleted fragments of IE2 promoter gene were amplified and cloned inside the pGL3-Basic vector reporter plasmid. The plasmids obtained were then transfected in BESCs and treated with IFN-γ. The luciferase assay showed a reduction of IFN-γ response for deletions between -680 and -455 in IE2 promoter. All these results confirm the hypothesis that IFN-γ mediated BoHV-4 replication restriction in BESCs is IE2 gene expression dependent. Through these experiments, a new and more complete scenario, regarding the onset of bovine uterine diseases, could be defined. During the post partum period, cows are particularly prone to the development of uterine diseases, because of the direct penetration of environmental bacteria inside uterine lumen. The initial defense of mammalian endometrium against these pathogens is based on the innate immune system, including Toll-Like Receptors (TLRs), antimicrobial peptides (AMPs) and acute phase proteins. Microorganisms are detected by Toll Like Receptors (TLRs), exposed on the surface of bovine epithelial cells, and their activation allow the synthesis of several pro-inflammatory cytokines and chemokines, which mobilize and activate immune cells. Inflamed uterus attracts BoHV-4 persistently infected macrophages from the periphery to the site of inflammation, where BoHV-4 replication can be reactivated. TNF-α, produced by epithelial and stromal cells, and IFN-γ, secreted by uterine resident Natural Killer (NK), Natural Killer T (NK T) and T Lymphocytes cells, were demonstrated to act in a opposite manner on BoHV-4 infection, the first one increasing and the second one restricting its replication, both of them modulating IE2 gene promoter activation and ORF50/Rta production. It becomes intuitive that, in normoergic animals, BoHV-4 replication can be maintained under control by IFN-γ production while, in

animals with impaired IFN-γ axis response, inflammation can be shift from an acute status toward a chronic one.

3.2 *Perspectives on future research*

3.2.1 Introduction

As the right mechanism by which BoHV-4 infects the endometrial substrate is not still understood, it could be of interest to deeply investigate the interaction between the virus and the endometrial layer, analyzing the differences showed by BoHV-4 infected and uninfected bovine endometrial stromal cells, in terms of gene expression patterns. Basing on preliminary data obtained through RNA sequencing (RNAseq) analysis, it was noticed that several genes are up-regulated after BoHV-4 infection and that, among these genes, Metalloproteinase 1 is one of the most relevant, because of its possible implication in the development of bovine endometritis. Subsequent analyzes elicited by western-blot and real time techniques, were able to corroborate these results, paving the way for a new experimental approach, based on RNAseq technology, for the study of the onset of pathologies.

3.2.2 Matherials and methods

Endometrial cell isolation and primary cultures

Bovine uteri from post-pubertal non-pregnant BoHV-4 serum negative animals with no evidence of genital disease were collected at a local abattoir immediately after slaughter and kept on ice until further processing in the laboratory. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the ovarian morphology. Genital tracts with an ovarian Stage I corpus luteum were selected for endometrial cell isolation and culture and only the horn ipsilateral to the corpus luteum was used. Uteri were dissected and washed firstly with a 70% alcohol solution and then with sterile PBS solution, supplemented with 100 IU/ml of penicillin (Sigma), 100 µg/ml of streptomycin (Sigma) and 2.5 µg/ml of Amphotericin B (Sigma). The intercaruncular areas of the endometrium were cut into strips and placed into sterile Hank's balanced salt solution (HBSS; Sigma), supplemented with 100 IU/ml of penicillin (Sigma), 100 µg/ml of streptomycin (Sigma)

and 2.5 μg/ml of Amphotericin B (Sigma). Tissue was digested in 25 ml sterile filtered digestive solution, composed by 50 μg/ml of collagenase II (Sigma) diluted in HBSS solution, supplemented with 100 IU/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma) and 2.5 μg/ml of Amphotericin B (Sigma). Following a 1 h of incubation in a shaking water bath at 37 °C, the cell suspension was filtered through a 40 μm mesh (Fisher) to remove undigested material and the filtrate was resuspended in washing medium, composed by HBSS containing 10% FBS (Sigma), 100 IU/ml of penicillin (Sigma), 100 μg/ml of streptomycin (Sigma) and 2.5 μg/ml of Amphotericin B (Sigma). The suspension was centrifuged at 1400 r.p.m. for 7 min and, following two further washes in washing medium, the cells were resuspended in EMEM containing 10% FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin (Sigma) and 2.5 μg/ml of Amphotericin B (Sigma). The cells were plated and medium was changed 18 h after plating, in order to obtain selective attachment of the bovine endometrial stromal cells (BESCs). The purity of stromal cells was verified by function assays and by histology and the absence of immune cells confirmed by RT-PCR for the CD45 pan-leukocyte marker. The culture media was changed every 48 h until the cells reached confluence. All cultures were maintained at 37 °C with 5% CO_2 in air, in a humidified incubator.

Viral Infection

Primary stromal cells were infected at 1 M.O.I. of BoHV-4-EGFPΔTK (Donofrio et al., 2002) and incubated at 37 °C for 2 hours. Infected cells were then washed with serum free EMEM (Lonza) and then overlaid with EMEM containing 10% fetal bovine serum (FBS), 2 mM of l-glutamine (Sigma), 100 IU/ml of penicillin (SIGMA), 100 μg/ml of streptomycin (SIGMA) and 2.5 μg/ml of Amphotericin B (Sigma).

Reverse transcription PCR

Total RNA was isolated by Trizol (Invitrogen) from primary stromal cells infected or un-infected with 1 M.O.I. of BoHV-4ΔTKEGFP (Donofrio et al., 2002). Three micrograms of RNA were reverse transcribed using Ready to Go, T-Primed first –Strand Kit (Amersham Bioscience) according to the manufacturer's instructions. Bovine Matrix metalloproteinase 1 (bMMP-1) expression was analyzed at 12 hours after infection and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as normalizing control. PCR amplification was carried out in a final volume of 50 μl containing 10 mM Tris-hydrochloride pH 8.3, 0.2 mM deoxynucleotide triphosphates, 2.5mM MgCl2, 50 mM KCl, 5% Di Methyl Sulfoxide (DMSO) and 0.25 μM of each primer. The primers used for the amplification of bMMP-1 and GAPDH are listed in the table 3. One hundred nanogram of cDNA samples were amplified over 35 cycles, each cycle consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and chain elongation with 1U of Taq DNA Polymerase (Fermentas) at 72 °C for 30 sec with a final extension at 72°C for 5 min. The so generated amplicons were then checked in 2% agarose gel and visualized after ethidium bromide staining in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM tTris-Acetate, 1mM EDTA).

Real Time PCR

Total RNA was extracted from BoHV-4ΔTKEGFP (Donofrio et al., 2002) infected and un-infected stromal cells; then, RNA was reverse transcribed as described above. MMP1 and IE2 gene expression were evaluated by Real Time PCR using the StepOne Real-Time PCR System (Applied Biosystem) and Maxima SYBR Green qPCR Master Mix (2x Rox solution provided) starting with 50 ng of reverse transcribed total RNA. GAPDH expression was used as an internal control using the primers listed in table 3. PCR parameters were the following: an initial

denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 30 sec, and 72°C for 30 sec.

PRIMER NAME		SEQUENCE 5' -> 3'	PRODUCT SIZE (bp)
GAPDH	sense	GGC CTG AAC CAC GAG AAG TAT AA	103
GAPDH	anti-sense	CCC TCC ACG ATG CCA AAG T	
MMP-1	sense	TGT GGA GAC GGT GAA GAA ATA CCT	102
MMP-1	anti-sense	AGC TTT TCA GTT ATG AGG CCA CC	

TABLE 3: list of primers used in this work

Western Blotting

Protein cell supernatants were collected from 75cm^2 flasks of primary stromal cells infected or uninfected with 1 M.O.I. of BoHV-4ΔTKEGFP (Donofrio et al., 2002) at 48 hours after infection. 15 ml of supernatants were concentrated using Amicon Ultra 15 ml Filters Purification and Concentration (Millipore), through a 40 minutes centrifugation at 10000 r.p.m., as suggested by the manufacturers. Proteins were electrophoresed through a 12% SDS-PAGE gel and transferred to nylon membranes by electroblotting. Membranes were incubated with mouse anti bovine MMP-1 antibody (3B6, diluited 1:1000) (Thermo Scientific). As a secondary antibody an anti-mouse IgG horseradish peroxidase conjugated (A0545, SIGMA) was used and the signal was visualized by enhanced chemiluminescence (ECL kit, Pierce).

3.2.3 Results

MMP1 over-expression in BoHV-4 infected endometrial stromal cells

Referring to preliminary data concerning RNASeq analysis, it was of interest to investigate the real effect of BoHV-4 infection on Metalloproteinase 1 expression. For this purpose, primary endometrial stromal cells were infected with 1 M.O.I. of BoHV-4 EGFPΔTK and 12 hours after infection, MMP-1 expression was verify through PCR and qReal Time analyses (Fig. 37 A and B). Bovine GAPDH was used as internal control. MMP1 secretion in cell supernatant was evaluated 48 hours after infection of primary stromal cells with 1 M.O.I. of BoHV-4 EGFPΔTK (Fig. 37 C)

respect to mock uninfected control. All these experiments revealed how MMP1 transcription and translation increase in relation to BoHV-4 infection.

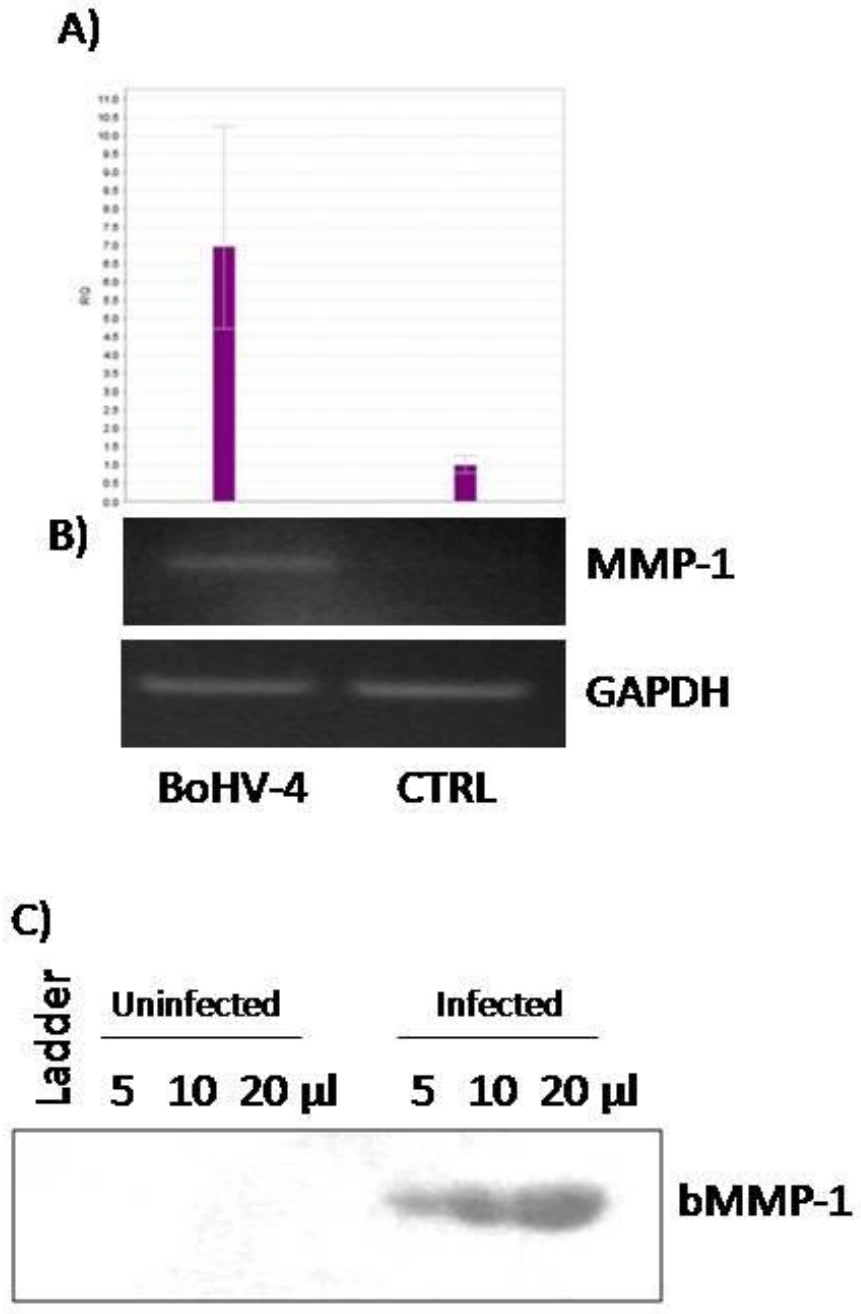

FIGURE 37: Analysis of MMP1 expression in BoHV-4-EGFPΔTK infected and uninfected BESCs. A) Quantitative real time PCR B) Semi quantitative reverse transcription PCR. Bovine GAPDH was employed as an internal control. **C) Western immune-blotting.**

3.2.4 Discussion

The role played by BoHV-4 in the development of bovine post-partum uterine diseases is still not well understood and the right mechanism by which the virus interacts with the stromal layer needs to be investigated. To better understand how endometrial stromal cells sense, interact and respond to BoHV-4 infection, it was of great interest to analyze the different expression patterns showed by infected and un-infected cells, in order to underline which genes are up-regulated or down-regulated after BoHV-4 infection. This type of analysis, which was possible thanks to the use of a novel RNA sequencing Technology (RNASeq), highlighted how BoHV-4 is able to induce the expression of several cellular genes, some of which are involved in cell-cycle regulation, in the activation of inflammation (Interleukin-8), and in extracellular matrix degradation, such as Matrix Metalloproteinase 1. As the involvment of Matrix Metalloproteinases in the development of several diseases was demonstrated, it could be of interest to analyze the role that the activation of Matrix Metalloproteinase 1, could play in the contest of BoHV-4 infection inside endometrium. Matrix metalloproteinases are conserved multi-domains endopeptidases, able to degrade different extracellular components (Mastroianni and Liuzzi 2007). Generally, they are produced like inactive enzymes, requiring chemical or catalytic changes to be activated, in response to a variety of inductions. MMPs play an important role both in remodeling extracellular matrix (ECM) and in the context of immune response activation, modulating chemokines and cytokines activity. As they are involved in several cell and inflammatory processes, the right balance between MMPs and their specific tissue inhibitors (TIMPs) expression is essential for the establishment of an health or pathogenic condition. In fact, dysregulation of MMPs expression or deficit of TIMPs production can determine the development of several chronic and degenerating diseases (Chang and Werb 2001) (Hernandez-Guillamon, Mawhirt et al. 2015) (Zhang, Chen et al. 2015) (Zhang, Chen et al. 2015) (Rabkin 2014). The correlation between dysregulation of MMPs and

different infectious diseases was also demonstrated (Elkington PT 2005). Among the viral and bacterial pathogens that can cause metalloproteinases over-production there are Human immunodeficiency Virus (HIV), Human T lymphotrophic Virus (HTLV-1), Hepatitis B virus as well as *Helicobacter pylori* and *Mycobacterium tuberculosis* (Elkington PT 2005) (Mastroianni and Liuzzi 2007). The involvement of MMPs during infections is still not well known. It should be hypothesized that MMPs can participate to tissue repair, when viruses as well as bacteria cause damages and lesions. In the same time, the fact that some viruses or bacteria are able to induce MMPs expression can be considered as a mechanism used by the pathogens to spread and diffuse through cells and tissues. Over-expression of MMP1 transcript and protein was observed inside endometrial stromal cells, at first with RNASeq analysis and then through qReal Time and traditional PCR techniques and westernblotting, as a consequence of BoHV-4 infection and an involvement of this enzyme in the development of bovine uterine disease could be hypothesized. Bos Taurus Metalloproteinase 1 (MMP1) is also known as interstitial collagenase because of its ability to cleave triple helical regions especially of type I, II and III collagens but also of type VII and X ones. It has a typical structure, consisting of a pre-domain, a pro-domain, a catalytic domain, a linker region and a hemopexin-like domain. MMP-1 is always produced as a interstitial collagenase precursor and its 2015 pb mRNA encodes for an inactive peptide of 469 aa (Tamura, Shimokawa et al. 1994). The pre-domain is a signal peptide of about 18 amino acids (18 aa) and its removal is indispensable for MMP-1 secretion in extracellular space (Sudbeck, Jeffrey et al. 1992). The pro-domain is an activation peptide of about 80 aa; it is usually cleaved during maturation of MMP-1 precursor, loosing every biological function after cleaving, but its removal is not always necessary for MMP-1 activation. Inside this domain there is the conserved sequence (PRCGVPD) responsible of catalytic Zinc ion binding, determinant for enzyme inhibition. For this reason, this region is important for that mechanism usually described as “cysteine switch” (Van Wart and Birkedal-Hansen 1990). In

fact, the dissociation of Zinc ion from cysteine is generally associated with MMPs activation. Connected with a small “linker region”, the N-terminal domain is characterized by the presence of four different Hemopoxin-like domains, which are primarily related to the interaction of metalloproteinases with MMPs inhibitors. Bovine MMP-1, as well as other metalloproteinases, is related to endometrium physiology. Although MMPs production is mostly studied in the context of human uterus, it was demonstrated that MMP-1 expression usually increased during menstruation, because it plays an important role in the breakdown of endometrium (Wathes, Cheng et al. 2011), and during uterine involution after parturition, which was especially observed in rats (Manase, Endo et al. 2006). It was observed that MMP-1, 2, 3, 9 and 13 are the mostly ones expressed inside bovine endometrium during late gestation (Kizaki, Ushizawa et al. 2008) and that MMP-1 and MMP-13 are specifically down-regulated immediately in post-partum uterus. Furthermore, MMP-1 over-expression was observed inside bovine endometrium during pro-inflammatory processes, which can overcome in the case of metritis or endometritis, developed after bacterial or viral infections (Swangchan-Uthai, Lavender et al. 2012). In fact, it was demonstrated that LPS, usually produced during gram negative bacterial infections, significantly increased expression of MMP-1 and MMP-13, which are also produced with high levels in the endometrium of cows with severe negative energy balance status (NEB). For these reasons, cows with NEB and uterine infections can delay the mechanisms of endometrial repair in postpartum period because of alteration of MMPs activity (Swangchan-Uthai, Lavender et al. 2012). In this context, BoHV-4 mediated MMP1 over-expression can be probably explained as a cooperating factor determinant for the establishment of an uterine disease, because of the delay in the tissue remodeling and repairing after parturition. Moreover, the virus can use MMP1 ability to degrade ECM in order to spread inside endometrium and, in the same time, the increased permeability of blood vessels, caused by MMP1 activity, makes possible a better penetration of the bacteria,

responsible of the onset of uterine pathology, in the bloodstream, causing the passage from a local inflammation to a systemic one. This could be another way by which BoHV-4 and bacterial infections inside endometrium can be related. Future investigations are oriented to understand the way by which BoHV-4 is able to induce MMP1 expression as well as the right mechanism through which this enzyme acts. Finally, it will be of primary interest to investigate which are the other genes over-expressed after BoHV-4 infection and which one could be their role in the onset of bovine uterine diseases.

4 CHAPTER 4

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Appendix: List of papers published during the 3 years of the PhD course on "Medical and Veterinary Science" (2013-2015):

1. **Bovine herpesvirus 4-based vector delivering a hybrid rat/human HER-2 oncoantigen efficiently protects mice from autochthonous Her-2+ mammary cancer.** **Jacca S**, Rolih V, Quaglino E, Franceschi V, Tebaldi G, Bolli E, Rosamilia A, Ottonello S, Cavallo F, Donofrio G. OncoImmunology 2015. doi:10.1080/2162402x.2015.1082705.
2. **BoHV-4 immediate early 1 gene is a dispensable gene and its product is not a bone marrow stromal cell antigen 2 counteracting factor.**
   Franceschi V, Capocefalo A, **Jacca S**, Rosamilia A, Cavirani S, Xu F, Qiao W, Donofrio G. BMC Vet Res. 2015 Aug 27;11:224. doi: 10.1186/s12917-015-0540-4.
3. **BoHV-4-Based Vector Single Heterologous Antigen Delivery Protects STAT1(-/-) Mice from Monkeypoxvirus Lethal Challenge.**
   Franceschi V, Parker S, **Jacca S**, Crump RW, Doronin K, Hembrador E, Pompilio D, Tebaldi G, Estep RD, Wong SW, Buller MR, Donofrio G. PLoS Negl Trop Dis. 2015 Jun 18;9(6):e0003850. doi: 10.1371/journal.pntd.0003850. eCollection 2015 Jun.
4. **Interferon gamma-mediated BoHV-4 replication restriction in bovine endometrial stromal cells is host IDO1 gene expression independent and BoHV-4 IE2 gene expression dependent.**
   Jacca S, Franceschi V, Agosti M, Cavirani S, Mistretta F, Donofrio G. Biol Reprod. 2014 Nov;91(5):112. doi: 10.1095/biolreprod.114.123000. Epub 2014 Oct 1.
5. **Generation and characterization of the first immortalized alpaca cell line suitable for diagnostic and immunization studies.**
   Franceschi V, **Jacca S**, Sassu EL, Stellari FF, van Santen VL, Donofrio G. PLoS One. 2014 Aug 20;9(8):e105643. doi: 10.1371/journal.pone.0105643. eCollection 2014.

6. **Retinal transduction profiles by high-capacity viral vectors.**

Puppo A, Cesi G, Marrocco E, Piccolo P, **Jacca S**, Shayakhmetov DM, Parks RJ, Davidson BL, Colloca S, Brunetti-Pierri N, Ng P, Donofrio G, Auricchio A. Gene Ther. 2014 Oct;21(10):855-65. doi: 10.1038/gt.2014.57. Epub 2014 Jul 3.

7. ***In vivo* image analysis of BoHV-4-based vector in mice.**

Franceschi V, Stellari FF, Mangia C, **Jacca S**, Lavrentiadou S, Cavirani S, Heikenwalder M, Donofrio G. PLoS One. 2014 Apr 21;9(4):e95779. doi: 10.1371/journal.pone.0095779. eCollection 2014.

8. **Enlightened Mannhemia haemolytica lung inflammation in bovinized mice.**

Stellari FF, Lavrentiadou S, Ruscitti F, **Jacca S**, Franceschi V, Civelli M, Carnini C, Villetti G, Donofrio G. Vet Res. 2014 Jan 25;45:8. doi: 10.1186/1297-9716-45-8.

9. **Efficient heterologous antigen gene delivery and expression by a replication-attenuated BoHV-4-based vaccine vector.**

Capocefalo A, Mangia C, Franceschi V, **Jacca S**, van Santen VL, Donofrio G. Vaccine. 2013 Aug 20;31(37):3906-14. doi: 10.1016/j.vaccine.2013.06.052. Epub 2013 Jul 3.

10. **Bovine endometrial stromal cells support tumor necrosis factor alpha-induced bovine herpesvirus type 4 enhanced replication.**

Jacca S, Franceschi V, Colagiorgi A, Sheldon M, Donofrio G.Biol Reprod. 2013 May 31;88(5):135. doi: 10.1095/biolreprod.112.106740. Print 2013 May.