Molecular Immunology 111 (2019) 136–144

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Modulation of cathelicidins, IFNβ and TNFα by bovine alpha-herpesviruses is dependent on the stage of the infectious cycle

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ARTICLE INFO

Keywords:
Cathelicidins
Cytokines
Bovine
Alpha-Herpesviruses
Expression patterns
Respiratory system

ABSTRACT

Production of antimicrobial peptides cathelicidins, interferons and cytokines is an important feature in airway epithelial host defense. The innate immune response to alpha-herpesvirus infection at the sites of primary replication has not been fully studied. Thus, the aim of this study was to determine the expression of innate immune components, cathelicidins, IFNβ, TNFα and TNF receptors (TNFRI and TNFRII) during acute infection and reactivation of bovine herpesvirus type 1 (BoHV-1) and 5 (BoHV-5) in the respiratory tract and lymphoid tissue of their natural host. We found that BoHV infection modulates mainly the expression of BMAP28, a key cathelicidin in cattle. It was downregulated by both viruses in retropharyngeal lymph nodes of acutely infected-calves, and it was accompanied by a lower expression of IFNβ, TNFα and TNFRI. BoHV-5 showed a pronounced role in the downregulation of BMAP28, even in nasal mucosa and lung. However, during reactivation, BoHV-5 upregulated both BMAP28 and IFNβ in retropharyngeal lymph nodes. Acute replication induced also TNFα mRNA and protein synthesis, and expression of TNFRI and II was positively regulated during both acute infection and reactivation, particularly in the trachea. Moreover, BMAP27 was detected during BoHV-1 reactivation suggesting a potential role at this stage. Thus, cathelicidins are implicated in alpha-herpesvirus infections of the bovine respiratory system and the response is distinct during BoHV-1 and BoHV-5 acute infection and reactivation. This demonstrates that these viruses modulate differentially the components of innate immune response, possibly influencing their pathogenesis. This study provides an initial pilot analysis of factors that might be implicated in alpha-herpesvirus infection of the bovine respiratory system.

1. Introduction

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are closely related alpha-herpesviruses that replicate in the epithelium of the upper respiratory tract. BoHV-1 is associated with infectious bovine rhinotracheitis (IBR), an infection of the upper respiratory tract characterized by nasal and ocular discharges, fever, low milk production and anorexia (Muylkens et al., 2007). BoHV-1 causes immunosuppression, and the respiratory infection can lead to pneumonia and death due to the action of opportunistic microorganisms (Tikoo et al., 1995). Abortion due to BoHV-1 in cows and fatal systemic infections in young calves may also occur (Miller et al., 1991). BoHV-5 is the primary etiological agent of non-suppurative necrotizing meningoencephalitis in calves (Perez et al., 2002). It has occasionally been isolated from aborted bovine foetuses (Schudel et al., 1986) and from the genital and respiratory tracts of cattle (Schudel et al., 1986; Esteves et al., 2003; Kirkland et al., 2009). From the primary site of replication, alpha-herpesviruses travel toward the corresponding nerve ganglia to establish lifelong latency. Virus reactivation leads to virus transmission and spread (Del Médico Zajac et al., 2010).

Cathelicidins are small cationic peptides which are mainly expressed by epithelial and innate immune cells (Zanetti, 2004) and play important roles in airway epithelial host defenses (Schrumpf et al., 2017). In the respiratory tract, cathelicidins can directly inhibit

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https://doi.org/10.1016/j.molimm.2019.04.022
Received 13 February 2019; Received in revised form 24 April 2019; Accepted 25 April 2019
Available online 01 May 2019

infection while triggering recruitment of immune cells (Tecle et al., 2010). Cathelicidins are abundantly expressed in mammalians, including cattle (Kościuczuk et al., 2012). Whereas humans or mice have a single cathelicidin, there is a variety of structurally diverse cathelicidins in cattle (Zanetti, 2005). Among them, cathelicidins BMAP27 and BMAP28 have a broad-spectrum antimicrobial activity against bacteria, viruses and parasites (Benincasa et al., 2003; Haines et al., 2003, Young-Speirs et al., 2018). These peptides have direct antiviral activity against respiratory syncytial virus *in vitro* and protective function *in vivo* in mice and humans (Currie et al., 2016). BMAP28 also provides *in vitro* protection against human herpes simplex virus type 1 (HSV-1) (Benincasa et al., 2003). Other cathelicidin functions include chemotaxis, epithelial cell activation, epithelial wound repair and activation of cytokine secretion.

Interferons (IFNs) are key defenses against herpesviral infections (Paludan et al., 2011; Gaajetaan et al., 2012). IFNβ, but not IFNα, demonstrated antiviral effect against HSV-1 following stimulation of dendritic cells (Gaajetaan et al., 2012). Induction of this particular type I IFN is also important in BoHV infections (Perez et al., 2008; Marin et al., 2014a). Expression of tumor necrosis factor alpha (TNFα) and its receptors was associated with bovine leukemia virus disease progression in cattle (Kabeya et al., 2001; Konnai et al., 2006) and early viral elimination (Kabeya et al., 1999). The biological role of TNFα is defined by the cross-talk between its receptors, TNFRI and TNFRII. Whereas TNFRI has been associated with proliferation, apoptosis and necroptosis, TNFRII is associated with anti-inflammatory responses (Cabal-Hierro and Lazo, 2012). Little is known about herpesvirus replication and host innate immune responses at the primary replication site of the bovine respiratory tract. Our aim was to determine the expression of innate immune components, cathelicidins, IFNβ, TNFα and TNF receptors during acute infection and reactivation of BoHV-1 and BoHV-5 in the respiratory tract and lymphoid tissue of their natural host.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby Bovine Kidney (MDBK) cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used for this study. MDBK cells were propagated in Minimum Essential Medium (Eagle) with Earle salts (E-MEM) (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% foetal bovine serum (Bioser, Buenos Aires, Argentina), certified free from adventitious viruses and antibodies, and antibiotic-antimycotics (Gibco, Langley, OK, USA) (100 U/ml penicillin G, 100 µg/mL streptomycin sulphate, and 0.025 µg/mL amphotericin B). Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Virus reference strain Cooper (BoHV-1.1) and the Argentinean BoHV-5 field strain (identified as 97/613), both in the eighth passage, were used for animal challenges. The isolate 97/613 was recovered from the brain of a 2-year-old cow with necrotizing encephalitis (Perez et al., 2002). Virus identification was confirmed by isolation in cell culture, followed by direct immunofluorescence using a polyclonal antibody against BoHV (American BioResearch, Sevierville, TN, USA) and by real-time PCR with high resolution melting (HRM) analysis (Marin et al., 2016a). Virus strains for inoculum preparation were propagated in MDBK cells for 24 h. Supernatants were harvested and stored at −80 °C until use. Viral titres were determined by the endpoint titration method and expressed as tissue culture infective doses ($TCID_{50}$) (Reed and Muench, 1938).

2.2. Experimental design and BoHV challenge

All procedures for animal handling and experimentation were performed according to the Animal Welfare Committee of the University of the Centre of Buenos Aires Province (Res. 087/02).

Crossbred calves, 6–8 months old, free of detectable antibodies to BoHV-1, BoHV-5 and bovine viral diarrhoea virus were used. Calves were randomly assigned to one of six groups. In Group 1 (*BoHV-1 primary acute infection*), 2 calves were intranasally inoculated with a high dose ($10^{6.3}$ $TCID_{50}$ in 10 mL) of BoHV-1 and in Group 2 (*BoHV-5 primary acute infection*) other 2 calves with $10^{6.3}$ $TCID_{50}$ (in 10 mL) of BoHV-5. These calves were sacrificed at 6 days post-infection (dpi), a time considered the peak of acute infection (Perez et al., 2002). Group 3 (*BoHV-1 reactivation*) consisted of 2 calves that were intranasally inoculated with a low dose (10^3 $TCID_{50}$ in 10 mL) of BoHV-1 and Group 4 (*BoHV-5 reactivation*) of other 2 calves intranasally inoculated with 10^3 $TCID_{50}$ (in 10 mL) of BoHV-5. It is known that this viral dose allows the survival of calves and the establishment of latency (Perez et al., 2002). The animals in Groups 3 and 4 received an intravenous dose of dexamethasone (0.1 mg/kg bodyweight; Dexametona, Schering Plough) at 20 dpi and two additional intramuscular doses 24 and 48 h later (Inman et al., 2002). Calves in these groups were sacrificed at 25 dpi. In Group 5 (*uninfected control for acute infection*), 1 calf was intranasally inoculated with 10 mL MEM as placebo and killed at 6 dpi. In Group 6 (*uninfected control for reactivation)* 1 calf was intranasally inoculated with 10 mL MEM as placebo, treated with dexamethasone using the same regime as the calves in Groups 3 and 4 and sacrificed at 25 dpi. All calves were observed daily for the presence of nasal or ocular secretions or other clinical signs. Ocular and nasal secretions were periodically obtained for viral titres, as explained below.

2.3. Sample collection

After euthanasia, tissue sections from the epithelium of the nasal mucosa, trachea and bronchi, apical, middle and diaphragmatic lobes of the lungs and retropharyngeal, bronchial and mediastinal lymph nodes were aseptically and individually collected for viral isolation and viral DNA detection by real-time PCR (Marin et al., 2016b). Samples from the retropharyngeal lymph nodes and the respiratory tract, including epithelium of the nasal mucosa and trachea, and diaphragmatic lobe of the lungs, were obtained for gene and protein expression studies. These tissues were the main sites from which the viruses were isolated and/or the viral DNA was detected (Marin et al., 2016b). Retropharyngeal lymph nodes were also included in the analysis since lymph nodes draining the respiratory tract have shown a main transcriptional response at the peak of clinical signs after intranasal inoculation with BoHV-1 (Behura et al., 2017).

2.4. RNA extraction, DNase treatment and reverse transcription

Total RNA from the respiratory system was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and digested with DNase I Amplification Grade (Invitrogen, Carlsbad, CA, USA) for 30 min at 37 °C to remove any contaminating genomic DNA (gDNA). The quality and quantity of the resulting RNA were determined using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). Complementary DNA (cDNA) was synthesized using a reaction mixture containing 1 µg of total RNA, random hexamers (12 ng/µl) (Promega, Madison, WI, USA), and Moloney murine leukaemia virus reverse transcriptase (10 U/µl) (Promega, Madison, WI, USA). Negative controls, omitting the RNA or the reverse transcriptase, were included.

2.5. Transcriptional gene expression of cathelicidins, IFNβ, TNFα and TNF receptors

Real-time RT-PCR reactions for bovine cathelicidins (BMAP27 and BMAP28), IFNβ1, TNFα and, TNFRI and TNFRII were carried out using specific primers as detailed in Table 1. Expression of the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (McGuire et al., 2004). The PCR reactions

Table 1
Sequences of primers for mRNA relative quantification by real-time RT-PCR.

mRNA	Primer sense	Amplicon Size (base pairs)	5´-3´sequences	Reference
GAPDH	F[(a)]	112	TTCTGGCAAAGTGGACATCGT	McGuire et al. (2004)
	R[(b)]		CTTGACTGTGCCGTTGAACTTG	
BMAP27	F	163	ATGGGCTGGTGAAGCAATGTGTAG	Tomasinsig et al. (2010)
	R		TGGAGTAGCGGAATGACTGGAGAA	
BMAP28	F	141	TCGGGAGTAACTTCGACATCACCT	Tomasinsig et al. (2010)
	R		GGCCCACAATTCACCCAATTCTGA	
IFNβ1	F	175	AGGAGCTACAGCTTGCTTCG	Perez et al. (2008)
	R		TGACCAATATGGCATCTTCC	
TNFα	F	176	AGCCTCAAGTAACAAGCC	Konnai et al. (2006)
	R		TGAAGAGGACCTGTGAGT	
TNFRI	F	64	AAGCCCAAGCTCTACTCCATCA	New design
	R		GTTCTGGCTCCCCCTCTTTT	
TNFRII	F	142	CAGGCTGTGTTTACCCCCTA	Sakumoto et al. (2011)
	R		TGTCCAAGGTCATGTTGCAT	

[(a)] Forward primer.
[(b)] Reverse primer. The new primers were designed with the Primer Express Software, Applied Biosystems.

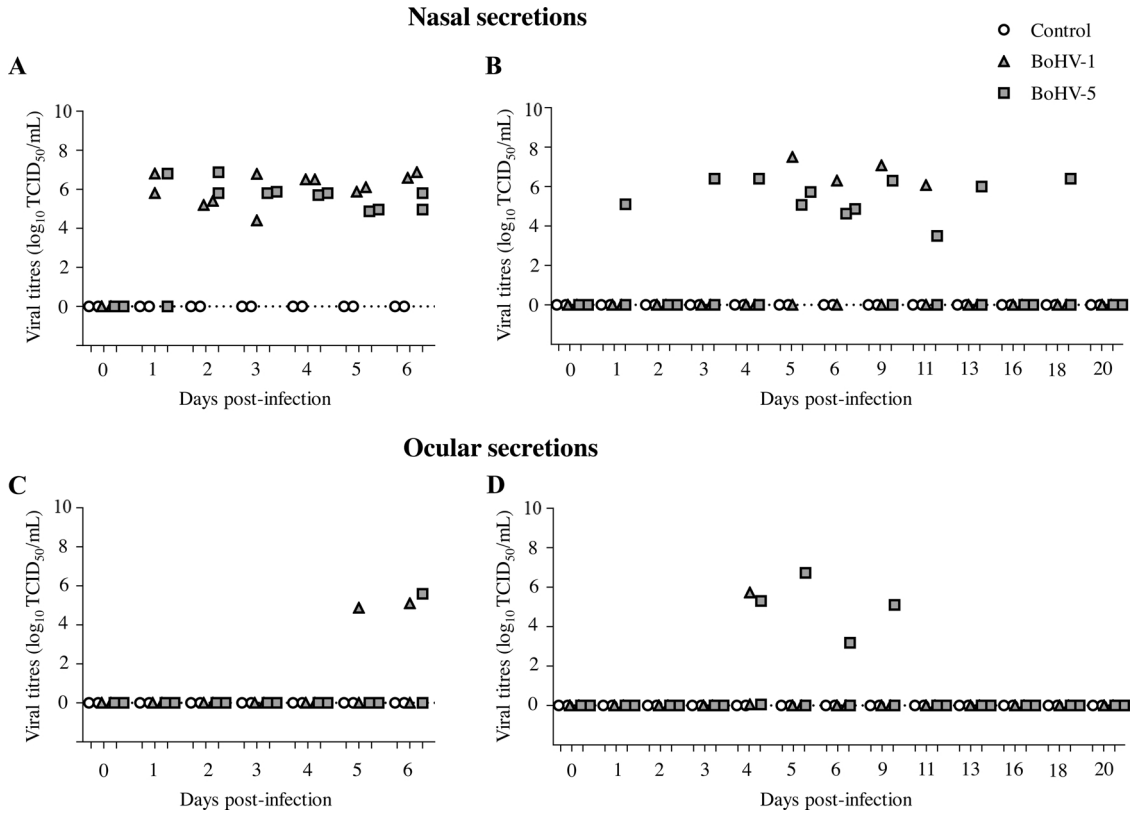

Fig. 1. Viral excretion in nasal (A and B) and ocular (C and D) secretions.
Ocular and nasal secretion were periodically obtained for animals inoculated with a high (A and C) and low (B and D) dose of BoHV-1 (n = 2) and BoHV-5 (n = 2) inoculum. Viral titres in the secretions were determined on different days post-infection by the endpoint titration method and expressed as tissue culture infective doses (log_{10} $TCID_{50}$/mL). Data represent the values obtained for individual calves in the group, at the indicated day and secretion type.

contained 800 nM specific forward and reverse primers, 1X PCR Master Mix (FastStart Universal SYBR Green Master Rox, Roche, Mannhein, Germany), and 1 µl of cDNA sample in a final volume of 20 µl. The amplification and detection of the specific products were carried out in an Applied Biosystems 7500 cycler, with the following amplification conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 20 s at 95 °C and 60 s at 60 °C. After amplification, a melting curve analysis was performed, which resulted in a single product-specific melting curve. Negative controls for cDNA synthesis and PCR procedures were included in all cases. The amplification efficiency was determined for each gene using 10-fold dilutions of the cDNA. The results are reported as the mean fold change of gene transcription levels in the regions of the respiratory system of infected calves over the levels detected in tissue sections of uninfected calves (control).

2.6. Tumor necrosis factor alpha protein detection

Protein levels of TNFα were determined by western blotting in respiratory tissues lysed with radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were determined by BCA assay (Pierce BCA protein assay kit, Thermo Scientific, Rockford, IL, USA). Each sample (30-µg protein) was mixed 1:1 with Laemmli sample buffer, denatured to 70 °C for 10 min, and separated on 10% SDS-PAGE gels. Proteins were transferred onto a PVDF membrane (BioRad, Hercules,

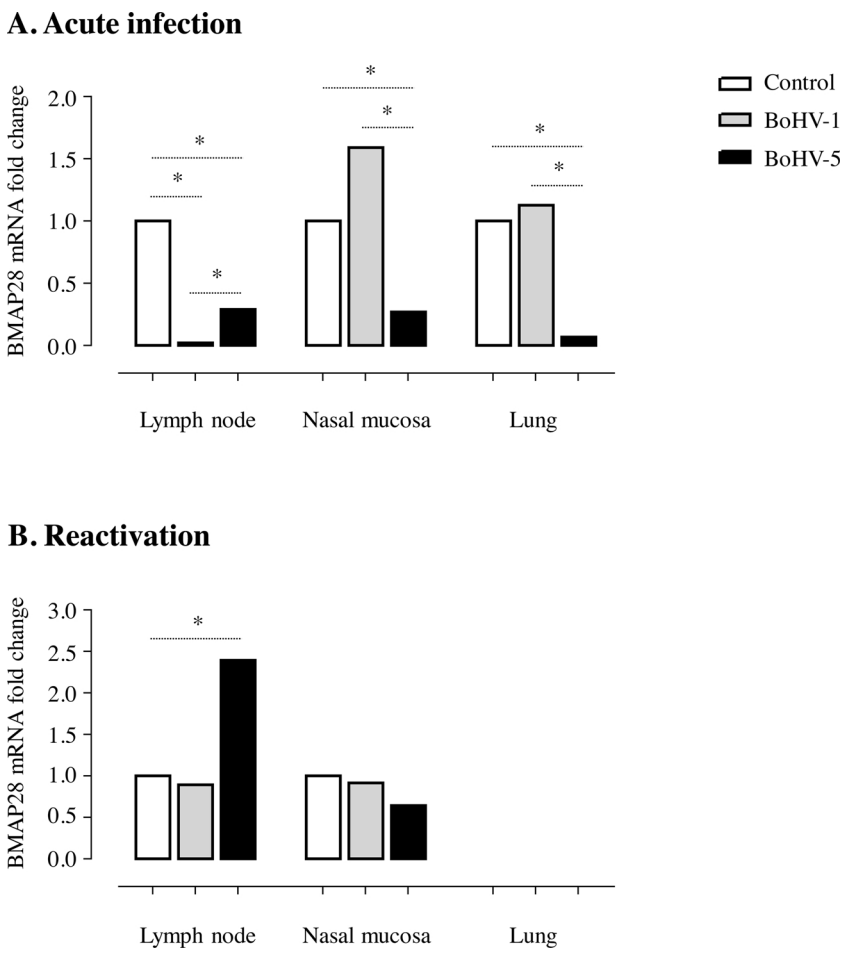

Fig. 2. BMAP28 expression is suppressed upon BoHV-1 and BoHV-5 acute infections (A) and induced upon BoHV-5 reactivation (B).
The results represent the mean fold change of BMAP28 transcription levels in specific areas of the respiratory tract and retropharyngeal lymph nodes of calves infected with BoHV-1 (n = 2) and BoHV-5 (n = 2) over levels detected in the same tissue area of uninfected calves, which served as the control groups. *: statistically significant differences ($P < 0.05$). mRNA relative levels and statistical significance of the differences in mRNA expression were obtained with the software REST (Qiagen Inc., Valencia, CA, USA). For analysis of data, the values obtained for each animal in the group, with 2 technical replicates per animal, were used to obtain a final mean fold-change value for infected groups over the values of the unifected group.

CA, USA) activated with methanol. The membrane was blocked with 5% skim milk powder dissolved in Tris buffered saline plus 0.1% Tween 20 solution (TBST) for 1 h. Membranes were probed with anti-TNFα (Abcam Inc., Cambridge, MA, USA; 1:500) or anti-GAPDH (Calbiochem, Billerica, MA, USA; 1:1000) antibodies at 4 °C overnight. After incubation with a horseradish-peroxidase-conjugate secondary anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:10,000) for 2 h at room temperature, blots were developed using the Clarity Western ECL Detection System (BioRad, Hercules, CA, USA). Image captures and densitometric analyses were performed with the ChemiDoc MP Imaging system and ImageLab 4.0.1 software (BioRad, Hercules, CA, USA), respectively. Normalization was carried out using GAPDH protein levels as reference. The results are reported as the mean fold change of target expression in infected groups compared with the uninfected control group. Independent experiments were conducted three times.

2.7. Statistical analysis

The relative expression analysis of the target genes was performed using the Relative Expression Software Tool (REST, Qiagen Inc., Valencia, CA, USA) for evaluating group differences for significance with a pair-wise fixed reallocation randomization (Pfaffl et al., 2002). The real-time RT-PCR efficiency for each gene was determined by a linear regression model according to the equation: $E = 10[-1/\text{slope}]$.

Paired two-tailed Student's *t*-test was used for comparison between TNFα protein levels in uninfected and infected groups. *P* values less than 0.05 were considered statistically significant and the statistical analysis was performed with Graph Pad Prism software (Graph Pad 4.0.3, CA, USA).

3. Results

3.1. Both BoHV-1 and BoHV-5 developed similar clinical disease in experimentally infected calves

BoHV-1 and BoHV-5 were isolated in nasal secretions from 1 to 6 dpi in acutely infected calves (Fig. 1A). Calves acutely infected with BoHV-1 showed profuse nasal secretions at 1 and 3–5 dpi. In calves acutely infected with BoHV-5, abundant nasal secretions were present from 2 to 5 dpi. Animals inoculated with a low dose-inoculum excreted virus intermittently, from 5 to 11 dpi for BoHV-1 and from 1 to 18 dpi for BoHV-5 (Fig. 1B). Viruses were present in ocular secretions at 5 and 6 dpi after challenge with high doses of BoHV-1 and BoHV-5 (Fig. 1C), whereas in calves inoculated with a low dose-inoculum, viruses were identified in ocular secretions at 4 and from 4 to 9 dpi, respectively (Fig. 1D).

In calves infected with BoHV-1 reactivated with dexamethasone at 20 dpi (Marin et al., 2016b), nasal secretions appeared at 23–24 dpi. Ocular secretions were evident during the period of primary infection, but not during reactivation. After inoculation with a low-dose BoHV-5 and reactivated with dexamethasone, nasal secretions were present at 23 and 24 dpi. Viral ocular secretions were rarely observed; they were present in only one calf at 3 dpi acutely infected with BoHV-5 (data not shown). Infected animals did not show neurological signs or severe respiratory distress. As expected, respiratory or neurological signs were not observed in uninfected animals. These findings showed that calves undergoing acute infection or reactivation from latency of BoHV-1 and BoHV-5 developed comparable clinical conditions.

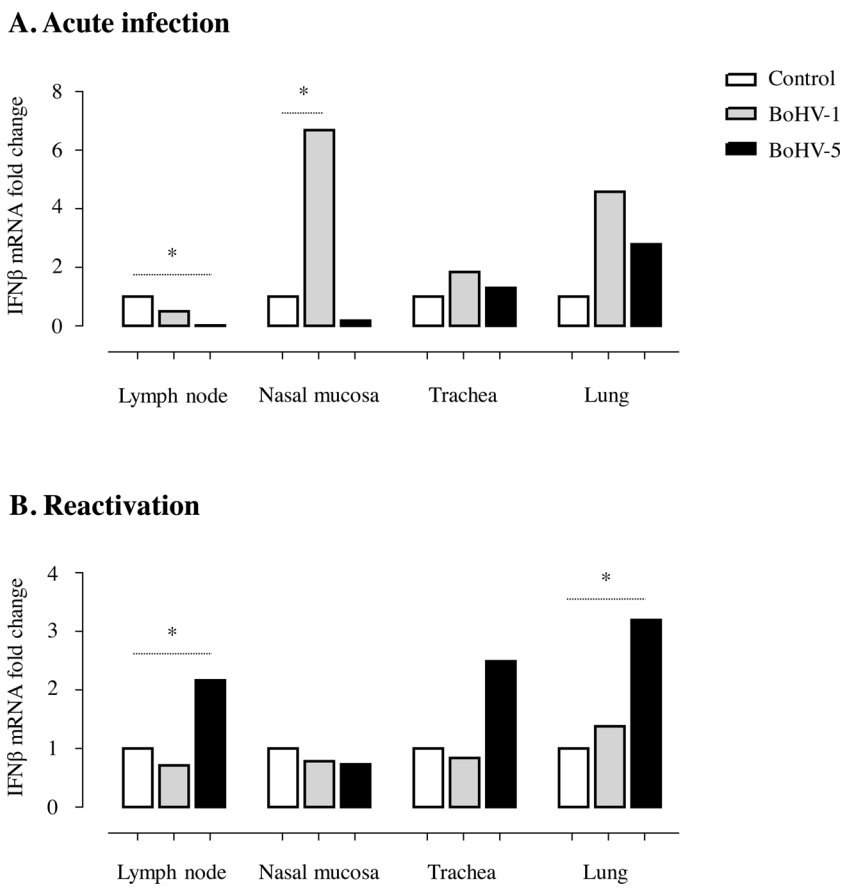

Fig. 3. BoHV-1 and BoHV-5 modulated IFNβ during acute infection (A) but only BoHV-5 increased the levels during reactivation (B).
The results represent the mean fold change of IFNβ transcription levels in specific areas of the respiratory tract and lymphoid tissue of calves infected with BoHV-1 (n = 2) and BoHV-5 (n = 2) over levels detected in tissue sections of uninfected calves, which served as the control groups. *: statistically significant differences ($P < 0.05$) with respect to the uninfected control. mRNA relative levels and statistical significance of the differences in mRNA expression were obtained with the software REST (Qiagen Inc., Valencia, CA, USA). For analysis of data, the values obtained for each animal in the group, with 2 technical replicates per animal, were used to obtain a final mean fold-change value for infected groups over the values of the unifected group.

3.2. Cathelicidin BMAP28 is downregulated during BoHV-5 and BoHV-1 acute infection

Alpha-helical BMAP cathelicidins are key molecules protecting mucosal surfaces against pathogen invasion since they are able to enhance innate immune responses induced by nucleic acids in epithelial and circulating immune cells (Baumann et al., 2017). We showed that infection with BoHV downregulated the transcriptional expression of BMAP28 along the respiratory tract during acute infection (Fig. 2A). In retropharyngeal lymph nodes, both BoHV-1 and BoHV-5 downregulated BMAP28, with BoHV-1 showing more pronounced downregulatory effects only in this tissue (0.02-fold and 0.29-fold, respectively) ($P < 0.05$). Moreover, acute infection with BoHV-5 lowered BMAP28 gene transcriptional levels in nasal mucosa and lungs (0.27-fold and 0.07-fold, respectively). Upon viral reactivation, BoHV-5 induced BMAP28 in retropharyngeal lymph nodes (2.39-fold) ($P < 0.05$) but no changes were observed in nasal mucosa ($P > 0.05$) and BMAP28 mRNA was undetected in lungs (Fig. 2B).

In tracheal epithelium, BoHV-1 and BoHV-5 induced only detectable levels of BMAP28 mRNA whereas it was undetected in uninfected calves (data not shown). BMAP27 expression was barely detected in retropharyngeal lymph nodes from calves acutely-infected with either BoHV-1 or BoHV-5 and after BoHV-1 reactivation, whereas it was absent in this tissue in uninfected animals or in nasal mucosa, trachea and lungs of uninfected and infected calves (data not shown).

3.3. Differential induction of IFNβ expression by BoHV-1 or BoHV-5 acute infection and reactivation

It has been observed that impaired ability to stimulate type I IFN responses predisposes cattle to alpha-herpesviral infections (Andersen et al., 2015), with INFβ being key during early BoHV infection (Perez et al., 2008). In our study, IFNβ expression was consistently detected in the respiratory tract and retropharyngeal lymph nodes of uninfected and infected calves (Fig. 3). In acute infection (6 dpi), BoHV-1 increased IFNβ mRNA levels in the nasal epithelium (6.69-fold; $P < 0.05$), whereas a decrease was detected during BoHV-5 infection of retropharyngeal lymph nodes (0.02-fold) ($P < 0.05$) (Fig. 3A). After dexamethasone-induced reactivation, IFNβ expression was upregulated by BoHV-5 in retropharyngeal lymph nodes (2.17-fold) and lungs (3.2-fold) (Fig. 3B). Thus, IFNβ response was more evident in the lower respiratory tract during BoHV-5 reactivation.

3.4. TNFα is upregulated in tracheal epithelium by acute BoHV-1 and BoHV-5 infection

TNFα is a pivotal proinflammatory cytokine (Biron and Sen, 2001), although its role in BoHV infections has not been fully established. BoHV-1 and BoHV-5 induced TNFα mRNA (6.5- and 32.94-fold, respectively) ($P < 0.05$) in tracheal epithelium during acute infection (Fig. 4A). Likewise, TNFα protein levels increased in the tracheal epithelium from BoHV-1 and BoHV-5 acutely infected calves (Fig. 4B).

During BoHV-1 and BoHV-5 reactivation, TNFα mRNA levels in retropharyngeal lymph nodes decreased (0.06- and 0.36-fold, respectively) ($P < 0.05$) (Fig. 4A). A decrease in relative protein levels was also shown in retropharyngeal lymph nodes of either BoHV-1 or BoHV-5 infections and in trachea after BoHV-5 reactivation (Fig. 4B). Nevertheless it must be considered that TNFα mRNA could not be detected in the trachea.

TNFα gene expression in nasal mucosa and lungs was induced only during BoHV-1 reactivation whereas cytokine expression was undetected in other uninfected or infected tissues. On the other hand, TNFα protein levels were unmodified in nasal mucosa and lung (data not shown).

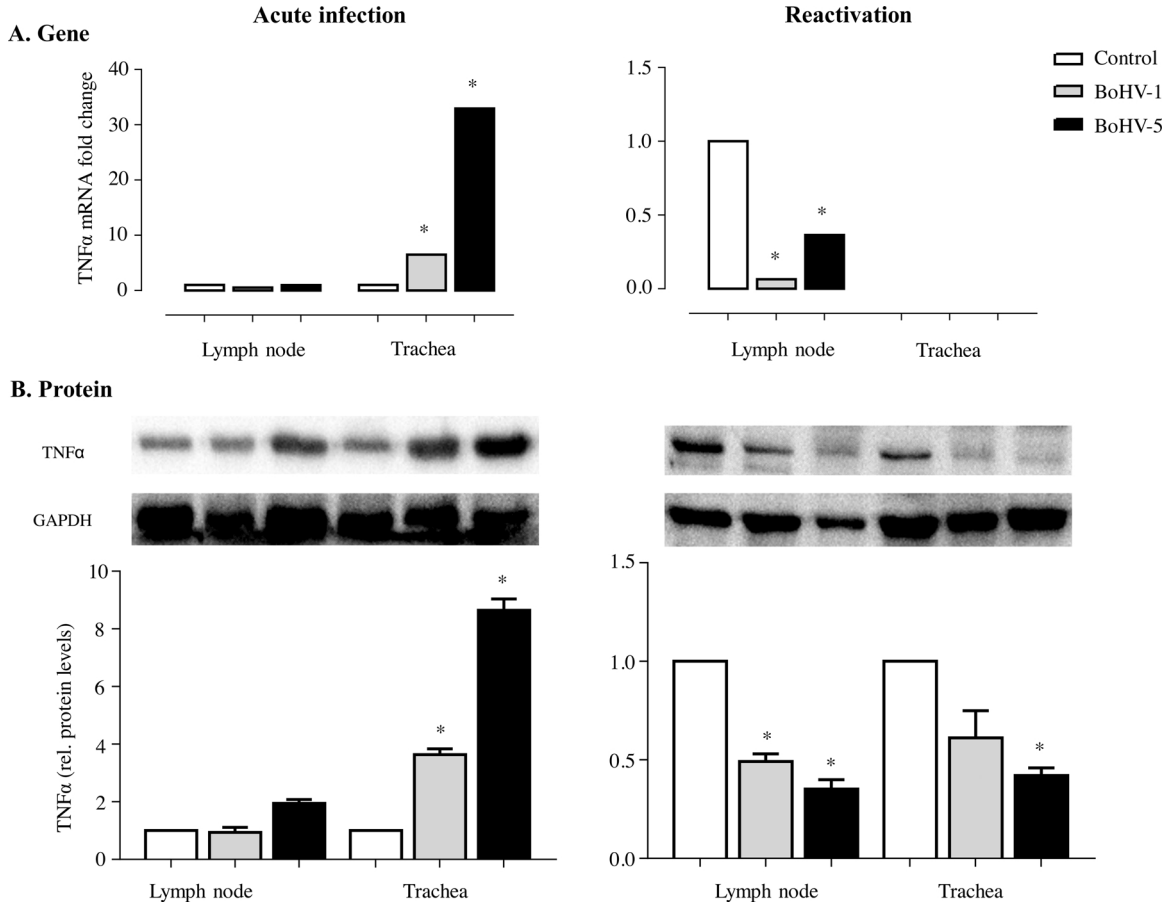

Fig. 4. TNFα expression is modulated by BoHV-1 and BoHV-5 infection in lymph nodes and tracheal epithelium.
Relative gene expression was determined by RT-qPCR (A). Protein expression was measured by western blotting (B). Bars represent densitometric analysis of protein bands. Representative blots from three independent experiments are shown above the corresponding bar. Results represent mean fold change of TNFα transcription levels (A) and protein concentrations (B) in specific tissues of calves infected with BoHV-1 (n = 2) and BoHV-5 (n = 2) versus levels detected in tissue sections of uninfected calves (control). *: statistically significant differences ($P < 0.05$) with respect to the uninfected control. mRNA relative levels and statistical significance of the differences in mRNA expression were obtained with the software REST (Qiagen Inc., Valencia, CA, USA). Paired two-tailed Student's *t*-test was used for protein levels comparison; error bars represent SD. For analysis of data, the values obtained for each animal in the group, with 2 technical replicates per animal for RT-qPCR and 3 experimental replicates for western blotting were used to obtain a mean fold-change value for infected groups over the values of the unifected group.

3.5. TNF receptors (TNFRI and TNFRII) are differentially modulated by BoHV-1 and BoHV-5 infection

Lack of TNFα signaling through its receptors makes mice more susceptible to acute infection but it seems not to alter reactivation of HSV-1 (Mohankrishnan et al., 2018). We found TNFRI as the main receptor modulated during both acute infection and viral reactivation. Downregulation in TNFRI mRNA was observed during BoHV-5 acute infection in retropharyngeal lymph nodes (0.5-fold; $P < 0.05$) (Fig. 5A). TNFRI mRNA was upregulated during acute infection (3.42- to 6.45-fold) in the epithelium of the nasal mucosa from animals infected with both viruses and, in trachea of BoHV-5-infected calves ($P < 0.05$) (Fig. 5A). Reactivation of any of the viruses induced a downregulation of TNFRI in lymphoid tissue and nasal mucosa and in lungs of BoHV-1-infected calves (Fig. 5B). However, a marked TNFRI upregulation was evident in the trachea after infection with any of the viruses and in the lung during BoHV-5 reactivation (12- to 16-fold; $P < 0.05$) (Fig. 5B).

TNFRII was upregulated (2–3 fold) during acute infection in trachea (Fig. 5A) and upon BoHV-5 reactivation in lymph nodes ($P < 0.05$) (Fig. 5B). In nasal mucosa, TNFRII was detected only in infected animals, while was barely detected in lungs.

4. Discussion

BoHV-1 is a major respiratory virus in cattle. Most virus isolates from cases of neurological disease have been identified as BoHV-5. Nevertheless, BoHV-1 and BoHV-5 can be isolated from very similar clinical conditions (Silva et al., 2007). Both herpesviruses multiply on the tracheal epithelium of acutely-infected animals (Marin et al., 2016b). In this work, we described the responses of cathelicidin antimicrobial peptides and viral cytokines during BoHV-1 and BoHV-5 acute infection and reactivation in the respiratory tract.

Cathelicidins are relevant components of the innate immune defense which are responsible for attracting and activating inflammatory cells, such as neutrophils, B lymphocytes and macrophages (Tecle et al., 2010). Indeed, cathelicidin production is key in controlling some intracellular pathogens, such as *Mycobacterium tuberculosis*, during primary infection (Liu et al., 2007; Castañeda-Delgado et al., 2010) and reactivation (Gonzalez-Curiel et al., 2011). We found that BMAP28, a key cathelicidin in cattle, was downregulated in retropharyngeal lymph nodes of calves acutely infected with BoHV-1, while in those acutely infected with BoHV-5 the decrease in the expression levels occurred in both retropharyngeal lymph nodes and respiratory tract. Both viruses seem to impede cathelicidin transcription as a potential mechanism of evasion. Although BoHV-1 and BoHV-5 are closely related alphaherpesviruses, with a high genomic homology (Delhon et al., 2003),

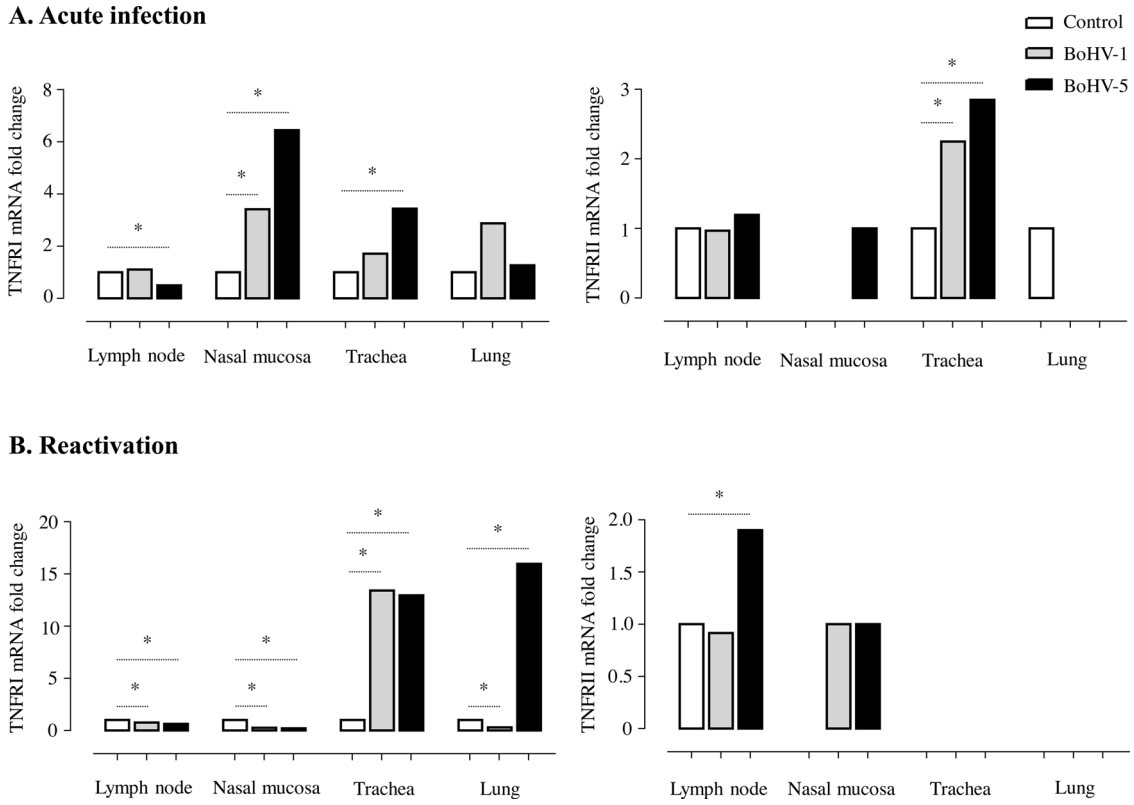

Fig. 5. TNF receptors (TNFRI and TNFRII) expression are modulated by BoHV-1 and BoHV-5 acute infection (A) and reactivation (B).
The results represent the mean fold change of TNFRI and TNFRII transcription levels in specific areas of the respiratory tract of infected calves over levels detected in tissue sections of uninfected calves, which served as the control groups. *: statistically significant differences ($P < 0.05$) with respect to the uninfected control. mRNA relative levels and statistical significance of the differences in mRNA expression were obtained with the software REST (Qiagen Inc., Valencia, CA, USA). For analysis of data, the values obtained for each animal in the group, with 2 technical replicates per animal, were used to obtain a final mean fold-change value for infected groups over the values of the unifected group.

BoHV-5 showed a more pronounced role downregulating BMAP28 during acute infection. Indeed, we recently observed that cathelicidin transcription decreases only in BoHV-5-infected cultures of fetal bovine lung at 6 h post-infection (hpi). This is not evident at 24 hpi. Furhtermore, differences are not observed for BoHV-1 infection at 6 and 24 hpi (Burucúa et al., unpublished data). A generalized decrease of BMAP28 in lymph nodes during primary infection by bovine herpesviruses coincides with lower expression of IFNβ and to a lesser extent with the expression of TNFα and TNFRI.

During reactivation, the BMAP28 response was different; BoHV-5 upregulated both BMAP28 and IFNβ in retropharyngeal lymph nodes. Increased BMAP28 and IFNβ mRNA transcription after BoHV-5 reactivation may correspond with the presence of BoHV-5 DNA in retropharyngeal lymph nodes and lymph nodes in the respiratory tract at this stage (Marin et al., 2016b). Despite the difference in the immunomodulatory role during active replication, both calves with BoHV-1 and BoHV-5 acute infection or reactivation from latency developed comparable clinical conditions and did not show neurological signs or severe respiratory distress, probably due to the short period of time between inoculations an euthanasia.

Acute infections of BoHV-1 and BoHV-5 have been distinguished by differential activation patterns of the innate immune mechanisms. At this stage, we previously demonstrated that it was particularly evident an up-regulation of other innate immune components, the Toll-like receptors (TLRs) 3 and 7–9, in the respiratory tract of cattle during reactivation (Marin et al., 2016b). Moreover, TLR3 and TLR7 stimulation, along with BMAP28 upregulation, protected *in vitro* bovine fetal lung cells infected with bovine alpha-herpesviruses (Burucúa et al., 2018). Although we found that BoHV infection modulates mainly the expression of BMAP28 in the respiratory tract, BMAP27 was detected in infected lymph nodes during BoHV-1 reactivation. This indicates the relevance of BMAP28 (Baumann et al., 2017) but also a potential role for BMAP27 during reactivation.

IFNβ is a relevant cytokine produced during infection by bovine alpha-herpesviruses (Perez et al., 2008; Marin et al., 2014b). Expression of this cytokine was evident in nasal mucosa under acute infection of BoHV-1 and in lung after reactivation of BoHV-5. Moreover, acute replication of BoHV-1 and BoHV-5 induced the synthesis of TNFα and TNFα receptors, TNFRI and II, were positively regulated during acute infection in nasal mucosa and trachea and also during reactivation, particularly in trachea. Amplified TNFα synthesis in the presence of active viruses is an attempt to contain the viral infection, associated with early viral clearence (Kabeya et al., 1999, 2001). In concordance, TNFRII has been associated with the regulation of the inflammatory response in the respiratory tissue, mainly the lung (Grau et al., 1996) and it is likely that the same might occur in other sites of the respiratory tract, particularly at the stages of active virus replication. On the other hand, in this work it was observed an important TNFRI downregulation in lymph nodes during BoHV-5 acute infection. However, this was more evident during BoHV-1 and BoHV-5 reactivation, in lymph nodes, nasal mucosa and lung. Likewise, HSV-1 induces the degradation of TNFRI mRNA, suggesting that by this mechanism the virus precludes the replenishment of naturally decaying TNFRI (Liang and Roizman, 2006). Therefore, the present work shows that virus infection regulates TNFα and the expression of its receptors to establish respiratory infections.

Overall, this study demonstrates that cathelicidins, togheter with relevant cytokines, are implicated in alpha-herpesvirus infections of the bovine respiratory system and the response is different during BoHV-1 and BoHV-5 acute infection and reactivation. It provides an initial pilot analysis of factors that might be implicated in alpha-herpesvirus

infection of the bovine respiratory system. However, the size of the experimental groups in this study is limited. Therefore, further work will be necessary to confirm our results. Understanding the action of antimicrobial peptides in respiratory infections and their immunomodulating properties would be useful for preventive and therapeutic strategies for two closely related pathogens of cattle.

Conflict of interest statement

The authors declare no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial and personal relationships that could inappropriately influence this work.

Acknowledgements

This work was financially supported by National Institute of Agricultural Technology (INTA, Argentina), Specific Projects PNSA-1115053 and PNSA-1115054 and PICT2008-1421 and 2014-0386 (ANPCyT, Argentina). We especially thank María Rosa Leunda and Susana Pereyra for their contribution and support to the study.

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