Research in Veterinary Science 96 (2014) 196–203

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Toll-like receptor activation and expression in bovine alpha-herpesvirus infections

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ARTICLE INFO

Article history:
Received 10 July 2013
Accepted 30 November 2013

Keywords:
Toll-like receptor
Bovine herpesvirus
Agonist
Expression patterns

ABSTRACT

The involvement of Toll-like receptors (TLRs) in bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) infections has not been analyzed. In this study, the role of TLR signaling on virus replication was investigated. Blood leukocytes consistently express TLRs. Thus, our approach was to study *in vitro* the effects of agonist stimulation of TLRs expressed by peripheral blood leukocytes on BoHV-1 and BoHV-5 replication. Furthermore, the patterns of TLRs 3, 7–9 expression on virus-infected-bovine leukocytes were analyzed. Only Imiquimod (TLR7/8 agonist) showed anti-viral activity on infected MDBK cells. This is the first evidence that the timely activation of TLR7/8 signaling is effective in impairing BoHV-1 and 5 replication, thereby providing an experimental indication that Imiquimod may be a promising immune modulator. This work describes, for the first time, the expression patterns of TLRs in BoHV-1- or BoHV-5-infected-bovine leukocytes, suggesting the involvement of TLR7 and TLR9 in the recognition of these viruses.

1. Introduction

Within the family *Herpesviridae*, the alpha-herpesviruses represent the largest sub-family, which comprises many different, closely related, human and animal pathogens. A key attribute of these viruses is their ability to establish life-long latent infections, mainly in the sensory ganglionic neurons of the peripheral nervous system (Muylkens et al., 2007). Viral persistence in the infected host implies that herpesviruses have evolved mechanisms to delay and avoid their recognition and elimination by the immune system (Favoreel, 2008). Initiation of the replication and establishment of infection are strongly influenced by the early stages of the virus-host interactions. Therefore, to successfully colonize the host, herpesviruses need to actively evade and modulate the host responses during all stages of infection. The immunological control of herpesviruses is achieved by both the innate and the adaptive immune systems, with type I interferons (IFNs) having a crucial role in the innate antiviral immune response that mediates the containment of herpesvirus infections (Paludan et al., 2011).

IFNs and other pro-inflammatory cytokines are rapidly induced by the innate immune system in response to pathogen invasion. The innate immune mechanisms are activated following the sensing of infections through the pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) were the first PRRs to be discovered and are the best characterized. TLRs are type I transmembrane glycoproteins that have an extracellular domain containing leucine-rich repeats, which mediate the recognition of PAMPs, a transmembrane domain, as well as a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, which interacts with downstream signaling molecules (Botos et al., 2011). TLRs can be broadly divided into two subgroups based on their cellular localization and the respective PAMPs they recognize. The first group comprises TLRs 1, 2, 4, 5, 6 and 11, which are expressed on the cell surface and mainly recognize microbial membrane components, such as lipids, lipoproteins and proteins. The second group comprises TLRs 3, 7, 8 and 9, which are expressed in intracellular vesicles, such as endosomes, lysosomes and the endoplasmic reticulum. This latter group of TLRs recognizes microbial nucleic acids, mainly of viral origin. Double-stranded RNA (dsRNA) is recognized by TLR3, single-stranded RNA (ssRNA) is detected by TLR7/8 and TLR9 recognizes the un-methylated CpG di-nucleotides in DNA molecules. These components are present in the viral genome, or they are generated during the replication of many viruses (Borrow et al., 2010). Therefore, the TLRs in this group constitute a powerful sensor system to detect viral invasion.

Upon ligand recognition, TLRs stimulate the strong production of a wide variety of cytokines. The expression of a specific cytokine depends on the recognized PAMP and the participating TLR. Thus,

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http://dx.doi.org/10.1016/j.rvsc.2013.11.015

cytokine production leads to an antiviral state by preventing virus replication (Boo and Yang, 2010). One of the major products of TLR activation is the production of type I IFNs, key components to mount a proper and robust immune response to herpesviral infections (Paludan et al., 2011; Gaajetaan et al., 2012). Gaajetaan et al. (2012) determined that IFN-α would be dispensable for TLR-induced antiviral effects against herpes simplex virus type 1 (HSV-1) and that IFN-β would be more important that IFN-α. They demonstrated that the expression of IFN-β, but not of IFN-α, was strongly enhanced following stimulation of dendritic cells with different TLR agonists and this finding correlated with the antiviral effect of the TLR ligands in HSV-1 infection. In contrast to humans or mice, cattle contain three different IFN-β genes that are differentially regulated because they have distinct promoters (Wilson et al., 1983). Induction of this particular type I IFN is also an important feature of bovine herpesvirus (BoHV) infections (Pérez et al., 2008).

Studies of the effect of TLR activation on BoHV replication have not been reported. Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are two closely related alpha-herpesviruses that infect cattle. BoHV-1 causes significant economic losses to the cattle industry worldwide (Muylkens et al., 2007) since it is responsible for a variety of clinical syndromes, including respiratory disease, conjunctivitis, abortion and genital infections. BoHV-5 is highly prevalent in South America, and it is the primary etiological agent of non-suppurative meningoencephalitis (Pérez et al., 2002). Although the expression of TLRs in several bovine tissues has been described (Menzies and Ingham, 2006), the association of these receptors to the development of infectious diseases has not been extensively evaluated. Cell lines routinely used for bovine herpesviruses studies do not express TLRs. However, peripheral blood cells of the immune system can be cultured and they represent a useful TLR-responsive model for examining TLR signaling events. Peripheral blood leukocytes (PBLs) express all known TLRs (Zarember and Godowski, 2002; Guo et al., 2009; Gaikwad et al., 2012). Nevertheless, TLR expression patterns among different bovine leukocyte populations are still largely unknown (Guo et al., 2009; Gaikwad et al., 2012). Although several TLRs appear to be more restricted to B cells, professional phagocytes express the greatest variety of TLR mRNAs (Zarember and Godowski, 2002). In the present study we used PBLs to analyze the role of TLR signaling on the replication of BoHV-1 and -5. Our approach was to study *in vitro* the effects of agonist stimulation of TLRs expressed by PBLs on BoHV-1 and BoHV-5 replication. Furthermore, the changes in the expression levels of TLRs 3, 7, 8 and 9 messenger RNA in BoHV-1 and -5-infected-bovine leukocytes were analyzed.

2. Materials and methods

2.1. Cell cultures

The Madin-Darby Bovine Kidney (MDBK) cell line from the American Type Culture Collection (ATCC, Rockville, MD, USA) was propagated in minimum essential medium (Eagle), with Earle's salts (MEM-E) (Sigma–Aldrich, Saint Louis, MO, USA). The medium was supplemented with 10% fetal bovine serum (Bioser, Buenos Aires, Argentina), free from viruses and antibodies, and with an antibiotic–antimycotic solution (Gibco, Langley, OK, USA) containing 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 0.025 μg/ml amphotericin B. PBLs were obtained from BoHV-1 and -5 seronegative calves. The anti-coagulated blood was centrifuged and the erythrocytes were lysed by treatment with ammonium chloride (pH 6.8). PBLs were then washed with phosphate buffered saline and re-suspended in E-MEM supplemented as previously described. The percentages of the cell type components of the leukocyte population were determined by differential counting. MDBK cells and PBLs were incubated at 37 °C in a 5% CO_2 atmosphere. Cell viability was measured by trypan blue exclusion assay.

2.2. Virus strains

The reference strain Los Angeles 38 (LA38) (BoHV-1.1) and the Argentinean BoHV-5 field strain (97/613) were used for this study. The isolate 97/613 was recovered from the brain of a 2-year-old calf with clinical encephalitis. 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 nested PCR (Campos et al., 2009). Viral stocks were then amplified in MDBK cells in T-25 flasks (Greiner Bio-one, Frickenhausen, Germany) (1×10^5 cells/ml) for 24 h. The supernatants were harvested and frozen at −80 °C. Virus titers were determined by the end-point titration method and expressed as $TCID_{50}$/ml, according to the method of Reed and Müench (1938).

2.3. Effects of TLR activation on BoHV-1 and 5 replication

2.3.1. Experimental design

To test whether TLR 3, 7, 8 and 9 stimulation has an effect on BoHV-1 and -5 replication, bovine PBLs were initially stimulated with TLR agonists. Supernatants from stimulated and non-stimulated PBLs were harvested and added to MDBK cells which had been previously infected with BoHV-1 or BoHV-5 strains. Twenty-four hours later, viral yield was quantified.

To confirm that the addition of the agonists effectively induced a change in the profile of cytokines produced by PBLs, the expression of IFN-β1, IFN-β2 and IFN-β3 was determined. Furthermore, TLR expression in MDBK cells was also evaluated to corroborate that the effect on viral replication was exclusively a consequence of the signaling induced after stimulation of PBLs and not to a signaling cascade mediated by TLRs that could have been initiated in the infected cell line. Adequate non-infected and un-stimulated controls were included in each experiment.

2.3.2. TLR stimulation, viral infection of MDBK cells and viral quantitation

PBLs were seeded in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of 1×10^6 cell/ml and stimulated with the following TLR agonists: Poly(I:C) (10 μg/ml) (TLR3) (Alexopoulou et al., 2001; Matsumoto et al., 2002), Imiquimod (5 μg/ml), CL075 (5 μg/ml) and ssPolyU/LyoVec (1 μg/ml) (TLR7/8) (Hemmi et al., 2002; Diebold et al., 2004; Heil et al., 2004; Gorden et al., 2005) and ODN 2006 (10 μg/ml) (TLR9; class B CpG ODN) (Hemmi et al., 2000; Bauer et al., 2001), or a combination of these agonists. All TLR agonists were used according to manufacturer recommended concentrations (InvivoGen, San Diego, CA, USA) for inducing optimal TLR activity. PBLs were incubated at 37 °C with 5% CO_2, and the supernatants were harvested at two different time points: 6 and 24 h post-stimulation (hps), which is considered the optimal time for the analysis of the afferent phase in the production of cytokines and immune-mediators (Jordan and Ritter, 2002). Supernatants from stimulated PBL cultures were added to MDBK cells grown in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of 1×10^6 cells/ml. MDBK cells had been previously infected for 0, 4, 6 or 24 h with BoHV-1 or BoHV-5 strains at a multiplicity of infection (MOI) of 0.1. Previous studies on the kinetics of BoHV-1 and BoHV-5 infection of MDBK cells demonstrated that these time-points are suitable for the analysis proposed (Marin et al., 2012). Twenty-four hours later, the supernatants were harvested and frozen at −80 °C for further viral quantification. Virus titers were calculated using the method of

Reed and Müench (1938) and expressed as $TCID_{50}$/ml. Three replicates of each experiment were performed to establish the effects of TLR stimulation on viral replication for each treatment. As a negative control, the supernatants from non-stimulated PBLs were added to BoHV-1- or BoHV-5-infected-MDBK cells.

2.3.3. IFN-β mRNA expression in PBLs

Total RNA from non-stimulated and stimulated PBLs 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 was 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 leukemia virus reverse transcriptase (10 U/μl) (Promega, Madison, WI, USA), following the procedures suggested by the manufacturer. Negative controls, omitting the RNA or the reverse transcriptase, were included.

An aliquot (3 μl) of the cDNA was used for each PCR, using primers specific for bovine IFN-β1, IFN-β2 and IFN-β3 (Pérez et al., 2008) and for bovine glyceraldehyde-phosphate-dehydrogenase (GAPDH) (McGuire et al., 2004), which was used as an internal control. The PCRs reactions were carried out in a total of 25 μl containing 0.8 mM specific forward and reverse primers, 3 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 0.4 mM each dNTP (Promega, Madison, WI, USA), 1.5 U Taq DNA polymerase (Promega, Madison, WI, USA) and 1× PCR buffer (Promega, Madison, WI, USA). Amplification was carried out as follows: 95 °C 5 min, 32 cycles by denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min and extending at 72 °C for 1 min and one extension cycle at 72 °C for 7 min. PCR products were visualized on 1.6% agarose gels stained with SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA, USA).

2.4. TLR expression profiles in PBLs infected with BoHV-1 and BoHV-5

2.4.1. Viral infection of PBL

In vitro-infected bovine PBLs were used as a source of bovine TLR mRNA to establish and optimize a Real time RT-PCR procedure to measure the relative expression levels of TLR3, TLR7, TLR8 and TLR9. Lymphocytes and monocytes are susceptible to BoHV-1 infection and viral DNA and antigen can be detected after several months post-challenge (Wang et al., 2001). Therefore, these cell types, which are also known to consistently express TLRs, represent a good initial model to understand the interaction of BoHV-1 and 5 and the receptors of the innate immune system. PBLs were seeded in 24-well plates (Greiner Bio-one, Frickenhausen, Germany) at a concentration of 6×10^6 cells/ml and infected for 24 h with either LA38 BoHV-1 or 97/613 BoHV-5 strains, at an MOI of 0.1. Six replicates of the infected PBLs were used for each experiment. Uninfected PBL cultures were used as a negative control group. To demonstrate culture infection, PBLs were seeded on chamber slides (Nunc, Naperville, IL, USA) and infected under the same experimental conditions. After 24 h of incubation at 37 °C, 5% CO_2 the media was poured out, cells were fixed with acetone for 20 min at −20 °C and incubated with an anti-BoHV polyclonal antibody conjugated with fluorescein isothiocyanate (American BioResearch, Sevierville, TN, USA) for 35 min at 37 °C.

2.4.2. Primers and probes

The primers and probes were designed for bovine TLRs, based on sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/), using the Primer Premier software (PREMIER Biosoft International, Palo Alto, CA, USA). The sense and antisense primers for TLR3 and TLR9 were placed in two consecutive exons of the respective gene. The probe spanned the junction of the two exons, covered by the forward and reverse primer to ensure proper discrimination between cDNA and gDNA. Primers and probes for TLR7 and TLR8 were designed on the only exon that comprises these gene coding regions. GAPDH was used as a housekeeping gene (McGuire et al., 2004). Each probe was labeled at the 5′-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′-end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). Details of the primers and probes commercially synthesized (Eurofins MWG Operon, Huntsville, AL, USA) are provided in Table 1.

2.4.3. TLR mRNA expression analysis

Total RNA from infected PBLs was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. DNase treatment and reverse transcription were carried out as described above. Real time RT-PCR reactions for bovine GAPDH and TLR3, TLR7, TLR8 and TLR9 were run in separate wells. The PCR reactions contained 800 nM specific forward and reverse primers, 200 nM specific probe, 1× PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems, Branchburg, NJ, USA) and 1 μl of cDNA sample in a final volume of 25 μl. The amplification and detection of the specific products were carried out using an Applied Biosystems 7500 cycler, with the following amplification conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 20 s at 95 °C and 60 s at 60 °C. In all cases, the experiments were conducted in duplicate. 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 cDNA. The results are reported as the mean fold change of TLR transcription levels over uninfected PBLs, which served as the control group.

2.5. TLR expression in MDBK cells

TLR mRNA expression in MDBK cells was evaluated as described for expression profiles in infected PBLs. The assay was run by triplicate and as positive control for each Real time RT-PCR reaction, cDNA from PBL cultures were used. Amplification of GAPDH was also used as an internal control.

2.6. Statistical analysis

Virus titers in each group were determined as described in Section 2. Statistical differences in these viral titers were calculated by analyzing the interactions among the viruses, TLR agonists and time of infection at each stimulation time point. Comparisons of least square means between the agonist treatment(s) and the control group were performed using the Tukey–Kramer test, conducted for each combination of the time of infection and virus. The MIXED procedure (SAS, Institute Inc., Cary, NC, USA) was used, and the level of significance was 0.05.

Quantification of the TLR mRNA relative levels and the statistical significance of the differences ($P < 0.05$) in TLR mRNA expression levels were analyzed with the Relative Expression Software Tool (REST, Qiagen Inc., Valencia, CA, USA), which calculated the group-wise comparisons and the statistical analyses of relative expression results for Real time PCR (Pfaffl et al., 2002). Real time RT-PCR efficiency for each gene was determined by a linear regression model, according to the equation: $E = 10[-1/\text{slope}]$.

Table 1
Sequences of primers and probes for Real Time RT-PCR.

mRNA	Primer or probe	Amplicon size (base pairs)	5′–3′Sequences
GAPDH	F[a]	112	TTCTGGCAAAGTGGACATCGT
	R[b]		CTTGACTGTGCCGTTGAACTTG
	Probe		ACATGGTCTACATGTTCCAGTATGATTCCACCC
TLR3	F	143	CAGGTCAACAGTCCCGAA
	R		GCAGCACATTCCCCACAT
	Probe		ACAGTAAGTGAAGGCAAAACCATGAGCAGA
TLR7	F	144	TAAAACTCTGCCCTGTGATG
	R		CCTGCTATGTGGTTAATGGT
	Probe		ATTCCTGCCAATGCCACCAAC
TLR8	F	117	TTATTGCAGAATGTAATGGTCG
	R		GAAAGGATTCATTCGTTACCC
	Probe		ACCCCAAACAGTGGACAAAGATGTGACG
TLR9	F	113	AACCTGCCCGCCAGACCCT
	R		GCCAGGGCCACTGCCAGTG
	Probe		ATTCCCTGTCATGGGCCCCTACTGTGCCCC

[a] Forward primer.
[b] Reverse primer.

Table 2
BoHV-1 and -5 titres ($log_{10}TCID_{50}$/ml) after treatment of infected MDBK cells with supernatants of peripheral blood leukocytes stimulated with TLR agonists. The values are the average of three replicates. Means and standard error are shown.

Virus	Hours of stimulation	Hours of infection	Agonist							Standard error
			Control	Poly(I:C)	Imiquimod	CL075	ssPolyU/LyoVec	ODN 2006	Combined	
BoHV-1	6	0	4.2 a	4.8 b	3.4 c	4.4 ab	4.8 b	4.9 b	4.7 ab	0.197
		4	4.5 a	4.8 a	3.8 b	4.6 a	5.4 c	6.0 d	5.5 c	
		6	4.4 ab	4.7 a	4.1 b	5.6 c	6.3 d	6.8 d	6.5 d	
		24	5.3 ab	5.3 ab	4.9 a	5.6 b	6.5 c	6.3 c	6.6 c	
	24	0	4.2 a	4.5 ab	3.2 c	5.2 d	4.9 bd	4.9 bd	4.9 bd	0.197
		4	4.5 a	4.9 ab	3.5 c	4.6 a	5.4 bd	6.3 e	5.8 de	
		6	4.4 a	5.2 b	3.7 c	4.7 ab	6.0 d	7.0 e	6.5 de	
		24	5.3 a	5.8 b	5.4 ab	6.4 c	6.7 c	6.5 c	6.7 c	
BoHV-5	6	0	2.8 a	3.6 b	2.6 c	4.8 d	4.5 d	3.5 b	2.2 c	0.187
		4	3.9 a	4.8 b	3.1 c	3.8 a	5.1 b	5.0 b	4.6 b	
		6	3.8 a	4.9 b	3.3 c	3.9 a	4.9 b	5.6 d	5.0 b	
		24	4.8 ab	4.5 b	5.1 a	5 ab	6.3 c	5.9cd	5.7 d	
	24	0	2.8 a	3.0 a	2.6 a	4.0 b	2.5 ac	3.0 a	1.9 c	0.187
		4	3.9 a	3.7 a	2.6 b	3.9 a	4.7 c	4.9 c	4.1 a	
		6	3.8 a	3.8 a	2.7 b	3.9 a	4.7 c	4.8 c	4.1 a	
		24	4.8 a	5.4 b	4.5 a	4.8 a	5.4 b	6.1 c	4.7 a	

Lower case letters (a, b, c, d, e) indicate comparisons between viral titers after treatment with different TLR agonists for each virus and time of stimulation and infection ($P < 0.05$).

3. Results

3.1. Effects of TLR stimulation on BoHV-1 and BoHV-5 replication

To test whether there was an effect of agonists stimulation of TLRs on BoHV-1 and -5 replication, bovine PBLs were stimulated with TLR 3, 7, 8 and 9 agonists for 6 or 24 h. PBL cultures were consistently composed by lymphocytes (86–90%), neutrophils (7–12%) and monocytes (2–3%). Supernatants from the stimulated PBLs were harvested and added to MDBK cells, which had previously been infected for 0, 4, 6 or 24 h with BoHV-1 or BoHV-5 strains. Twenty-four hours later, the supernatants were harvested and virus titers were calculated and expressed as $TCID_{50}$/ml (Table 2). As a negative control, supernatants from non-stimulated PBLs were added to BoHV-1- or BoHV-5-infected MDBK cells.

To determine whether agonist TLR stimulation induced an effective change in cytokine gene expression, IFN-β1, IFN-β2 and IFN-β3 levels in bovines PBLs were analyzed. In non-stimulated cultures, IFN subtype expression was not detected. When bovine PBL cultures were treated with the different agonists for 6 or 24 h, the expression of at least one of the IFN subtypes was evidenced. IFN-β1 and IFN-β2 mRNA was detected in all stimulated cultures, except for ssPolyU/LyoVec- or ODN2006-stimulated PBLs in which expression of IFN-β1 was not observed at 6 or 24 hps, respectively. Poly(I:C) stimulation for 6 h or CL075 treatment for 24 h, did not induce IFN-β2 mRNA expression. IFN-β3 mRNA was detected in cultures treated with Imiquimod or with a combination of agonists for 6 and 24 h. In addition, this IFN subtype was detected in PBL cultures stimulated with CL075 and ODN2006 for 24 h (Fig. 1).

Among the TLR ligands, only the supernatants obtained after Imiquimod (TLR7/8 agonist) stimulation of bovine PBLs demonstrated antiviral activity against BoHV-1 and -5 on infected MDBK cells. All other agonists had a positive effect on BoHV-1 and BoHV-5 replication. Stimulation with Imiquimod for 6 and 24 h significantly decreased ($P < 0.05$) the extracellular BoHV-1 and -5 yields during the first hours of viral infection. In contrast, treatment at the later stages of the viral replication cycle (i.e., 24 h after the

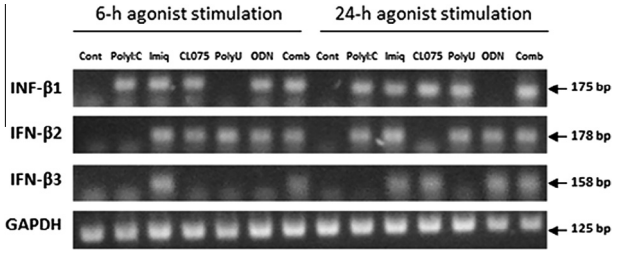

Fig. 1. IFN-β subtypes mRNA expression in bovine PBL cultures stimulated with TLR agonists for 6 or 24 h. Cont: non-stimulated control cultures; PolyI:C: Poly(I:C), TLR3 agonist; Imiq: Imiquimod, TLR7/8 agonist; CL075: TLR7/8 agonist; PolyU: ssPolyU/LyoVec; TLR7/8 agonist; ODN: ODN2006, TLR9 agonist; Comb: cultures stimulated with a combination of agonists. RT-PCR amplification products had the expected molecular weights (IFN-β1: 175 base pairs [bp]; IFN-β2: 178 bp; IFN-β3: 158 bp; GAPDH: 125 bp).

initiation of BoHV infection) completely failed to execute a protective effect, thereby resulting in virus titers similar to those observed in non-treated cells (Table 2).

In MDBK cells treated with supernatants from Poly(I:C)-stimulated PBLs for 6 h, an increase of BoHV-1 and -5 titers was detected when treatment was performed at the time of infection (Time 0) (Table 2). Similar results were obtained when the supernatants were added to MDBK cells at 4 and 6 h after BoHV-5 infection (Table 2). Twenty-four hours stimulation of PBLs with the specific TLR3 agonist had no significant effect ($P > 0.05$) on the production of virus particles during the first hours of infection. However, treatment with these supernatants induced a significant increase ($P < 0.05$) in virus replication in MDBK cells that were infected for 6 and 24 h with BoHV-1, as well as cells infected for 24 h with BoHV-5 (Table 2).

With some exceptions, the supernatants from the PBLs that were treated with CL075, a TLR7/8 agonist, induced an increase in the extracellular BoHV-1 and -5 titers only when they were added at the time of MDBK infection (Table 2). In contrast, treatment of cells with other TLR7/8 agonists, such as ssPolyU/LyoVec, resulted in a significant increase ($P < 0.05$) in the replication of both viruses except when supernatant collected at 24 h was added at the same time of BoHV-5 inoculation of MDBK cells. Similar results were obtained when the supernatants from the PBLs stimulated with the TLR9 agonist were used. Supernatants treated with these agonists induced the largest differences in virus titers when compared to mock-infected cultures (i.e., one- to twofold increase). Combination of agonists resulted in significantly higher BoHV-1 titers ($P < 0.05$) when compared with mock-infected cultures. The magnitude of the increase was similar to that obtained with ssPolyU/LyoVec (TLR7/8 agonist) and ODN 2006 (TLR9 agonist). In the case of BoHV-5, combined agonists had a detrimental effect on viral replication, primarily when supernatants were added to MDBK cells at the time of infection (0 h pre-infection [hpi]). However, supernatants collected after 6 h of stimulation added at 4, 6 and 24 hpi induced a significant increase ($P < 0.05$) in BoHV-5 replication. No effect was detected with the supernatants collected after 24 h of stimulation (Table 2).

Finally, TLR expression in non-infected, un-stimulated MDBK cells was assessed to demonstrate that the effect on viral replication was only a consequence of agonist stimulation of PBLs and not due to direct TLR stimulation by residual agonists in the collected supernatants added to MDBK cells. Our data indicated that TLR3, 7–9 mRNA is not detectable in this cell line. C_T values for TLR3, 7–9 expression in uninfected MDBK were not recorded. Expression of these receptors was readily detected in positive controls (cDNA from PBL cultures, which consistently express TLRs mRNA). GAPDH, used as internal control, was amplified in MDBK cells and PBL cultures.

3.2. TLR expression profiles in PBLs infected with BoHV-1 and BoHV-5

To determine whether BoHV-1 and -5 infection has an effect on TLR expression in bovine PBLs, the levels of TLR mRNA were quantified after virus infection of PBL cultures for 24 h. TLR mRNA expression levels from the infected PBLs were compared to the expression of these receptors in the uninfected PBL cultures. PBL cultures were composed by cell populations mentioned previously for agonist stimulation assays. Culture infection was confirmed by observation of BoHV positive immunofluorescence of the cells after 24-h of infection.

GAPDH expression levels remained constant in both the infected and uninfected cells and a linear relationship between the amount of the template and C_T values was observed when the amplification efficiency for each gene was determined (data not shown). Our data indicate that TLR mRNA is detectable in bovine PBLs and that, in most cases, BoHV-1 or BoHV-5 infection induces TLR expression (Fig. 2). TLR7 is significantly up-regulated ($P < 0.05$) after BoHV-1 and -5 infection of PBLs (in comparison to uninfected PBLs), by a mean factor of 3.4 (standard error (SE) range is 1.2–10.1) and 5.4 (SE range is 3.5–9.2), respectively. TLR9 is significantly up-regulated ($P < 0.05$) in BoHV-5-infected PBLs by a mean factor of 2.7 (SE range is 1.4–4.7) above the control level. No statistically significant differences ($P > 0.05$) were found for the expression levels of TLR9 in BoHV-1-infected PBLs when compared to uninfected cultures. The expression levels of TLR3 and TLR8 were not affected ($P > 0.05$) by either BoHV-1 or -5 infections (Fig. 2).

4. Discussion

TLRs are an important family of receptors that participate in the detection of PAMPs of the invading pathogens and in the initiation of the innate immune response. TLRs and their signaling pathways are emerging as novel therapeutic targets. This study is an initial point analysis of viral TLRs which might be involved in herpesviral infections in cattle. The participation of the innate immune system in several infectious diseases is now widely recognized. However, the link between TLRs and viral animal diseases has not been extensively analyzed yet. As a primary goal of our study, we established the use of BoHV-1 and -5-infected-MDBK cells as a tool for studying the effects of TLR activation on the replication of these viruses. MDBK is a widely used cell line that supports BoHV replication (Marin et al., 2012). It has been observed that MDBK cells are less susceptible to BoHV-1 induced apoptosis, when compared to immune cells, such as lymphocytes and monocytes (Hanon et al., 1998). Nevertheless, as it was observed in the present work, like many established cell lines of non-immune origin, MDBK cells do not express TLRs. However, peripheral blood cells of the immune system can be cultured and they are a useful tool to analyze the effects of secreted innate immune mediators. For this study, bovine PBLs were used since they are cell effectors of the immune response and, as discussed below, it was demonstrated that these cells constitutively express the four studied TLRs.

It is well recognized that certain TLR ligands are able to stimulate the release of type I IFN. However, information on their real antiviral potential is lacking. The results obtained on the role of the TLR system in the control of BoHV-1 and -5 replication demonstrated that stimulation with Imiquimod, a TLR7/8 agonist, induces antiviral activity against BoHV-1 and -5 on infected MDBK cells. Imiquimod is recognized as showing antiviral activity. However, its effect on replication of herpesviruses and the signaling pathways involved are often different for each virus and model of study. Kan et al. (2012) determined that Imiquimod suppresses replication of HSV-1, a close relative to BoHV, in the human cell line FL. However, in contrast to our results, the expression of IFN

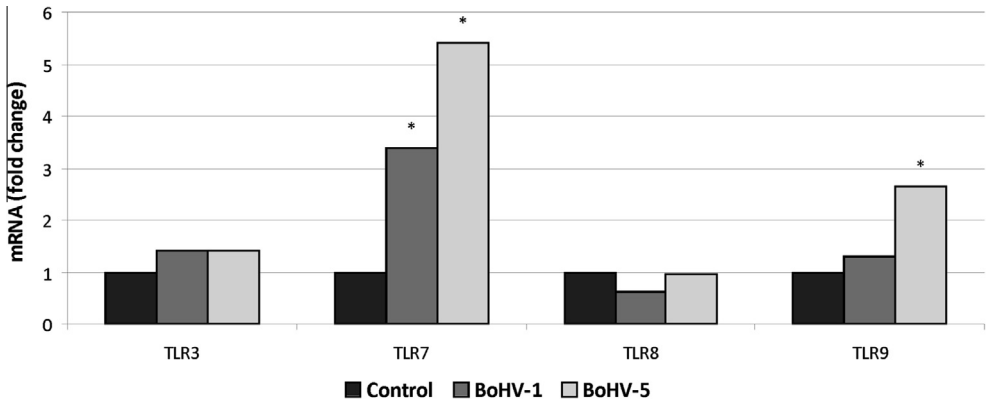

Fig. 2. Relative TLR expression levels in PBLs infected with BoHV-1 or BoHV-5. Asterisks indicate statistically significant differences ($P < 0.05$) in relation to un-infected PBLs (control). Six replicates of each experiment were performed. mRNA relative levels and statistical significance of the differences in TLR mRNA expression were obtained with the software REST (Qiagen Inc., Valencia, CA, USA).

and TLR genes was not altered, suggesting that the IFN system did not contribute to the anti-HSV-1 effect of Imiquimod. On the other hand, it has been reported that agonists specific for TLR7/8, including Imiquimod, reactivate latent Kaposi's sarcoma-associated herpesvirus (KSHV) and induce viral lytic gene transcription and replication (Gregory et al., 2009). Moreover, Valente et al. (2012) observed that TLR7 activation with Imiquimod stimulates the expression of Epstein–Barr Virus (EBV) latent membrane protein-1 suggesting that this mechanism may be present *in vivo* exacerbating the development of lupus.

In this study, the supernatants from TLRs 3- and 9-stimulated PBLs, as well as those from PBLs stimulated with a second TLR7/8 agonist (distinct from Imiquimod), failed to halt viral replication. Instead, at certain times, post-infection addition of these agonists increased the extracellular BoHV-1 and -5 yields. Thus, our findings demonstrate a link between innate immune activation and herpesviral replication, which suggests that the agonists evaluated, with exception of Imiquimod, can serve as biological trigger for BoHV replication. In contrast with our results, Gaajetaan et al. (2012) demonstrated that stimulation of dendritic cells and fibroblasts with different TLR ligands, in particular Poly(I:C) and CpG (TLRs 3 and 9 agonists), indeed inhibits HSV-1 infection, remarking that the biological effects of TLR activation are complex. However, HSV grows optimally in cells where NF-κB signaling has been activated (Takeda et al., 2011). The recognition of TLRs that occurs after HSV-1 infection leads to a robust NF-κB activation which then induces the up-regulation of a wide array of cytokines, chemokines and IFNs. NF-κB cascade downstream of TLR9 can be hijacked by HSV-1 and diverted for its own replication (Takeda et al., 2011). Emerging studies suggest that other herpesviruses may also usurp host innate immune responses to promote viral transcription and lytic replication (Dong et al., 2010; Meyer et al., 2013). A thorough analysis of cytokine expression induced after TLR stimulation was not performed in this work. Nevertheless, the differences observed would be a consequence of the specific activation of particular immunological effectors after stimulation by each agonist. Imiquimod was the only TLR agonist that specifically induced expression of all IFN-β subtypes, mainly IFN-β3. As previously described by Pérez et al. (2008), induction of this type I IFN subtype is an important feature of BoHV-1 infections. The results of this study indicate that the time at which Imiquimod was added to the cultures was crucial for establishing an antiviral state. The antiviral effect was evident when the agonists were added simultaneously or during the first hours of BoHV-1 or BoHV-5 infection. However, they failed to modulate viral yield when the treatment was performed at later stages of the viral replication cycle (i.e., 24 hpi). IFNs and other pro-inflammatory cytokines induced by TLR activation are rapidly synthesized (Liang et al., 2011). Thus, at early times following infection, cells are able to quickly respond to block viral replication. Similar results regarding the timing of TLR activation on impairing viral replication have been observed with other viruses (Liang et al., 2011). In contrast, the anti-viral response was not different after treatment with supernatants obtained from PBLs that were stimulated with Imiquimod for 6 or 24 h. These results are in agreement with the analysis of IFN-β expression, which demonstrated that at both stimulation time-points the three cytokine subtypes were induced.

A protective effect of TLRs against human herpesvirus infections has already been documented. For example, ligands for TLR3, 7 and 9 significantly inhibited HSV-2 replication both in *in vivo* and *in vitro* (Svensson et al., 2007; Nazli et al., 2009). Interestingly, recent studies suggest that, in certain situations, the activation of specific TLR responses by microorganisms might serve as an escape mechanism from the host's defenses, including the immune evasion by amplification of viral replication (Dong et al., 2010; Takeda et al., 2011). In this study, only Imiquimod had an anti-viral effect that led to the impairment of BoHV-1 and -5 replication. Other TLR7/8 ligands, such as CL075 and ssPolyU/LyoVec, enhanced virus replication. As demonstrated by Butchi et al. (2008), the individual TLR7/8 agonists can widely differ in their abilities to induce an immune response. Similar effects on the enhanced BoHV-1 and -5 replication were observed with TLR3 and TLR9 ligands. CpG ODN motifs are species-specific and those with different sequences have been shown to induce various types or levels of immunostimulatory responses. In general, motifs with an "optimal" activity in humans are also active in the various domestic species, whereas motifs displaying the highest activity in the murine system show little or no activity (Werling et al., 2009). Therefore, the effect obtained depends on the ODN class employed. ODN 2006 is a class B CpG ODN, specific for human TLR9 and it has been demonstrated to be optimal for effective stimulating immune responses in cattle (Zhang et al., 2003). The nature and/or magnitude of the changes observed in this work were dependent on the ligand, as well as on the time of stimulation and the stage of the viral cycle at which the stimulated supernatants were added to the cells. According to Favoreel (2008), the ability of the virus to persist in the infected host implies that herpesviruses have evolved mechanisms to delay and avoid their recognition and elimination by the immune system. Therefore, our results suggest that BoHV-1 and BoHV-5 might modulate TLR signaling to evade the immune system. However, it will be necessary to emphasize the importance of future work that will investigate the mechanisms of host defenses against *in vivo* BoHV-1 and BoHV-5 infections.

The timing and potency of the cellular and immunological events that occur immediately post-infection are crucial determinants that govern the changes induced by pathogens in host cells, which are often accompanied by marked changes in gene expression due to the host- and/or pathogen-mediated reprogramming of the transcriptome during infection (Jenner and Young, 2005). The main focus of our study was to investigate the changes in TLR expression patterns induced by BoHV-1 or BoHV-5 infection. Menzies and Ingham (2006) demonstrated that homologues of human TLRs 1–10 exist in cattle and sheep. However, information regarding tissue distribution and expression patterns in these species is limited. The expression of TLRs is mainly detected in immune cells or cell types that are likely to first encounter antigens and it has also been shown that the tissues involved in immune function have the most diverse repertoire of TLRs (Zarember and Godowski, 2002). In this work, we studied the expression of TLR3, TLR7, TLR8 and TLR9 in bovine PBLs. After determining the TLR expression levels in un-infected bovine PBLs, it was tested whether viral infection induced changes in their patterns of expression. All of the analyzed TLRs were expressed in un-infected PBLs. In these cells, the up-regulation of TLR7 following infection by BoHV-1 or BoHV-5 and the up-regulation of TLR9 following infection by BoHV-5 during a 24-h period were the most striking findings. The ligand for TLR7 is ssRNA, either viral genomic RNA or RNAs that originated during viral replication (Borrow et al., 2010). The enhanced levels of viral transcripts generated during the productive replicative cycle of BoHV-1 and BoHV-5 may be responsible for the up-regulation of TLR7. Pérez (2006) also showed that TLR7 expression is up-regulated in bovine tonsils and trigeminal ganglion at the end of the acute infection of calves infected with BoHV-1. TLR9 is a receptor for sensing unmethylated CpG sequence motifs of DNA viruses. These motifs are found abundantly in some viral genomes, such as herpesviruses. For example, murine cytomegalovirus and HSV induce TLR9 expression (Boo and Yang, 2010). Contrary to the results from this study, primary KSHV infection of monocytes increases TLR3 expression (Jacobs et al., 2013)). Even though statistically significant differences were not detected in TLR3 expression levels, a slight induction of receptor expression was detected in virus-infected cells, which might be relevant from a biological perspective. Similar observations can be made regarding TLR9 expression in BoHV-1-infected cells. Pérez (2006) found that TLR3 and TLR9 expression in tonsils and trigeminal ganglion of BoHV-1-infected calves were down-regulated or maintained to levels which are similar to mock-infected calves. It is recognized that small increases in TLR mRNA levels might induce striking changes in cytokine levels (Lang et al., 2003) and that mRNA and protein levels do not always reflect their functional activity (Hopkins and Sriskandan, 2005).

The information on the expression levels of bovine TLRs is limited. Presently, only a few studies (Griebel et al., 2005; Menzies and Ingham, 2006; Schneberger et al., 2011) have been published that specifically focus on viral TLRs in cattle. However, their relationship with BoHV infection has not been determined. This work reports, for the first time, the expression of TLR mRNAs in bovine peripheral blood immune cells infected with BoHV-1 and -5 strains. This study describes the involvement of at least TLR7 and TLR9 in the recognition of BoHV-1 and BoHV-5. As reported by Zucchini et al. (2008), the overlapping functions of TLR7 and TLR9 for the innate defense against murine cytomegalovirus (an herpesvirus) uncover a new insight into the immune sensing of infectious DNA viruses. TLR7 is a known mediator of immune responses to ssRNA viruses. However, very little is known about the role of this receptor in the recognition of a DNA virus. Interestingly, the cytoplasmic receptor for the uncapped 5′-phosphorylated RNA, RIG-I (retinoic acid-inducible gene I), has been recently demonstrated to trigger IFN-α/β responses in cells infected by EBV *in vitro* (Samanta et al., 2006), further supporting a possible role for RNA sensing in the detection of and response to a DNA virus infection. In support of the findings of the present study, some preliminary data (Pérez, 2006) on TLR expression in bovine immune and non-immune cells have also shown that BoHV-1 can up-regulate TLR7 expression. Thus, this work also suggests a probable role for TLR7 in the infection by a bovine DNA virus.

In summary, the results from this study are the first evidence that the timely activation of TLR7/8 signaling is effective in impairing BoHV replication on MDBK cells. These results provide an experimental clue that Imiquimod may be a promising immune-modulator against BoHV-1 and BoHV-5 infection. TLRs 7 and 8 are highly homologous, and their distinct roles in innate immune responses are still being analyzed (Heil et al., 2004). Zhu et al. (2009) cloned and characterized bovine TLR8 and found that it is highly responsive to two TLR7 ligands, Imiquimod and gardiquimod. However, these responses appeared to be mediated primarily by TLR7, rather than TLR8. This agrees with our *in vitro* studies in which BoHV-1 and -5 infection of bovine PBLs increased TLR7 mRNA expression levels but not TLR8 mRNA. Therefore, our study links TLR7 to the recognition of a DNA virus infection. The effect of TLR7 on the replication of other BoHV strains and in other cells will be the focus of future investigations. Our findings regarding the other TLRs analyzed also contribute to the understanding and knowledge of BoHV-1 and BoHV-5 pathogenesis. Modulation of TLR expression may be an additional tool that contributes to viral persistence in the host. Furthermore, gaining knowledge on the immune mechanisms that participate in the response to viral infection is crucial for the development of antiviral drugs and vaccines aimed to prevent and/or control herpesvirus infections.

Conflict of interest statement

The authors declared 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 INTA, Specific Projects AESA-201711 and AESA-203981. We especially thank Dr. Adriana Cano for her collaborative efforts to conduct the statistical analyses and Dr. Andrea Verna and Susana Pereyra for their contribution and support to the study. Dr. Ana Bratanich is gratefully acknowledged for her help in review this work.

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