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

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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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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