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Short communication

Bovine herpesvirus 1 productive infection stimulates inflammasome formation and caspase 1 activity

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ABSTRACT

Bovine herpesvirus 1 (BoHV-1), a significant viral pathogen of cattle, causes inflammation in affected tissue during acute infection. Consequently, we tested whether productively infected bovine cells stimulate inflammasome formation. Expression of two components required for inflammasome formation, the DNA sensor IFI16 (gamma-interferon-inducible protein 16) and NLRP3 (NOD-like receptor family, pyrin domain containing 3), were induced in bovine kidney cells by eight hours after infection. IFI16 was detected in punctate granules localized to the cytoplasm and nucleus. During productive infection, more than ten times more cells were caspase 1 positive, which is activated following inflammasome formation. Two caspase 1 inhibitors had no effect on productive infection. Conversely, another caspase 1 inhibitor, glyburide, significantly inhibited virus infection suggesting it had off-target effects on related enzymes or interfered with infection via non-enzymatic mechanisms. Collectively, these studies demonstrated that BoHV-1 infection stimulated inflammasome formation, which we predict is important for clinical symptoms in cattle.

Bovine herpesvirus 1 (BoHV-1) is an *alpha-herpesvirinae* subfamily member that causes significant economical losses to the cattle industry. Infection of cattle with BoHV-1 can lead to conjunctivitis, genital disorders, abortions and bovine respiratory disease complex, a life threatening respiratory tract infection, reviewed by (Jones, 2009; Jones and Chowdhury, 2007). The ability of BoHV-1 to induce immune suppression in cattle is important for its pathogenic potential, reviewed in (Jones, 2009). For example, infection inhibits cell-mediated immunity (Carter et al., 1989; Griebel et al., 1987a,b, 1990), CD8+ T cell recognition of infected cells (Hariharan et al., 1993; Hinkley et al., 1998; Koppers-Lalic et al., 2005; Nataraj et al., 1997), and induces apoptosis in CD4+ T cells (Eskra and Splitter, 1997; Winkler et al., 1999). Furthermore, two viral regulatory proteins, bICP0 and bICP27, inhibit interferon dependent transcription (da Silva and Jones, 2012; Henderson et al., 2005; Jones, 2009; Saira et al., 2007; Saira and Jones, 2009). Finally, infection erodes mucosal surfaces within the upper respiratory tract, and consequently promotes establishment of bacterial pathogens in the lower respiratory tract (Highlander et al., 2000; Highlander, 2001; Zecchinon and Desmecht, 2005).

The focus of these studies was to examine the effect that BoHV-1 had on inflammasome formation. The rational for these studies stem from observations demonstrating that acute infection of calves leads to inflammation in the trachea, trigeminal ganglia, tonsils, and tissues within the ocular cavity (Perez et al., 2005, 2006; Winkler et al., 2002, 2000). It is also clear that other viruses, including Epstein-Barr virus and herpes simplex virus type 1 (HSV-1), activate the inflammasome during productive infection (Gastaldello et al., 2013; Johnson et al., 2013). For these studies, bovine kidney cells (CRIB) were infected with BoHV-1 and at various times after infection expression of cellular proteins that promote inflammasome formation were examined (IFI16 and NLRP3) (Latz et al., 2013). Polyclonal antibodies that recognize human, mouse, and rat proteins were used for these studies to ensure the possibility that they recognized the respective bovine protein. IFI16 and NLRP3 were consistently induced by 8 h after infection (Fig. 1A). The IFI16 antibody detected three prominent proteins, which are consistent with the size of IFI16 proteins expressed in rodents and humans (68, 75, 95 kD). The bovine IFI16 ORF is nearly the same size as other mammalian IFI16 proteins, and has several conserved domains (C. Jones, unpublished data). The NLRP3 antibody detected a prominent 72 kD band after infection, also consistent with the size of the NLRP3 protein expressed in humans and rodents. Expression of the bICP0 protein was readily

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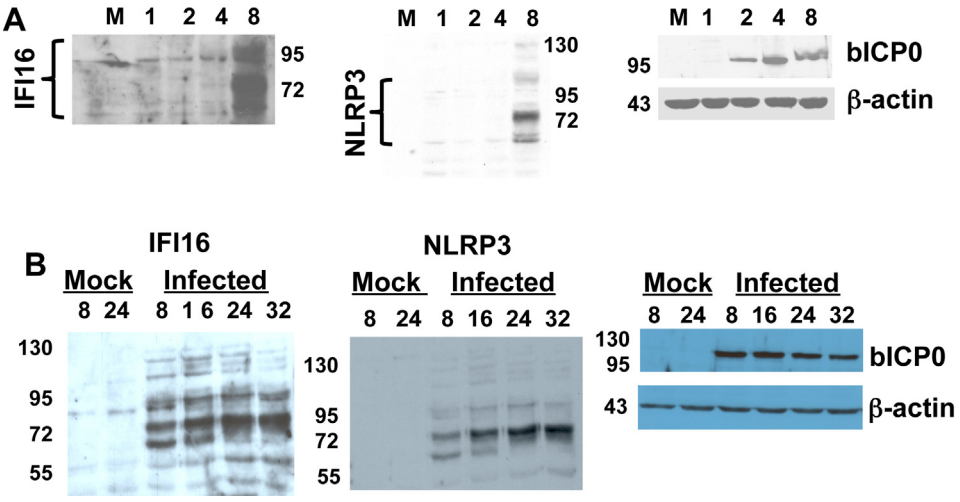

Fig. 1. BoHV-1 infection induces IFI16 and NLRP3 protein expression. Panel A: CRIB cells were infected with BoHV-1 (1 plaque forming unit/cell of the Cooper Strain) for 1, 2, 4, or 8 h after infection. Total cell lysate was prepared as previously described (Workman et al., 2012; Workman and Jones, 2011). As a control, cell lysate from mock-infected cells was used (lane M). Panel B: CRIB cells were infected as described in panel A for 8, 16, 24, or 32 h after infection. As controls, cell lysate from mock-infected cells that were cultured for 8 or 24 h were examined (Mock lane). Proteins from each sample in Panels A and B (100 μg protein) were separated on a SDS-polyacrylamide gel and proteins subsequently transferred to a membrane. Western blot analysis was performed using antibodies directed against IFI16 (sc-6050, Santa Cruz Biotechnology), NLRP3 (ab4207, Abcam), bICP0 (peptide specific antibody), and β-actin as previously described (Workman et al., 2012; Workman and Jones, 2010, 2011). The results in panels A and B are consistent with 5 independent experiments.

detected at 2 h after infection as well as 1 h after infection when the blot was exposed for longer periods of time (data not shown). Although bICP0 was only detected in infected cells, β-actin protein expression was detected at similar levels in all samples. At 16, 24, and 32 h after infection, the levels of IFI16 and NLRP3 were similar to 8 h after infection (Fig. 1B). In summary, these results suggested that accumulation of late viral gene products triggered inflammasome formation or a viral-encoded or – induced factor interfered with inflammasome formation during early stages of infection.

Confocal microscopy was performed to determine the localization of IFI16 in CRIB cells after infection. Several studies demonstrated IFI16 is present in the nucleus; conversely other studies concluded IFI16 is localized to the cytoplasm (Duan et al., 2011; Gariano et al., 2012; Orzalli et al., 2012; Unterholzner et al., 2010; Veeranki and Choubey, 2012). At 16 h after infection, most of the IFI16 was detected in granules localized to areas surrounding the nucleus (Fig. 2). Close inspection of the images also suggested that a small percentage of these granules were present in a subset of nuclei. As expected, IFI16 and bICP0 were not detected in mock-infected cells, and bICP0 was primarily localized to the nucleus. Biochemical fractionation of infected cells further suggested that IFI16 may be present in the nuclear fraction of infected cells at 16 h after infection (Fig. 2B). The cytoplasmic fraction also contained IFI16, which was consistent with the confocal microscopy studies. Histone 3 (H3) was primarily detected in the nuclear extract at 16 h after infection as expected. As previously demonstrated in primary bovine cells infected with BHV-1 (Frizzo da Silva and Jones, 2011), a subset of bICP0 was detected in the cytoplasm; however most of the bICP0 protein was detected in the nuclear fraction. Evidence is accumulating for a functional role of IFI16 in controlling HSV-1 infection because IFI16 requires an intact nuclear localization signal to bind to HSV-1 DNA in infected cells and activate IFN-β expression (Li et al., 2012). An independent study demonstrated that IFI16 sensing of HSV-1 DNA is nuclear in human foreskin fibroblast cells (Orzalli et al., 2012). It will be of interest to determine whether IFI16 interacts with BoHV-1 DNA during productive infection and influences viral replication.

Caspase 1 cleavage and activation is the hallmark of inflammasome formation, reviewed by (Grant and Dixit, 2013; Latz et al., 2013; Stutz et al., 2009). Therefore, it was of interest to determine whether caspase 1 was cleaved and activated following infection. We initially tested whether caspase 1 was cleaved using polyclonal antibodies. These antibodies did not yield reliable results: perhaps because they do not recognize bovine caspase 1 (data not shown). Consequently, we measured caspase 1 activity in infected cells using a fluorescent peptide (Immunochemistry Technologies, Bloomington, MN) that specifically and covalently binds activated caspase 1. Approximately 10 times more fluorescent positive cells were observed at 16 h after infection when compared to uninfected cells (Fig. 3A). To confirm these studies, flow cytometry was used to quantify the number of caspase 1-activated cells after infection relative to mock-infected cells. In agreement with studies in Fig. 3A, there was a significant difference between the number of caspase 1 positive cells at 16 h after infection relative to mock-infected cells (Fig. 3B).

To test whether caspase 1 activation influences BoHV-1 productive infection, we examined the effect that caspase 1 inhibitors have on virus production. Three specific caspase 1 inhibitors were used for these studies: (1) glyburide, a sulfonylurea drug (Lamkani et al., 2009), (2) YVDA-CHO a peptide based inhibitor (ENZO Life Sciences), and VX-765 (Cellagen Technology) (Gastaldello et al., 2013; Lamkani et al., 2009). YVDA-CHO and VX-765 had little or no effect on virus yield in CRIB cells (Fig. 3C). In contrast, increasing concentrations of glyburide decreased the levels of infectious virus in CRIB cells suggesting glyburide has unknown off-target effects on BoHV-1 replication. Glyburide is widely used for type 2 diabetes in the United States, and has few side-effects on patients (Riddle, 2003). Consequently, it is of interest to understand its mechanism of action against BoHV-1 and whether it has anti-viral activities directed against human alpha-herpesviruses. Interestingly, a recent study demonstrated that caspase 1 inhibitors, including glyburide, inhibited Epstein-Barr virus replication, in part by blocking the cleavage of a large tegument protein (BPLF1) that has deneddylase activity (Gastaldello et al., 2013). BPLF1 is reported to be the homologue of HSV VP16 (Schmaus et al., 2004). However, it is not known whether the BoHV-1 VP16 protein has deneddylase activity. At the concentration used for the respective drugs, no major effects on cell growth or morphology were observed (data not shown). The