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Infection of goats with goatpox virus triggers host antiviral defense through activation of innate immune signaling

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ABSTRACT

Goatpox, caused by goatpox virus (GTPV), is one of the most serious infectious diseases associated with high morbidity and mortality in goats. However, little is known about involvement of host innate immunity during the GTPV infection. For this, goats were experimentally infected with GTPV. The results showed that GTPV infection significantly induced mRNA expression of type I interferon (IFN)-α and IFN-β in peripheral blood lymphocytes, spleen and lung. In addition, GTPV infection enhanced expression of several inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-18; and tumor necrosis factor-α (TNF-α). Strikingly, infection with GTPV activated signal transducers and activators of transcription 3 (STAT3), a critical cytokine signaling molecule. Interestingly, the virus infection induced expression of suppressor of cytokine signaling (SOCS)-1. Importantly, the infection resulted in an increased expression of some critical interferon-stimulated genes, such as interferon-induced transmembrane protein (IFITM) 1, IFITM3, interferon stimulated gene (ISG) 15 and ISG20. Furthermore, we found that infection with GTPV up-regulated expression of Toll-like receptor (TLR) 2 and TLR9. These results revealed that GTPV infection activated host innate immune signaling and thereby triggered antiviral innate immunity. The findings provide novel insights into complex mechanisms underlying GTPV–host interaction and pathogenesis of GTPV.

1. Introduction

Goatpox virus (GTPV), together with sheeppox virus and lumpy skin disease virus, belongs to genus Capripoxvirus under family Poxviridae, a family of large, complex dsDNA viruses that replicate in the cytoplasm of host cells (Diallo and Viljoen, 2007). Goatpox, caused by GTPV infection, is a highly fatal contagious viral disease in goats, characterised by pyrexia, oculo-nasal discharge and generalized pocks in the skin (Damon, 2007). The disease causes substantial economic loss due to high morbidity and mortality (Venkatesan et al., 2010). GTPV is one of 15 animal pathogens listed by World Organization for Animal Health (Babiuk et al., 2008). Outbreaks of the disease have recently been reported frequently (Bhanuprakash et al., 2010; Venkatesan et al., 2010; Verma et al., 2011; Yan et al., 2012). It is worth noting that several studies have indicated that the host specificity of sheeppox virus and GTPV strains is becoming weaker, resulting in an increase of their ability to infect both sheep and goat (Bhanuprakash et al., 2010; Yan et al., 2012). However, mechanisms underlying the interaction between host and these viruses are poorly understood. In particular, little information is available about the role of host immune response to GTPV infection in goats.

Innate immune response is the first line of defense against invading pathogens, limits their spread through recognition of conserved microbial molecules known as pathogen-associated molecular patterns (PAMPs) by a cluster of receptors called pattern-recognition receptors (PRRs) (Janeway and Medzhitov, 2002; Akira et al., 2006). Virus infection can initiate activation of nuclear factor-κB (NFκB) and/or interferon-regulatory factors (IRFs), which regulate production of a multitude of antiviral cytokines, including type I and type III interferons (IFNs) and proinflammatory cytokines (Honda and Taniguchi, 2006; Chen et al., 2013; Wei et al., 2014). Induced IFNs bind to their receptors to activate the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, resulting in increased expression of a large group of IFN-stimulated genes (ISGs) that play critical roles in defense against viral infection (Wei et al., 2014). However, to establish a successful infection, poxviruses have developed multiple strategies to evade or subvert key aspects of the host antiviral response. For example, it has been found that poxviruses are extraordinarily adept in encoding proteins that interdict extracellular and intracellular components of

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innate immune responses (Seet et al., 2003). Recently, several studies have described host innate antiviral immunity triggered by poxviruses (Brady and Bowie, 2014). Previous studies have identified that various proteins from *parapoxvirus* orf virus (ORFV002, ORFV024 and ORFV121) (Diel et al., 2011), *orthopoxvirus* vaccinia virus (A46R, A52R, N1L, B14, M2L and K1L) (Chen et al., 2008; Gedey et al., 2006; Shisler and Jin, 2004; Stack et al., 2005), cowpox virus (CrmB, CrmC, CrmD, CrmE and CP77) (Chang et al., 2009; Gileva et al., 2006), *leporipoxvirus myxoma* virus (M-T2, M150R and M013L) (Camus-Bouclainville et al., 2004; Rahman et al., 2009b; Xu et al., 2000), and *yatapoxvirus tanapox* virus (TPV-2L) (Rahman et al., 2009a) could inhibit NF-κB activation through targeting multiple molecules of the signaling pathways.

Although progresses in understanding innate immune response to a variety of poxviruses have been made, GTPV-induced host innate immunity in goat remains largely unknown. In this study, an experimental infection of GTPV in goats was performed to understand innate immune response to goatpox virus. We observed that GTPV infection increased the expression of type I IFNs in peripheral blood lymphocytes (PBLs), spleen and lung. In addition, GTPV infection increased the expression of inflammatory cytokines including IL-1β, IL-6, IL-18, and TNF-α. Infection with GTPV greatly enhanced the phosphorylation of STAT3 and induced the expression of suppressor of cytokine signaling-1. Importantly, some critical antiviral molecules, including interferon-induced transmembrane protein (IFITM) 1, IFITM3, ISG15 and ISG20 were significantly induced during the GTPV infection. Furthermore, elevated expression of TLR2 and TLR9 was identified in the infected host. Together, these experiments demonstrated that infection of goats with GTPV could trigger cytokine-dependent antiviral defense through activation of innate immune signaling.

2. Materials and methods

2.1. Ethics statement

The goat experimental design and protocols used in this study were approved by 'College of Animal Science, Fujian Agriculture and Forestry University Ethics Committee' (Authorization Number: PZCASFAFU2014003, Date of Approval: December 20, 2013). All goat experimental procedures were performed in accordance with Regulations for Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

2.2. PBL isolation and collection of tissue samples

Using antibody assay kit (Dong Songs), the titers of GTPV antibody were screened in five-month-old hybrid goats that were not immunized by goatpox vaccine previously. Afterwards, six GTPV-negative goats were selected and randomly divided into the control and experimental groups (three goats/group). For the control group, all animals were injected intradermally with cell culture supernatant. For the experimental group, 10 mL of blood sample was collected as control from each animal before GTPV infection. Subsequently, each goat was injected intradermally with 1.5 mL of cell culture supernatant containing GTPV FZ strain ($10^{8.5}$ $TCID_{50}$/mL) in the root of the tail and inner side of the right thigh, respectively. At 12, 24, 48, 72, 96, 120 and 144 h post inoculation (HPI), 10 mL of blood from each goat was collected in a sodium heparin-containing centrifuge tube and diluted with 20 mL of red blood cell (RBC) lysis buffer. The samples were then centrifuged at 1100 *g* for 15 min at room temperature. PBLs were collected and used for total RNA extraction. At 144 HPI, all goats were euthanized and necropsied for collection of tissue samples for analysis by reverse transcription-PCR (RT-PCR) and Western blotting. GTPV FZ strain used in this study was isolated originally from skin pox of a goat with naturally occurring goatpox and propagated on OFTu cells, as described previously (Zeng et al., 2014).

2.3. RT-PCR and quantitative real-time PCR

RT-PCR and quantitative real-time PCR (qPCR) were performed according to the manufacturer's instructions (Tiangen and TaKaRa). Briefly, total RNA was extracted from the tissues using TRIzol reagent (Tiangen), and eluted in RNase-Free dH_2O (TaKaRa) in a total volume of 50 μL. Concentration and quality of RNA were evaluated using a NanoDrop 2000 Spectrophotometer (Thermo). cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (TaKaRa, RR037A) in a 40 μL solution containing 2 μg of total RNA, 1 × PrimeScript Buffer, PrimeScriptRT Enzyme Mix I and 50 μM Oligo dT Primer, and the mixture was incubated at 37 °C for 15 min. PCR was performed using a program that included an initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles (95 °C for 45 s, 57 °C for 30 s and 72 °C for 45 s) and a final incubation at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 1.2% agarose gel containing 0.5 pg/mL of ethidium bromide.

A two-step qPCR was performed with the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad) using SYBR ® Premix Ex Taq™ (TaKaRa, RR420A). Each reaction containing 2 μL of cDNA template in a 20 μL volume was performed under the conditions of 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. For each gene, a minimum of the three replicates was used to calculate fold changes. Expression levels of genes tested were normalized to the housekeeping gene β-actin and the 2(−delta delta CT) method was applied for qPCR data analysis (Livak and Schmittgen, 2001). The quantitative standard curve was prepared from a pool of cDNA serial 10-fold dilutions of a stock containing 10^8 copies/μL for β-actin and all tested genes. The specificity was confirmed by observation of a single peak at the predicted temperature and the absence of nonspecific products or primer dimers. Primers used in this study are shown in Table 1.

2.4. Western blotting

Western blotting was performed as previously described (Ouyang et al., 2014; Wang et al., 2014). Briefly, tissue samples were homogenized and lysed in cell lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice, followed by centrifugation. The lysates were separated on 10% gels by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose (NC) membranes. The membranes were blocked in TBST containing 5% powdered milk for 3 h and incubated overnight with primary antibodies against phospho-Stat3 (Tyr705) (diluted 1:1000, Cell Signaling), Stat3 (diluted 1:1000, Cell Signaling), IFITM1/3 (diluted 1:1000, Proteintech), ISG15 (diluted 1:1000, Proteintech) and β-actin (diluted 1:1000, Beyotime, China) at 4 °C. Blots were washed and probed with secondary antibody goat anti-rabbit IgG conjugated with horseradish peroxidase (diluted 1:1000, Beyotime, China) at 37 °C for 2 h. The membranes were then detected by using a chemiluminescent substrate (Beyotime, China) and exposed to X-ray film.

2.5. Histopathological analysis

Samples were collected from an experimental goat at 144 HPI, fixed in 10% (v/v) buffered formalin. Subsequently, each tissue sample was rinsed thoroughly with water, dehydrated using a graded series of ethanol and embedded in paraffin. Then 5-μm thick sections were prepared and stained with hematoxylin and eosin (H&E). The slides were observed using a Nikon 55i microscope (Nikon).

2.6. Statistical analysis

Results were shown as mean values ± standard deviation (mean ± SD). Data were analyzed using a paired-samples Student's t test. A level of $P < 0.05$ was considered to be significant.