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Avian leukosis virus subgroup J triggers caspase-1-mediated inflammatory response in chick livers

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ABSTRACT

Many pathogens trigger caspase-1-mediated innate immune responses. *Avian leukosis virus* subgroup J (ALV-J) causes serious immunosuppression and diverse tumors in chicks. The caspase-1 inflammasome mechanism of response to ALV-J invading remains unclear. Here we investigated the expression of caspase-1, the inflammasome adaptor NLRP3, IL-1β and IL-18 in response to ALV-J infection in the liver of chick. We found caspase-1 mRNA expression was elevated at 5 dpi and peaked at 7 dpi in ALV-J infected animals. Corresponding to this, the expressions of NLRP3 and proinflammatory cytokines IL-1β and IL-18 were significantly increased at 5 or 7 dpi. In addition, caspase-1 protein expression and inflammatory cell infiltration were induced after virus infection. These results indicated that ALV-J infection could trigger the caspase-1- mediated inflammatory response in chicks. Thus, an understanding of the inflammatory responses can provide a better insight into the pathogenicity of ALV-J and a possible anti-virus target for ALV-J infection.

1. Introduction

Caspase-1, a cysteine protease, acts as an essential regulator of inflammatory responses and pyroptosis, a proinflammatory cell death program (Miao et al., 2010; Fink and Cookson, 2007; Lin et al., 2014). Caspase-1 is initially expressed as an inactive precursor and is activated within the inflammasome complex by microbial, chemical or environmental stimuli (Thomas et al., 2009; Ather et al., 2011; Tschopp and Martinon, 2004). Pathogenic infections trigger caspase-1 and play a central role in infections induced by *Streptococcus pneumoniae* (*S. pneumoniae*) (Fang et al., 2014) and *Listeria monocytogenes*, the latter which causes pyroptosis (Lin et al., 2014). Caspase-1 induces the regulation and maturation of the proinflammatory cytokines, IL-1β and IL-18. These cytokines induce many genes and activities in the innate immune response. Decreased activity of caspase-1 is accompanied by reduced secretion of IL-1β and IL-18 (Gonçalves et al., 2013). Mice lacking caspase-1 exhibit defective IL-1β and IL-18 production at the infection site and are resistant to cutaneous *Leishmania major* infection (Gurung et al., 2015) and endotoxic shock (Lamkanfi et al., 2010; Meissner et al., 2008).

The NOD-like receptor family, pyrin domain-containing 3 (NLRP3, also known as NALP3 or cryopyrin), acts as an intracellular sensor of activated pro-caspase-1 during certain viral infections, such as Rift Valley fever virus (RVFV) infection (Ermler et al., 2014) and dengue virus (DENV) infection (Callaway et al., 2015). Recent studies have shown that the NLRP3/caspase-1 inflammasome plays a critical role in viral infection by inducing caspase-1 activation and the subsequent processing and secretion of IL-1β and IL-18, *in vitro* (Kanneganti et al., 2006). *Avian leukosis virus* (ALV), an oncogenic retrovirus of chicks, causes a major infectious disease that impacts the poultry industry worldwide. ALV-J, a novel, virulent subgroup of ALV, can induce immunosuppression together with multiple and diverse tumors, and causes more serious infections in commercial layer flocks (Dong et al., 2015; Li et al., 2014; Gao et al., 2010). Therefore, the pathogenic mechanisms of ALV-J remain largely obscure and must be explored. Until now, no effective vaccine has been developed for prevention and treatment of the disease caused by the virus. The activation of caspase-1 and its inflammasome component in response to ALV-J infection remains unclear *in vivo*. In this study, we infected chicks with the ALV-J virus and analysed the changes in expression of caspase-1, NLRP3, IL-1β and IL-18 in liver tissues, at different days after virus and mock infection. We showed that viral load resulted in pathological changes with *in vivo*

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expression of the caspase-1 inflammatory response to ALV-J infection in the liver, one of the predominant target tissues of ALV-J. The results indicated that ALV-J could trigger the caspase-1 inflammatory response, which may benefit *in vivo* virus infection, thus improving our understanding of the pathogenicity of ALV-J.

2. Materials and methods

2.1. ALV-J strain and cell line

A strain of ALV-J was isolated from the liver of a commercial layer chick, naturally infected by ALV-J in the Anhui Province of China and was stored at −80 °C in our laboratory before propagation in DF-1 fibroblastic cells. DF-1 cells were cultured at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, USA), supplemented with 10% FCS, 100 units/ml penicillin and 100 units/ml streptomycin (Zhanga et al., 2014).

2.2. Animals and sample preparation

SPF postnatal day 1 chicks (Beijing Meiliyaweitong Experimental Animal Technology Co., Ltd., China) were randomly divided into two groups. Each experiment was performed with three chicks. Chicks in one group were subcutaneously infected via the neck with 0.2 ml of the ALV-J strain AJV-HF211 (approximately 10^4 TCID50 units per milliliter). The mock-infected animals were subcutaneously injected with cell-culture medium from DF-1 cell (DMEM). Approval for these animal studies was obtained from the Center for Animal Care and Use Committee of Anhui Agricultural University.

Total RNA were extracted from liver tissues of virus-infected and control chicks at 1, 3, 5, 7, 9, 13 and 17 dpi using Eastep™ Universal RNA Extraction Kit (Promega, Beijing, China), respectively. First-strand cDNAs were synthesised using TransScript First Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd., Beijing, China) and stored at −80 °C until further use.

2.3. Virus detection in the liver

Total RNAs were extracted from liver samples as described above. RT-PCR was performed with a pair of specific primers for amplifying a 545-bp sequence of ALV-J *pol* (H5) and *gp85* (H7) (Smith et al., 1998): H5: 5′-GGATGAGGTGACTAAGAAAG-3′; H7: 5′-CGAACCAAAGGTAACACACG-3′. The amplified products were sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

2.4. Histopathological detection in the liver

Preparation of liver tissue paraffin sections for histopathology was performed by the recognized method (Ramos-Vara et al., 2014). Sectioned samples were obtained from the livers infected with ALV-J (1, 7 and 17 dpi) and control livers, respectively. The sections fixed and treated were observed by routine H&E staining methods.

2.5. Polyclonal caspase-1 antibody preparation and immunohistochemical detection

The full-length cDNA sequence (852 bp) of caspase-1 was amplified by RT-PCR from the liver sample of a naturally infected chick. The primer sequences were as follows:

forward: 5′-CGGAATTCATGAGCAGGGCAAGATCTTCG-3′ (*Eco*R I);

reverse: 5′-ACGCGTCGACGTCAGAGGCCTGGGAAGAGATAGAAC-3′ (*Sal* I). A purified PCR product was inserted into the vector pMD18-T and sub-cloned into pET-32a (+) vector. The pET32a-caspase1 recombinant plasmid was introduced into *Escherichia coli* Rosetta (DE3) cells. The fusion protein expression was induced with 0.6 mmol/l IPTG, purified using a high-affinity nickel Sepharose™ 6 Fast Flow resin column (GE Healthcare, Piscataway, USA). The polyclonal rabbit anti-caspase-1(anti-Casp1) serum was prepared using a similar methodology as described in a previous paper (Liang et al., 2015). The specificity of anti-caspase-1 antibody was assessed by western blotting following over expression of caspase-1 upon pE-green fluorescent protein (GFP)-N1 vector transfection in DF-1 cells plated onto 6-well plates for 24 h, using Lipofectamine® 2000 reagent (Life Technologies, USA). Cells were then treated with 10 μg/ml LPS (Sigma–Aldrich) for 4 h and 5 mM ATP (Sigma–Aldrich) for 45 min before RIPA lysis. Pro-caspase-1 and active caspase-1 were detected with Pierce ECL Western Blotting Substrate (Thermo, USA) using Multi-channel imaging system (VersaDocTM4000MP, Bio-Rad).

The sections for immunohistochemistry were obtained from the same histopathological samples. The procedures and steps of tissue section preparation for immunohistochemical staining of caspase-1 were performed on chick livers at 1, 7 and 17 dpi. After endogenous peroxidase activity was quenched, antigen retrieval and immunohistochemical detection were performed with polyclonal antibody for caspase-1 (1:200 dilution), for 2 h at 37 °C. After incubation with the correspondent secondary antibody for 1 h at 37 °C, the slices were washed 3× with PBS at 10-min intervals. A 3, 3′-diaminobenzidine-tetrahydrochloride (DAB) kit (Sigma–Aldrich, USA) was used for coloring the slices, and they were subsequently re-dyed with haematoxylin.

2.6. Expression of caspase-1 inflammasome and cytokines by RT-qPCR

Total RNA was isolated and cDNA was synthesized as described in Section 2.2. RT-qPCR was performed using the 7500 real-time PCR system (Applied Biosystems, USA) and the TransStrat TipTop Green qPCR SuperMix (TransGen Biotech Co., Ltd., Beijing, China). Relative expression levels of caspase-1, NLRP3, IL-1β, IL-18, IFN-γ and IFN–β in the infected and control livers were normalized using the $2^{-\Delta\Delta Ct}$ formula and the endogenous housekeeping gene β-actin. The primers for specific amplification are shown in Table 1.

2.7. Statistical analysis

All values were compared using the Student's *t* test. Differences between groups were considered significant when the *p*-value was <0.05.

3. Results

3.1. Detection of viral invasion and pathological changes in the liver

The ALV-J *pol-gp85* gene fragment (545 bp) could be detected in liver samples from 9 to 17 dpi using RT-PCR (Fig. 1A). We then detected the inflammatory cells using liver paraffin sections, which were processed for routine H&E staining. As shown in Fig. 1B, the inflammatory cell proliferation was induced in liver tissues of infected animals, but not in those of control animals (Fig. 1B, a and b). Moreover, no difference in pathologies was observed between the virus infected and control chicks before 7 dpi (figure not shown). The recruitment of infiltrating inflammatory cells can be detected in the liver at 17 dpi (Fig. 1B, e–f).