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Modulation of the innate immune-related genes expression in H9N2 avian influenza virus-infected chicken macrophage-like cells (HD11) in response to *Escherichia coli* LPS stimulation

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ABSTRACT

Macrophages play important roles in mediating virus-induced innate immune responses and are thought to be involved in the pathogenesis of bacterial superinfections. The innate immune response initiated by both low pathogenicity AIV and bacterial superinfection in their avian host is not fully understood. We therefore determine the transcripts of innate immune-related genes following avian H9N2 AIV virus infection and *E. coli* LPS co-stimulation of avian macrophage-like cell line HD11 cells. More pronounced expression of pro-inflammatory cytokines (IL-6 and IL-1β) as well as the inflammatory chemokines (CXCLi1 and CXCLi2) was observed in virus infected plus LPS treated HD11 cells compared to H9N2 virus solely infected control. For two superinfection groups, the levels of genes examined in a prior H9N2 virus infection before secondary LPS treatment group were significantly higher as compared with simultaneous virus infection plus LPS stimulation group. Interestingly, similar high levels of IL-6 gene were observed between LPS sole stimulation group and two superinfection groups. Moreover, IL-10 and TGF-β3 mRNA levels in both superinfection groups were moderately upregulated compared to sole LPS stimulation group or virus alone infection group. Although TLR4 and MDA5 levels in virus alone infection group were significantly lower compared to that in both superinfection groups, TLR4 upregulation respond more rapid to virus sole infection compared to LPS plus virus superinfection. Collectively, innate immune-related genes respond more pronounced in LPS stimulation plus H9N2 virus infection HD11 cells compared to sole virus infection or LPS alone stimulation control cells.

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1. Introduction

H9N2 avian influenza viruses (AIV) are among the most commonly occurring in domestic poultry populations, with several outbreaks reported in Asia and North America since 1990 (Alexander, 2003). H9N2 subtype viruses are classified as low-pathogenicity avian influenza (LPAI) based on molecular characterization and pathotyping (Abolnik et al., 2007). Most LPAI viruses cause no or mild disease in avian species. It has long been acknowledged that a substantial fraction of influenza-related death and disability results not from primary viral disease but from secondary infection with bacteria unrelated to the virus (Stegemann-Koniszewski et al., 2013). Deaths to secondary bacterial infection commonly result from immunopathology, rather than bacterial infection, and once initiated, immunopathology in the form of sepsis and septic shock may progress independently of the bacterial burden itself (Bano et al., 2003; Small et al., 2010; Zavitz et al., 2010). Specifically, even if host suffering from heterologous infections are treated with sterilizing antibiotics, they still frequently succumb to pathologic inflammation. A number of mechanisms have been proposed by which influenza may predispose to superinfection with an unrelated or heterologous pathogen (Sun and Metzger, 2014), but the subsequent interaction between the host, virus, and bacteria remains an understudied area. Focusing on the interaction between LPAI viruses and secondary bacterial infection, much remain to be explored. Studying a panel of selected cytokines should contribute to the understanding of LPAI viruses-related pathogenesis of bacterial superinfections.

Macrophages play important roles in innate immunity, such as phagocytosis, containment of pathogens and the secretion of cytokines that contain infection and augment immune regulation (Twigg, 2004; Taylor et al., 2005). Previous studies demonstrated that chicken macrophages are susceptible to infection with LPAI H9N2 and H6N2 viruses and that infection may specifically modulate host adaptive immune responses negatively in avian species (Kaufmann et al., 2001; Xing et al., 2008). Liniger et al. (2012a) reported that chicken DF-1 fibroblasts and HD11 macrophage-like cells employ melanoma differentiation-associated protein 5 (MDA-5) to sense AIV infections. Chicken macrophages also express transcripts for TLRs 2–5, 7 and 21 (Iqbal et al.,

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2005; Barjesteh et al., 2014). Activation of the TLR4 pathway results in the expression of pro-inflammatory cytokines interleukin (IL)-6, IL-1β, IL-8, and IL-12 (Ferdous et al., 2008; Ferdous and Scott, 2015). Additionally, according to another group of researchers, macrophages constitutively express transcripts for both pro- and anti-inflammatory cytokines for anti-viral responses and bacterial antigen presentation (St Paul et al., 2012). It is well recognized that certain Gram-negative bacterial products such as lipopolysaccharide (LPS) can activate macrophages and other cells through their TLR4 (Haddadi et al., 2013; Gou et al., 2015). Chicken macrophages-like cells (HD11) have been used with success in the past as a model for avian influenza virus infection in avian hosts (Liniger et al., 2012a; Liniger et al., 2012b). In this study, we compared the innate immune-related genes profiles of HD11 cell lines in response to avian H9N2 virus infection or LPS stimulation. Furthermore, the changes of these genes expression in HD11 following primary viral infection and secondary *E. coli* LPS stimulation, or simultaneous virus infection and LPS treatment were also evaluated in the current study. This study may shed light on the underlying mechanisms responsible for the severity of systemic infection and inflammatory damage in low virulent avian H9N2 virus infected avian host caused by pathogenic avian *E. coli* superinfection.

2. Materials and methods

2.1. Cells

The chicken macrophage-like cell line HD11 cells (Beug et al., 1979) were cultured in Dulbecco's Modified Eagle's Medium (DMDM, Gibco-BRL, Grand Island, NY, USA) containing 4.0 mM L-glutamine and 4500 mg of glucose/l and supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) at 39 °C with 5% CO_2.

2.2. Virus

The virus strain used in this study, low-pathogenicity H9N2 avian influenza viruses strain (A/chicken/Shaanxi/11/2012), was isolated from Shaanxi province, China, in 2012 (Wang et al., 2013).

2.3. Experimental design

The transcripts of innate immune-related genes of HD11 cells was respectively detected in response to simultaneous avian H9N2 virus infection and LPS stimulation (VIC + LPS), as well as in response to a prior virus infection 24 h before secondary LPS treatment (VIC(24) + LPS). For VIC + LPS group, HD11 cells were seeded into 6-well plates at a concentration of 1×10^6 cells/ml and cultured at 39 °C for 24 h, and confluent HD11 cells were infected with H9N2 AIV at a multiplicity of infection (MOI) of 1, then, 2 ml culture medium containing *E. coli* O55:B5 LPS (5 μg/ml, AdipoGen, CA, USA) at a concentration of 5 μg/ml was added to each wells; For VIC(24) + LPS, confluent HD11 cells were prior infected with H9N2 AIV for 24 h, then, the HD11 cells were exposed to *E. coli* O55:B5 LPS. Additionally, the immune response of HD11 cells was also detected in sole avian H9N2 virus infected control group (VIC/VIC(24)) and LPS treated control group (LPS). The cells in above groups were collected for RNA extraction at indicated time point. Three biological triplicates were used in each group.

2.4. Evaluation of viral loads

The colonization of H9N2 AIV in HD11 cells was examined by SYBR green-based real-time PCR assays. Primers targeted to the M gene of H9N2 AIV, forward: 5′-TTCTAACCGAGGTCGAAAC-3′ (47–65), reverse: 5′-AAGCGTCTACGCTGCAGTCC-3′ (275–256) were used for PCR analysis. To perform real-time PCR, viral RNA was extracted from the cells with TRIzol reagent (Takara Biotechnology, Japan) then reverse transcribed into cDNA. The cDNA was further subcloned into the PMD ™19-T vector and then transformed into DH5α *E. coli*. The recombinant plasmid was identified by *Eco*RI and *Sal*I digestions. Serial 10-fold dilutions of positive recombined were applied as a positive quantitative template to establish the standard curve from 10^1 to 10^7 copies/μl. The real-time PCR reactions were performed as described previously (Wang et al., 2015; Qi et al., 2016). All amplifications were performed in triplicate. The obtained cycle threshold (Ct) values were plotted against the amount of RNA copy number to the standard curve.

2.5. Innate immune-related genes expression in avian H9N2 virus infected HD11 cells in response to LPS stimulation

The mRNA levels for genes examined, including CXCLi1, CXCLi2, IFN-α, IL-1β, IL-6, TGF-β3, IL-10, TLR4, MDA5, iNOS and β-actin genes, in virus solely infected or in LPS solely stimulated control HD11 cells and virus infected plus LPS stimulated cells were analyzed by a two-step real-time RT-PCR. Total RNA, which was prepared from the cells with TRIzol reagent (Takara Biotechnology, Japan), was used for each quantitative RT-PCR reaction with the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen biotech, China), following the manufacturer's instruction. Real-time PCR was conducted with 1 μg cDNA in a total volume of 20 μl with the iQ SYBR Green Supermix (Bio-Rad, CA, USA), following the manufacturer's instructions. Relative expression values were normalized using an internal β-actin control. The fold change of relative gene expression levels was calculated following the formula: $2^{-(\Delta Ct \text{ of gene} - \Delta Ct \text{ of } \beta\text{-actin})}$. The primers targets genes examined in this study have been previously reported and the sequences of the primers are given in Table 1 (Laurent et al., 2001; Chiang et al., 2009; Zhou et al., 2014; Karnati et al., 2015; Wang et al., 2015).

2.6. Statistical analysis

All experiments shown were performed a minimum of three times, and the data were calculated as the mean ± SEM. A two-tailed Student's *t*-test was used to determine statistical significance of selected samples.

3. Results

3.1. Kinetics of viral loads in HD11 cells in response to H9N2 AIV infection plus LPS stimulation

To investigate the replication of avian H9N2 virus in HD11 cells in response to pathogenic *E. coli* superinfection, the cells were simultaneously infected with characterized strain of H9N2 AIV and *E. coli* LPS, or were previously infected with H9N2 virus and then stimulated with *E. coli* LPS for secondary infection. For both superinfection groups, similar kinetics of viral replication was detected between viruses solely infected control group and viruses plus LPS stimulated group. As shown in Fig. 1A, a marked increase of virus loads was examined from 1 h to 8 h post LPS stimulation both in VIC control group and VIC + LPS group. Although VIC(24) + LPS group showed further increased the virus loads, similar kinetics of virus loads were also detected in VIC(24) control group (Fig. 1B).

3.2. Immune responses in HD11 cells following simultaneous H9N2 AIV infection and LPS stimulation

Expression patterns of all immune-related genes examined in VIC + LPS group were largely similar. Collectively, the expression levels of genes examined peaked at 2 h p.s. followed by a gradual decrease. It is interesting to note that, although the genes expression patterns were similar between VIC + LPS group and LPS group, the genes levels in co-stimulated cells were significantly higher compared to sole LPS stimulated cells (Fig. 2A). Infection with H9N2 virus alone (VIC) significantly upregulated pro-inflammatory genes expression, including the pro-inflammatory cytokines IL-1β and IFN-α as well as the inflammatory

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