



Gene expression changes in chicken NLRC5 signal pathway associated with *in vitro* avian leukosis virus subgroup J infection

L.L. Qiu¹, L. Xu¹, X.M. Guo¹, Z.T. Li¹, F. Wan¹, X.P. Liu², G.H. Chen¹ and G.B. Chang¹

¹College of Animal Science and Technology, Yangzhou University, Yangzhou, Jiangsu, China

²Poultry Institute, Chinese Academy of Agricultural Science, Yangzhou, Jiangsu, China

Corresponding author: G.B. Chang

E-mail: passioncgb@163.com

Genet. Mol. Res. 15 (1): gmr.15017640

Received September 15, 2015

Accepted December 29, 2015

Published March 18, 2016

DOI <http://dx.doi.org/10.4238/gmr.15017640>

ABSTRACT. Nucleotide-binding oligomerization domain-like receptors (NLRs) play a key role in the innate immune response as pattern-recognition receptors. However, the role of NLRC5, which is a member of the NLR family, in NF- κ B activation and MHC-I expression remains debatable. Infection with the J group avian leukosis virus (ALV-J) can result in immunosuppression and a subsequent increase in susceptibility to secondary infection. This results in huge economic losses to the poultry industry worldwide. Using quantitative real-time polymerase chain reaction (qRT-PCR), we investigated the mRNA expression levels of NLRC5 signal pathway-related genes in secondary chicken embryo fibroblasts 7 days after infection with ALV-J. The results indicated that, compared with the control groups, the expression levels of TLR7, MHC-I, and IL-18 increased significantly in the infected groups at 7 days post-infection (d.p.i.). The expression levels of NLRC5 and IL-6 were conspicuously downregulated at 7 d.p.i., but the expression levels of NF- κ B, STAT1, and STAT3 were not significantly altered. These results suggest that NLRC5 and some

genes involved in the NLRC5 pathway play a key role in antiviral immunity, typically the response to ALV-*J* infection. Moreover, MHC-I expression levels vary between different cell types.

Key words: Avian leukosis virus; Chicken embryo fibroblast; *NLRC5*; Signal pathway; Real-time PCR

INTRODUCTION

Avian leukosis, which is caused by avian leukosis viruses (ALVs), reduces growth rate and egg production in chickens (Gavora et al., 1980; Stedman and Brown, 1999). This results in significant economic losses in the broiler industry worldwide. ALVs are retroviruses that induce malignant neoplasms in poultry; those that infect chickens can be divided into seven subgroups according to their envelope antigens. ALV subgroup J (ALV-*J*) is linked to an increased incidence of tumor formation, immunosuppression, and ensuing high mortality rates (Wang et al., 2011). Despite current purification strategies, ALV-*J* remains a serious problem in the poultry industry because there is no effective means of dealing with it.

Nucleotide-binding oligomerization domain-like receptors (NLRs) are intracellular receptors that can respond to pathogen attacks or cellular stress. Emerging evidence suggests that the NLR family members also play a crucial role in antiviral responses (Lamkanfi and Kanneganti, 2012; Zhao and Shao, 2012). NLRC5, the largest member of the NLR protein family, has been identified as a critical regulator of innate and adaptive immune responses (Yao and Qian, 2013). As with other NLRs, NLRC5 contains three structural domains: the N-terminal atypical caspase activation and recruitment domain; the centrally located NACHT domain; and 27 leucine-rich repeats at the C-terminal (Lian et al., 2012). Recently, NLRC5 has been shown to play a role not only in the regulation of the inflammasome signaling pathway, but also in the NF- κ B pathway, and antiviral innate immune responses (Cui et al., 2010; Kuenzel et al., 2010; Neerincx et al., 2010; Allen et al., 2011). Although NLRC5 plays a key role in antiviral responses, its role in innate immunity remains controversial and unresolved; recent research has offered discrepant functions for NLRC5 in innate and adaptive immunity (Cui et al., 2010; Kumar et al., 2011), which require further studies to resolve.

In the current study, we compared the expression of genes that related to the NLRC5 signal pathway in ALV-*J*-infected and control groups, and investigated gene expression changes in the NLRC5 signal pathway, with the aim of providing a new basis for the better understanding of NLRC5 function in the chicken immune response.

MATERIAL AND METHODS

Infection of chicken embryo fibroblasts (CEFs) with ALV-*J* virus

Primary cultures of CEFs derived from 10-day-old specific-pathogen-free chicken embryos (Jinan Sais Poultry Co., Ltd., Shandong Province, China) were deposited in 25-cm² cell-culture flasks. After several days, the cells were passaged and seeded at a concentration of approximately 10⁶ cells/well on 6-well culture plates. Throughout derivation and culture, the cells were grown in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) in a humidified incubator at 37°C and 5% CO₂. The cells were then infected with 100 TCID₅₀ (50% tissue culture infective dose) ALV-*J* strain, obtained from Shandong

Agricultural University, China. After 3 h of incubation, the virus was removed and the cells were further incubated in media with 1% FBS. Host-virus interactions were tested in duplicate wells, which were harvested 0, 24, and 168 h post-infection for RNA extraction.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from each sample using the TRIzol method and the concentration and quality of RNA were detected using a NanoDrop 1000 spectrophotometer (Thermo Fisher). cDNA was synthesized using a FastQuant RT Kit (with gDNase) (Tiangen, China) according to the manufacturer instructions, and stored at -20°C until required for real-time PCR testing.

Real-time PCR

According to the sequences of the NLRC5 pathway-related and housekeeping genes published on the GenBank database, primers were designed using the Oligo7.0 software and synthesized by the Shanghai Sangon Biotechnology Company (Table 1).

Table 1. Primers used for real-time polymerase chain reaction (PCR).

Gene symbol	Primer sequences (5'-3')	Accession No.	Amplification (bp)	Annealing temperature (°C)
<i>GP37</i>	F: TGCCTGCGTGGTTATTATTC R: AATGGTGAGGTCGCTGACTGT	NC_015116.1	198	60
<i>TLR7</i>	F: CCTGACCCTGACTATTAACCAT R: CGTAAAGTAGCAGGAAGACCC	NM_001011691	246	60
<i>NF-κB</i>	F: TGAACGGATCCGCACCAATA R: CGTTCACCCACACCTGGAAG	NM_205129.1	107	60
<i>MHC-I</i>	F: ACAAGTACCAGTGCCGCGTG R: CGCGATGTTGTAGCCCTTCC	NM_001097530.1	197	60
<i>IL-6</i>	F: GCTACAGCACAAGCACCTG R: GACTTCAGATTGGCGAGGAG	AJ250838	112	60
<i>IL-18</i>	F: AGGTGAAATCTGGCAGTGAAT R: ACCTGGACGCTGAATGCAA	AJ276026	94	63.5
<i>STAT1</i>	F: CCGATACACATGGCAATGATAA R: TGCATCAAGCTCCTTCTGTTTA	NM_001012914	147	60
<i>STAT3</i>	F: TAGTGCTGCTCCGTATCTGAAG R: CAGGTCAATGGTATTGCTGAAG	NM_001030931.1	73	60
<i>NLRC5</i>	F: TGAGCTACACGTCAGGAAGGA R: GCTCTGCAGAATGGACACAA	JQ044414.1	193	60
<i>GAPDH</i>	F: CGATCTGAACATCATGGTTTAC R: TCTGCCCATTTGATGTTGC	NM_204305	151	60

Real-time PCRs were performed on an ABI 7500 Real-time PCR Detection System (Applied Biosystems, USA) with Ultra SYBR Mixture reagent (with ROX) (CoWin Biotechnology Company, China). The 20-μL reaction volume comprised 10 μL 2X Ultra SYBR Mixture (with ROX), 0.5 μM each primer, and 2 μL cDNA. The following PCR cycling profile was used: one single step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, ending with a melting curve analysis of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s.

Statistical analysis of data

Statistical significance was determined by the *t*-test using the SPSS v13.0 software, and a value of 0.05 was considered significant when compared with the respective controls. The results are represented as $2^{-\Delta\Delta Ct}$.

RESULTS

mRNA expression levels of ALV-J transmembrane protein GP37

The transmembrane protein encoded by the *GP37* gene plays a critical role when virus fusion with the cell membrane occurs. In this study, the *GP37* gene was used for the detection of ALV-J load at 1 and 7 days post-infection by relative PCR quantitative testing. ALV-J load was measured as the ALV-J relative copy number.

The results revealed that at 7 days after ALV-J infection, the relative expression levels of the ALV-J *GP37* gene, which encodes a transmembrane protein, were considerably elevated (Figure 1). Therefore, we investigated the NLRC5-mediated antiviral pathway and inflammatory cytokines in CEF cells infected with avian leukosis virus at 7 days.

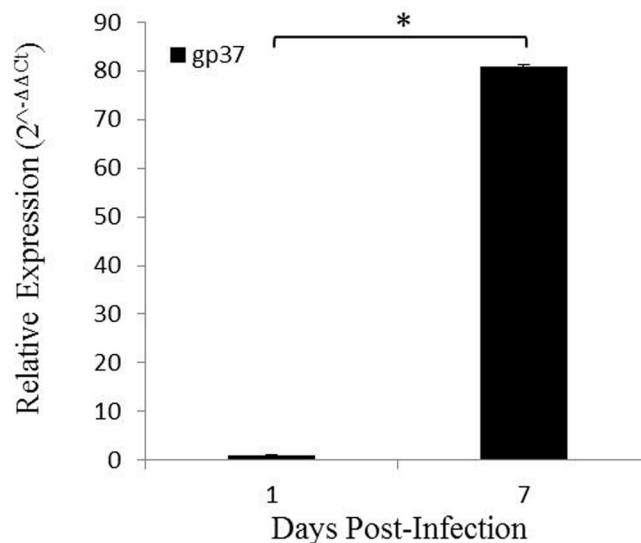


Figure 1. Levels of J group avian leukosis virus (ALV-J) *GP37* mRNA transcription at 1 and 7 days after ALV-J infection (* $P < 0.05$).

Gene expression changes in the NLRC5 signal pathway at 7 days post-ALV-J infection

As can be seen from Figure 2, the expression levels of TLR7, MHC-I, and IL-18 were significantly upregulated in ALV-J-infected secondary CEF cells compared with the negative controls at 7 days post infection (d.p.i.) ($P < 0.05$). The expression levels of NLRC5 and IL-6 showed sharp downregulation ($P < 0.05$), and STAT1 expression decreased in infected groups compared with control groups at 7 d.p.i. However, there were no significant differences in the expression levels of NF- κ B and STAT3 between the virus-treated and control groups at 7 d.p.i. in the secondary CEF cells.

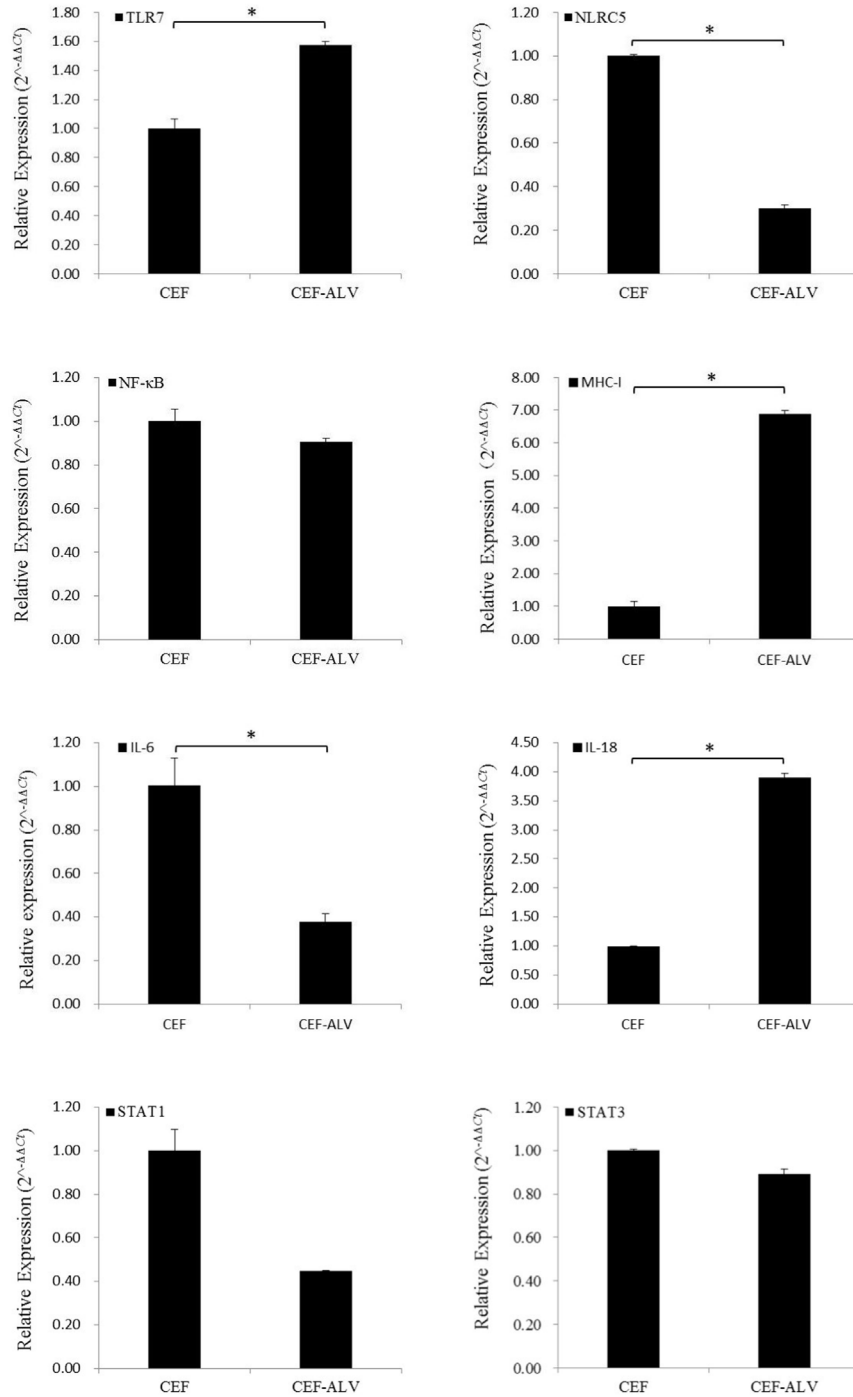


Figure 2. Gene mRNA expression changes (i.e., control group levels/infected group levels) in chicken embryo fibroblast (CEF) cells at 7 days after J group avian leukosis virus (ALV-J) infection (*P < 0.05).

DISCUSSION

Viral RNA is recognized by Toll-like receptors and RIG-I-like receptors; TLR7 recognizes single-strand RNA and induces the innate immune response (Oshiumi et al., 2011). TLR7 transduces the virus infection signal and triggers downstream signaling events, such as the activation of NF- κ B and inflammatory cytokines (Zeng et al., 2014). In this study, two cytokines, IL-6 and IL-18, were chosen for detection and analysis because they are the most important signaling molecules in the immune response. IL-6 is a multifunctional cytokine and is closely involved in oncogenesis owing to its influence on the differentiation of CD4⁺ cells (McGovern et al., 2012; Tippenhauer et al., 2013; Moon et al., 2014). IL-18 is a proinflammatory cytokine, and can be expressed extensively in a wide variety of cells.

In the present study, the significant increases in the expression levels of TLR7, MHC-I, and IL-18 at 7 d.p.i. in CEF cells might be attributable to the rapid replication of ALV-J, which induces antiviral immunity, as shown in Figure 1. In this study, we observed an IL-18 expression pattern that was similar to that reported by Gao et al. (2015) in ALV-J infections *in vivo*. The discrepancies between the two observations might be attributable to the different objects infected by the virus at different time-points. Research has shown that NLRC5 might negatively regulate NF- κ B-dependent responses (Cui et al., 2010). However, in this study, at 7 d.p.i. the expression of NLRC5 was conspicuously downregulated, although there was no significant change in the level of NF- κ B expression. This might depend on the detection time-point, which we may have missed.

MHC-I plays a vital role in the immune defense against viruses and tumors. Two recent reports revealed a potential involvement of NLRC5 in MHC class I gene expression, but arrived at opposing conclusions (Benko et al., 2010; Meissner et al., 2010). Benko et al. (2010) showed that NLRC5 is a negative modulator in RAW264.7, whereas Meissner et al. (2010) found that NLRC5 might enhance MHC-I expression by activating the promoters in HeLa, HEK293T, and Jurkat cells. In this study, the expression levels of MHC-I at 7 d.p.i. increased sharply while NLRC5 expression levels conspicuously decreased; we agree with Benko et al. (2010). In NLRC5-deficient B cells from the spleen and CD11c⁺ dendritic cells, the expression of MHC-I decreased (Biswas et al., 2012). Obviously, MHC-I is involved in antiviral immunity, and we can conclude from this study that the type of host cell influences the outcome.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#31172199 and #31301966) and the Science & Technology Pillar Program of Jiangsu (#BE2013392).

REFERENCES

- Allen IC, Moore CB, Schneider M, Lei Y, et al. (2011). NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF- κ B signaling pathways. *Immunity* 34: 854-865. <http://dx.doi.org/10.1016/j.immuni.2011.03.026>
- Benko S, Magalhaes JG, Philpott DJ and Girardin SE (2010). NLRC5 limits the activation of inflammatory pathways. *J. Immunol.* 185: 1681-1691. <http://dx.doi.org/10.4049/jimmunol.0903900>
- Biswas A, Meissner TB, Kawai T and Kobayashi KS (2012). Cutting edge: impaired MHC class I expression in mice deficient for Nlr5/class I transactivator. *J. Immunol.* 189: 516-520. <http://dx.doi.org/10.4049/jimmunol.1200064>

- Cui J, Zhu L, Xia X, Wang HY, et al. (2010). NLRC5 negatively regulates the NF-kappaB and type I interferon signaling pathways. *Cell* 141: 483-496. <http://dx.doi.org/10.1016/j.cell.2010.03.040>
- Gao Y, Liu Y, Guan X, Li X, et al. (2015). Differential expression of immune-related cytokine genes in response to J group avian leukosis virus infection in vivo. *Mol. Immunol.* 64: 106-111. <http://dx.doi.org/10.1016/j.molimm.2014.11.004>
- Gavora JS, Spencer JL, Gowe RS and Harris DL (1980). Lymphoid leukosis virus infection: effects on production and mortality and consequences in selection for high egg production. *Poult. Sci.* 59: 2165-2178. <http://dx.doi.org/10.3382/ps.0592165>
- Kuenzel S, Till A, Winkler M, Häslner R, et al. (2010). The nucleotide-binding oligomerization domain-like receptor NLRC5 is involved in IFN-dependent antiviral immune responses. *J. Immunol.* 184: 1990-2000. <http://dx.doi.org/10.4049/jimmunol.0900557>
- Kumar H, Pandey S, Zou J, Kumagai Y, et al. (2011). NLRC5 deficiency does not influence cytokine induction by virus and bacteria infections. *J. Immunol.* 186: 994-1000. <http://dx.doi.org/10.4049/jimmunol.1002094>
- Lamkanfi M and Kanneganti TD (2012). Regulation of immune pathways by the NOD-like receptor NLRC5. *Immunobiology* 217: 13-16. <http://dx.doi.org/10.1016/j.imbio.2011.08.011>
- Lian L, Ciraci C, Chang G, Hu J, et al. (2012). NLRC5 knockdown in chicken macrophages alters response to LPS and poly (I:C) stimulation. *BMC Vet. Res.* 8: 23. <http://dx.doi.org/10.1186/1746-6148-8-23>
- McGovern JL, Nguyen DX, Notley CA, Mauri C, et al. (2012). Th17 cells are restrained by Treg cells via the inhibition of interleukin-6 in patients with rheumatoid arthritis responding to anti-tumor necrosis factor antibody therapy. *Arthritis Rheum.* 64: 3129-3138. <http://dx.doi.org/10.1002/art.34565>
- Meissner TB, Li A, Biswas A, Lee KH, et al. (2010). NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci. USA* 107: 13794-13799. <http://dx.doi.org/10.1073/pnas.1008684107>
- Moon YM, Lee J, Lee SY, Her YM, et al. (2014). Gene associated with retinoid-interferon-induced mortality 19 attenuates murine autoimmune arthritis by regulation of th17 and treg cells. *Arthritis Rheumatol.* 66: 569-578. <http://dx.doi.org/10.1002/art.38267>
- Neerincx A, Lautz K, Menning M, Kremmer E, et al. (2010). A role for the human nucleotide-binding domain, leucine-rich repeat-containing family member NLRC5 in antiviral responses. *J. Biol. Chem.* 285: 26223-26232. <http://dx.doi.org/10.1074/jbc.M110.109736>
- Oshiumi H, Matsumoto M and Seya T (2011). [Innate immune response to RNA virus infection]. *Uirusu* 61: 153-161. <http://dx.doi.org/10.2222/jsv.61.153>
- Stedman NL and Brown TP (1999). Body weight suppression in broilers naturally infected with avian leukosis virus subgroup J. *Avian Dis.* 43: 604-610. <http://dx.doi.org/10.2307/1592664>
- Tippenhauer M, Heller DE, Weigend S and Rautenschlein S (2013). The host genotype influences infectious bursal disease virus pathogenesis in chickens by modulation of T cells responses and cytokine gene expression. *Dev. Comp. Immunol.* 40: 1-10. <http://dx.doi.org/10.1016/j.dci.2012.10.013>
- Wang F, Wang X, Chen H, Liu J, et al. (2011). The critical time of avian leukosis virus subgroup J-mediated immunosuppression during early stage infection in specific pathogen-free chickens. *J. Vet. Sci.* 12: 235-241. <http://dx.doi.org/10.4142/jvs.2011.12.3.235>
- Yao Y and Qian Y (2013). Expression regulation and function of NLRC5. *Protein Cell* 4: 168-175. <http://dx.doi.org/10.1007/s13238-012-2109-3>
- Zeng S, Feng Z and Liu Y and Yang Z (2014). The induction of TLRs-mediated antiviral signaling pathway and inflammatory cytokine in chicken embryo fibroblasts infected with Newcastle disease virus. *J. Sichuan Agric. Univ* 32: 436-441.
- Zhao Y and Shao F (2012). NLRC5: a NOD-like receptor protein with many faces in immune regulation. *Cell Res.* 22: 1099-1101. <http://dx.doi.org/10.1038/cr.2012.83>