dPRLR Cause the Differences in Immune Responses Between Early and Late Feathered Chickens After ALV-J Infection

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Research

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Abstract

Background: The late feathering (LF) chickens are more susceptible to avian leukosis virus subgroup J (ALV-J) infection than are early feathering (EF) chickens. To understand the differences in immune responses between EF and LF chickens after infection with ALV-J, we monitored the levels of prolactin (PRL) and growth hormone (GH), and immunoglobulin IgG and IgM in serum in LF and EF chickens for 8 weeks. Six birds with positive or negative ALV-J infection from EF or LF, respectively. Moreover, we analyzed the expression of immune-related genes in the spleen and the expression of prolactin receptor (*PRLR*), sperm flagellar 2 (*SPEF2*), and parts of *PRLR* (*dPRLR*) in the spleen, thymus, bone marrow, cecal tonsils, and DF-1 cells by qRT-PCR.

Results: The results showed that ALV-J infection affected the expression of prolactin, growth hormone, IgG and IgM in serum. No matter LF and EF chickens were infected with ALV-J or not, the levels of the two hormones and the two immunoglobulins in the serum of EF chickens were higher than those of LF chickens ($P < 0.05$). However, the expression of immune-related genes in the spleen of LF positive chickens was higher than that of EF positive chickens. In the four immune organs, the *PRLR* and *SPEF2* expression of LF chickens was also higher than that of EF chickens. Furthermore, the *dPRLR* expression of LF positive chickens was higher than that of LF negative chickens. After being infected with ALV-J, the expression of *PRLR* in DF-1 cells was significantly increased. Besides, overexpression of *PRLR* or *dPRLR* in DF-1 cells promoted the replication of the ALV-J virus.

Conclusion: Overall, the levels of PRL, GH, IgG and IgM in the serum along with the expression of some immune-related genes in the spleen were all different in LF and EF chickens after being infected with ALV-J. These findings revealed that the susceptibility of LF chickens to ALV-J might be induced by *dPRLR*.

Background

Avian leukosis virus (ALV) is an oncogenic retrovirus that causes immunosuppression and neoplastic diseases [1]. According to the host range, the interference of viral envelopes and patterns of cross-neutralization, ALVs can be divided into 10 subgroups (from A to J) [2]. The ALV-J was first detected in the late 1980s and subsequently spread globally [3, 4]. To date, ALV-J has been found to infect many egg-type stocks as well as the local Chinese breeds, resulting in significant economic losses in the poultry industry [4–7].

Sex identification of chicks can be based on the difference in rates of feather growth, which can be divided into an early feathering (EF) type and a late feathering (LF) type. Both EF and LF contain prolactin receptor (*PRLR*) and sperm flagellar 2 (*SPEF2*) genes in their genetic structures. However, LF chickens have one more fusion gene, *dSPEF2/dPRLR*, and endogenous retroviruses 21 (*ev21*), compared with EF chickens [8–10]. Breeders use different feather growth rates for breeding, and found that EF and LF chickens are responded differently to ALV-J infection. Some White-Leghorn breeders have reported reduced production and slightly higher mortality in female progeny from dams carrying the *ev21* gene

[11]. Compared to EF chicken lines, breeders have difficulty in eradicating ALV from most pure LF lines [12]. The existence of *ev21* increases susceptibility of chickens to ALV infection, and affects production performance and tumorigenesis [13–15].

However, a recent study has revealed that some LF chickens lack the *ev21* gene and some EF chickens harbor the *ev21* gene [16]. Furthermore, *dPRLR* encodes a new prolactin (PRL) functional receptor, which is widely expressed in all chicken tissues, and the pattern of spatiotemporal expression is likely to that of the original *PRLR* gene. The important thing is that PRLR and dPRLR were shown to be functionally coupled to the intracellular JAK/STAT signaling pathway in vitro [17]. As we know, PRL functions by firstly binding to its receptors and activating the JAK/STAT signaling pathway [18]. Growth hormone (GH) and PRL have similar structures and functions. Their receptors and signal transduction pathways are fundamentally the same [19]. After being infected with ALV-J, it is not clear whether the levels of the two hormones of PRL and GH and the two immunoglobulin of IgG and IgM in the serum of LF and EF chickens, as well as the expression of some immune-related genes in the spleen are different. On the other hand, both *PRLR* and *dPRLR* genes are present in LF chickens. Whether these two genes have any effect on the immune responses of LF chickens infected with ALV-J remains unknown.

To understand the difference of immune responses between EF and LF chickens after being infected with ALV-J, we detected the levels of PRL, GH, IgG and IgM in the serum of positive and negative individuals of these two types of chicken for eight consecutive weeks. Furthermore, we analyzed the expression of some immune-related genes in the spleen to illustrate the different immune responses of EF and LF chickens after infection with ALV-J.

Materials And Methods

Cells and Antibodies

Chicken embryo fibroblast cell lines, DF-1 cells, were obtained from ATCC (Manassas, USA) and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1% penicillin/streptomycin at 37 °C in an atmosphere of 5% CO₂. The ALV-J envelope protein that is a specific mouse anti-monoclonal antibody, JE9, was kindly provided by Prof. Aijian Qin, Yangzhou University. Goat anti-mouse IgG labeled with FITC was purchased from Bioss (China), while the ALV antigen-capture enzyme-linked immunosorbent assay kit was purchased from IDEXX (USA).

Animals

The 140 d LF and EF yellow chickens were sourced from Chinese chicken farms in Guangdong Province, China. Virus isolation and identification were performed in DF-1 cells as we described previously [20]. The virus identification primer sequences are listed in Table S1. The virus identification and ALV-J viremia results are shown in Fig. 1S and Table S2.

We used molecular identification methods to identify the feathering genotypes of the samples. Using primers designed by Tixier-Boichard *et al.* [21], we amplified the *ev21* gene in our DNA samples. The expected PCR product of EF chickens is only a 515 bp band, while for LF chickens it is two bands of 390 and 515 bp. We designed a pair of primers for *dSPEF2/dPRLR* gene amplification. A 1434 bp target fragment should be obtained in LF chickens but not in EF chickens. The primers are listed in Table S1. The feathering genotype detection results of LF and EF chickens are shown in Fig. 2S.

Based on the results of virus isolation and identification and the feathering genotype detection, 6 ALV-J positive LF chickens, 6 ALV-J negative LF chickens, 6 ALV-J positive EF chickens and 6 ALV-J negative EF chickens were selected. All animals were from the same farm. These selected chickens were raised separately, with a consistent feeding protocol between all individuals.

Sample collection

Once each week for eight weeks, we aseptically collected 2 mL of anticoagulated blood from each individual. The plasma was then separated by centrifugation at 2000 rpm at 4°C for 15 min and stored at -80°C. All the samples were collected at the same time: 10:00 am every Monday of each week. Eight weeks later, the spleen, bone marrow, thymus and cecal tonsil of each chickens were collected and stored at -80°C. The plasma samples were analyzed with a p27 test for each collection, and the cells supernatant p27 test results were shown in Table S2.

Determining of the levels of PRL, GH, IgG and IgM in the serum by ELISA

The levels of the PRL in the serum was measured using enzyme-linked immunoassay (ELISA) kits for PRL (CLOUD-CLONE, Wuhan, China), following the manufacture's protocol. The levels of GH, IgG and IgM were detected in the serum by specific ELISA kits purchased from Shanghai Enzyme-Linked Biotechnology Co. Ltd (Shanghai, China), following the manufacture's protocol.

Cell's infection and overexpression

The infection of DF-1 cell with ALV-J was carried out as our previously described [22]. After incubation for 24 h and 48 h in culture, we collected cells and extracted RNA, and then measured the *PRLR* and *SPEF2* expression by qRT-PCR. The laboratory ALV-J strain SCAU-HN06 was kindly provided by Prof. Weisheng Cao (South China Agricultural University, Guangzhou, China).

According to the *PRLR* sequence (NM_204854.1) on the NCBI database, Wuhan Genecreate Industrial Co., Ltd was commissioned to construct the pcDNA3.1-*PRLR* and the pcDNA3.1-*dPRLR* plasmid. Then we followed the method described by Li *et al.*[22]. for cells transfection and cells infection. After incubation for 24 h and 48 h in culture, we collected cells and extracted RNA, and then measured the ALV-J virus expression by quantitative real-time polymerase chain reactions (qRT-PCR).

RNA isolation and cDNA synthesis

Total RNA was extracted from tissues with RNAiso reagent (Takara, Japan) according to the manufacturer's protocol. The integrity and quantity of RNA were assessed using 1% agarose gel electrophoresis and spectrophotometry (ND-2000, USA), respectively. cDNA was synthesized using MonAmp™ RTIII All-in-One Mix (Monad Co., LTD Guangzhou, China), following the manufacturer's protocol. The synthesized cDNA was stored at -20°C until subsequent analysis using qRT-PCR.

Quantitative real-time PCR

Immune-related differentially expressed genes, *TLR4*, *TLR7*, *MDA5*, *SOCS3*, *VIP*, *IL-10*, *IRF1*, *NFκB*, *TNFα* and *IL-1β*, were selected, and the expression of each gene was detected in the spleen of the LF and EF chickens. The MonAmp™ SYBR® Green qPCR Mix (Monad Co., LTD Guangzhou, China) was used for qRT-PCR in an ABI 7500 Real-Time Detection instrument (Applied Biosystems, USA) according to the manufacturer's protocol. Relative gene expression was measured by applications of qRT-PCR for each sample and the nuclear gene GAPDH was used as a control. The primers used in the qRT-PCR are shown in Table S1.

Western Blotting Assay

Western Blotting (WB) Assay were performed as previously described [23]. The antibodies and their dilutions used for WB were as follows: anti-ALV-J envelope protein specific monoclonal antibody JE9 (kindly provided by Prof. Aijian Qin, Yangzhou University, 1:1000). Rabbit Anti-beta-Actin antibody (Boss, China; 1:500), Goat Anti-Rabbit IgG H&L/HRP antibody (Boss, China; 1:500).

Statistical analyses

Statistical comparisons were performed using GraphPad Prism 5 (GraphPad Software Inc., USA). The data were presented as means ± one standard error of the mean (SEM). The statistical analyses were performed using one-factor analysis of variance, and statistical significance was represented by *P*-values. *P* < 0.05 was considered statistically significant, and *P*-value bands of statistical significance were denoted as: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Results

The PRL and GH levels in the serum of LF chickens are lower than those of EF chickens, regardless of being infected or not.

Comparing LF negative with EF negative chickens, the PRL levels of EF chickens, were higher than those of LF chickens except for the fifth, seventh and eighth weeks (Fig. 1A). However, the PRL levels of EF positive chickens were higher than those of LF positive chickens in the third, the second and eighth had no difference, and were lower than those of LF positive chickens in other times (Fig. 1B). PRL and GH are similar in structure and function, and their receptors and signal transduction pathways are basically the same. Therefore, we also detected the serum GH content in the serum of LF and EF chickens. Intriguingly, regardless of ALV-J infection, the GH levels in the serum of LF chickens was always lower than those of

EF chickens (Fig. 1C, D). One explanation of these results is that PRL can bind to both PRLR and DPLR in LF chickens, which may lead to the lower PRL levels in LF chickens compared to EF chickens.

The IgG and IgM levels in the serum of LF chickens are always lower than those of EF chickens

Immunoglobulin refers to an animal protein with antibody activity. IgM is the first secreted antibody, while IgG is the most abundant antibody in the serum. Immunoglobulins play an important role in the function of the immune system. PRL can promote lymphocyte mitosis in a dose-dependent manner [24] and stimulate the multiplying of chicken splenocytes and thymocytes. It is noteworthy that, regardless of whether chickens were infected with ALV-J or not, the IgG (Fig. 2A, B) and IgM (Fig. 2C, D) levels of LF chickens were always significantly lower than those of EF chickens. This may be caused by the lower PRL levels in LF chickens compared to EF chickens, which may fail to increase the levels of IgM and IgG in the serum.

The expression of most of immune-related genes in the spleens of LF chickens is higher than that of EF chickens

In negative chickens, except for *MDA5*, the expression of other genes in EF chickens was lower than that in LF chickens. However, the differences of the expression of *TLR7*, *IRF1* and *NFκB* between EF and LF chickens were not significant ($P > 0.05$) (Fig. 3A). In positive chickens, except for *IRF1*, the expression of other genes in EF chickens was significantly lower than that in LF chickens (Fig. 3B). This is because that PRL combines with PRLR and dPRLR to stimulate the expression of immune-related genes through the JAK-STAT signaling pathway. The LF chickens showed higher expression of most of the above immune-related genes because of the existing of dPRLR.

The expression of *PRLR*, *SPEF2* and *dPRLR* in immune organs of LF chickens is significantly higher than that of EF chickens

We analyzed the expression of *PRLR*, *SPEF2* and *dPRLR* genes in the four immune organs, the spleen, bone marrow, thymus and cecal tonsil, by qRT-PCR. Results showed that the expression of *PRLR* and *SPEF2* genes in each immune organ was significantly higher in LF chickens than in EF chickens ($P < 0.05$). Compared with the LF chicken, the *PRLR* and *SPEF2* expression in the four immune organs of EF chicken was very low (Fig. 4A, B). In the spleen and thymus, the *PRLR* expression in LF negative individuals was higher than that in LF positive individuals. While in bone marrow and cecal tonsils, the *PRLR* expression in LF positive individuals was higher than that in LF negative individuals (Fig. 4A). The *SPEF2* expression of LF positive individuals was higher than LF negative individuals in the spleen, bone marrow and cecal tonsils, except thymus (Fig. 4B). The *dPRLR* expression in the spleen and bone marrow was higher in LF positive individuals than in LF negative individuals. However, there is no difference of the *dPRLR* expression in thymus and cecal tonsils between EF and LF chickens (Fig. 4C).

After infection with ALV-J, the *PRLR* and *SPEF2* expression in DF-1 cells was shown in Fig. 6. The *PRLR* expression in the infection group was significantly higher than that of the control group at 24 h and 48 h

($P \leq 0.05$) (Fig. 4D). The *SPEF2* expression in infection group was significantly higher than that in the control group at 24 h ($P \leq 0.01$) (Fig. 4E).

Overexpression of *PRLR* or *dPRLR* promotes ALV-J virus replication

Next, we overexpressed the *PRLR* or *dPRLR* gene in vitro to investigate the role of the *PRLR* or *dPRLR* gene on ALV-J replication in chickens. Results showed that overexpression of *PRLR* (Fig. 5A, B) or *dPRLR* (Fig. 5C, D) promoted the expression of ALV-J *gp85* gene at 24 h and 48 h, indicating that *PRLR* and *dPRLR* significantly promoted ALV-J virus replication in chickens.

Discussion

Ev21 has been used as a molecular marker for LF detection in chickens. It is believed that the harboring of *ev21* is the cause of LF chickens' susceptibility to ALV-J [13, 25, 26]. From the 1980s on, some researchers have reported reduced performance and slightly higher mortality in female progeny of dams carrying the *ev21* gene [11, 12]. Harris *et al.* [13] reported that decreased egg productivity is associated with an increased incidence of ALV-J. Fadly and Smith [27] thought that the status of infection with *ev21* may affect ALV-J infection and tumor development. Williams *et al.* [28] showed that harboring *ev21* influences ALV-J viremia and antibody production in some White Leghorn chickens. After inoculation with ALV-J at hatch, 5% of chickens lacking *ev21* are viremia tolerant compared with 54% of chickens harboring *ev21* [28]. The incidence of tumors in chickens harboring *ev21* (13.8%) is significantly higher ($P < 0.01$) than that in chickens lacking *ev21* (2.6%) [29]. All these studies demonstrated that chickens harboring *ev21* are more susceptible to ALV-J infection than are chickens lacking *ev21*.

In this study, we found that the levels of GH, IgG and IgM in LF chickens (harboring *ev21*) were significantly lower than that in EF chickens (lacking *ev21*), regardless of being infected with ALV-J. In LF chickens, the PRL levels in serum were generally lower than those in EF chickens. Results further confirmed the difference of ALV-J infection in LF and EF chickens. However, the expression of most immune-related genes in LF chicken's spleen was higher than that in EF chickens. Therefore, we speculated that the *dPRLR* gene might regulate the immune response of LF chickens.

Some LF chickens lack the *ev21* gene and some EF chickens harbor the *ev21* gene [16]. *dPRLR* is likely to encode a novel functional receptor for PRL and is widely expressed in all chicken tissues, and its spatiotemporal expression pattern is likely to that of the *PRLR* gene [17]. Similar to the *PRLR* gene, the activation of *dPRLR* can significantly increase the levels of STAT5 phosphorylation [17]. In LF chickens, the PRL levels in the serum were generally lower than those in EF chickens, but the expression of most immune-related genes, *TLR4*, *TLR7*, *MDA5*, *SOCS3*, *VIP*, *IL-10*, *IRF1*, *NFkB*, *TNFa* and *IL-1β*, in the spleen were significantly higher than those in EF chickens. PRL and GH are similar in structure and function, and their receptors and signal transduction pathways are basically the same. GH can also regulate the immune response through the JAK/STAT signal pathway. When PRL levels decrease, the body may compensate by increasing GH levels. It was found that PRL can promote lymphocyte mitosis [24, 30].

Thus, the IgG and IgM levels in the serum of EF chickens were observed to be higher than that of LF chickens. This may be because that there is one more *dPRLR* gene in LF chickens than in EF chickens.

We found that the expression of *PRLR* and *SPEF2* in the four immune organs in LF chickens was significantly higher than that in EF chickens. The expression of *dPRLR* in the spleen and bone marrow in LF positive individuals was higher than that in LF negative individuals, and the expression of *PRLR* in DF-1 cells was significantly increased after being infected with ALV-J. Likely to the *PRLR*, the activation of *dPRLR* significantly increases the levels of STAT5 phosphorylation [17]. The JAK/STAT signaling pathway is a signal transduction pathway stimulated by a variety of cytokines, and is involved in multiple immune signaling pathways [31, 32]. These results indicated that *PRLR* and *dPRLR* play an important role in ALV-J infection. Okamura *et al.* [33] proved that the transcription of *dSPEF2* gene possibly represses the expression of *dPRLR* mRNA and alters alternative splicing bias in the 5' UTR of *PRL* receptor mRNAs to increase translation efficiency. The immune functions of *SPEF2* and *dSPEF2* genes are still unclear.

Receptors for growth factors, cytokines or hormones are clearly potential targets as viral receptors in that they are already adapted to bind to specific circulating ligands, and subsequently promote viral entry and survival by receptor-mediated endocytosis [34]. Receptors known to be used by viruses include insulin-like growth factor 1 receptor (IGF1R), platelet derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) [35, 36]. Recently, Griffiths *et al.* [37] found that IGF1R is an entry receptor for the respiratory syncytial virus (RSV). RSV glycoprotein F binds to the IGF1R, triggering activation of protein kinase C ζ , which recruits nucleolin from the nuclei of cells to the cell surface where it probably facilitates entry of RSV into the cell [37]. To infect a host cell, a virus has first to bind to receptors on the host cell surface. *PRLR* and *dPRLR* clearly have potential as viral receptors. It is widely distributed in many tissues. Our present results demonstrated that overexpression of *PRLR* or *dPRLR* promoted the expression of ALV-J *gp85* gene. Therefore, we speculated that *PRLR* and *dPRLR* are the receptors of ALV-J virus. The presence of the *dPRLR* gene might be the reason why LF chickens are susceptible to ALV-J.

Conclusions

In conclusion, after infection with ALV-J, the levels of PRL, GH, IgG and IgM in the serum along with the expression of some immune-related genes in the spleen were all different in LF and EF chickens. The reason why LF chickens are susceptible to ALV-J is probably due to the presence of the *dPRLR* gene.

Abbreviations

ALV-J: avian leukosis virus subgroup J; PRL: prolactin; LF: late feathering chickens; EF: early feathering chickens; GH: growth hormone; *PRLR*: prolactin receptor; *SPEF2*: sperm flagellar 2; *dPRLR*: parts of *PRLR*; *ev21*: endogenous retroviruses 21; JE9: anti-ALV-J envelope protein specific monoclonal antibody; qRT-PCR: quantitative real-time polymerase chain reactions.

Declarations

Ethical statement

All animal experiments in this study were conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of South China Agriculture University (No: SCAU 2018c008) and in accordance with the Animal Protection Law of the People's Republic of China.

Authors' contributions

G.M. designed the study, drafted the paper, carried out experiments, and analyzed data. B.H. helped by providing useful discussion and language correction and helped with performing some of the experiments. Q.Z., Z.R., W.L., J.L., Y.S., Z.M., and Z.Z. helped with performing some of the experiments. Z.W. and M.S. helped with valuable suggestions and comments. X.Z. participated in the design, manuscript writing, and final approval of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are publicly available.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have approved the final manuscript.

Competing interests

The authors declare no conflict of interest

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Figures

Figure 1

The PRL and GH levels in the plasma of sampled chickens. (A) PRL levels in the serum of LF negative and EF negative chickens. (B) PRL levels in the serum of LF positive and EF positive chickens. (C) GH levels in the serum of LF negative and EF negative chickens. (D) GH levels in the serum of LF positive and EF positive chickens. LF positive, late feathering chickens infected with ALV-J; LF negative, late feathering chickens uninfected with ALV-J; EF positive, early feathering chickens infected with ALV-J; EF negative, early feathering chickens uninfected with ALV-J; n, number of samples. The error bars represent one standard error of the mean (SEM) (*P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001).

Figure 2

The IgG and IgM levels in the plasma of sampled chickens. (A) IgG levels in the serum of LF negative and EF negative chickens. (B) IgG levels in the serum of LF positive and EF positive chickens. (C) IgM levels in the serum of LF negative and EF negative chickens. (D) IgM levels in the serum of LF positive and EF positive chickens.

Figure 3

The expression of immune-related genes in the spleen. (A) LF negative and EF negative chickens. (B) LF positive and EF positive chickens.

Figure 4

Expression of PRLR, SPEF2 and dPRLR genes in the four immune organs and DF-1 cells by qRT-PCR. (A) The expression of PRLR in the four immune organs of LF and EF chickens. (B) The expression of SPEF2in the four immune organs of LF and EF chickens. (C) The expression of dPRLR in the four

immune organs of LF chickens. (D) The expression of PRLR in DF-1 cells after being infected with ALV-J. (E) The expression of SPEF2 in DF-1 cells after being infected with ALV-J.

Figure 5

overexpression of PRLR or dPRLR promoted ALV-J virus replication. The expression of ALV-J gp85 gene in DF-1 cells after being infected with ALV-J by qRT-PCR (A) and WB (B) under overexpression of PRLR. The expression of ALV-J gp85 gene in DF-1 cells after being infected with ALV-J by qRT-PCR (C) and WB (D) under overexpression of dPRLR.

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