

Identification, cloning, and characterization of Cherry Valley duck CD4 and its antiviral immune responses

Tianxu Li,^{*,1} Rong Li,^{*,†,1} Tingting Zhang,[‡] Huihui Zhang,^{*} Xingdong Song,^{*} Xinyu Zhai,^{*} Jinchao Wang,^{*} Bin Xing,^{*} Xiaolan Hou,^{*} and Liangmeng Wei^{*,‡,2}

**Sino-German Cooperative Research Centre for Zoonosis of Animal Origin of Shandong Province, Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Shandong Provincial Engineering Technology Research Center of Animal Disease Control and Prevention, College of Animal Science and Veterinary Medicine, Shandong Agricultural University, 61 Daizong Street, Tai'an City, Shandong Province 271018, China; †College of Marine Science and Engineering, Qingdao Agricultural University, Qingdao, Shandong Province 266109, China; and ‡Collaborative Innovation Center for the Origin and Control of Emerging Infectious Diseases, College of Basic Medical Sciences, Shandong First Medical University, Tai'an City, Shandong Province 271000, China*

ABSTRACT CD4 protein is a single chain transmembrane glycoprotein and has a broad functionality beyond cell-mediated immunity. In this study, we cloned the full-length coding sequence (CDS) of duck CD4 (duCD4) and analyzed its sequence and structure, and expression levels in several tissues. It consists of 1,449 nucleotides and encodes a 482 amino acid protein. The putative protein of duCD4 consisted of an N-terminal signal peptide, three immunoglobulins and one immunoglobulins-like domain in its central, one terminal transmembrane region, and a C-terminal domain of the CD4 T cell receptor. The duCD4 also has the typical signature "CXC" of CD4s. The multiple sequence alignment

suggests duCD4 has four potential N-glycosylation sites and the phylogenetic analysis suggests duCD4 shares greater similarity with avian than other vertebrates. Quantitative real-time PCR analysis showed that duCD4 mRNA transcripts are widely distributed in the healthy Cherry Valley duck, and the highest level in the thymus. During the virus infection, the obvious change of duCD4 expression was observed in the spleen, lung and brain, which suggesting that duCD4 could be involved in the host's immune response to multiple types of viruses. Our research studied the characterization, tissue distribution, and antiviral immune responses of duCD4.

Key words: Cherry Valley duck, CD4, cloning, tissue distribution, virus infection

2021 Poultry Science 100:101262

<https://doi.org/10.1016/j.psj.2021.101262>

INTRODUCTION

The interaction of T lymphocyte with the antigen presenting cells is the cornerstone of adaptive immunity. T cells that live in the peripheral blood or secondary lymphoid organs were distinguished by the mutually exclusive expression of either CD4 or CD8 (Benoist and Mathis, 1999). According to the molecules expressed on the cell surface, the alpha/beta T lymphocytes were further divided into a CD4⁺ T helper population and the

CD8⁺ cytotoxic T cell population (Konig et al., 1992; Germain, 2002). Expression of CD4 and CD8 is very important for cell-mediated immune defense as well as T-cell development in the thymus (Cammarota et al., 1992; Ma, et al., 2013). CD4 is a single chain transmembrane glycoprotein which belongs to the immunoglobulin superfamily (Maddon, et al., 1985; Parnes, 1989). It assists T cell receptors in communicating with antigen presenting cells through an interaction with the major histocompatibility complex class II molecules a2 and b2 domains (Doyle and Strominger, 1987; Cammarota, et al., 1992; Konig et al., 1995). This interaction activates the signal of T cell which was transduced by the protein tyrosine kinase p56^{lck}, and this protein could binds to the cytoplasmic domain of CD4 with a conserved site of Ick motif (Veillette et al., 1989; Glaichenhaus et al., 1991). Study has pointed out that the CD4 could be integrated into the membrane and alters the membrane's stiffness (Bui and Nguyen, 2016). In mammals, the CD4⁺ T cell is crucial in

© 2021 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received February 8, 2021.

Accepted May 12, 2021.

¹Tianxu Li and Rong Li equally contributed to this paper.

²Corresponding author: lmwei@sdau.edu.cn

response and recognition of pathogens, and plays an important role in the immune system.

Compared with the information available about the chicken, the known about the immune system of other waterfowl is little. However, the duck as the natural reservoir for influenza (Wei et al., 2014) and other avian viruses such as duck Tembusu virus (DTMUV) (Cao et al., 2011), novel duck reovirus (NDRV) (Li et al., 2016c), and duck plague virus (DPV) (Lian et al., 2011; Huang et al., 2014) has the largest waterfowl market in China. Virus infection could induce the expression of antigen-specific CD4⁺ T cells and these virus-specific CD4⁺ T cells play a crucial role in controlling viral infections (Whitmire, 2011; Phares et al., 2012). Previous study also suggested that even the uninfected CD4⁺ T cells also have a broader and more profound depletion via multiple indirect effects of infection (Matrajt et al., 2014). However, to date, little is known about the sequence and immune biological activity of T cell surface proteins CD4 in duck.

Here, we describe the molecular cloning of the CD4 cDNA from the Cherry Valley duck. The model demonstrates that despite the low sequence identity in duck and mammalian CD4, several similar structural features have been conserved. Furthermore, the amino acid (AA) sequence, the structural and phylogenetic analysis of duCD4, and tissue distribution in adult Cherry Valley duck was studied. Moreover, the immunological function of duCD4 during DTMUV, novel duck reovirus NDRV, and duck plague virus DPV infection was identified. This study will expand our knowledge of immune response of the waterfowl, and the results of this study will reveal the role of duCD4 during the viral infection.

MATERIALS AND METHODS

Animals and Virus Strains

Healthy 1 d of age Cherry Valley ducks were purchased from a duck farm (Tai'an, Shandong, China) and housed in isolators (did not receive any vaccinated) until at three weeks old. Serum samples from 1 d of age ducks were tested by ELISA to verify that all ducks were serologically negative for DTMUV. Both NDRV and DPV were negative by quantitative real-time PCR (qRT-PCR).

DTMUV, NDRV, and DPV-GM viral strains used in this study were all isolated from infected ducks from farms, and virus stocks were propagated in duck embryo fibroblasts (Yan et al., 2011; Li et al., 2016b, 2018). Viral titers were determined as the median tissue culture infective dose (TCID₅₀)/mL by infection with duck embryo fibroblasts and the calculation method was used by the Reed and Muench (Reed and Muench, 1938).

Cloning and Bioinformatic Analysis of duCD4

Total RNA from duck spleen was extracted using FastPureCell/Tissue total RNA isolation kit (RC112,

Table 1. Primer sequences used in this study.

Primer name	Nucleotide sequence (5'-3')	Purpose
duCD4-F	ATGGAGCTGTGTGGGTACAGC	Gene cloning
duCD4-R	CTACTTATTCATCCGGTGCT	
q-duCD4-F	ATGGAGAGCTGCGTGAAACT	qRT-PCR
q-duCD4-R	TGCTTCAGCTGGATTTTGTG	
q-β-actin-F	GGTATCGGCAGCAGTCTTA	qRT-PCR
q-β-actin-R	TTCACAGAGGCGAGTAACTT	

Abbreviations: F, forward primer; R, reverse primer; q, qRT-PCR.

Vazyme, Nan'jing, China) and reverse transcribed to cDNA using HiScript II One-Step RT-PCR kit (R223-01, Vazyme). The duCD4 gene was amplified by PCR from cDNA using Rapid Taq Master Mix (P222, Vazyme), primers (duCD4-F and duCD4-R) used to amplify the gene are listed in Table 1. The PCR products were sent to the Tsingke Biotechnology Co., Ltd. (Qing'dao, China) for DNA sequencing. Open reading frame and AA sequence of duCD4 cDNA were conducted by use of DNASTar (Burland, 2000) and the Sequence Manipulation Suite (<http://www.bioinformatics.org/sms/>). The sequences of animals were retrieved from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>). And the phylogenetic tree was built by using the MEGA-X software and based on the Neighbor-Joining method with bootstrapping over 1,000 replicates (Kumar et al., 2018). The predicted structure of AA sequence was performed using the SMART program (<http://smart.embl-heidelberg.de/>) (Schultz et al., 1998). Alignment of AA sequence was performed using the Clustal X program (Thompson et al., 1997) and edited with Boxshade (https://embnet.vital-it.ch/software/BOX_form.html).

Animal Experiments

In order to evaluate the expression of CD4 in normal tissues, 3 healthy 3-wk-old ducks were euthanized, and tissues were collected including heart, liver, spleen, lung, kidney, brain, cerebellum, brainstem, thymus, bursa of Fabricius, trachea, esophagus, gizzard, proventriculus, skin, muscle, duodenum, jejunum, ileum, and cecum. And to analyze the change in duCD4 mRNA expression level after DTMUV, NDRV, and DPV infection, three-week-old ducks were randomly divided into four groups of fifteen. Groups A, B, and C were intramuscularly injected with DTMUV (0.4 mL 10^{5.2} TCID₅₀/mL per duck) (Li et al., 2015), NDRV (0.5 mL 10^{4.5} TCID₅₀/mL per duck) (Li et al., 2016c) and DPV (0.3 mL 10^{6.5} TCID₅₀/mL per duck) (Li et al., 2016a), respectively. Group D ducks were inoculated with 0.5 mL sterile phosphate buffer solution in the same manner as a negative control group. Three duck per group was used each time at 1-, 3-, and 5-days postinfection (dpi), respectively, and the spleen, brain and lung were collected and stored in liquid nitrogen for total RNA extraction. Relative duCD4 mRNA expression of these samples was studied using qRT-PCR. The rest of ducks were observed for clinical symptoms to 9 dpi and euthanized with an

Table 2. Reference sequences information of CD4.

Species	GeneBank accession numbers
<i>Anser anser</i>	AFG26508.1
<i>Gallus gallus</i>	ABA55042.1
<i>Columba livia</i>	XP_013223720.1
<i>Taeniopygia guttata</i>	XP_012425328.1
<i>Meleagris gallopavo</i>	NP_001290125.1
<i>Homo sapiens</i>	NP_000607.1
<i>Pan troglodytes</i>	NP_001009043.1
<i>Macaca mulatta</i>	BAA09671.1
<i>Aotus nancymaae</i>	AKM95886.1
<i>Saimiri sciureus</i>	BAA13131.1
<i>Oryctolagus cuniculus</i>	NP_001075782.1
<i>Mus musculus</i>	NP_038516.1
<i>Rattus norvegicus</i>	NP_036837.1
<i>Felis catus</i>	NP_001009250.1
<i>Canis lupus familiaris</i>	NP_001003252.1
<i>Mustela putorius furo</i>	ABS50090.1
<i>Bos grunniens</i>	AJP16709.1
<i>Sus scrofa</i>	NP_001001908.2
<i>Macropus eugenii</i>	ABR22561.1
<i>Monodelphis domestica</i>	ABG81321.1
<i>Chelonia mydas</i>	XP_007069709.3
<i>Pelodiscus sinensis</i>	XP_006124249.1
<i>Alligator sinensis</i>	XP_014382635.1
<i>Cyprinus carpio</i>	ABD58988.1
<i>Lateolabrax japonicus</i>	AFK73394.1
<i>Oncorhynchus mykiss</i>	AA42068.1

intravenous injection of sodium pentobarbital (100 mg/kg body weight) (Li et al., 2015). All of the samples were stored at -80°C for total RNA extraction.

QRT-PCR

One set of the specific primers (Table 1) was designed according to the sequence of the predicted *Anas Platyrhynchos* CD4 from the NCBI (XM_013096823.1). In addition, the expressions of duCD4 were quantified using qRT-PCR with the ChamQ SYBR qPCR Master Mix (Q311, Vazyme) and analyzed with the 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA). The specificity of PCR amplification for all primer sets was verified from the dissociation curves. The qRT-PCR consisted of 20 μ L volume and conditions as follows: one cycle of pre-denatured at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s and extension at 60°C for 34 s, then a dissociation curve was analyzed (95°C for 10s, 65°C for 10 s, 97°C for 1 s). Expression levels of the duCD4 and endogenous house-keeping gene β -actin were analyzed using the $2^{-\Delta\Delta C_t}$ and $2^{-\Delta C_t}$ method (Pfaffl, 2001). All qRT-PCR reactions were performed in triplicate.

Statistical Analysis

Data were expressed as mean \pm SD from 3 separate experiments. Significance was determined by one-way ANOVA using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). For all tests, $P < 0.05$ was considered statistically significant, $P < 0.01$ was highly significant, and $P < 0.001$ was extremely significant.

RESULTS

Cloning and Structural Analysis of duCD4

The full-length CDs of duCD4 were amplified using the primers duCD4-F and duCD4-R (Table 1), and it consisted of a single ORF of 1449 bp which encodes 482 AAs. And the sequence has been uploaded to NCBI with the GenBank number of KX588247.1. Multiple alignment analysis revealed that duCD4 had four potential N-glycosylation sites (NISF, NATA, NGTK and NYTV) (Figure 1A). Our results also indicated that the duCD4 contained the conserved Lck motif (CXC) of the CD4 typical signature (Figure 1A). The protein domains of duCD4 were predicted using SMART program, and the results indicated that duCD4 contained 7 characteristic domains: one signal peptide at its N-terminus (AA1-27), 3 immunoglobulins (IG) (AA28-121, 126-214, 221-333) and one IG-like domain (AA338-424) at the central, one transmembrane domain (AA429-451), and one C-terminal domain of the CD4 T cell receptor (AA455-482) (Figure 1B).

Sequence Comparison and Phylogenetic Analysis of duCD4

To confirm the evolutionary relationship of duCD4, the phylogenetic tree with the AA sequence of duCD4 and other CD4s was shown in Figure 2A. The phylogenetic tree indicated that all these CD4s AA sequences could cluster into four major branches as mammal, reptile, bird and fish. And the duCD4 was classified into the avian species, especially closely related to *Anser anser* CD4, but distant from fish CD4 molecules. These results indicated that the duCD4 AA sequence has a closer relationship to the CD4s of birds. To have a further study of CD4 from the Cherry Valley duck, the AA sequence of duCD4 was compared with the other birds, fishes, mammals as well as reptiles. And the multiple sequence alignment analysis showed that duCD4 shared 85.4% identity with *Anser anser* CD4, 62.2% identity with *Gallus gallus* and *Meleagris gallopavo* CD4, 62.1% identity with *Columba livia* CD4, 26.2% identity with *Homo sapiens* CD4, and 25.8% identity with *Mus musculus* CD4 (Figure 2B). The CD4 gene identity was conserved in mammalians, but significantly different between mammalian and duck, with only 26% homology (Figure 2B).

Tissue Distribution of duCD4

To investigate the tissue distribution of duCD4, total RNA was extracted separately from heart, liver, spleen, lung, kidney, brain, cerebellum, brainstem, thymus, bursa of Fabricius, trachea, esophagus, gizzard, proventriculus, skin, muscle, duodenum, jejunum, ileum, and cecum. QRT-PCR was used to analyze the duCD4 mRNA expression level. As showed in Figure 3, duCD4 has a broad expression profile of all the tested tissues, and thymus showed highest expression level followed by

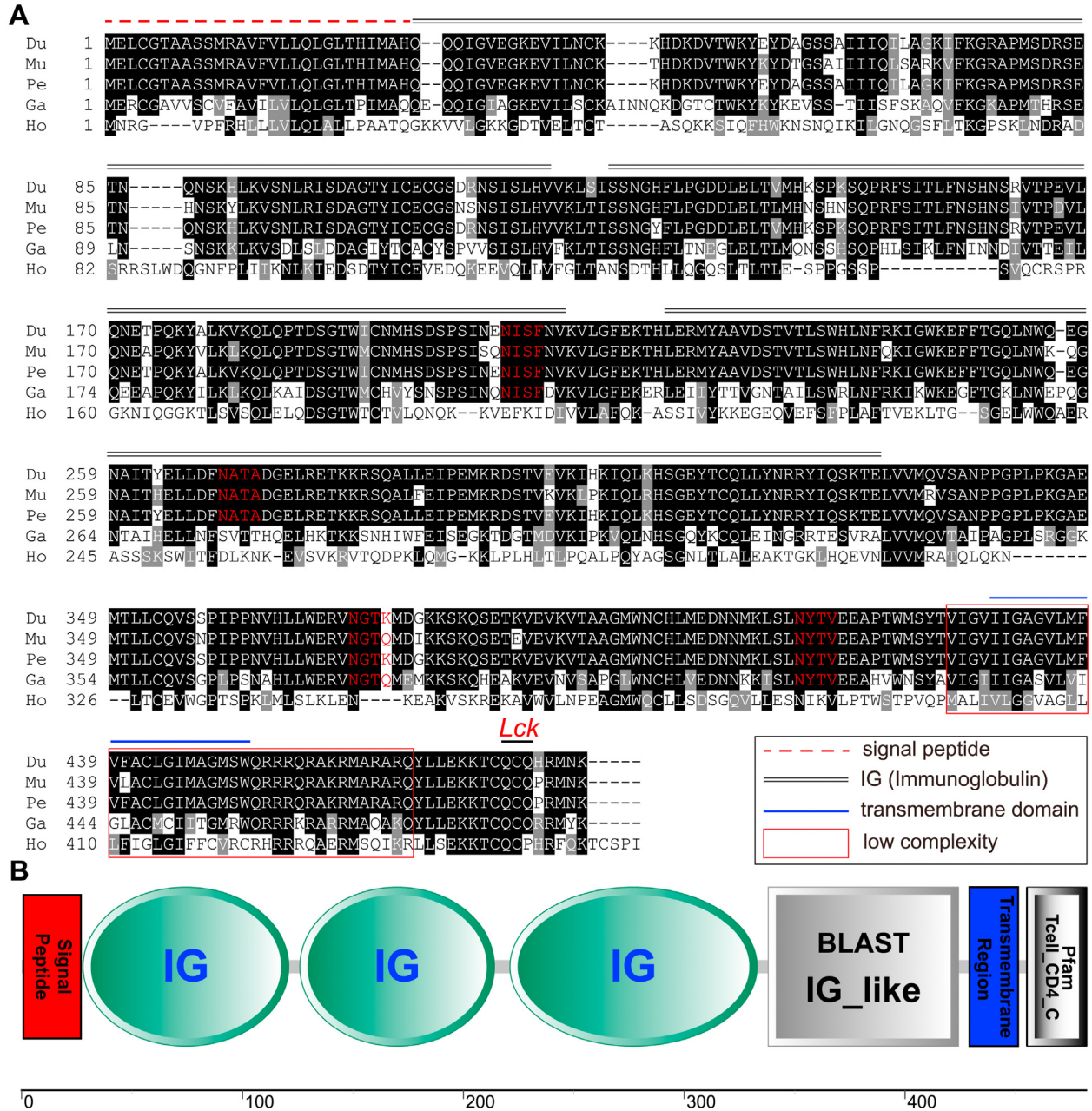


Figure 1. (A) Alignment of the deduced AA sequence of duCD4 with other animals. Black shading indicates AA identity; gray shading indicates similarity (50% threshold). The red font indicated the four potential N-glycosylation sites. Lck represented the conserved CXC motif which may bind the tyrosine protein kinase p56^{lck}. (B) Prediction of duCD4 protein domains by the SMART program. DuCD4 contains one signal peptide at its N-terminus (AA1-27), three IG (AA28-121, 126-214, 221-333) and one IG-like domain (AA338-424) at the central, one transmembrane domain (AA429-451), and one C-terminal domain of the CD4 T cell receptor (AA455-482). Abbreviations: Du, Cherry Valley Duck; Ga, *Gallus gallus*; Ho, *Homo sapiens*; IG, immunoglobulins; Mu, Muscovy ducks (AAW63065.1); Pe, Pekin ducks (AAW63061.1).

heart and trachea. However, the lower expressions were observed in cerebellum and gizzard. The broad expression profile of duCD4 across the different tissues suggests that the role of duCD4 might have certain universality in the immune response.

Expression Profiles of duCD4 in the Viral Infected Ducks

In order to investigate whether the duCD4 could be involved in the host's antiviral immune response to the multiple types of viruses, the mRNA expression level of

duCD4 was detected in the spleen, brain, and lung during the virus infection. After infection with these three viruses, the duCD4 expression has clearly decreased in the spleen at 1, 3, and 5 dpi (Figure 4A, 4D and 4G). Among them, the most significant downregulation occurred in the DTMUV-infected group (Figure 4G), and the down-regulation folds in the DPV-infected group increased with the later time points (Figure 4D). In the lung, the duCD4 expression was significantly upregulated at the indicated time in the DPV-infected ducks, and reached its peak at 5 dpi (13.63-fold, $P < 0.01$; Figure 4E). However, the duCD4 mRNAs were only up-regulated at 1 dpi, and then downregulated at 3

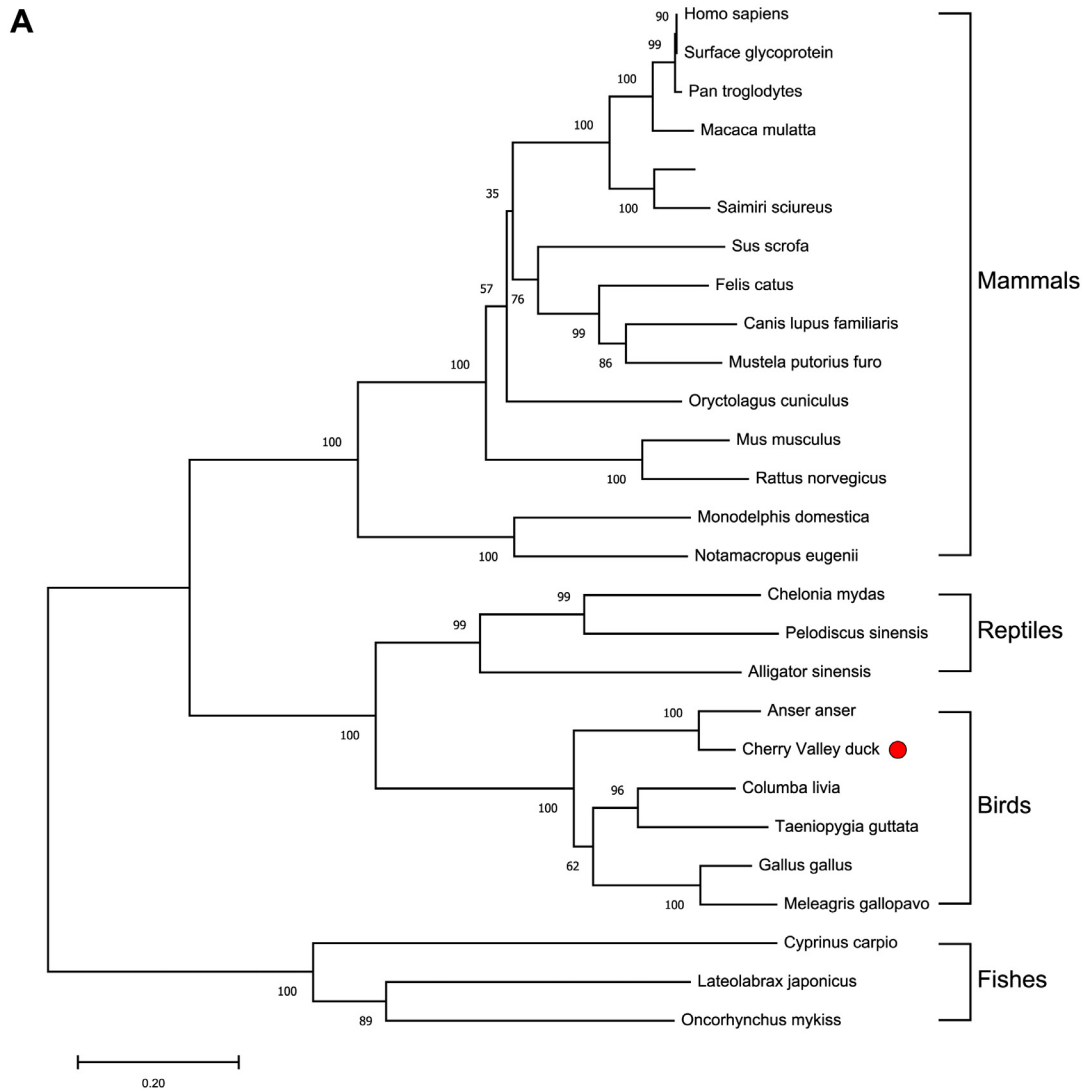


Figure 2. Phylogenetic analysis and sequence similarity of CD4. (A) The phylogenetic tree of the AA sequence of duCD4 and other animals, a neighbor-joining tree was generated using MEGA-X and a 1000 bootstrap analysis was performed. The scale bar is 0.20. GenBank accession numbers are shown in Table 2. (B) Sequence similarity analysis of CD4 among different species. The program was performed using the MegAlign software.

and 5 dpi both in the NDRV and DTMUV-infected group lung (Figure 4B and 4H). In the brain, the duCD4 transcripts hold at the background level in the DPV and DTMUV-infected ducks ($P > 0.05$; Figure 4F and 4I). However, the expression of duCD4 was upregulated at

the indicated time and reached the peak at 1 dpi with 10.32-fold in the NDRV-infected groups ($P < 0.01$; Figure 4C). On the whole, these results indicated that duCD4 could be involved in the host's immune response to multiple types of viruses.

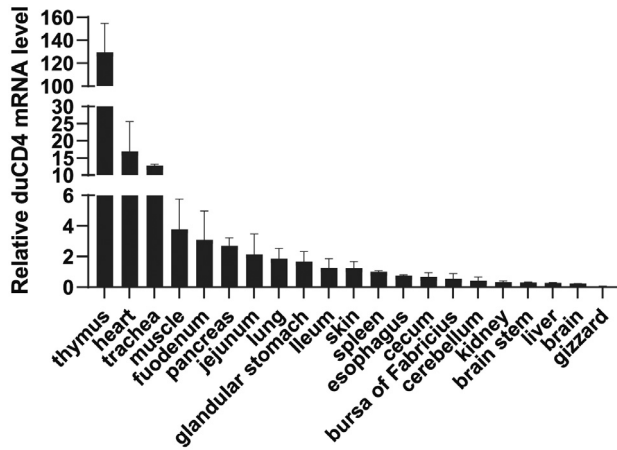


Figure 3. The relative mRNA levels were normalized to the expression of the β -actin gene from various tissues. Data were normalized to the spleen and error bars indicated SD. Abbreviation: duCD4, duck CD4.

DISCUSSION

In the past decades, the study of T cell responses to pathogen infection has progressed significant (Harty et al., 2000). Virus-specific CD4 as a transmembrane glycoprotein was found in helper T cells and thymocyte (Maddon et al., 1985; Parnes, 1989). This molecule has an essential importance in both mammalian and avian immune system.

In the current study, CD4 was identified, cloned and characterized for the first time from Cherry Valley duck. The duCD4 contains a 1,449 bp ORF and has seven characteristic structure domains: one signal peptide, three IG, one IG-like domain, one transmembrane domain, and one C-terminal domain of the CD4 T cell receptor. Multiple sequence alignments indicated that the AA sequence of duCD4 had four potential N-glycosylation sites (NISF, NATA, NGTK, and NYTV) (Figure 1). The conserved CXC motif as a typical CD4 signature is located in the cytoplasmic tails of CD4, and mediates the binding to tyrosine protein kinase p56lck by means of a Zn clasp structure (Kim et al., 2003). And the phylogenetic analysis suggests that duCD4 only exhibited 26.2% identities to *Homo sapiens*, 62.2% to *Gallus gallus*, and 85.4% to *Anser anser*. Notably, the homology between duck and *Anser anser* was greatest, followed by *Gallus gallus* (Figure 2B). Similar result was also observed in the phylogenetic tree (Figure 2A). These results reveal that duCD4 has a close genetic relationship to other birds, especially the *Anser anser* and *Gallus gallus*.

Since CD4 is an essential protein of immunity, study of its tissue distribution will contribute to a better understanding of its function. In this study, although duCD4 was expressed in all tested tissues, the highest expression was found in the thymus, followed by the heart and trachea. As the central immune organ, the thymus is

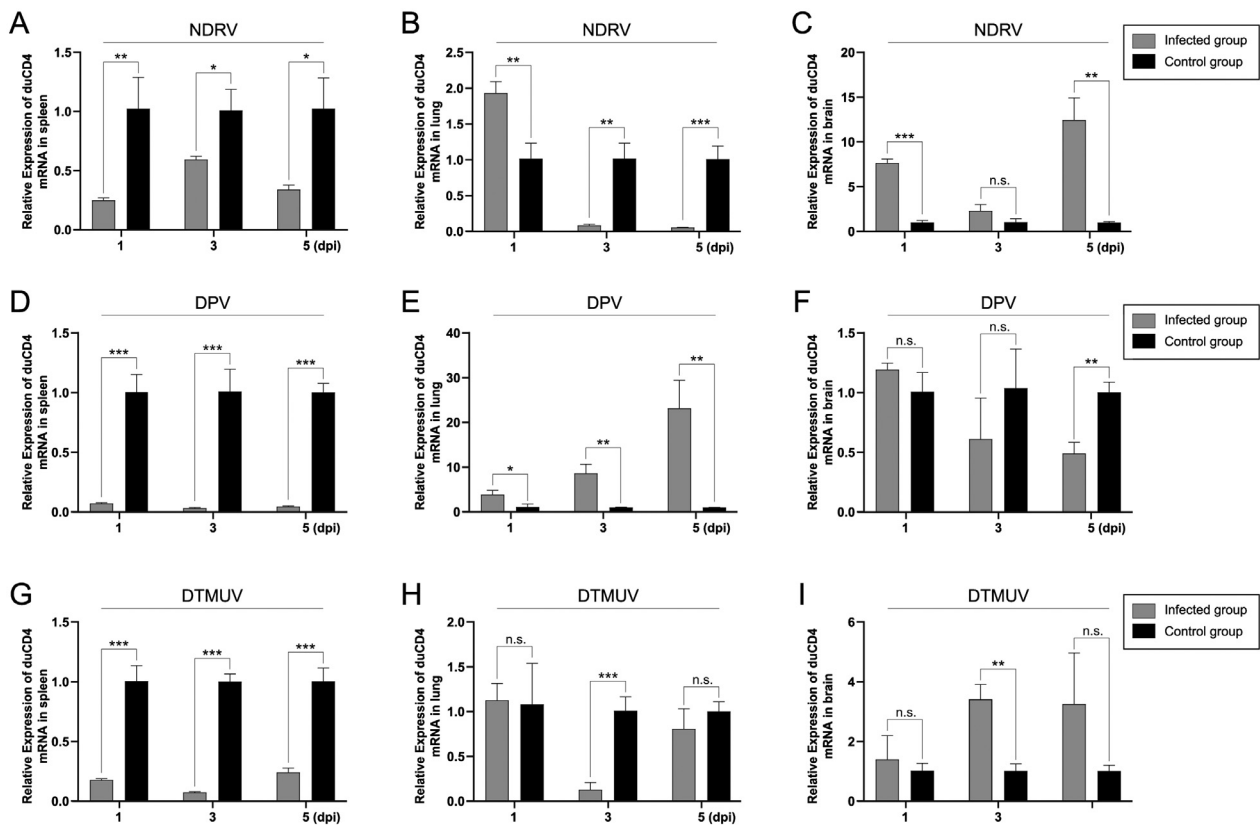


Figure 4. Analysis of duCD4 transcript at early stages of viral infection (A) NDRV-spleen, (B) NDRV-lung, (C) NDRV-brain, (D) DPV-spleen, (E) DPV-lung, (F) DPV-brain, (G) DTMUV-spleen, (H) DTMUV-lung, (I) DTMUV-brain. The relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method. All samples were analyzed triplicate and all data were expressed as means \pm SD. Student's *t* test were performed to evaluate differences. *Significant difference ($P < 0.05$); **Highly significant difference ($P < 0.01$); ***Extremely significant ($P < 0.001$); n.s., no significant difference.

the place where T cells mature; thus, the thymus has a highest expression of T cells, this is similar to the results of mammal CD4 and chicken (Maddon et al., 1987; Koskinen et al., 1999). In addition, the expression of duCD4 was also observed in the brain, and the brain CD4 expression has also been reported (Funke et al., 1987). The expression of duCD4 was low in liver and cerebellum, which were identical to the adult goose CD4 tissue distribution (Yan et al., 2013). In our study, we find that the duck cellular immune function is primarily dependent on the thymus, while the other immune organs also offer support to immune protection. Of course, the development of tissues and organs from different animals, as well as different stages, directly affects the tissue distribution. The detailed biological function of the tissue distribution of duCD4 also will need further study.

The cytotoxic T cells are cornerstone of the host's antiviral immune system by acting through the granule secretory pathway and/or the Fas-mediated pathway (Pardo et al., 2009). Here, the duCD4 antiviral defense efforts were further studied after DTMUV, NDRV and DPV infection. The clinical findings of DTMUV, NDRV, and DPV were characterized by the digestive and neurologic symptoms (Li, et al., 2015, 2016a, c), and activating innate immune pathways to triggering inflammatory response (Hou, et al., 2020). The immune related organs, as well as the virus target organs, were chosen for the tissues to be tested in our study. We found that the expression level of duCD4 changed significantly after these three viral infections. The expression of duCD4 was significantly downregulated in the spleen in these 3 virus-infected duck (Figure 4A, 4D and 4G), but this does not agree with reports on the goose CD4 (Yan, et al., 2013). Presumably, this change was due to the migration of CD4⁺ T lymphocyte from the immune organ (such as the spleen) into the peripheral immune organs during viral infection. Relatively, the duCD4 expression remained at the background level in the brain of the DPV-infected and DTMUV-infected ducks (Figure 4F and 4I), but in the NDRV-infected group, the expression of duCD4 was upregulated 10.32-fold and 8.72-fold at 1 and 5 dpi, respectively (Figure 4C). This phenomenon suggests that there is no effect for the duCD4 expression in the brain post DTMUV and DPV infection, but in the initial infection, the NDRV has a bigger effect on the duCD4 expression. In the lung, the duCD4 expression was upregulated at each of the tested 3 d in the DPV-infected group. This suggests that the lung duCD4 has been involved in the antiviral immune response of the DPV infection. But, in the NDRV-infected groups, the duCD4 expression was diminishing from the 1 to 5 dpi (Figure 4B), indicating that the lung duCD4 could be involved in the antiviral immune response at the early stages of NDRV infection. Of course, significant reduction of lung duCD4 at 5 dpi also indicates the duCD4 plays an important role in the process of antiviral infection. In the DTMUV-infected duck, duCD4 expression was upregulated at 1 dpi then downregulated at 3 dpi, but stable at the background level at 5 dpi. Due to the complex regulatory mechanism of the

signaling pathway, the different expression of duCD4 may be due to different viruses and different activation pathways. This is similar to our previous studies about immune-related genes expression during the infection of these 3 viruses. For example, IL-6, an important inflammatory cytokine, was slightly down-regulated in the spleen and significantly upregulated in the brain during DPV infection (Li, et al., 2016a). However, this inflammatory cytokine was significantly down-regulated in both liver and spleen during NDRV infection (Li, et al., 2016c). All of these results indicate that the above three viral infection has changed the original expression of duCD4 from organs in vivo, it is suggested that duCD4 may involve in the antiviral immune response through an unknown mechanism, and what is clear is that the specific role of duCD4 was different in the process of each different virus infection. Reports have pointed out that the CD4⁺ T-cells also have a close relationship to bacterial infections (Bacher, et al., 2014), and we will also test similar features of duCD4 in the future study.

In conclusion, the duCD4 was identified and its function was analyzed in this study. The expression of duCD4 in healthy ducks was quite extensive. The relative expression of duCD4 has changed quite significantly during the viral infection in vivo. Together, our finding is providing new insights into the duCD4 molecule and immunological characteristics as well as contributes to understanding of T cell-mediated immunity responses of ducks during viral infections. These results contribute to better understanding the antiviral immune system of ducks.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31972664), the Higher Education Support Program of Youth Innovation and Technology of Shandong Province, China (2019KJF022), the Local Science and Technology Development Fund Project Guided by the Central Government of Shandong Province (YDZX20203700004857), the Project of Natural Science Foundation of Shandong Province (ZR2017JL018), the China Postdoctoral Science Foundation (2018M632268 and 2019T120404) and the Shandong "Double Tops" Program (SYL2017-YSTD11). This animal study was reviewed and approved by Shandong Agricultural University Animal Care and Use Committee (no. SDAUA-2019-008).

Ethical statement: This animal study was reviewed and approved by Shandong Agricultural University Animal Care and Use Committee (no. SDAUA-2019-008).

Author Contributions: Tianxu Li and Rong Li wrote the manuscript and performed the most of the experiments. Tingting Zhang and Xiaolan Hou performed the experiment and wrote the discussion. Xinyu Zhai, Jinchao Wang and Bin Xing helped with the animal experiments and helped with sampling. Huihui Zhang and Xingdong Song collected the samples and extracted the sample RNA. Liangmeng Wei designed the study and polished the article.

DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

- Bacher, P., O. Kniemeyer, J. Teutschbein, M. Thon, M. Vodisch, D. Wartenberg, D. H. Scharf, N. Koester-Eiserfunke, M. Schutte, S. Dubel, M. Assenmacher, A. A. Brakhage, and A. Scheffold. 2014. Identification of immunogenic antigens from *Aspergillus fumigatus* by direct multiparameter characterization of specific conventional and regulatory CD4+ T cells. *J. Immunol.* 193:3332–3343.
- Benoist, C., and D. Mathis. 1999. T-lymphocyte differentiation and biology. *Fundam. Immunol.* 4:376.
- Bui, V. C., and T. H. Nguyen. 2016. The role of CD4 on mechanical properties of live cell membrane. *J. Biomed. Mater. Res. A.* 104:239–244.
- Burland, T. G. 2000. DNASTAR's Lasergene sequence analysis software. *Methods Mol. Biol.* 132:71–91.
- Camarota, G., A. Scheirle, B. Takacs, D. M. Doran, R. Knorr, W. Bannwarth, J. Guardiola, and F. Sinigaglia. 1992. Identification of a CD4 binding site on the beta 2 domain of HLA-DR molecules. *Nature.* 356:799–801.
- Cao, Z., C. Zhang, Y. Liu, Y. Liu, W. Ye, J. Han, G. Ma, D. Zhang, F. Xu, X. Gao, Y. Tang, S. Shi, C. Wan, C. Zhang, B. He, M. Yang, X. Lu, Y. Huang, Y. Diao, X. Ma, and D. Zhang. 2011. Tembusu virus in ducks, China. *Emerg. Infect. Dis.* 17:1873–1875.
- Doyle, C., and J. L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature.* 330:256–259.
- Funke, I., A. Hahn, E. P. Rieber, E. Weiss, and G. Riethmuller. 1987. The cellular receptor (CD4) of the human immunodeficiency virus is expressed on neurons and glial cells in human brain. *J. Exp. Med.* 165:1230–1235.
- Germain, R. N. 2002. T-cell development and the CD4-CD8 lineage decision. *Nat. Rev. Immunol.* 2:309–322.
- Glaichenhaus, N., N. Shastri, D. R. Littman, and J. M. Turner. 1991. Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. *Cell.* 64:511–520.
- Harty, J. T., A. R. Tvinnereim, and D. W. White. 2000. CD8+ T cell effector mechanisms in resistance to infection. *Annu. Rev. Immunol.* 18:275–308.
- Hou, X., G. Liu, H. Zhang, X. Hu, X. Zhang, F. Han, H. Cui, J. Luo, R. Guo, R. Li, N. Li, and L. Wei. 2020. High-mobility group box 1 protein (HMGB1) from Cherry Valley duck mediates signaling pathways and antiviral activity. *Vet. Res.* 51:12.
- Huang, J., R. Jia, M. Wang, B. Shu, X. Yu, D. Zhu, S. Chen, Z. Yin, X. Chen, and A. Cheng. 2014. An attenuated duck plague virus (DPV) vaccine induces both systemic and mucosal immune responses to protect ducks against virulent DPV infection. *Clin. Vaccine Immunol.* 21:457–462.
- Kim, P. W., Z. Y. Sun, S. C. Blacklow, G. Wagner, and M. J. Eck. 2003. A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. *Science.* 301:1725–1728.
- Konig, R., L. Y. Huang, and R. N. Germain. 1992. MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8. *Nature.* 356:796–798.
- Konig, R., X. Shen, and R. N. Germain. 1995. Involvement of both major histocompatibility complex class II alpha and beta chains in CD4 function indicates a role for ordered oligomerization in T cell activation. *J. Exp. Med.* 182:779–787.
- Koskinen, R., U. Lamminmaki, C. A. Tregaskes, J. Salomonsen, J. R. Young, and O. Vainio. 1999. Cloning and modeling of the first nonmammalian CD4. *J. Immunol.* 162:4115–4121.
- Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35:1547–1549.
- Li, N., T. Hong, R. Li, M. Guo, Y. Wang, J. Zhang, J. Liu, Y. Cai, S. Liu, T. Chai, and L. Wei. 2016a. Pathogenicity of duck plague and innate immune responses of the Cherry Valley ducks to duck plague virus. *Sci. Rep.* 6:32183.
- Li, N., T. Hong, R. Li, Y. Wang, M. Guo, Z. Cao, Y. Cai, S. Liu, T. Chai, and L. Wei. 2016b. Cherry valley ducks mitochondrial antiviral-signaling protein-mediated signaling pathway and antiviral activity research. *Front. Immunol.* 7:377.
- Li, N., T. Hong, Y. Wang, Y. Wang, K. Yu, Y. Cai, S. Liu, L. Wei, and T. Chai. 2016c. The pathogenicity of novel duck reovirus in Cherry Valley ducks. *Vet. Microbiol.* 192:181–185.
- Li, N., Y. Wang, R. Li, J. Liu, J. Zhang, Y. Cai, S. Liu, T. Chai, and L. Wei. 2015. Immune responses of ducks infected with duck Tembusu virus. *Front. Microbiol.* 6:425.
- Li, Z., Y. Cai, G. Liang, S. El-Ashram, M. Mei, W. Huang, X. Li, W. Li, C. He, and S. Huang. 2018. Detection of Novel duck reovirus (NDRV) using visual reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Sci. Rep.* 8:14039.
- Lian, B., A. Cheng, M. Wang, D. Zhu, Q. Luo, R. Jia, F. Liu, X. Han, and X. Chen. 2011. Induction of immune responses in ducks with a DNA vaccine encoding duck plague virus glycoprotein C. *Viol. J.* 8:214.
- Ma, D., Y. Wei, and F. Liu. 2013. Regulatory mechanisms of thymus and T cell development. *Dev. Comp. Immunol.* 39:91–102.
- Maddon, P. J., D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, and R. Axel. 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell.* 42:93–104.
- Maddon, P. J., S. M. Molineaux, D. E. Maddon, K. A. Zimmerman, M. Godfrey, F. W. Alt, L. Chess, and R. Axel. 1987. Structure and expression of the human and mouse T4 genes. *Proc. Natl. Acad. Sci. U S A.* 84:9155–9159.
- Matrajt, L., P. M. Younan, H. P. Kiem, and J. T. Schiffer. 2014. The majority of CD4+ T-cell depletion during acute simian-human immunodeficiency virus SHIV89.6P infection occurs in uninfected cells. *J. Virol.* 88:3202–3212.
- Pardo, J., J. I. Aguilo, A. Anel, P. Martin, L. Joeckel, C. Borner, R. Wallich, A. Mullbacher, C. J. Froelich, and M. M. Simon. 2009. The biology of cytotoxic cell granule exocytosis pathway: granzymes have evolved to induce cell death and inflammation. *Microbes. Infect.* 11:452–459.
- Parnes, J. R. 1989. Molecular biology and function of CD4 and CD8. *Adv. Immunol.* 44:265–311.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* 29:e45.
- Phares, T. W., S. A. Stohlman, M. Hwang, B. Min, D. R. Hinton, and C. C. Bergmann. 2012. CD4 T cells promote CD8 T cell immunity at the priming and effector site during viral encephalitis. *J. Virol.* 86:2416–2427.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* 27:493–497.
- Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting. 1998. SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U S A.* 95:5857–5864.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic. Acids. Res.* 25:4876–4882.
- Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. *Nature.* 338:257–259.
- Wei, L., J. Cui, Y. Song, S. Zhang, F. Han, R. Yuan, L. Gong, P. Jiao, and M. Liao. 2014. Duck MDA5 functions in innate immunity against H5N1 highly pathogenic avian influenza virus infections. *Vet. Res.* 45:66.
- Whitmire, J. K. 2011. Induction and function of virus-specific CD4+ T cell responses. *Virology.* 411:216–228.
- Yan, P., Y. Zhao, X. Zhang, D. Xu, X. Dai, Q. Teng, L. Yan, J. Zhou, X. Ji, S. Zhang, G. Liu, Y. Zhou, Y. Kawaoka, G. Tong, and Z. Li. 2011. An infectious disease of ducks caused by a newly emerged Tembusu virus strain in mainland China. *Virology.* 417:1–8.
- Yan, X., F. Liu, S. Chen, Q. Zhao, Y. Qi, M. Wang, R. Jia, D. Zhu, X. Chen, and A. Cheng. 2013. Molecular cloning, characterization and tissue expression of CD4 in Chinese goose. *Gene.* 519:298–304.