Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.066

RESEARCH ARTICLE

Production of Monoclonal Antibodies against Chicken Interleukin-4 and Development of a Capture Enzyme-Linked Immunosorbent Assay

Hua Dai[1, 3], Jun-hua Chen[1, 2], Ying Shen[3], Xiao-hui Zhou[4], Zheng-zhong Xu[1,2], Xiang Chen[1,2] and Xin-an Jiao[1,2]*

[1]Jiangsu Key Lab of Zoonosis, Yangzhou University, Yangzhou, Jiangsu, China; [2]Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, Jiangsu, China; [3]Medical School, Yangzhou University, Yangzhou, Jiangsu, China; [4]Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT, USA
*Corresponding author: jiao@yzu.edu.cn

ARTICLE HISTORY (18-117)

Received: March 24, 2018
Revised: June 26, 2018
Accepted: June 28, 2018
Published online: August 06, 2018

Key words:
Chicken Interleukin-4
Enzyme-linked immunosorbent assay
Monoclonal antibodies

A B S T R A C T

The purpose of this study was to generate monoclonal antibodies (mAb) specific for chicken interleukin-4 (ChIL-4) and develop a capture ELISA for the detection of ChIL-4. Five mAbs against ChIL-4 were generated by immunizing Balb/c mice with recombinant ChIL-4 (rChIL-4). As determined in ELISA, immunofluorescent assay and western blotting, each mAb reacted specifically with rChIL-4 derived from baculovirus and *Escherichia coli* (*E. coli*) expression systems. In order to identify a suitable pair of mAbs for the development of ChIL-4 capture ELISA, mAbs were tested as both capture and revealing antibodies. To achieve the highest sensitivity of the capture ELISA, mAb 20C9 was used as the capture antibody and biotinylated mAb 16D8 was used as the revealing antibody, which allowed the detection of ChIL-4 low to 500 pg/ml. The specificity of ELISA was verified using rChIL-4 derived from baculovirus expression system and irrelevant proteins as control. The results showed that this ELISA is suitable to detect rChIL-4 in different forms and would provide a sensitive tool to measure the in vitro release of ChIL-4 in birds.

To Cite This Article: Dai H, Chen JH, Shen Y, Zhou X, Xu ZZ, Chen X and Jiao XA, 2018. Production of monoclonal antibodies against chicken interleukin-4 and development of a capture enzyme-linked immunosorbent assay. Pak Vet J, 38(3): 286-290. http://dx.doi.org/10.29261/pakvetj/2018.066

INTRODUCTION

IL-4 is a lymphokine secreted predominantly by Th2 cells, and plays a key role in initiation and progression of a Th2 type response (Paul, 1991; Zhu, 2015; Bao and Reinhardt, 2015; Kidane *et al.*, 2016; Fox *et al*., 2018). IL-4 is a crucial regulator in humoral and adaptive immunity when facing extracellular pathogens, helminth parasites infection, it also has the function of switching off the inflammatory response to prevent excessive production of inflammatory cytokine and host damage (Brown and Hural, 1997; Ma *et al.*, 2005; Yang *et el.*, 2013; Matisz *et al.*, 2015; Serezani *et al*., 2017; Fernandez *et al*., 2018).

Chickens are an important food protein source worldwide; however, as a reservoir for foodborne pathogens, they remain to be a potential threat to human health (Xing *et al.*, 2008; Hermans *et al*., 2012; Kallon *et al*., 2013; Tan *et al*., 2018). Therefore, it is urgent to develop useful and convenient assay to evaluate chicken immune status especially after innoculation or infection. Previous studies have shown that chicken IL-4 (ChIL-4) works well as an immune adjuvant (Sawant *et al.*, 2011; Annamalai and Selvaraj, 2012; Kalaiyarasu *et al.*, 2016; Khajeh *et al.*, 2016). Compared to chicken IFN-γ (ChIFN-γ), ChIL-4 was recently identified (Avery *et al*., 2004). So far, only a few studies have been conducted to evaluate the immune enhancing property of avian IL-4 at the transcriptional or cellular level (Sawant *et al.*, 2011; Chaussé *et al*., 2014; Płowiec *et al*., 2015; Tan *et al*., 2018). Thus, it is necessary to generate antibodies which are specific for ChIL-4 and can be used for the detection and functional studies of ChIL-4. In addition, ELISAs are highly sensitive, specific and easy to perform especially for large scale use.

The purpose of this study was to generate monoclonal antibodies (mAbs) against recombinant ChIL-4 (rChIL-4) and develop a capture ELISA assay to specifically detect ChIL-4. Based on two mAbs generated here, the ChIL-4 capture ELISA was established. This assay could be a

potent tool for future studies on the role of ChIL-4 in immune response in poultry.

MATERIALS AND METHODS

Animals: Balb/c mice and specific pathogen free (SPF) white leghorn chickens were provided by the Comparative Medical Center of Yangzhou University. Animals were housed and treated following the approval by the institutional animal experimental committee.

Recombinant proteins and native ChIL-4: The production of rChIL-4 (His-ChlL-4, GST-ChIL-4) derived from *Escherichia coli* (*E. coli*) and Bac-ChIL-4 from baculovirus expression system were described previously (Dai *et al.*, 2008, 2017). Recombinant proteins: bovine IL-4 (His-BovIL-4), chicken IFN-α (His-ChIFN-α) and chicken IFN-γ (His-ChIFN-γ) are described elsewhere (Dai *et al.*, 2006, 2009; Chen *et al.*, 2011). Native ChIL-4 was produced from the supernatants of splenocytes stimulated with phorbol myristate acetate (PMA) and ionomycin. Spleen were removed from the SPF chickens and prepared into single cell suspension in RPMI 1640 medium containing 10% inactivated fetal bovine serum, 100 U penicillin/ml, 100 μg streptomycin/ml. cells were adjusted to 10^7 cells/ml, then 2.5×10^6 cells per well were seeded into flat-bottomed 24-well plates. Equal volumes of medium with PMA (50 ng/ml) and ionomycin (100 ng/ml) were added in triplicates, negative controls received the same amount of medium only. After 72 h incubation at 41°C with 5% CO_2, supernatants were harvested from each well for the measurement of ChIL-4 generated.

Generation of monoclonal antibodies: Eight-week-old Balb/c mice were immunized with 100 μg of GST-ChIL-4 emulsified with Freud's adjuvant by subcutaneous injections. Secondary immunization was given at a two-week interval. A booster injection was performed intravenously with the same antigen (50 μg). Three days later, spleen cells from the immunized mice were prepared and fused with sp2/0 cells. After fusing, cells were diluted in DMEM medium containing hypoxanthine-aminopterin-thymidine (HAT medium) and plated in 96-well culture plates on a feeder cell layer at 37°C with 5% CO_2. Hybridomas were tested by indirect ELISA (see below). Positive Hybridomas were cloned by limiting dilution. The isotype of each mAb was determined. Ascites was produced in Balb/c mice, mAbs were purified and biotinylated by standard methods (GenScript Biotechnique Company, China).

Enzyme-linked immunosorbent assays (ELISA): Microtiter plates were coated with 1 μg/ml of His-ChIL-4 in 0.05 M/L carbonate buffer overnight at 4°C, 50 μl/well. Hybridoma supernatants (50 μl/well) were added for 1 h at 37°C, serum from immunized mice and non-immunized mice were used as positive or negative controls, respectively. Horseradish peroxidase (HRP)-conjugated secondary antibody was added and HRP activity was measured with tetramethylbenzidine (TMB) substrate, reactions were stopped with 50 μl of 2 M H_2SO_4 and absorbance was measured.

Western blotting analysis: Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. After blocking, membrane was incubated with ChIL-4 mAbs for 1 h at 37°C and HRP-conjugated goat anti-mouse secondary antibody was added for a further 30 min at 37°C. After washing, membrane was developed with detection reagent and signals were detected.

Immunofluorescence: Plasmid pVAX1-ChIL-4 (Dai *et al*., 2008) and pVAX1 vector was transfected into Cos-7 cells using lipofectamine 2 000 reagent, respectively. Plates were washed and fixed, mAbs was added for 1 h and stained by FITC-conjugated goat anti-mouse IgG(H+L) secondary antibody. Fluorescence of each well was examined under the microscope.

Capture ELISA: A capture ELISA, using anti-ChIL-4 mAbs, was developed in this study. ELISA plates were pre-coated with 100 μl of purified capture mAbs (2.5-40 μg/ml) in 0.01 M PBS by 24 h incubation at 4°C. Subsequently, plates were washed and blocked with 200 μl of PBS-T containing 2% BSA overnight at 4°C. After blocking, 100 μl of serial diluted His-ChIL-4 (0-8 μg/ml) were added to individual wells with 2 h incubation at 37°C. For the detection of native ChIL-4, supernatants (100 μl/well) from the cultured splenocytes stimulated or non-stimulated with PMA plus ionomycin were added. Unbounded protein was removed by PBS-T washing. Followed by addition of biotinylated detecting mAb into the plates with the concentration of 20 ng/ml (100 μl/well) for 1 h incubation at 37°C. After washing, streptavidin-HRP diluted in optimal concentration was added and incubated for 30 min at 37°C, then exposed to TMB substrate solution. Reaction was stopped with 2 M H_2SO_4 and absorbance of each well was measured.

Statistical analysis: Statistical analysis was performed using Graphpad Prism 5 software. Analysis of data was performed using T-test or one-way ANOVA. Results were considered statistically significant if $P\leq0.05$.

RESULTS

Generation and characterization of mAb to ChIL-4: To obtain reagents for the detection of ChIL-4, mice were immunized with GST-tagged rChIL-4. After fusing, hybridomas were screened by His-ChIL-4 coated ELISA plates. Five hybridomas (16D8, 17D7, 18F7, 19F1, 20C9) were cloned and selected for further study based upon their strong reactivity against ChIL-4 with indirect ELISA. The isotype of mAb 16D8 was IgG2a, while the rest mAbs were IgG1. The specificity of the 5 mAbs to ChIL-4 was confirmed by indirect ELISA, immunofluorescence assay (IFA) and western blotting. The result of ELISA showed that all mAbs could react with rChIL-4 but not recombinant ChIFN-α, ChIFN-γ and BovIL-4 (Table 1). IFA results revealed that mAbs could detect the rChIL-4 expressed by Cos-7 cells after the transfection with plasmid pVAX1-ChIL-4 (Fig. 1). Western blotting analysis further revealed that All mAbs could only recognize His-ChIL-4, GST-ChIL-4 and Bac-ChIL-4 at 18 kDa band, 38 kDa band and 18 kDa band,

respectively, no reaction was observed with recombinant bacteria BL21(DE3)-(pET) induced with IPTG (Fig. 2).

Establishment of a ChIL-4 capture ELISA: In a checkboard analysis, all 5 mAbs were tested as capture or revealing antibodies to set up an antigen-capture ELISA system. For each combination, His-ChIL-4 was assayed. The result revealed that the capture ELISA system was very sensitive when mAb 20C9 was used as capture antibody and biotinylated mAb 16D8 was used as revealing reagent (Fig. 3). Subsequently, this pair of mAbs was optimized and the detection limit of His-ChIL-4 could be as low as 0.5 ng/ml with low back ground when the concentration of coating mAb 20C9 was used to 10 μg/ml and revealing mAb 16D8 was adjusted to 0.2 μg/ml (Fig. 4A). The capture ELISA established here showed a good relationship between OD450 value and ChIL-4 protein concentration in the samples detected (Fig. 4B). Then, this capture ELISA was further tested by the detection of Bac-ChIL-4 derived from baculovirus expression system, and the results also showed a suitability for the measurement of Bac-ChIL-4 (Fig. 5A). According to the formula established as above, the concentration of Bac-ChIL-4 produced is about 11.4 ng/ml. Then the specificity of ELISA developed as above was analyzed with some unrelated proteins including ChIFN-α, ChIFN-γ and BovIL-4. The results revealed that the capture ELISA is sensitive and specific to detect trace amount of ChIL-4 protein in samples (Fig. 5B). Then the capture ELISA was used to detect native ChIL-4 secreted in the supernatant of splenocytes stimulated with PMA and ionomycin, the results showed that the ELISA could detect native ChIL-4 with trace amounts in samples (Fig. 6).

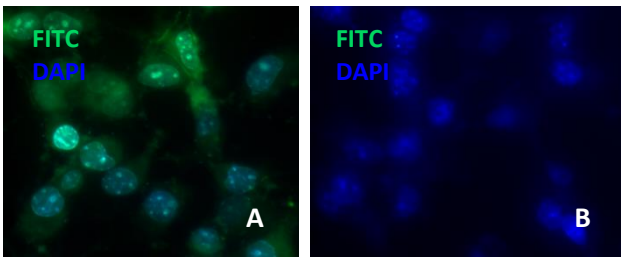

Fig. 1: Immunofluorescence assay (IFA) analysis of the ability of mAb in recognizing ChIL-4 expressed by Cos-7 cells after transfection; (A. Cos-7 cells transfected with pVAX1-ChIL-4 was incubated with mAbs; B. Cos-7 cells transfected with pVAX1 vector was incubated with mAbs as a control).

Table 1: Characterization of the specificity of monoclonal antibodies for ChIL-4

mAb	Reactivity of cytokines $OD_{450\ nm}$			
	His-ChIL-4	His-BovIL-4	His-ChIFN-α	His-ChIFN-γ
16D8	1.610±0.015	0.080±0.005	0.069±0.011	0.078±0.010
17D7	1.169±0.057	0.067±0.004	0.065±0.007	0.071±0.006
18F7	1.000±0.049	0.071±0.009	0.073±0.009	0.082±0.007
19F1	1.313±0.052	0.087±0.012	0.075±0.002	0.089±0.002
20C9	1.368±0.032	0.067±0.006	0.080±0.015	0.077±0.006

Note: Recombinant ChIL-4, BovIL-4, ChIFN-α and ChIFN-γ obtained by *E. coli* used as coating antigens (2 μg/ml) in the indirect ELISA, respectively. The indicated mAbs (1 mg/ml) were incubated in the antigen coated wells and their binding ability was revealed by reacting with HRP-labelled goat anti-mouse IgG.

DISCUSSION

IL-4 is an important indicator of humoral immunity, and has the function to modulate Th1/ Th2 balance. An understanding of biological properties of chicken Th2 cytokines will be important to study the humoral immune responses of chicken especially after immunization or in disease. However, chicken cytokines have not been extensively studied, and most of them are not well known, such as Th2 cytokines. Therefore, it is urgent to develop a convenient and stable assay to detect this key Th2 cytokine on protein level.

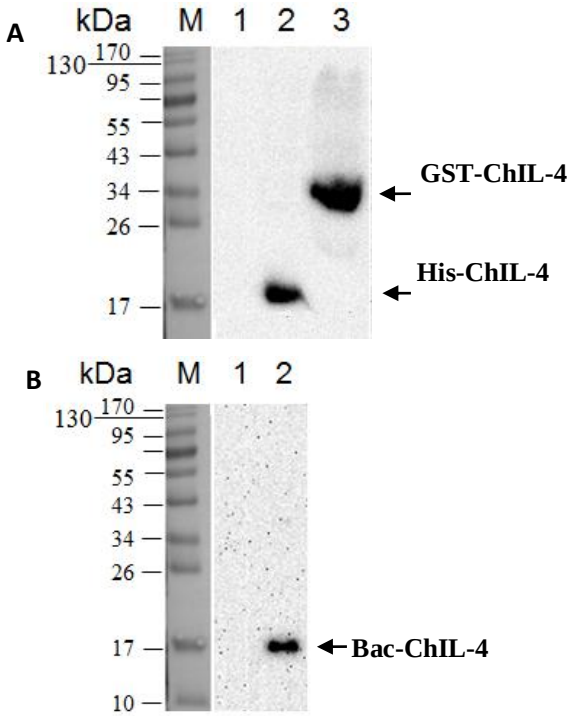

Fig. 2: Analysis of the mAbs specific to ChIL-4 by western blotting; (Recombinant ChIL-4 protein derived from *E. Coli* and baculovirus expression system were separated by SDS-PAGE, then transferred to PVDF membrane, the membranes were incubated with various mAbs, followed by addition of HRP-conjugated secondary antibody and ECL as substrate. (A. Lane 1. Recombinant bacteria BL21(DE3)-(pET) induced with IPTG, Lane 2, 3. Recombinant protein GST-ChIL-4, His-ChIL-4; B. Lane 1. Supernatants of sf9 cells transfected by wild baculovirus as a control, Lane 2. Recombinant Bac-ChIL4).

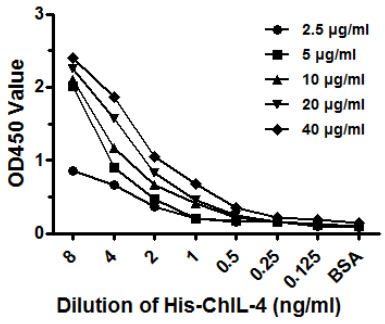

Fig. 3: The establishment of ChIL-4 capture ELISA; (mAb 20C9 with different concentration (2.5-40 μg/ml) was coated in microtiter plates, and serial dilutions of recombinant protein (His-ChIL-4) were added into the plates, biotinylated mAb 16D8 was used as the revealing reagent. After the addition of HRP-conjugated streptavidin, revealing and stopping steps, OD450 value of each well was read.)

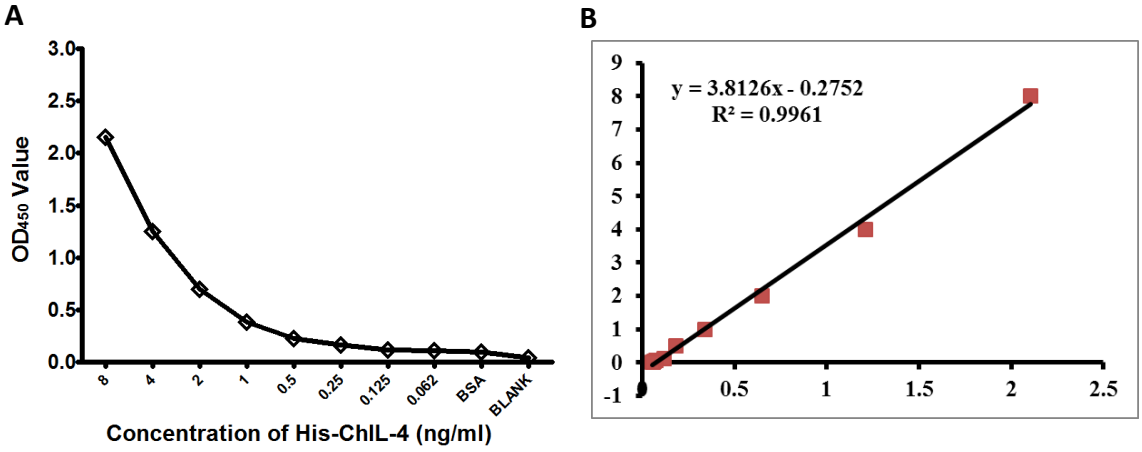

Fig. 4: Sensitivity of the capture ELISA was confirmed using His-ChIL-4; (A. Serial diluted His-ChIL-4 were used as antigen to detect the sensitivity of ChIL-4 capture ELISA; B. A linear relationship formula was set up according to the sensitivity test of the capture ELISA).

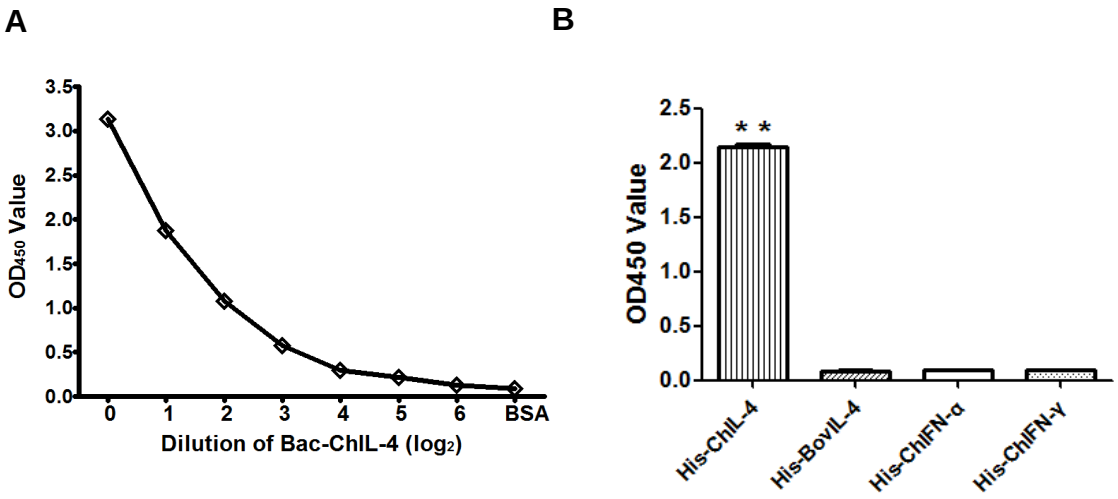

Fig. 5: The specificity of ChIL-4 capture ELISA was verified by detecting recombinant ChIL-4 in different forms; (A. Recombinant ChIL-4 derived from baculovirus expression system was used as detecting antigen in this capture ELISA; B. Recombinant proteins as His-ChIL-4, His-BovIL-4, His-ChIFN-α, His-ChIFN-γ were tested in this ELISA to verify its specificity in detecting ChIL-4, **P＜0.001.)

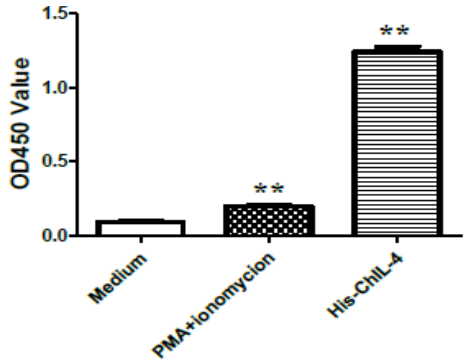

Fig. 6: The capture ELISA was able to detect native ChIL-4 produced by chicken splenocytes activated with PMA plus ionomycin in cultured samples, **P<0.001.

Our strategy relied on previous studies that mAbs with good biological activity could be developed by using recombinant antigens and also could be used to establish a potent capture ELISA (Scott *et al.*, 2006; Dai *et al.*, 2016). In this study, five mAbs against ChIL-4 were generated, and the specificity of these mAbs was analyzed by ELISA, IFA and western blotting analysis. The results showed that all five mAbs could distinguish and bind rChIL-4 but not the control proteins. These results indicated that the mAbs generated in this study are specific to ChIL-4. We further showed that mAb pair of 16D8 and 20C9 was the most suitable mAb combination (20C9 was used as the capture antibody and biotinylated 16D8 was used as revealing reagent), which could detect rChIL-4 as low as 0.5 ng/ml with very low background. Thus, this capture ELSIA was utilized to detect native ChIL-4 produced by mitogen-activated splenocytes. The results showed that this ELISA could detect native ChIL-4, but OD value is relatively low, indicating this ELISA still needs to be modified to increase its sensitivity to meet the field-scale need, because the level of ChIL-4 is very low in serum or samples.

Conclusions: Th2 cytokine responses are typical immune reactions to infections, allergies and asthma, and are characterized by the production of the cytokine IL-4 by subsets of Th2 cells. Our results demonstrated that this capture ELISA could detect both recombinant and native forms of ChIL-4, We concluded that the ELISA developed in this study is likely to be useful for measuring native ChIL-4 in biological samples and for studying the function of native forms.

Acknowledgements: This work was supported by grants from National Natural Science Foundation of China under agreement number 30972171 and 81472815.

Authors contribution: In this article, DH and CJH contributed equally to this work; DH, CJH and JX designed review/project/study; DH, CJH, XZZ executed the experiment CX and SY helped to make recombinant proteins; DH wrote the manuscript; ZXH and JX critically revised the manuscript; All authors approved the final version.

REFERENCES

Annamalai T and Selvaraj RK, 2012. Interleukin 4 increases CCR9 expression and homing of lymphocytes to gut-associated lymphoid tissue in chickens. Vet Immunol Immunopathol 145:257-63.

Annamalai T and Selvaraj RK, 2012. Effects of in ovo interleukin-4-plasmid injection on anticoccidia immune response in a coccidia infection model of chickens. Poult Sci 91:1326-34.

Avery S, Rothwell L, Degen WD, *et al*., 2004. Characterization of the first nonmammalian T2 cytokine gene cluster: the cluster contains functional single-copy genes for IL-3, IL-4, IL-13, and GM-CSF, a gene for IL-5 that appears to be a pseudogene, and a gene encoding another cytokinelike transcript, KK34. J Interferon Cytokine Res 24:600-10.

Bao K and Reinhardt RL, 2015. The differential expression of IL- 4 and IL-13 and its impact on type-2 immunity. Cytokine 75:25-37.

Brown MA and Hural J, 1997. Functions of IL-4 and control of its expression. Crit Rev Immunol 77:1-32.

Chaussé AM, Grépinet O, Bottreau E, *et al*., 2014. Susceptibility to Salmonella carrier-state: a possible Th2 response in susceptible chicks. Vet Immunol Immunopathol 159:16-28.

Chen X, Zhang CQ, Xu ZZ, *et al*., 2011. Prokaryotic expression of recombinant bovine IL-4 and development of monoclonal antibodies against bovine IL-4. Chi J Cell Mol Immunol 27:653-6.

Dai H, Jiao XA, Pan ZM, *et al*., 2006. Preparation and characterization of monoclonal antibodies against chicken interferon-γ. Chi J Cell Mol Immunol 22:510-2.

Dai H, Zheng JY, Chen JH, *et al*., 2008. Cloning and expression of chicken interleukin-4 gene. Chi J Prev Vet Med 30:473-7.

Dai H, Zheng JY, Chen JH, *et al*., 2009. Prokaryotic expression and activity assay of recombinant chicken interferon-alpha. Chi J Prev Vet Med 31:804-7.

Dai H, Xu ZZ, Wang M, *et al*., 2016. Development of a double-monoclonal-antibody sandwich ELISA: Tool for chicken interferon-γ detection ex vivo. Can J Vet Res 80:134-40.

Dai H, Chen JH, Xu ZZ, *et al*., 2017. Expression of chicken (Gallus gallus) IL-4 in baculovirus vector system and its bioactivity analysis. J Agric Biotech 25:1349-55.

Fernandez CP, Afrin F, Flores RA, *et al*., 2018. Identification of duck IL-4 and its inhibitory effect on IL-17A expression in R. anatipestifer-stimulated splenic lymphocytes. Mol Immunol 95:20-9.

Fox A, Harland KL, Kedzierska K, *et al*., 2018. Exposure of human $CD8^+$ T cells to type-2 cytokines impairs division and differentiation and induces limited polarization. Front Immunol 9:1141.

Hermans D, Pasmans F, Messens W, *et al*., 2012. Poultry as a host for the zoonotic pathogen Campylobacter jejuni. Vector Borne Zoonotic Dis 12:89-98.

Kalaiyarasu S, Bhatia S, Mishra N, *et al*., 2016. Elevated level of pro inflammatory cytokine and chemokine expression in chicken bone marrow and monocyte derived dendritic cells following LPS induced maturation. Cytokine 85:140-7.

Kallon S, Li X, Ji J, *et al*., 2013. Astragalus polysaccharide enhances immunity and inhibits H9N2 avian influenza virus in vitro and in vivo. J Anim Sci Biotechnol 4:22.

Khajeh H, Bahari A, Lagzian M, *et al*., 2016. Functional and key gene expression analyses of chicken monocyte-derived dendritic cells with recombinant interleukin 4. Iran J Allergy Asthma Immunol 15:508-14.

Kidane FA, Bilic I, Mitra T, *et al*., 2016. In situ hybridization to detect and localize signature cytokines of T-helper (Th) 1 and Th2 immune responses in chicken tissues. Vet Immunol Immunopathol 175:51-6.

Ma S, Huang Y, Davis A, *et al*., 2005. Production of biologically active human interleukin-4 in transgenic tobacco and potato. Plant Biotechnol J 3:309-18.

Matisz CE, Leung G, Reyes JL, *et al*., 2015. Adoptive transfer of helminth antigen-pulsed dendritic cells protects against the development of experimental colitis in mice. Eur J Immunol 45:3126-39.

Paul WE, 1991. Interleukin 4: a prototypic immunoregulatory lymphokine. Blood 77:1859-70.

Płowiec A, Sławińska A, Siwek MZ, *et al*., 2015. Effect of in ovo administration of inulin and Lactococcuslactis on immune-related gene expression in broiler chickens. Am J Vet Res 76:975-82.

Sawant PM, Verma PC, Subudhi PK, *et al*., 2011. Immunomodulation of bivalent Newcastle disease DNA vaccine induced immune response by co-delivery of chicken IFN-γ and IL-4 genes. Vet Immunol Immunopathol 144:36-44.

Scott TR and LiLLehoj HS, 2006. Monoclonal antibodies against chicken interleukin-6. Vet Immunol Immunopathol 114:173-7.

Serezani APM, Bozdogan G, Sehra S, *et al*., 2017. IL-4 impairs wound healing potential in the skin by repressing fibronectin expression. J Allergy Clin Immunol 139:142-51.

Tan L, Zhang Y, Qiao C, *et al*., 2018. NDV entry into dendritic cells through macropinocytosis and suppression of T lymphocyte proliferation. Virology 518:126-35.

Yang X, Xiao YL, Chen JL, *et al*., 2013. Potentiation of immunity of piglets to mycoplasma hyopneumoniae vaccine with fused gene for pig IL-4 and IL-6 embodied in chitosan nanoparticles. Procedia in Vaccinology 7:15-22.

Xing Z, Cardona CJ, Li J, *et al*., 2008. Modulation of the immune responses in chicken by low-pathogenicity avian influenza virus H9N2. J Gen Virol 89:1288-99.

Zhu J, 2015. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine 75:14-24.