


# Research Note: Characterization of monoclonal antibodies and development of sandwich ELISA for detecting chicken IL7

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**ABSTRACT** IL7 is a hematopoietic growth factor required for development and maintenance of lymphocytes including T cells, B cells, and natural killer cells. Recently, chicken IL7 (**chIL7**) has been cloned and studied in viral and parasite infection models. However, no monoclonal antibodies (**mAb**) that specifically detect chIL7 have been developed so far. In this study, recombinant chIL7 that expressed for immunization and mAb against chIL7 were developed and characterized to assess their immunologic properties. Five

mAb exhibiting specific binding to chIL7 were generated and investigated for their applicability by Western blot, ELISA, and neutralization assays. A sandwich ELISA mAb pair that enables the measurement of chIL7 protein levels in biological samples from *Eimeria*-infected chickens was identified and several mAb neutralized chicken primary thymocyte proliferation mediated by chIL7. The mAb developed in this study will be valuable reagents for fundamental and applied immunological studies in poultry.

**Key words:** IL7, chicken, monoclonal antibody, sandwich ELISA, thymocyte proliferation assay

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## INTRODUCTION

IL7 was originally discovered as a pre-B cell growth factor involved in immune and hematopoietic systems (Namen et al., 1988a; Schluns et al., 2000). Murine IL7 was first isolated as a soluble growth factor derived from long-term murine bone marrow cultures with a property of pre-B cell proliferation and initially described as lymphopoietin 1 (Namen et al., 1988a). Later, it was cloned from Whitlock Witte-derived stromal clonal cell line (IxN/A6) and its bioactivity assessed by stimulation of lymphoid progenitor proliferation, then renamed as IL7 (Namen et al., 1988b). Thereafter, a cDNA clone, which encodes human IL7 ortholog, was isolated by cross hybridization of a human expression library. It stimulated murine pre-B cell proliferation and enriched human B lineage progenitors

(Goodwin et al., 1989). IL7 is secreted by stromal tissues, including epithelial cells in thymus and bone marrow (Sakata et al., 1990; Wiles et al., 1992), intestinal epithelia cells (Madrigal Estebas et al., 1997), keratinocytes (Heufer et al., 1993), liver (Golden Mason et al., 2001), dendritic (de Saint Vis et al., 1998; Sorg et al., 1998), and follicular dendritic cells (Kroncke et al., 1996). Indeed, IL7 has been isolated from mouse (Namen et al., 1998a,b), human (Goodwin et al., 1989), ovine (Barcham et al., 1995), swine (Ueha et al., 2001), puffer fish (Kono et al., 2008), and bovine (Lijo et al., 2016) and in all cases induced lymphocyte proliferation *ex vivo*. Chicken IL7 (**chIL7**) was cloned and shown 30% homology at the amino acid level with its human ortholog. Like in mammals, chIL7 induced proliferation of murine B cells and chicken primary lymphocytes isolated from the spleen and bursa. Moreover, treatment of chIL7 reduced infectious bursal disease virus burden in the bursa and morbidity in chickens challenged with infectious bursal disease virus (Huo et al., 2016a). As a potent adjuvant, it has been shown that chIL7 increased the immunogenicity of infectious bursal disease virus DNA and inactivated vaccines (Huo et al., 2016a,b; Cui et al., 2018) and recombinant coccidiosis DNA vaccines in *Eimeria acervulina*-infected chickens (Panebra and Lillehoj, 2019). Although, chIL7 plays an important role in immune protection, reliable

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bioassays are not available to detect and quantify bioactive IL7 in avian species owing to the lack of specific antibodies. In this study, we developed monoclonal antibodies (**mAb**) that specifically bind to chIL7 and a sandwich ELISA to quantify its levels in biological samples.

## MATERIALS AND METHODS

### **Production of Recombinant chIL7**

The coding region of chIL7 was amplified from a cDNA built of pooled tissues (intraepithelial lymphocytes, thymus, brain, heart, liver, bursa, and spleen) of *Eimeria maxima*-infected chickens by reverse-transcriptase PCR using restriction enzyme anchored specific primers (forward: 5'-CGGGATCCATGTCC-CATGCCTTTTTTGATCTA TCTTTC-3', reverse: 5'- CCAAGCTTTCACCTT-GAAATTATTTTTTCAAATTTATTCC-3', restriction enzymes, BamHI and HindIII, respectively, are underlined). Amplicon was cloned into pCR2.1 TOPO, sequenced and queried on BLAST database. After the sequence was confirmed, it was subcloned into pET32a (+) vector and expressed into *Escherichia coli* BL21 cells. BL21-expressed recombinant chIL7 (**rchIL7**) protein isolated on a Ni-NTA Sepharose column was used as an immunogen to generate anti-chIL7 mAb in C57BL/6 mouse (Jackson Laboratories, Bar Harbor, ME). Simultaneously, mammalian-expressed rchIL7 protein was obtained from pcDNA3.1(+) chIL7-transfected Chinese hamster ovary cell supernatant, purified by affinity chromatography and used in neutralization thymocyte proliferation assay. Both rchIL7 proteins were produced by GenScript Inc. (Piscataway, NJ), and their concentrations were quantified by bicinchoninic acid assay (Thermo Scientific Pierce, MA). Yield and purity of rchIL7 were evaluated on 12% sodium dodecyl sulfate/polyacrylamide gels. Human and mouse IL7 recombinant proteins (PeproTech, NJ) were used as specificity controls in chIL7 sandwich ELISA standardization.

### **Production and Purification of chIL7 mAb**

All procedures using mice including immunization and cell fusion were conducted by GenScript Inc. (Piscataway, NJ) (<https://www.genscript.com/genscript-received-olaw-office-of-laboratory-animal-welfare-approval-for-animal-welfare-assurance.html>). Briefly, BL21-expressed chIL7 (1.5-2 mg) were used for Balb/c mice (N = 5) prime and booster immunizations. Mice with higher anti-chIL7 antibody titers as determined by indirect ELISA were selected for fusion. Fused hybridomas secreting chIL7 mAb were grown, screened, and isotyped by indirect ELISA (Kim et al., 2017). *E. coli*-expressed chIL7 antigen (1 µg/well) was coated on 96-well microtiter plates overnight at 4°C, followed by blocking with PBS/1.0% BSA for 1 h and washed with PBS/0.05%

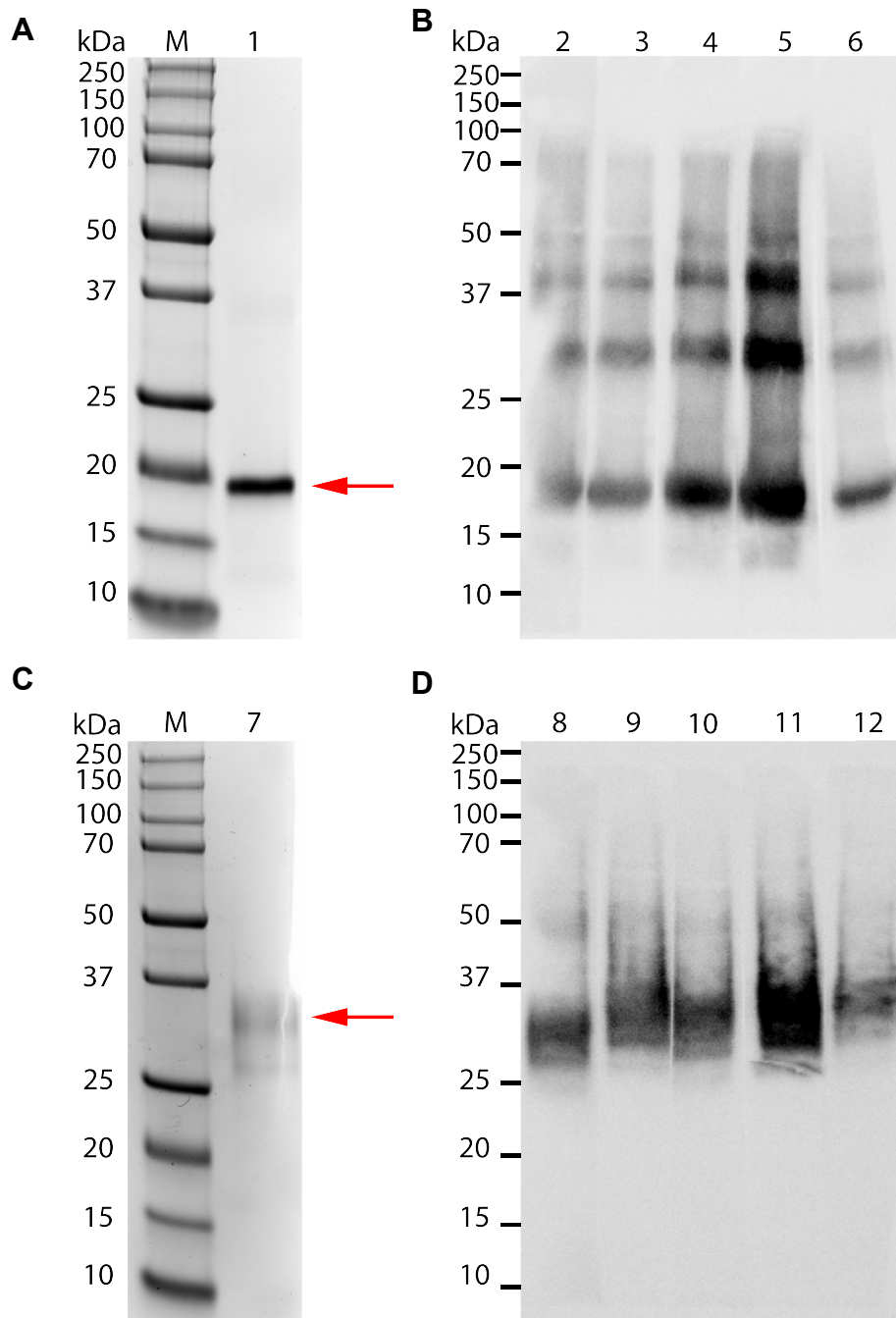
Tween 20 (PBS/T). After blocking, the plates were incubated at room temperature for 1 h with 100 µL/well undiluted hybridoma culture supernatant and then washed 5 times with PBS/T. Recombinant chicken IL4 was used as a negative control. Detection using horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (1/10,000 dilution), 3,3',5,5'-tetramethylbenzidine substrate, and H<sub>2</sub>O<sub>2</sub> (all from Sigma-Aldrich, St. Louis, MO) was carried out at room temperature for 20 min and the reaction stopped by adding 0.05 mL/well of 2N H<sub>2</sub>SO<sub>4</sub>. Optical density was measured at 450 nm using a microplate reader (BioTek, Winooski, VT). Reacting hybridomas were cloned by limiting dilution, and secreted mAb were further isotyped using an IsoQuick kit for Mouse Monoclonal Isotyping (Sigma-Aldrich, St. Louis, MO). Monoclonal antibodies purification from hybridoma cell culture supernatants were performed by affinity chromatography on Protein G agarose columns following the manufacturer's recommendation (Pierce, Rockford, IL). Purified mAb were biotinylated using an EZ-Link NHS-Biotin kit (Pierce, Rockford, IL) as per the manufacturer's instructions.

### **Western Blot Analysis**

*E. coli*-expressed chIL7 (1 µg/well) was separated on 12% sodium dodecyl sulfate/polyacrylamide and blotted onto polyvinylidene fluoride membrane. Blots were blocked using SuperBlock Blocking Buffer (Thermo-Fisher Scientific, Waltham, MA) and washed with 1X Tris/borate/saline buffer/0.05% Tween 20. Membranes were added to anti-chIL7 mAb at 1 µg/mL and incubated at 4°C overnight. After thorough washes, blots were added to goat anti-mouse IgG horseradish peroxidase-conjugated (Sigma-Aldrich, St. Louis, MO) (1:10,000) mixture and incubated at room temperature for 1 h. Blots were washed and immunoreactivity visualized by Clarity Western ECL Substrate and ChemDoc Imaging System (Bio-Rad, Hercules, CA).

### **Establishment of Sandwich ELISA**

All chIL7 mAb selected from screening step were tested as capture or detecting antibodies to identify compatible mAb sandwich pairs. To establish sandwich ELISA, each chIL7-purified mAb (10 µg/mL) was coated on a high-binding 96-well microtiter plates (Corning, MA) and incubated overnight at 4°C. The plates were thoroughly washed with PBS/T and blocked with 1% BSA/PBS and incubated at room temperature for 1 h. Afterward, either BL21-expressed chIL-7 recombinant protein (0.5 µg/mL) (standardization) or 0.1 mL serum sample (1:5 dilution in 0.1% BSA/PBS) was included and incubated for 2 h at 37°C. After washing with PBS/T, the plates were incubated with 0.1 mL biotin-labeled detecting mAb (1 µg/mL in 0.1% BSA/PBS) at 37°C for 1 h. The plates were again washed and later incubated with 0.1 mL avidin horseradish peroxidase in PBS/0.1%



**Figure 1.** Reactivity assessment of chicken IL7 mAb to BL21-derived or Chinese hamster ovary cells-derived recombinant chIL7 by Western blot. One  $\mu\text{g}$  per lane (1 and 7) BL21-derived (A) or Chinese hamster ovary cells-derived (C) rchIL7 were resolved in 12% SDS-PAGE and probed with the following chIL7 mAb: 2 and 8, 1B7; 3 and 9, 3D3; 4 and 10, 1H6; 5 and 11, 10H3; and 6 and 12, 4F12 (B and D). Abbreviations: mAb, monoclonal antibodies; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide.

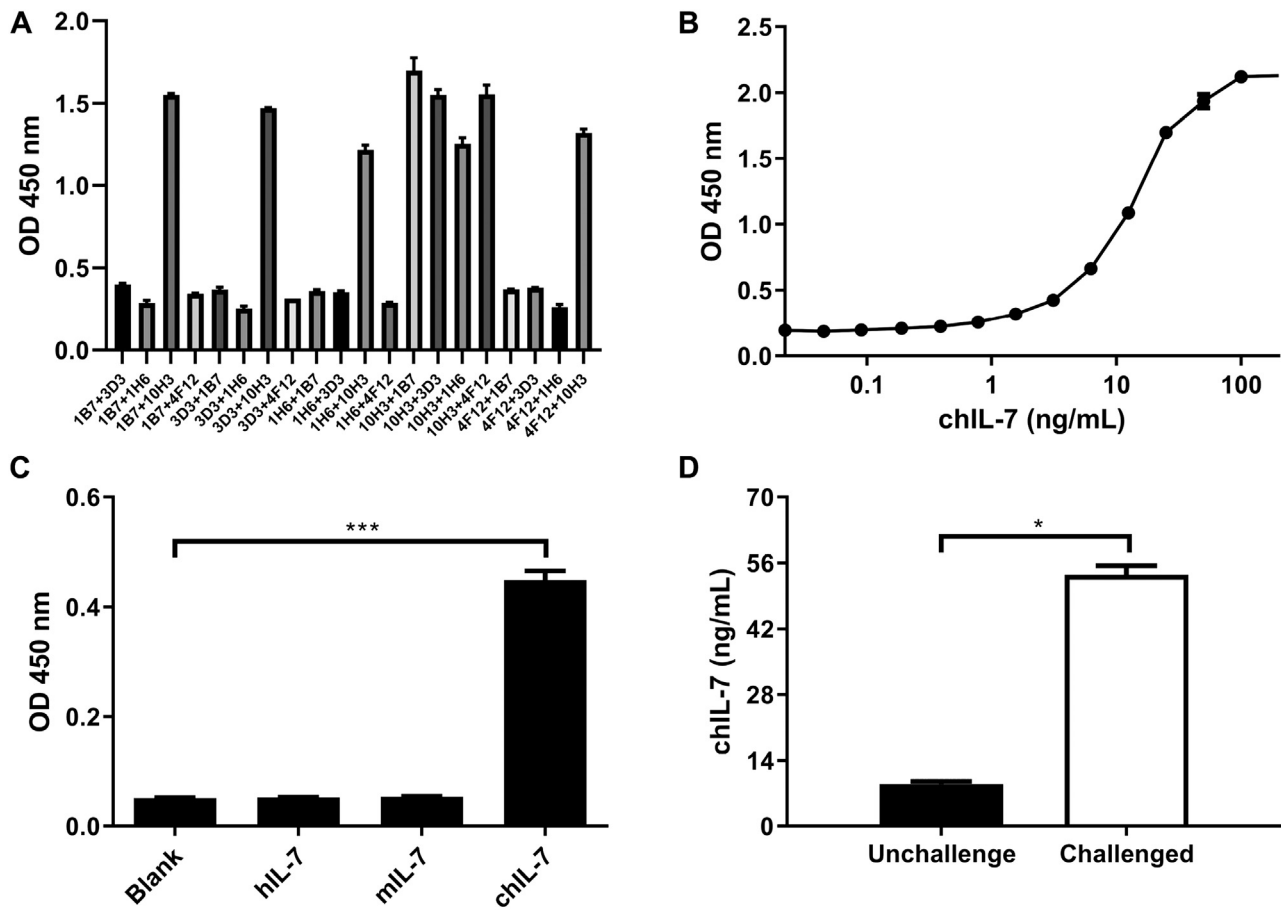
BSA (Sigma-Aldrich, St. Louis, MO) (1:10,000) at 37°C for 1 h and developed with Ultra 3,3',5,5'-tetramethylbenzidine peroxidase substrate solution (eBioscience, San Diego, CA) for 20 min and desired positive reaction stopped by addition of 0.05 mL 2N  $\text{H}_2\text{SO}_4$ . The optical density at 450nm was measured using a microplate reader ELx-800 (Biotek, Winooski, VT).

Sandwich ELISA sensitivity was assessed by a standard curve using chIL7 recombinant protein serial 2-fold dilutions from 200 ng to 22 pg. Meanwhile, specificity was evaluated using human and mouse IL7

recombinant proteins (both from PREPRO TECH, Rocky Hill, NJ) at 1  $\mu\text{g}/\text{mL}$ .

### **Neutralization of Thymocyte Proliferation Assay**

To evaluate if the newly developed mAb could neutralize chIL7-mediated thymocytes proliferation, an ex vivo thymocyte proliferation assay (Armitage et al., 1990; Panebra and Lillehoj, 2019) was used. The thymus



**Figure 2.** Pairing chIL7 mAb, sandwich ELISA, standard curve, specificity and detection of chIL7 levels in chicken unchallenged and challenge with *Eimeria acervulina*. (A) ChIL7 mAb pairing by sandwich ELISA. Plot of chIL7 mAb binding with different affinities to BL-21-derived chIL7 as assayed by sandwich ELISA. (B) ChIL7 sandwich ELISA standard curve using a mAb pair, 1B7 and biotinylated 10H3. (C) ChIL7 sandwich ELISA specificity. \*\*\* $P < 0.001$  as compared with the blank. (D) ChIL7 levels in bloodstream of chickens challenged by *E. acervulina*. Fourteen-day-old chickens were challenged with *E. acervulina* ( $1 \times 10^4$  sporulated oocysts), blood was collected at 9 d after challenge, and chIL7 protein levels were measured by sandwich ELISA. Samples were run in triplicates. \* $P < 0.05$  as compared with the unchallenged group. Data are represented mean  $\pm$  SD of triplicate from 3 independent experiments. Abbreviations: ChIL7, chicken IL7; mAb, mAb, monoclonal antibodies.

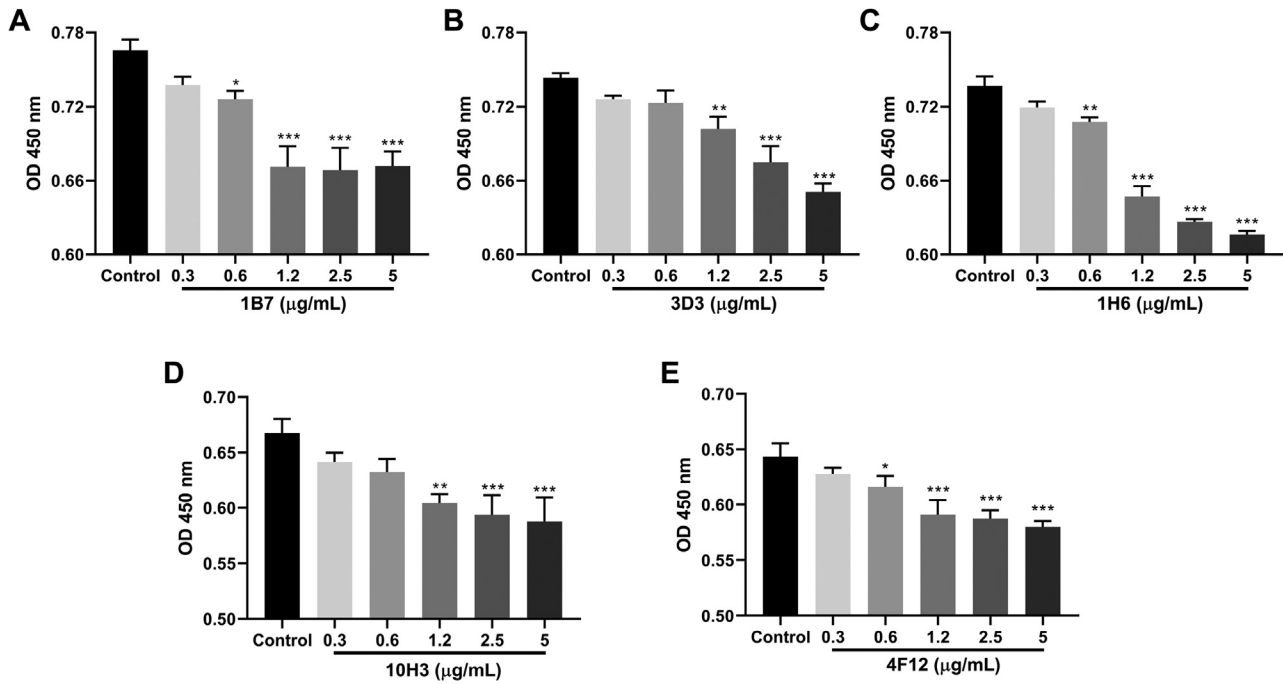
was excised from 2- to 4-wk-old healthy broiler chickens, trimmed, and sieved through a 100- $\mu$ m strainer. Enriched thymic cells were harvested by centrifugation at  $250 \times g$  for 10 min and washed twice with Hanks' Balanced Salt Solution/2% inactivated chicken sera (Sigma-Aldrich, St. Louis, MO). Thymic pellet was resuspended in complete media Roswell Park Memorial Institute 1640 containing 10% fetal bovine serum, 5% inactivated chicken sera, 1 mmol sodium pyruvate, 4 mmol glutamine, and Pen-Strep; layered slowly over Histopaque-1077 gradient and centrifuged at  $500 \times g$  for 30 min without brake. The thymocytes-enriched band was isolated, washed twice with complete media, and counted using trypan blue exclusion dye. Chinese hamster ovary-derived rchIL7 0.05 mL (0.1  $\mu$ g/mL) were preincubated (in triplicate) with 0.05 mL chIL7 mAb (1B7, 3D3, 1H6, 10H3 or 4F12; all at different concentrations ranging from 0.3 to 5  $\mu$ g/mL) at 41°C for 2 h. Then, they were added to the enriched thymocytes ( $2 \times 10^7$  cells/mL) and incubated at 41°C for 48 h. Afterward, Cell Counting Kit-8 reagent (Dojindo, Rockville, MD) was added (20  $\mu$ l/well), incubated at 41°C for 4 h, and optical density readings were recorded at 450 nm.

## Parasite Infection

Chickens were either unchallenged ( $N = 10$ ) or challenged ( $N = 10$ ) with  $1 \times 10^4$  sporulated oocysts/mL *E. acervulina* (ARS strain 12) by oral gavage on the third wk after hatch. One wk after *E. acervulina* challenge, chickens were euthanized by cervical dislocation, and blood samples were obtained by cardiac puncture. After overnight coagulation, serum samples were obtained by spinning down at  $1,000 \times g$  for 10 min, aliquoted, and stored at -20 °C. Chicken IL7 levels were monitored by sandwich ELISA as described previously. Animal trial procedures and experimental details were approved by the Beltsville Institutional Animal Care and Use Committee, Agriculture Research Services, USDA (Protocol numbers #18-019 and #19-018).

## Statistical Analysis

All data were expressed as mean  $\pm$  SD unless otherwise specified. Analyses were performed using the GraphPad Prism, version 5, software (GraphPad



**Figure 3.** Neutralization of chicken thymocytes proliferation. Chinese hamster ovary cells-derived rchIL7 and chIL7 mAb were preincubated and added to chicken thymocytes. The proliferation was measured with CCK-8 reagent. Data represented mean  $\pm$  SD of triplicate from 2 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$  as compared with the nontreated sample. Abbreviations: CCK-8, Cell Counting Kit-8; mAb, monoclonal antibodies.

Software Inc., La Jolla, CA). Statistical differences were evaluated by using 1-way ANOVA followed by Tukey's test. Differences were considered statistically significant when  $P$  values were  $< 0.05$ .

## RESULTS AND DISCUSSION

### Production of rchIL7 and Its mAb

Five mouse hybridomas (designated as 1B7, 1H6, 3D3, 4F12, and 10H3) secreting mAb specific for chIL7 protein were identified and cloned based on their strong ELISA reactivity in indirect ELISA. All clones were isotyped as IgG<sub>2a</sub> and light kappa chain (data not shown). The reactivity of mAb in Western blot is shown in Figures 1A and 1B. Recombinant chIL7 protein expressed in *E. coli* showed an expected molecular weight of 19 kDa, but mAb detected 19, 30, and 60 kDa suggesting dimer formation during the expression (Figures 1A and 1B). Another rchIL7 protein expressed from Chinese hamster ovary cells exhibited 30 kDa in size and smeared pattern in Western blot indicating highly glycosylated protein (Figures 1C and 1D). Taken together, our findings suggest that the mAb were successfully developed and specific for chIL-7.

### Pairing of mAb and Sandwich ELISA

Chicken IL7 mAb pairings were assessed in the sandwich ELISA format to find out the pairs that can be used for quantification of chIL7 by using purified unlabeled and biotinylated chIL7 mAb as capture and detecting antibodies, respectively. The assay

showed that 8 pairs (1B7/10H3, 3D3/10H3, 1H6/10H3, 10H3/1B7, 10H3/3D3, 10H3/1H6, 10H3/4F12, and 4F12/10H3, capture antibody/detection antibody) were found to be working in chIL7 quantification (Figure 2A). Further characterization in sandwich ELISA revealed that the sensitivity of the assay allowed us to detect the lowest levels of rchIL7 ( $\leq 1$  ng/mL), when 1B7 mAb was used as a capture antibody at 10  $\mu$ g/mL and biotinylated 10H3 was used as a detecting antibody at 1  $\mu$ g/mL (Figure 2B). It is also shown that our sandwich ELISA presented high specificity because neither human nor mouse recombinant IL7 protein (1  $\mu$ g/mL each) was recognized by any chIL7 mAb tested (Figure 2C). These data suggest that the pairs found in this study recognize different epitope on chIL7 so that they could specifically detect chIL7.

### Detection of chIL7 in Biological Samples

The newly developed sandwich ELISA was used to monitor chIL7 protein levels in sera samples. As avian coccidiosis has been known to elicit the B and T cell-mediated immune responses, we infected chickens with *E. acervulina* and collected sera samples to detect chIL-7 in biological samples. As expected, unchallenged control chickens showed around 10 ng/mL circulating chIL7, while chickens challenged with *E. acervulina* sporulated oocyst exhibited approximately 50 ng/mL chIL7 in serum (Figure 2D). These results indicate that the pair of mAb, 1B7 and biotinylated 10H3, could detect native circulating chIL7 in serum, and coccidiosis induced an increase in chIL7 at protein level.

## Neutralization of *chIL7* Activity by *Its* mAb

Chicken IL7 is well known as a potent regulator of lymphopoietic cellular proliferation (Schluns et al., 2000; Panebra and Lillehoj, 2019). To evaluate the activity of *chIL7* mAb for their neutralization activity, *rchIL7* (5 ng) was preincubated with *chIL7* mAb ranging from 0.3 to 5  $\mu$ g before stimulation of primary thymocytes. Figure 3 shows a dose-dependent neutralization of thymocytes proliferative response with all *chIL7* mAb ( $P < 0.05$ ).

In this study, considering the critical role of IL7 in T cell survival and its limited availability *in vivo*, we have developed a sensitive sandwich ELISA to monitor circulating *chIL7* levels in coccidiosis-infected chickens. The mAb against *chIL7* developed in this study could be valuable immune reagents to identify *chIL7*-secreting cells (e.g., flow cytometry and immunohistochemistry) or to quantify circulating levels (e.g., ELISA) in biological samples. Furthermore, *chIL7* mAb can be used to block *chIL7*-induced signal transduction pathway (i.e., JAK1, JAK3, Bcl-2, SOCS, and pSTAT5) and to monitor gene expression in target cells (e.g., Western blot and quantitative polymerase chain reaction). Further work is still needed to fully understand the role of *chIL7* on B cell or T cell development and proliferation and maintaining homeostasis of immune system in chickens.

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## DISCLOSURES

The authors declare no conflicts of interest.

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