

# Molecular cloning of chicken IL-7 and characterization of its antiviral activity against IBDV in vivo

Shanshan Huo,<sup>\*,†</sup> Liyue Wang,<sup>\*,†</sup> Yonghong Zhang,<sup>\*,†</sup> Jianlou Zhang,<sup>\*</sup> Yuzhu Zuo,<sup>\*</sup> Jian Xu,<sup>‡</sup> Dan Cui,<sup>\*</sup> Xiujin Li,<sup>‡,1</sup> and Fei Zhong<sup>\*,†,1</sup>

<sup>\*</sup>College of Veterinary Medicine, Agricultural University of Hebei, Hebei Engineering and Technology Research Center of Veterinary Biotechnology, Baoding 071001, China; <sup>†</sup>State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, 730046, China; and <sup>‡</sup>Department of Biotechnology, College of Environmental and Chemical Engineering, Yanshan University, Qinhuangdao 066004, China

**ABSTRACT** Mammalian interleukin-7 (IL-7) is able to stimulate lymphocyte proliferation and maturation, and reverse immunosuppression. However, whether poultry IL-7 has similar functions remains unclear. Chicken infectious bursal disease virus (IBDV) causes serious immunosuppression in chicken due to virus-induced immune disorder. Whether chicken IL-7 (chIL-7) has the ability to restore the immunity during IBDV-induced immunosuppression is not clear. To test this, we amplified chIL-7 gene by RT-PCR, prepared recombinant chIL-7 using HEK293T cells and treated the

chicken with the chIL-7 prior to IBDV infection. Our results indicate that chIL-7 promoted mouse B cell proliferation in vitro, and significantly reduced virus titer in bursal tissue and chicken morbidity of IBDV-infected chicken. Mechanically, chIL-7 induced chicken lymphocyte proliferation and interferon- $\gamma$  production, but down-regulated TGF- $\beta$  expression, suggesting that chIL-7 has the ability to reverse IBDV-induced immunosuppression and might be a potential therapeutic agent for prevention and treatment of infectious bursal disease.

**Key words:** chicken interleukin-7, eukaryotic expression, IBDV, prevention, treatment

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## INTRODUCTION

Interleukin 7 (IL-7), an important cytokine derived from thymic stromal cells, was first identified as a pre-B cell growth factor (Namen et al., 1988; Goodwin et al., 1989; Namen et al., 1998), which is essential for initiating and maintaining activities of immune and hematopoietic systems (Chazen et al., 1989; Schluns and Lefrançois, 2003). IL-7 is crucial for B cell differentiation, proliferation, maturation, and maintenance (Komschlies et al., 1995; Namen et al., 1998), and can thus facilitate the recovery of B cell number after immunological stresses, such as radiation and immunosuppression (Chazen et al., 1989; Miln and Paige, 2006). Consistently, blocking IL-7 by antibody results in rapid

reduction of B cell number in bone marrow and thymus (Grabstein, et al., 1993). In addition to its role in B cells, IL-7 also stimulates T cell development, proliferation, and homeostatic regulation (Hickman et al., 1990; Fry and Mackall, 2001). Due to its potent immunity-stimulating ability, recombinant IL-7 has been widely used in humans to treat a number of disorders with immunosuppressive properties, such as AIDS (Beq et al., 2004; Leone et al., 2009), hepatitis (Hou et al., 2015), and cancer (Sportès et al., 2010). In line with this notion, it was shown that recombinant human IL-7 significantly increases CD4<sup>+</sup> and CD8<sup>+</sup> cell proliferation in HIV-infected patients (Ferrari et al., 1995; Levy et al., 2009). Although mammalian IL-7 can promote lymphocyte proliferation and maturation, it remains elusive whether poultry IL-7 possesses the similar biological functions as its homologs in mammals.

Infectious bursal disease (IBD) is one of the most serious infectious diseases caused by infectious bursal disease virus (IBDV) (Müller et al., 2003) in the poultry industry. IBDV attacks the chicken immune system by destroying B and T cells, leading to lymphocyte decline and subsequent systemic immune dysfunction (Sharma et al., 2000). The failure of maintaining lymphocyte numbers and populations in IBDV-infected chicken increases the infection susceptibility to other

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<sup>1</sup>Corresponding author: [Lixiujin9824@aliyun.com](mailto:Lixiujin9824@aliyun.com) (XL); [feizhong2013@126.com](mailto:feizhong2013@126.com) (FZ)

pathogens, resulting in chicken death due to secondary infection (Ingrao et al., 2013). So far, no effective treatment is available to control this disease. Given that mammalian IL-7 has strong immunostimulating properties, we hypothesized that poultry IL-7 is able to antagonize IBDV-induced immunosuppression and thus possess anti-IBDV activity. In this study, we amplified chicken IL-7 gene, prepared recombinant chIL-7, evaluated its anti-IBDV activity in vivo and investigated the molecular mechanism involved.

## MATERIALS AND METHODS

### Cells, Viruses and Chickens

Human embryonic kidney (HEK) 293T cells were purchased from the Cell Culture Center of Peking Union Medical University (Beijing, China). Mouse immature B lymphocytes (2E8 cells) and UMNSAH/DF-1 chicken embryonic fibroblast cells (DF-1 cells) were purchased from ATCC (Manassas, VA). The virulent IBDV strain HB-1 serotype-1 were kindly presented by Dr. Wei-quan Liu in China Agricultural University. Specific pathogen-free (SPF) chickens (21-day-old White Leghorn layers) were purchased from a local chicken farm. The chickens were kept in the specific isolator in an environmentally controlled room with a 12/12 hour light/dark cycle. Animal experiments were performed based on the Guide for the Care and Use of Laboratory Animals of the Agricultural University of Hebei.

### chIL-7 Gene Amplification and Vector Construction

Chicken IL-7 gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) from chicken spleen with following primers (AM931037). Forward: 5'CGGGGTACC(*KpnI*)ACCATGTCCCCATGCCTTTTTTATGATC and reverse: 5'CCCGCTCGAG(*XhoI*)ACACCTTGAAATTATTTTTTAAATTTATTC. The PCR cycling conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1.5 min, followed by final extension at 72°C for 10 min. The amplified PCR product was cloned into a T-vector pMD19-T(TaKaRa) and then transferred into pcDNA3.1A plasmid to construct Myc/His-tag-fused chIL-7 gene eukaryotic expression vector, pcDNA-chIL-7/MH.

### Expression and Purification of Recombinant chIL-7

HEK293T cell transfection, recombinant chIL-7 expression and purification with Ni-NTA agarose beads were performed as previously described (Wen et al., 2013). Briefly, the pcDNA-chIL-7/MH plasmids were transfected into HEK293T cells for transient expression mediated by calcium phosphate method. After expres-

sion for 48 h in the expression medium (100 µg/mL of BSA and 176 µg/mL butyric acid in Dulbecco's modified Eagle's medium [DMEM]), the expressed chIL-7 was absorbed with Ni-NTA agarose beads. After washing with the wash buffer (20 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8.0). Bound chIL-7 was eluted with elution buffer (20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 8.0). The eluted chIL-7 was dialyzed against PBS (pH 8.0) to eliminate the imidazole.

### Western Blot

The recombinant chIL-7 in the culture medium was detected with Western blot as previously described (Wen et al., 2013) using Anti-Myc mAb (Santa Cruz Biotechnologies). For the intracellular chIL-7 detection, the transfected cells were lysed with lysis buffer (5 mM Tris-HCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 1% NP-40, 150 mM NaCl and protease inhibitors) and then subjected to Western blot described as above.

### Identification of chIL-7 Bioactivity

The bioactivity of recombinant chIL-7 was determined using mouse immature B lymphocyte 2E8, an IL-7-dependent proliferation-cell line (Van der Spek et al., 2002). The cells were cultured in RPMI-1640 complete medium at 37°C and 5% CO<sub>2</sub> in-air atmosphere. The cells were seeded into 96-well flat-bottomed plate at the density of 3×10<sup>4</sup> cells/well with 100 µL of VIVO-15 serum-free medium. Different doses (30, 60, 90, 120, 150 pg/mL) of human IL-7 as positive controls and chIL-7 were individually incubated with the 2E8 cells for 48 h at 37°C. The cell proliferation index (PI) was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and the chIL-7 bioactivity was evaluated based on the PI values.

### Lymphocyte Proliferation Assay

Mouse 2E8 cells, or the splenic and bursal lymphocytes isolated from different treated chickens (IBDV-infected, chIL-7-treated IBDV-infected, and uninfected chickens) were stimulated with chIL-7 (0, 0.1, 0.2, 0.5 µg/mL) or concanavalin A (Con A, 100 ng/mL) for 48 h in a 96-well plate. Lymphocyte proliferation was measured by MTT method.

### Enzyme-Linked Immunosorbent Assay (ELISA)

Chicken interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) in the cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using Chicken IFN-γ ELISA kit (HENGFELI, China) and Chicken TGF-β ELISA kit (FKBIO, China), following the manufacturer's instructions.

## Real-time PCR

Chicken IFN- $\gamma$  and TGF- $\beta$  mRNA expressions in chicken splenic tissues were measured by qRT-PCR. Total RNA was purified using Trizol reagent (Invitrogen) from splenic tissues. The cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) with the iCycler iQ RT-PCR detection system (Bio-Rad), with 40 cycles of real-time data collection at 95°C for 30 s, 50°C (IFN- $\gamma$ ) or 62°C (TGF- $\beta$ ) for 1 min and 72°C for 1 min, followed by melt-curve analysis to verify the presence of a single product, using the designed primers, IFN- $\gamma$  (GenBank: GQ421600): sense 5'CTGCAGGTAGTCTAAATCTTGTTTC, antisense 5'TGTCCAGTTCTTCAGTTTCTCT AC; TGF- $\beta$  (GenBank: JQ423909): sense 5'GGAGGCGGCCAA AAAGAAAC, antisense 5'CCAATACTCATCGGGT CCATCC.

## Virus Propagation and Titer Determination

IBDV were propagated in DF-1 chicken embryonic fibroblast cells as previously described by Kibenge et al. (1997). Briefly, DF-1 cells were grown in DMEM complete medium at 37°C in a 5% CO<sub>2</sub> in-air atmosphere and infected with the pathogenic IBDV strain when the cell density reached 80 to 90% confluence. The cells were harvested at 96 h post infection, the IBDV were released from the cells through more than 3 freeze-thaw cycles and purified using the modified method of Dobos (Dobos et al., 1979). Virus titers were determined by Reed-Muench method and expressed as the 50% tissue culture infective dose (TCID<sub>50</sub>).

## Animal Experiments

A total of 192 SPF white Leghorn layers (21-day-old) were randomly divided into 8 groups of 24 birds each. Chickens in group 1 and 5, as the controls, were pre-treated with bovine serum albumin (BSA) at 2.5 mg/kg/day for 12 d. Chickens in groups 2, 3, and 4, and groups 6, 7, and 8 were pre-treated with chIL-7 at 1, 2.5, and 5 mg/kg/d for 12 d, respectively. All of the chickens were challenged by intramuscular injection with virulent IBDV (0.1 mL of  $5 \times 10^6$  TCID<sub>50</sub>) at 3 d after chIL-7 (or BSA) treatment. Chickens in groups 1 to 4 were kept for clinical and mortality observation. IBDV titers in the chicken bursal tissue in groups 5 to 8 at the different time (0, 2, 4, 6, 8 d) post infection were measured with TCID<sub>50</sub>. The lymphocyte proliferation, IFN- $\gamma$ , and TGF- $\beta$  productions in splenic and bursal tissues at 8 d post infection were analyzed by MTT and ELISA, respectively.

## Chicken Splenic and Bursal Lymphocyte Isolation and Culture

Chicken splenic and bursal lymphocyte isolation was performed as previously described with slight modifica-

tions (Compton and Wickliffe, 1999). Briefly, splenic or bursal tissue was aseptically removed from the chicken and minced into small pieces in RPMI-1640 with sterilized scissors. The pieces were crushed through a cell sieve with a plunger. The homogenate was filtered through 100- $\mu$ m nylon mesh and washed twice with PBS. The lymphocytes were separated by density gradient centrifugation over Ficoll-Hypaque and washed twice with RPMI-1640, and cultured in RPMI-1640 complete medium in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

## Statistics

The significance of differences between experimental groups was evaluated by one-way analysis of variance (ANOVA) with Dunnett's post-comparison test for multiple groups and Student's *t*-test was used for a single comparison of the 2 groups, respectively. All experiments were performed at least 3 times.

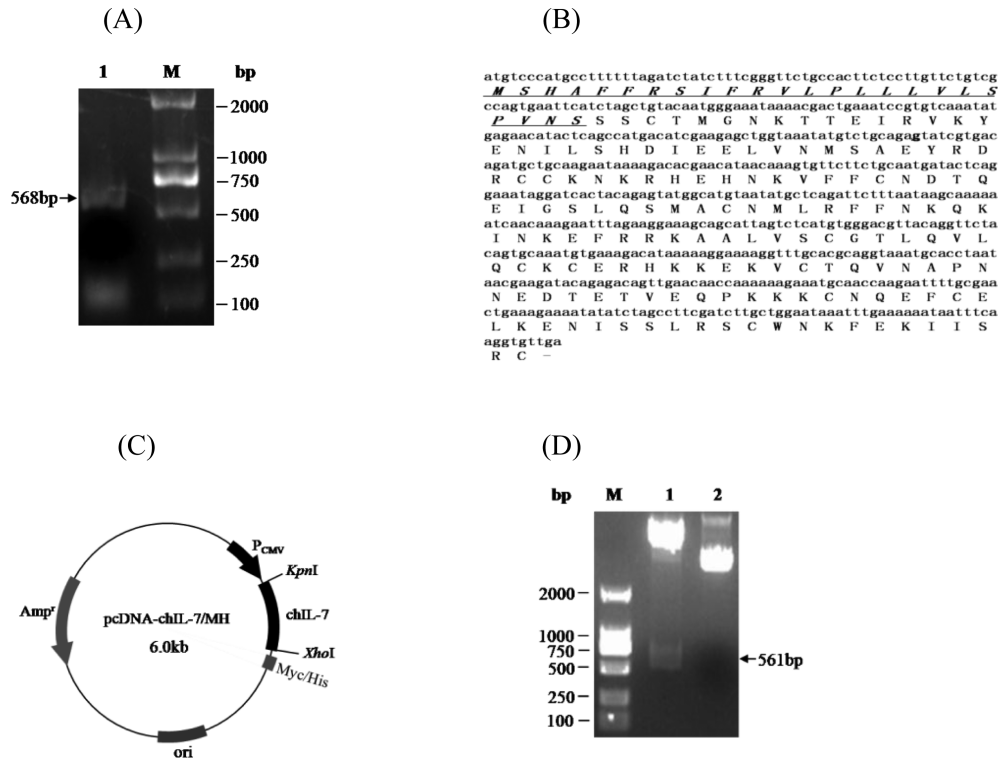
## RESULTS

### Chicken IL-7 Gene Amplification, Sequence Analysis and Expression Vector Construction

We amplified *chIL-7* gene (568 bp) from chicken spleen by RT-PCR (Figure 1A) and then cloned it into the pMD19-T vector. The sequencing results showed that the cloned *chIL-7* gene was consistent with the sequence in GenBank except for one base substitution (A<sub>171</sub>→G<sub>171</sub>), which, however, did not alter encoded amino acid (Figure 1B). By comparison with other species, we found that chIL-7 amino acid sequences showed high homology with other avian species, about 60 - 99% (Table 1), but low homology with non-avian species (about 30%) (Table 1). chIL-7 gene in pMD19-T vector was transferred into pcDNA3.1A plasmid to construct Myc/His-tag-fused chIL-7 eukaryotic expression vector, pcDNA-chIL7/MH (Figure 1C). The restriction analysis showed that the expression vector was correct (Figure 1D).

### Recombinant chIL-7 Can Be Expressed in a Secretory Manner in HEK293T Cells.

To test whether pcDNA-chIL7/MH vector can mediate chIL-7 secretory expression, we used HEK293T cells to express chIL-7, purified with Ni-NTA Agarose beads and identified with Western blot. As shown in Figure 2A and 2B, the specific hybridized band (about 25 kDa) were detected in the culture supernatant from pcDNA-chIL7/MH-transfected cells, but not from pcDNA3.1A empty vector-transfected cells, indicating that the chIL-7 vector could mediate chIL-7 expression in eukaryotic cells in a secretory manner. The size of the recombinant chIL-7 was bigger than that of the calculated molecular weight (18 kDa) based on amino acids, suggesting the chIL-7 might be a



**Figure 1.** ChIL-7 gene amplification and its eukaryotic expression vector construction. (A) Chicken IL-7 cDNA gene amplified by RT-PCR: Lane 1, IL-7 gene PCR product; M, DL2000<sup>TM</sup> DNA marker. (B) The gene sequence of chicken IL-7 and its encoded amino acids: The italic letters present the signal peptide. (C) Eukaryotic expression vector of chIL-7 gene driven by CMV promoter. (D) Restriction analysis for the chIL-7 expression vector: Lane 1, *Xho* I and *Kpn* I double digestion; Lane 2, chIL-7 expression vector; M, DL2000<sup>TM</sup> DNA marker.

**Table 1.** Homology of chIL-7 with non-avian and avian species.

Species	GenBank No.	Homology (%)
Non-avian animals		
Human	J04156.1	30
Mouse	NM_008371	32
Ovine	NM_173924	31
Porcine	NM_214135	31
Avian animals		
Chicken	AM931037	99.8
Cristata	XM_009703214	98
Apteryx	LK064683	72

glycosylated protein since 4 potential N-glycosylated sites exist in chIL-7 sequence. To check intracellular recombinant chIL-7 in the HEK293T cells, the transfected cells were lysed with cell lysis buffer and subjected to Western blot. Results in Figure 2A showed that the recombinant chIL-7 with the similar size were detected both in cell lysate and culture medium (Figure 2C).

### Recombinant chIL-7 Promotes Lymphocyte Proliferation

To determine whether the recombinant chIL-7 possesses bioactivity, we took advantage of the MTT method to measure proliferation of mouse immature B lymphocyte 2E8 with or without chIL-7 treatment. Results showed that the chIL-7 dose-dependently in-

creased 2E8 B lymphocyte stimulation index (Figure 3), indicating that recombinant chIL-7 is biologically active.

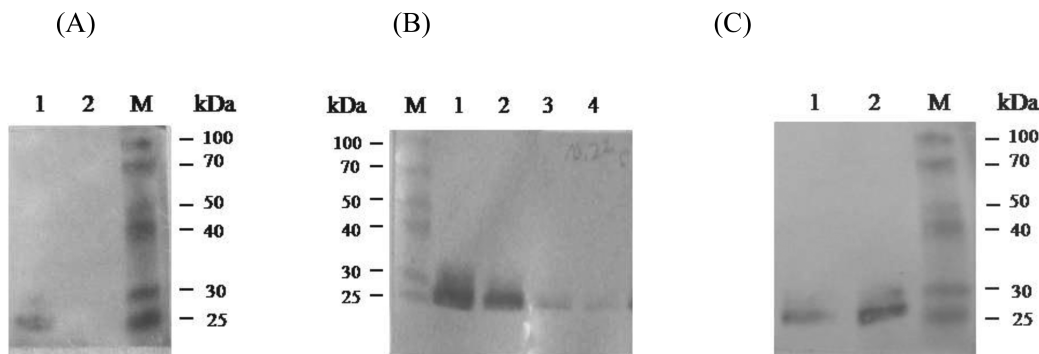
### ChIL-7 Promotes Chicken Immunity Against IBDV

To investigate the anti-IBDV immunity of recombinant chIL-7 in vivo, we pre-treated chickens with different doses of chIL-7 for 12 d, followed by IBDV infection at 3 d after chIL-7 treatment. IBDV titers in the bursal tissue under the different doses of IL-7 treatment at the different time were measured. The mortality of the infected chickens was calculated at 2, 4, 6, 8 d after IBDV infection. Results showed that chIL-7 dose-dependently reduced IBDV titers in the bursal tissues during the tested periods compared with that of untreated chickens (Figure 4A), suggesting that chIL-7 could inhibit IBDV replication in vivo. Moreover, chIL-7 also drastically reduced the mortalities of the IBDV-infected chickens (Figure 4B), suggesting the therapeutic potential of chIL-7 against IBDV.

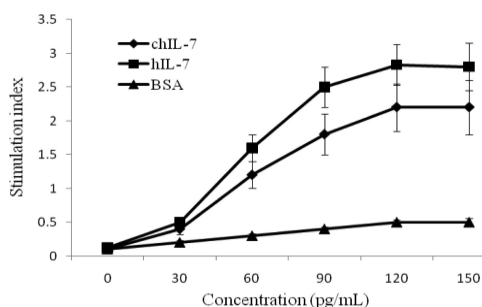
### ChIL-7 Stimulates Chicken Lymphocyte Proliferation and IFN- $\gamma$ Production

To investigate the effects of chIL-7 on lymphocyte proliferation and IFN- $\gamma$  production, the bursal and splenic lymphocytes were isolated from the





**Figure 2.** Detection of the recombinant chicken IL-7 by Western Blot. (A) Detection of chIL-7 in the culture medium from pcDNA-chIL7/MH vector-transfected cells (Lane 1) and pcDNA3.1A empty vector-transfected cells (Lane 2); M, prestained protein markers. (B) Purified chIL-7 proteins eluted from Ni-NTA Agarose column: M, prestained protein markers; Lanes 1-4, elutes 1-4 of chIL-7. (C) Detection of chIL-7 from the cell lysate (Lane 1) and the culture medium (Lane 2); M, prestained protein markers.



**Figure 3.** The stimulation index of chIL-7 on 2E8 cells at different doses measured by MTT.

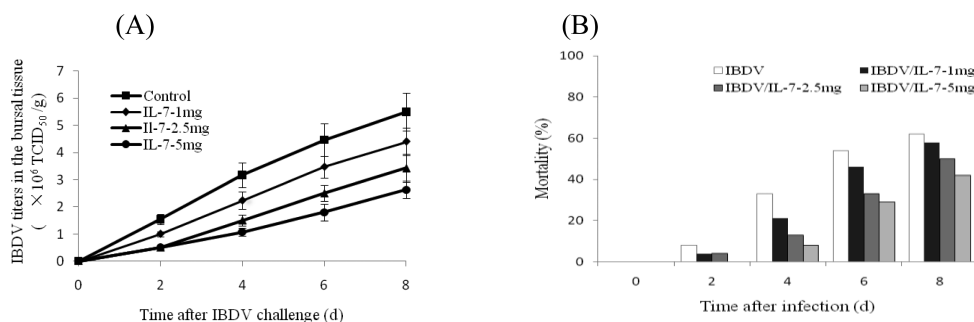
untreated chickens and stimulated with different doses of chIL-7 for 48 h. As shown in Figure 5A and B, chIL-7 induced proliferation of bursal and splenic lymphocyte in a dose-dependent manner. To further confirm this *ex vivo*, the isolated splenic lymphocytes from chIL-7-treated IBDV-infected chickens were stimulated with Con A (100 ng/mL) for 48 h. Consistent with the *in vitro* results, chIL-7 also promoted lymphocyte proliferation of IBDV-infected chickens (Figure 5C).

To test whether chIL-7 can stimulate IFN- $\gamma$  production from chicken lymphocytes *in vitro*, we treated splenic lymphocytes from naive chickens with chIL-7 and found that chIL-7 dose-dependently promoted IFN- $\gamma$  production (Figure 5D). To confirm this *in vivo*, we analyzed the IFN- $\gamma$  production in the chicken splenic

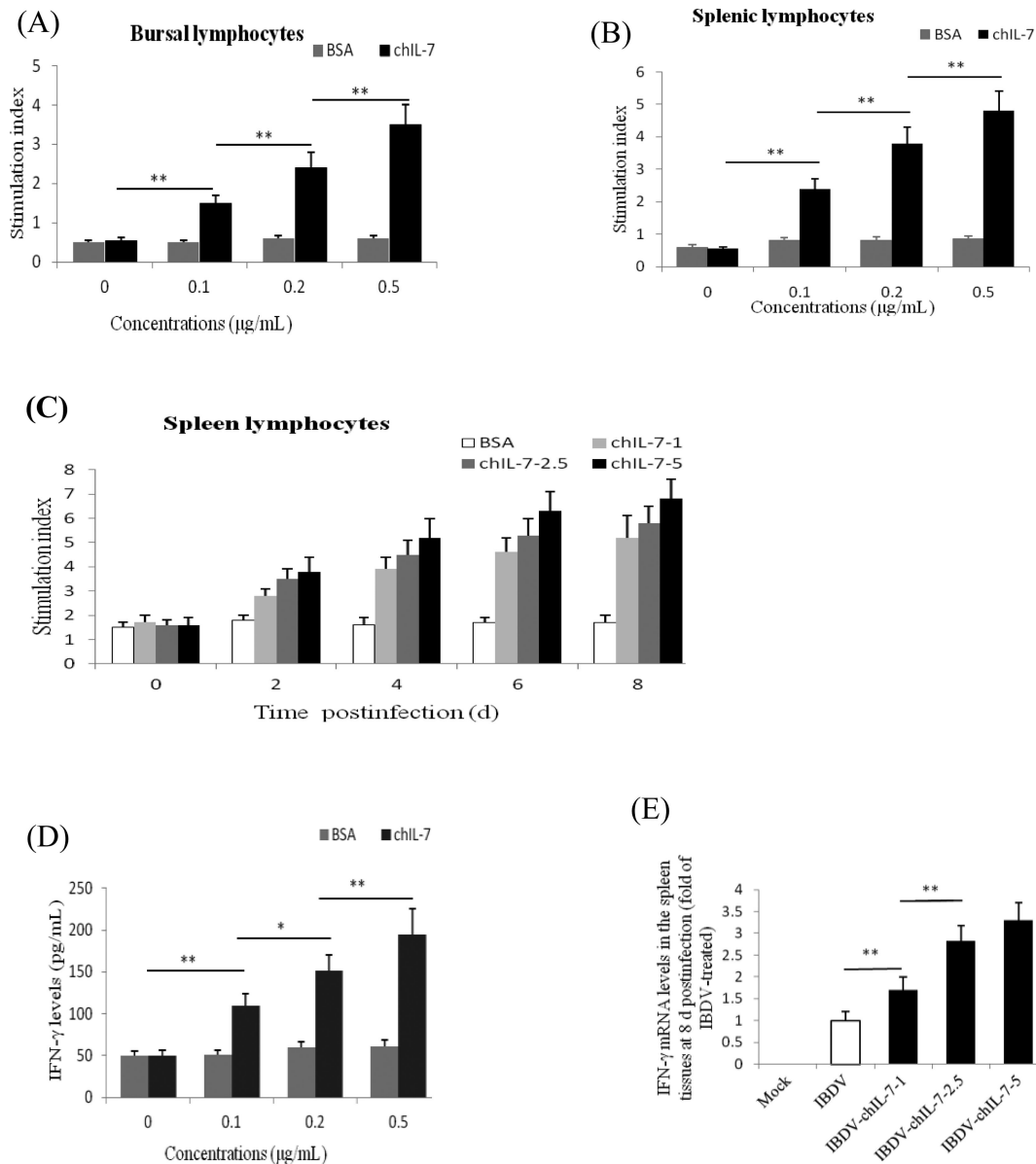
tissues at 8 d after IBDV infection with Real-time PCR and showed that chIL-7 also stimulated IFN- $\gamma$  production in the splenic tissues in a dose-dependent manner (Figure 5E). These results collectively suggest that chIL-7 stimulates chicken lymphocyte proliferation and IFN- $\gamma$  production, which might contribute to its anti-IBDV ability in chickens.

### Chicken IL-7 Suppresses Chicken TGF- $\beta$ Expression

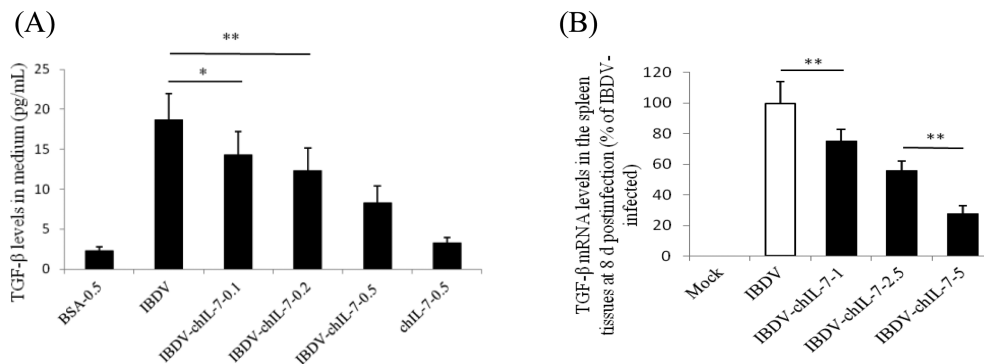
TGF- $\beta$  is a potent immunosuppressive cytokine regulating immune homostasis (Akhurst and Hata, 2012). Previous report showed that IBDV could stimulate TGF- $\beta$  expression in chicken lymphoid tissues, which was thought to promote IBDV-induced immunosuppression in chickens (Sharma et al., 1994; Eldaghayes et al., 2006). We reasoned that chIL-7 might inhibit IBDV-induced TGF- $\beta$  expression, resulting in enhancement of antiviral activity against IBDV. To test this, we treated splenic lymphocytes from naive chickens with chIL-7 and analyzed TGF- $\beta$  concentration in the culture supernatant by ELISA. Meanwhile, we analyzed TGF- $\beta$  mRNA levels in splenic tissues of chIL-7-treated IBDV-infected chickens 8 d post infection by real-time PCR. Consistent with ELISA results, chIL-7 significantly inhibited TGF- $\beta$  expression in splenic lymphocytes in a dose-dependent manner *in vitro* (Figure 6A),



**Figure 4.** The effects of chIL-7 on IBDV titers in bursal tissues and survival of IBDV-infected chicken. (A) The effects of chIL-7 on IBDV titers in bursal tissues of IBDV-infected chicken. (B) The effects of chIL-7 at different doses (1, 2.5, and 5 mg/kg/d) on the mortality of IBDV-infected chicken. The experimental data are presented as means  $\pm$  standard deviation.



**Figure 5.** The effects of chIL-7 on lymphocyte proliferation and INF- $\gamma$  production. Effects of chIL-7 on bursal (A) and splenic (B) lymphocyte proliferations isolated from IBDV-uninfected chicken measured by MTT. (C) Effects of chIL-7 on splenic lymphocyte proliferation isolated from IBDV-infected chicken, stimulated with Con A for 48 h, measured by MTT. (D) Effects of chIL-7 on INF- $\gamma$  productions in the culture medium of the splenic lymphocytes isolated from IBDV-uninfected chicken measured by ELISA. (E) Effects of chIL-7 on INF- $\gamma$  mRNA expression in splenic tissues of IBDV-infected chicken measured by real-time PCR. The experimental data are presented as means  $\pm$  standard deviation. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .



**Figure 6.** The effects of chIL-7 on chicken TGF- $\beta$  expression in vitro and in vivo. (A) Effects of chIL-7 on TGF- $\beta$  expression of splenic lymphocytes isolated from IBDV-uninfected chicken measured by ELISA. (B) Effects of chIL-7 on TGF- $\beta$  mRNA levels in splenic tissues of IBDV-infected chicken for 8 d measured by real-time PCR. The experimental data are presented as means  $\pm$  standard deviation. \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

and also inhibited TGF- $\beta$  expression in splenic tissues of IBDV-infected chickens *ex vivo* (Figure 6B). These results indicate that chIL-7 might possess the capacity to inhibit TGF- $\beta$ -mediated immunosuppression, which promotes its anti-IBDV immunity in chickens.

## DISCUSSION

In this study, we cloned chIL-7 cDNA gene, prepared recombinant chIL-7 using HEK293T eukaryotic cell line, analyzed its regulatory functions on splenic lymphocyte proliferation and IFN- $\gamma$  production, and evaluated its antiviral activity against IBDV *in vivo*. Our results indicated that, like non-avian species, chIL-7 has the ability to stimulate lymphocyte (2E8 B cells) proliferation and also suggested that the mouse 2E8 B cells can be used to evaluate chIL-7 biological activity since the mouse 2E8 B cells have been extensively used to test human and mouse IL-7 biological activity (van der Spek et al., 2002). The HEK293T cell, a eukaryotic cell line, was identified to be a suitable expression cell line for recombinant chIL-7 preparation. We initially used *E. coli* expression system to prepare the chIL-7, but found that its biological activity was very low (data not shown), probably due to the lack of post-translation modification in the *E. coli* system.

We found that chIL-7 could stimulate mouse 2E8 B cells and chicken bursal and splenic lymphocyte proliferation and IFN- $\gamma$  production, suggesting that chIL-7, a multifunctional cytokine, has the capacity to promote B and T cell-mediated immune responses, which might contribute to the chicken immune restoration from immunosuppression.

IBD is a viral immunosuppressive disease caused by IBDV. The IBDV not only destroys B cell population (Hulse and Romero, 2004), but also attacks T cells by inducing T cell-mediated cellular immunosuppression (Yeh et al., 2002; Ingraio et al., 2013; Wang et al., 2014). Therefore, to stimulate and restore B and T cell-mediated immune responses in IBDV-induced immunosuppression in chicken is critical for IBD prevention and treatment (Rautenschlein et al., 2002). Coincidentally, chIL-7 could promote B and T cell immune responses by stimulating their proliferation and IFN- $\gamma$  production, which presumably promotes its antiviral activity against IBDV *in vivo*. Furthermore, chIL-7 also inhibits TGF- $\beta$  expression, which at least partially explains the immunity-promoting effect of chIL-7. Similar to human IL-7, chIL-7 is capable of promoting host immunity and antagonizing immune suppression by stimulating lymphocyte proliferation and IFN- $\gamma$  production, and retarding inhibitory cytokine expression. Moreover, as IBDV-induced TGF- $\beta$  expression in chicken lymphoid organs is crucial for IBDV-induced immunosuppression, the chIL-7-mediated inhibition of TGF- $\beta$  expression should help to restore chicken antiviral immunity against IBDV. Together, these results suggest that chIL-7 might be a potential

therapeutic agent for certain virus-induced immunosuppression diseases in chickens.

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