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Cloning, in vitro expression and bioactivity of duck interleukin-18

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Abstract

The encoding sequence for duck *IL-18* was obtained, using reverse transcription-polymerase chain reaction, from mRNA harvested from Con A-stimulated Gushi (GS) duck splenic mononuclear cells. Recombinant duck IL-18 (rduIL-18) was produced in a prokaryotic expression system. In vitro bioactivity of rduIL-18 was determined in a lymphocyte proliferation assay and in vivo bioactivity of rduIL-18 was assessed by addition to a vaccine. Monoclonal antibody (mAb) and polyclonal antibodies (pAbs) specific for rduIL-18 were generated and subsequently characterized by ELISA, Western blot and neutralizing assays. Sequence analysis of GS duck *IL-18* demonstrated an open reading frame (ORF) of 603 base pairs encoding for a 200 amino acid precursor protein. The duck encoding sequence shares 85.3% similarity to the chicken equivalent, at the nucleotide level. A His-duIL-18 fusion protein was recognized in Western blot by mAbs against duck and chicken IL-18 (chIL-18), but not by mAb against human IL-18. Recombinant duIL-18 induced in vitro proliferation of Con A-stimulated duck splenocytes and enhanced the immune response of ducks vaccinated with an inactivated oil emulsion vaccine against avian influenza virus. PAb and mAb 5B2 against rduIL-18 had neutralizing ability, inhibiting the biological activities of both recombinant duIL-18 and endogenous duIL-18. The results indicate that rduIL-18 has the potential to be used as an immunoadjuvant, and the mAb against rduIL-18 further facilitates basic immunobiological studies of the role of IL-18 in the avian immune system.

Keywords: Duck; Interleukin-18; In vitro expression; In vivo bioactivity

1. Introduction

Interleukin-18 (IL-18), previously known as interferon-gamma (IFN-γ) inducing factor (IGIF), is an important cytokine with multiple functions in innate and acquired immunity (Dinarello and Fantuzzi, 2003; Gracie et al., 2003). Structurally, IL-18 is closely related to IL-1 and, like IL-1β, is synthesized as a biologically inactive precursor protein lacking a typical signal peptide. This molecule is cleaved by the proteolytic enzyme, caspase-1 (IL-1β converting enzyme, ICE), to

Abbreviations: aa, amino acid(s); cDNA, complementary DNA; DTT, methyl thiazolyl tetrazolium; Con A, concanavalin A; IFN, interferon; IL, interleukin; MW, molecular weight; NK, natural killer; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; mAb, monoclonal antibody; pAbs, polyclonal antibodies; HRP, horseradish peroxidase; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

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produce bioactive mature IL-18. Caspase-1 cleavage has been found to facilitate the secretion of mature IL-18 from the cytosol across the cell membrane (Fantuzzi and Dinarello, 1999; Gu et al., 1997).

Functionally, IL-18, similar to IL-12, facilitates Th1 immune responses. However, depending on the cytokine constellation, IL-18 may also promote Th2-type responses (Nakano et al., 2003; Pollock et al., 2003) and possibly antibody formation. Especially in the induction phase of an immune response, evoked either by infection or adjuvant-triggered immunization, IL-18 can act as an endogenous pro-inflammatory initiator of immune responsiveness (Wei et al., 2004; Akhiani et al., 2004; Kinoshita et al., 2004). In addition, the biological effects of IL-18 include its ability to induce T and NK cells to produce IFN-γ synergistically with IL-12 (Robinson et al., 1997; Micallef et al., 1996).

Schneider et al. (2000) were the first to obtained chicken *IL-18* cDNA and expressed in *Escherichia coli*. Expression-induced chicken spleen cells to produce IFN-γ, proving that chicken IL-18 has biological activity. Further research on chicken IL-18 has also been reported (Degen et al., 2005; Gobel et al., 2003; Puehler et al., 2003; Kaiser, 2002). Besides the two cDNA sequences of White Pekin duck (WP) and White Pekin super M2 hybrid (WM) duck IL-18 (duIL-18) (GenBank accession nos. DQ490137 and AF336122), no information is available on the *IL-18* gene of duck. Gushi layer (GS) duck is a Chinese layer breed originating from Henan province. The objectives of this study were to clone the cDNA sequence of GS duck *IL-18* and to express the duck *IL-18* gene in a prokaryotic expression system, to prepare monoclonal and polyclonal antibodies (pAbs) against recombinant duck IL-18 (rduIL-18) and to determine the bioactivity of the rduIL-18 protein and Abs against rduIL-18.

2. Materials and methods

2.1. Animals

GS duck embryonated eggs were obtained from Gushi Duck Breeding Co. Ltd. (Beijing, China). Eggs were hatched in our laboratory and ducklings were kept in a special room with feed and water *ad libitum*. In addition, 35-day-old, specific pathogen-free (SPF) Leghorn chickens were purchased from Beijing Merial Vital Laboratory Animal Technology Co. Ltd. (Beijing, China) and 4-week-old SPF BALB/c mice were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). New Zealand white rabbits (NZW rabbit) were purchased from Laboratory Animal Center of Henan Academy of Medicine (China). The use of all laboratory animals and animal subjects in this study was approved by the scientific ethical committee of Henan University.

2.2. Isolation of splenic mononuclear cells (SMCs) and preparation of endogenous duIL-18 protein

Ducks were sacrificed at 46 days old by intravenous inoculation of barbiturate. Spleens were collected aseptically and rinsed with Ca^{2+}- and Mg^{2+}-free PBS (717 mM K_2HPO_4, 283 mM KH_2PO_4, pH 7.2), minced and then passed through a 120-mesh nylon screen to obtain a homogeneous cell suspension. The spleen cells were collected after centrifugation at 250 × *g* for 10 min and resuspended in Hank's balanced salt solution (HBSS). The cell suspension was then overlaid on an equal volume of Histopaque-1077 (Sigma, St. Louis, MO, USA). An interface rich in mononuclear cells was recovered after centrifugation at 500 × *g* for 20 min. Cells were washed three times in serum-free RPMI 1640 (GIBCO BRL, Gaithersburg, MD, USA) and planted in six-well cell-culture plates in complete RPMI 1640 medium at a concentration of 2×10^6 cells/ml. The cells were stimulated with Con A (Sigma) at a final concentration of 20 μg/ml at 37 °C in 5% CO_2 for 0, 4, 8, 12, 16, 20, 24, 28, 32 and 48 h. The cell-culture supernatants were harvested at each time-point by centrifugation. Chicken SMCs for subsequent bioactivity assay were processed the same way as duck SMCs.

For each cell supernatant sample, the residual Con A in the supernatant was removed by incubating with 0.1 M α-methyl D-mannoside for 30 min. Ammonium sulfate was added to each supernatant with 90% saturation and stirred overnight at 4 °C. The precipitate was collected after centrifugation at 25,000 × *g* for 30 min at 4 °C, dissolved in PBS and dialyzed in PBS. Finally, the purified proteins were sterilized by passing through a filter (0.2 μm), and stored at −20 °C after further concentration using the polyethylene glycol 8000 method.

2.3. RT-PCR and sequencing

A pair of the specific oligonucleotide primers 610 bp in length was designed and synthesized according to duck *IL-18* cDNA (GenBank accession no. AF336122): upstream primer 5′-CCGATGAGATGTGAATTGATG-3′; downstream primer 5′-CCTAGTAATCACAGGTTG-TAC-3′. SMCs, stimulated for 24 h with Con A at a final concentration of 20 μg/ml, were harvested by centrifugation at 500 × *g* for 5 min at 4 °C, followed by washing

three times with PBS. Total cellular RNA was extracted with Trizol reagent (GIBCO). The cDNAs were synthesized from total cellular RNA using a special oligonucleotide primer (5′-CCTAGTAATCACAGG-TTGTAC-3′). The GS duck *IL-18* nucleic acid sequence was then amplified by PCR. The RT-PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, San Diego, CA, USA) and sequenced. The nucleotide sequence of GS duck *IL-18*, plus that of chicken and mammalian *IL-18* from Genebank (accession nos.: chicken, AY775780; human, AY044641; pig, AF191088; goat, AY524275; sheep, NM001009263; horse, Y11131; mouse, E17139; rat, NM019165; cat, NM001009213; bovine, NM174091; dog, NM001003169), was analyzed by DNASTAR 5.0 software (DNASTAR Inc., Madison, WI, USA).

2.4. Expression, purification and renaturation of rduIL-18

A cDNA sequence encoding the mature duck IL-18 molecule was amplified by PCR using the primers 'IL-18 BamHI sense' 5′-CGAGGATCCGCCTTCTC-TAAGGAA-3′ and 'IL-18 HindIII antisense' 5′-CCCAAGCTTTCACAGGTTGTACCT-3′. The product was digested with BamHI and HindIII and inserted into the bacterial expression vector pQE-30 (Qiagen, Toronto, Canada) previously digested with the same enzymes. The resulting plasmid, pQE30duIL-18, was used to transform *E. coli* M15.

The *E. coli* M15 strain containing pQE30duIL-18 was grown at 37 °C for approximately 2 h and induced by isopropylthio-β-D-galactoside (IPTG) with a final concentration of 1.0 mM for 5 h to produce the rduIL-18 protein. The bacteria were harvested by centrifugation at 12,000 × *g* for 5 min. An aliquot of bacterial pellet was resuspended in 2× SDS-PAGE sample buffer, boiled for 5 min and recentrifuged for 10 min at 12,000 × *g*. The sample was analyzed by SDS-PAGE as described previously (Zhou et al., 2004). Purification and renaturation of rduIL-18 was conducted as described previously (Sambrook et al., 1989). Potential lipopolysaccharide (LPS) contaminants were removed by incubation with polymyxin B Sepharose resin (Sigma) at 4 °C for 30 min. After centrifugation, the purified rduIL-18 was sterilized; a final concentration of rduIL-18 was determined and stored at −20 °C after concentration using the polyethylene glycol 8000 method. The protein was tested for biological activity by lymphocyte proliferation assay. The bacterial protein from *E. coli* M15 strain transformed with empty pQE30 vector was prepared and used as protein control.

2.5. Western blot analysis

The expressed proteins were subjected to SDS-PAGE using a 15% gel and then blotted to a nitrocellulose membrane (Amersham Pharmacia Biotech, NJ, USA) as described previously (Zhou et al., 2004). The membrane was blocked in TBS-T (25 mM Tris–HCl, 125 mM NaCl, 0.1% Tween-20, pH 8.0) containing 5% skimmed milk for 2 h at 37 °C. After washing five times in TBS (25 mM Tris–HCl, 125 mM NaCl, pH 8.0), the blots were incubated with anti-human IL-18 mAb (1:3000; Invitrogen), anti-chIL-18 mAb (1:3000, produced by our laboratory, unpublished data). After washing three times with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:4000; Southern Biotechnology Associates Inc., Birmingham, AL, USA) in TBS-T containing 5% skimmed milk. TMB (Promega, NJ, USA) was added to the corresponding reaction for color development.

2.6. Preparation and characterization of monoclonal antibody against rduIL-18 protein

Five SPF BALB/c mice were immunized intraperitoneally (i.p.) and subcutaneously (s.c.) with rduIL-18 (50 μg rduIL-18 per mouse) in complete Freund's adjuvant (CFA; Sigma). The mice were boosted with rduIL-18 (50 μg/mouse) in incomplete Freund's adjuvant (IFA) 3 weeks after the first immunization by i.p. injection. Two weeks after the second immunization, the vaccinated mice were again injected i.p. with 0.1 mg of the purified rduIL-18. The mice were subsequently euthanized 3 days later and the spleen cells were harvested. Mouse spleen cells were fused with non-secreting mouse myeloma SP2/0 cells using standard procedures. The fused cells were detected by indirect ELISA, as described previously (Miyamoto et al., 2001), using His, cell lysate of *E. coli* M15, recombinant chIL-18 (expressed in *E. coli* BL21 strain by our group; unpublished data) and rduIL-18 as antigens. The subtype analysis of each mAb was performed with standard procedures illuminated by the protocol of SBA Clonotyping TM System/HRP (Southern Biotechnology Associates Inc., Birmingham, USA). Ascites containing mAb to rduIL-18 was prepared in the female mice injected i.p. with 2,6,10,14-tetramethyl pentadecane and 5×10^6 hybridoma cells suspended in serum-free RMPI 1640 medium. The mAbs against rduIL-18 were identified by ELISA and Western blot, as described above. Ascite fluids were further purified by

fast protein liquid chromatography (FPLC® system; Amersham Pharmacia) according to manufacture's instructions.

2.7. Production of rabbit antiserum against recombinant duIL-18

Antiserum against rduIL-18 (pAb) was generated by a biweekly immunization of NZW rabbits with CFA, the purified rduIL-18 emulsion (100 μg of the recombinant duIL-18 per rabbit). Immunized rabbits were bled 14 days after the final injection and the serum was collected. Antibody was purified on fast protein liquid chromatography, as described above.

2.8. Antigen-capture ELISA

For quantification of rduIL-18 expressed in *E. coli* M15 and supernatants from Con A-stimulated SMC, an Ag-capture ELISA was developed, as described previously (Miyamoto et al., 2001), with some modifications using purified rduIL-18 as positive control. Briefly, 96-well flat-bottom plates (NUNC, Roskilde, Denmark) were coated with mAb (10 μg/ml, 100 μl/well) to rduIL-18 in 0.1 M carbonate buffer, pH 9.6 at 37 °C for 1 h and subsequently at 4 °C overnight. Each well was blocked with 200 μl PBS containing 3% BSA for 1 h at room temperature followed by three washes with PBS-T. One hundred microliters of culture supernatant from Con A-stimulated SMC or protein preparations containing rduIL-18 were added to each well and incubated for 4 h at room temperature. Rabbit polyclonal antibodies to rduIL-18 (100 μl/well) in PBS–0.1% BSA were added and incubated for 1 h at room temperature after five washes. After incubation with HRP-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) for 30 min at room temperature, the plates were washed six times. Peroxidase activity was detected by adding 100 μl/well of *o*-phenylenediamine (0.4 mg/ml in 0.05 M phosphate–citrate buffer, pH 5.0) substrate, incubated for 15 min and the reaction stopped by addition of 50 μl of 2 M sulfate acid. Reactions were monitored by an automated microtiter plate reader at 490 nm.

2.9. MTT assay

Duck and chicken lymphocyte proliferation under different stimulation conditions was detected by MTT test (Mosmann, 1983). Briefly, SMCs, stimulated by Con A for 24 h, were incubated in RPMI 1640 medium containing 0.1 M α-methyl D-mannoside for 30 min. The cells were then applied to Histopaque-1077 and live cells were selected after centrifugation. The enriched viable population was resuspended in RPMI 1640 containing 10% FCS and cultured at 5×10^6 cells/ml with the supernatant containing native duIL-18, as well as rduIL-18 protein, in triplicate in 96-well microtiter plates (100 μl/well). rchIL-18 was used as a positive control. The cells containing RPMI 1640–FCS only were used as a negative control. After incubation at 40 °C for 48 h, 20 μl MTT (5 mg/ml) was added to each well and reacted for 4 h. A 100-μl of lysis buffer (10% SDS–0.01 mol/l HCl) were added to each well. Plates were incubated for 20 h at 40 °C under 5% CO_2 and optical density values (OD) measured at 570 nm. A ODduIL-18/OD negative value of 1.5 or above was defined as positive criteria for rduIL-18 bioactivity.

Concentration of recombinant and endogenous duIL-18 was determined using the Ag-capture ELISA described in Section 2.8. Neutralizing ability of monoclonal and polyclonal antibodies against rduIL-18 was screened in 96-well plates, as described previously, with modifications (Zhou et al., 2003a,b), using the MTT assay mentioned above. Briefly, by fixing rduIL-18 with antibodies at different concentrations, rduIL-18 and supernatant of Con A-stimulated SMCs were mixed, respectively, with an equal volume of 10-fold serial dilutions of mAbs or pAb and incubated for 1 h at 37 °C. The mixture was added to each well of the plate with 100 μl of cells (5×10^6 cells/ml). Subsequent steps were performed as above. Neutralizing ability of monoclonal and polyclonal antibodies against rduIL-18 was calculated as follows:

$$\text{neutralizing ability}\ (\%) = \left[1 - \frac{OD_{neu} - OD_{neg}}{OD_{duIL\text{-}18} - OD_{neg}}\right] \times 100$$

with 1 indicating the biological activity of duIL-18 $[(OD_{duIL\text{-}18} - OD_{neg})/(OD_{duIL\text{-}18} - OD_{neg})]$ at 570 nm in the absence of neutralizing antibodies, while OD_{neu}, $OD_{duIL\text{-}18}$ and OD_{neg} represent OD values of lymphocyte proliferation in the presence of neutralization antibodies, duIL-18 positive and negative controls, respectively.

2.10. Duck immunization and antibody detection

Thirty-day-old GS ducks were divided into five groups, with six ducks per group. Groups 1–3 were, respectively, injected intramuscularly with a dose of

100, 150 and 200 ng rduIL-18; all ducks were immunized intramuscularly with the commercial inactivated oil emulsion vaccine against avian influenza virus (AIV, H5N1) (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China), according to the manufacture's instruction. Ducks in group 4 were intramuscularly vaccinated with the inactivated AIV oil emulsion vaccine (H5N1) only, while ducks in group 5 were intramuscularly immunized with the oil adjuvant as control. Maintained under the same feeding conditions, blood samples were collected via wing vein puncture at 1–4 weeks after immunization. The hemagglutinin-inhibition (HI) antibody titer against AIV was measured by the HI assay according to the manufacture's instruction (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China).

2.11. Statistics

The χ^2-test was used for the statistical analysis. Mean values and standard deviations were calculated and the differences evaluated by Student's *t*-test.

3. Results

3.1. Cloning and sequence analysis of duIL-18 cDNA

After agarose gel electrophoresis of the five PCR-amplified samples, a band migrating at approximately 600 bp was detected. The sequencing result revealed that GS duck *IL-18* cDNA was consistently 610 bp in length (GenBank accession no. DQ522948). OMIGA analysis of the encoding nucleotide sequence showed that there was an ORF of 603 bp in length encoding a predicted polypeptide of 200 amino acid residues. The predicted molecular weight for GS duck *IL-18* was 23.18 kDa and the theoretical isoelectric point value was 4.92.

The sequence of GS duck IL-18 differed by seven nucleotides from White Pekin super M2 hybrid (WM) IL-18 (98.8% similarity) and one nucleotide from White Pekin duck (WP) IL-18 (99.8%). The amino acid sequence of IL-18 from GS duck was compared with previously published sequences of IL-18 (Fig. 1). The sequence encoding duck IL-18 is phylogenetically more distant from those encoding for human, bovine, pig, goat, sheep, horse, mouse, rat or cat IL-18 (23.3–29.3%) than that encoding chicken IL-18, which exhibited 85.4% similarity to GS duck IL-18.

The predicted duck IL-18 protein did not demonstrate any hydrophobic signal-like sites. A 12 β-sheet

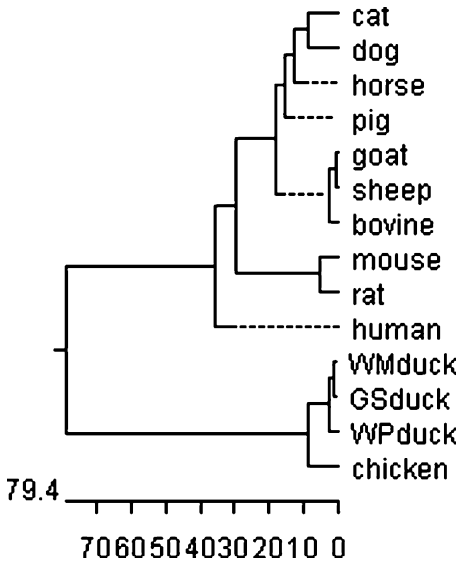

Fig. 1. Phylogenetic relationship of IL-18 amino acid sequences of several mammalian and avian species. The numbers of the staff gauge indicate the evolutionary distance of different species.

structure was predicted using a structurally based comparison with IL-18 from other species (Culhane et al., 1998; Argyle et al., 1999; Fournout et al., 2000); the predicted GS duck IL-18 amino acid sequence contains the IL-1 signature-like sequence ($FX_{12}FXSX_6$ FL) (Fig. 2) between amino acids 136 and 160 and an unusual leader sequence composed of 30 amino acids, which may be analogous to the IL-1β pro-domain that is cleaved for biological activity by ICE.

3.2. Expression, purification and molecular size of the rduIL-18 protein

The pQE30duIL-18 was constructed by cloning the mature duIL-18 ORF encoding 170 amino acids without the leader peptide sequence into the BamHI–HindIII site of pQE30. Sequencing the cloned insert showed an identical sequence to GS duck IL-18 ORF sequence. The whole cell lysate, obtained from the induced *E. coli* strain containing pQE30duIL-18 plasmid, contained a His-duIL-18 fusion protein with a weight of 19.76 kDa (Fig. 3A, lanes 3–5). The induced control bacterial extract containing only pQE30 did not show the expressed protein band (Fig. 3A, lane 1). For the purification of the fusion protein, the cells were sonicated and washed with TE2 and TE3, then denatured and renatured. The final concentration of rduIL-18 was approximately 0.424 mg/ml as determined via ultraviolet spectrophotometry. As shown in Fig. 3B, a band with a molecular weight of 19.76 kDa

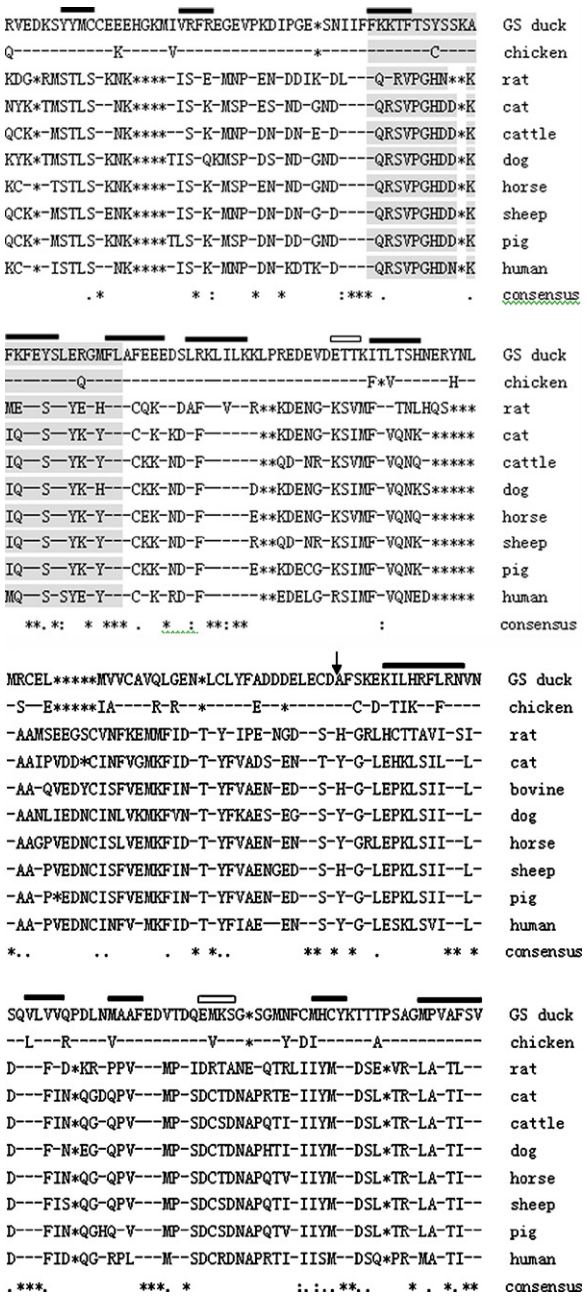

Fig. 2. Multiple alignment of the known IL-18 molecules. Conserved residues shared with the putative GS duck peptide are indicated with a dash (–) and gaps in the alignment are represented with '*'. Conservation of amino acid identity is indicated in the consensus line with '*', whereas ':' and '.' indicate high and low levels of amino acid similarity, respectively. The vertical arrowhead (↓) indicates the potential ICE cleavage site. The 12 β-sheets (solid horizontal bars) and two α-helices (open horizontal bars) in human IL-18 are shown above the alignment. The signature sequence is shaded.

was detected in the lysates of *E. coli* M15 strain containing pQE30duIL-18 by anti-duIL-18 mAb5B2 and anti-chIL-18 mAb (Fig. 3B, lanes 1 and 2), but not the pQE30 vector alone (data not shown).

3.3. Preparation and characteristics of monoclonal and polyclonal antibodies against rduIL-18 protein

The hybridoma cells were single-cell cloned and selected for further study based on their strong binding activity against rduIL-18 expressed in *E. coli* on ELISA. None of these antibodies showed any reaction against His and cell lysate of *E. coli* M15 strain as antigens. One hybridoma cell line (5B2) against rduIL-18 was obtained. The mAb 5B2 belongs to IgG1. The light chain of 5B2 is a κ-chain. Rabbit antiserum against rduIL-18, produced as described in Section 2.7, did not bind His and cell lysate of *E. coli* M15 strain.

3.4. Quantification of duck IL-18 by an Ag-capture ELISA

From the monoclonal and polyclonal antibodies against rduIL-18 described above, a sensitive Ag-capture ELISA for duck IL-18 was developed with mAb 5B2 to duIL-18 using the purified rduIL-18 protein as an antigen. The optimal coating concentration for mAb 5B2 was found to be 7.5 μg/ml and the optimal concentration of pAb was also 7.5 μg/ml. The concentration of the expressed rduIL-18 in the protein preparation expressed in *E. coli* was 2 ng/μl by the developed Ag-capture ELISA. No obvious signals were detected from the endogenous duIL-18 in unconcentrated supernatants from Con A-stimulated duck SMCs using Ag-capture ELISA, whereas using this ELISA method, the clear OD values were detected from the 100-fold concentrated supernatants from Con A-stimulated duck SMCs. Data in Fig. 4 show the duIL-18 levels in the concentrated supernatants from Con A-stimulated duck SMCs. Secretion of endogenous duIL-18 clearly increased in the first 8 h ($P < 0.05$) and slowly peaked at 24 h. When anti-hIL-18 mAb were coated, no obvious signals were detected from the endogenous duIL-18 in concentrated supernatants from Con A-stimulated duck SMCs at 24 h (data not shown).

3.5. In vitro proliferation of lymphocytes stimulated by rduIL-18

The biological activity of rduIL-18 was tested by in vitro proliferation of duck and chicken spleen cells. After the activated duck (5×10^6 cells/well) and

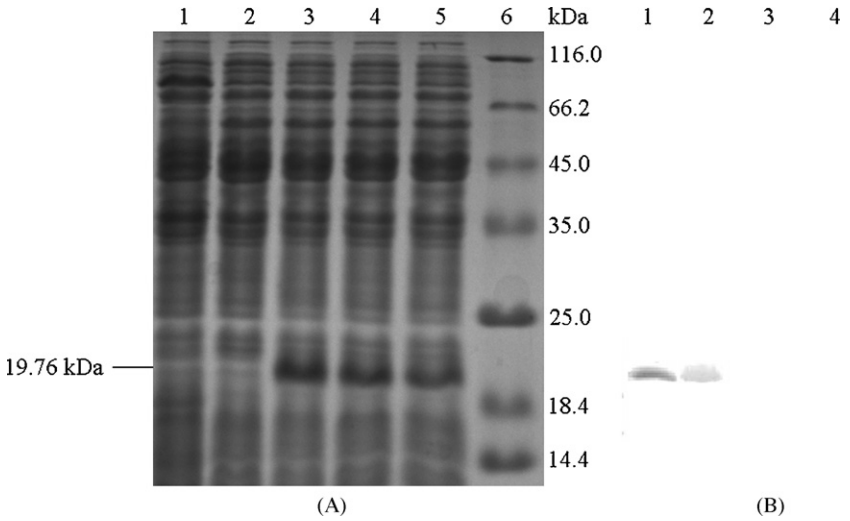

Fig. 3. Analysis of His-duIL-18 fusion protein expressed in *E. coli*. (A) SDS-PAGE analysis. Lane 1: cell lysates of bacteria transformed with empty pQE30 vector. Lane 2: cell lysates of *E. coli* M15 alone. Lanes 3–5: cell lysates of *E. coli* M15 transformed with pQE30duIL-18. Lane 6 represents the molecular weight marker. (B) Western blot analysis. Lanes 1 and 2 represent the rduIL-18 protein recognized by anti-duIL-18 mAb5B2 and anti-chIL-18 mAb. Lane 3 reveals that rduIL-18 does not recognize anti-hIL-18. Lane 4 shows that recombinant pig IL-18 does not recognize anti-duIL-18 mAb5B2.

chicken spleen cells (5×10^6 cells/well) were co-cultured with twofold serial dilutions of the protein preparations containing the expressed rduIL-18, protein control, RPMI-1640-FCS and concentrated supernatant from Con A-stimulated SMCs, respectively, the culture mixtures were reacted for 3 h with MTT. Data in Fig. 5A shows that 3.12 ng (1.56 μl) rduIL-18/well stimulated in vitro proliferation of duck lymphocytes, while for chicken lymphocytes, 25 ng (12.5 μl) rduIL-18/well was needed ($P < 0.05$). This result indicated that rduIL-18 induced the proliferation of chicken lymphocytes at a low level. In addition, data in Fig. 5 also show that the duck

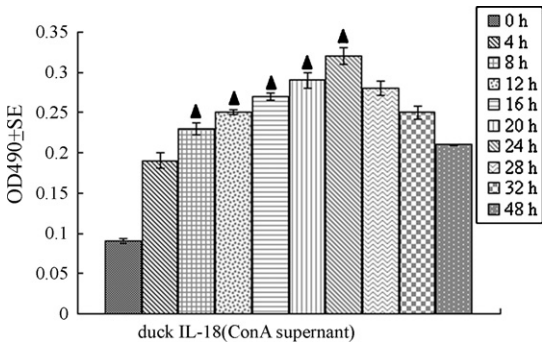

Fig. 4. Detection of duIL-18 secreted from Con A-stimulated duck splenocytes at different time-points in the antigen-capture ELISA assay ($n = 4$, i.e. number of times the test was repeated). Supernatants from each time-point were collected and concentrated. Values are expressed as mean counts ± standard error. (▲) Significantly different ($P < 0.05$) from secretion of endogenous duIL-18.

lymphocyte proliferation assay induced by rduIL-18 is typically dose-dependent. Similarly to rduIL-18, the biological activity of the rchIL-18 preparation was also tested; 3.2 ng rchIL-18/well stimulated in vitro proliferation of chicken lymphocytes, while for duck lymphocytes, 6.4 ng rchIL-18/well was needed ($P < 0.05$) (Fig. 5B).

3.6. Bioactivity of monoclonal and polyclonal antibodies against rduIL-18

Polyclonal and monoclonal antibodies were assayed for their ability to inhibit the proliferative activity of rduIL-18 and endogenous duIL-18 derived from Con A-stimulated duck SMCs, measured with the MTT assay described above. The mAb 5B2 against rduIL-18 showed a 66.5% neutralizing ability against rduIL-18, 61.3% for endogenous duIL-18 and 51.2% neutralizing ability against rchIL-18, whereas the inhibiting activity of rabbit anti-rduIL-18 serum against rduIL-18, endogenous duIL-18 and rchIL-18 was 67.3, 64.1 and 53.5%, respectively (Table 1). No significant differences were observed in bioactivity of monoclonal and polyclonal antibodies against rduIL-18.

3.7. In vivo immune response to AIV vaccine induced by rduIL-18

Following intramuscular inoculations with the rduIL-18 and inactivated oil emulsion AIV vaccine, blood

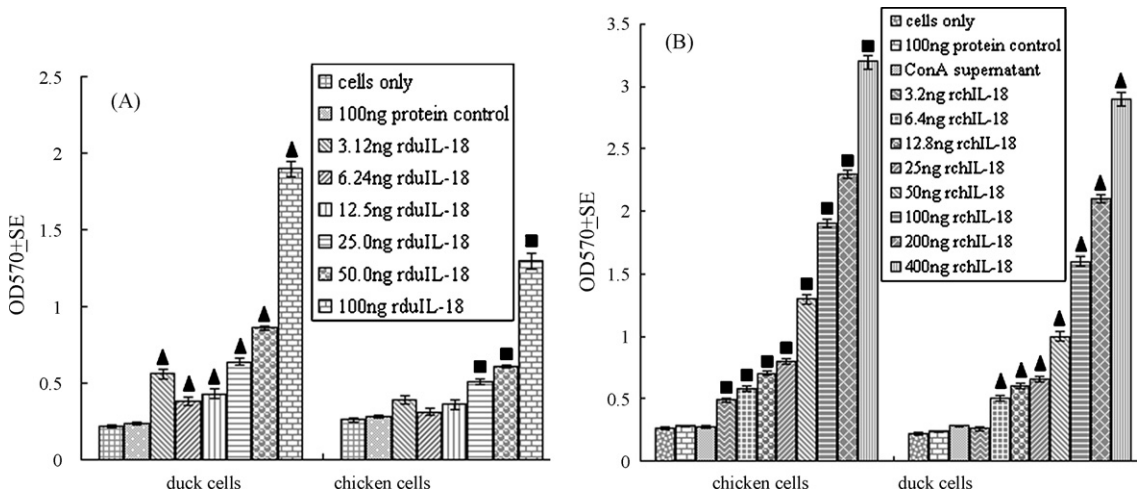

Fig. 5. In vitro lymphocyte proliferation assay of duck and chicken IL-18s. (A) Effect of rduIL-18 on duck and chicken lymphocytes under different concentrations ($n = 4$). (B) Effect of rchIL-18 on duck and chicken lymphocytes under different concentrations ($n = 4$, i.e. number of times the test was repeated). Cells without addition of any protein and protein preparation from bacteria transformed with empty pQE30 vector were used as negative control. The proliferation values are dose-responsive to rduIL-18 or rchIL-18 produced from prokaryotic system. Values are expressed as mean counts ± standard error. (▲) Significantly different ($P < 0.05$) from in vitro proliferation of duck lymphocytes. (■) Significantly different ($P < 0.05$) from in vitro proliferation of chicken lymphocytes.

samples were collected at specified times and the serum HI antibody to AIV was measured. As shown in Table 2, in ducks injected intramuscularly with rduIL-18 protein (150 or 200 ng per duck) and AIV vaccine 2 weeks after immunization, HI antibody titers to AIV reached 7.9–8.1 $\log_2$, while HI antibody titers to AIV were 6.1–6.3 $\log_2$ in ducks vaccinated with AIV vaccine or with 100 ng rduIL-18 and AIV vaccine only ($P < 0.05$). The results clearly showed that 150 ng rduIL-18/duck strengthened the in vivo immune responses induced by the inactivated oil emulsion AIV vaccine.

4. Discussion

Recombinant chIL-18 is able to enhance immunity (Gobel et al., 2003; Degen et al., 2005); however, data on duIL-18 have not been available, so far. In this study, the predicted protein of GS duIL-18 had a molecular weight of 23.18 kDa without signal peptide. A 12 β-sheet structure was predicted using a structurally based comparison with IL-18 from other species (Culhane et al., 1998; Argyle et al., 1999; Fournout et al., 2000). The predicted GS duck IL-18 amino acid sequence contained the IL-1 signature-like sequence ($FX_{12}FXSX_6$ FL) (Fig. 1) and the potential cleavage site on the precursor peptide by ICE was situated between *Asp*30 and *Tyr*31. Because there is no ICE in bacterium, the rduIL-18 without the leader peptide was successfully produced with the pQE30 expression system. By SDS-PAGE and Western blotting analysis, the observed molecular weight of the rduIL-18 fusion protein was 19.76 kDa. The yield of the rduIL-18 was about 0.424 mg/ml as determined via ultraviolet spectrophotometry.

In this study, the bioactivity of rduIL-18 was demonstrated using in vitro proliferation assay of duck lymphocytes and in vivo immune responses of AIV vaccine vaccination. Turkey and chicken IL-18 molecules will cross-react in functional assays (Kaiser, 2002). Duck lymphocytes stimulated with phytohe-

Table 1
Neutralizing activity of antibodies against rduIL-18 ($n = 4$)[a]

Ab	Isotype	Neutralizing ability (%)[b]			
		rduIL-18	Endogenous duIL-18	rchIL-18	Protein control
mAb5B2	IgG1	66.5 ± 0.2	61.3 ± 0.1	51.2 ± 0.3	0
pAb		67.3 ± 0.3	64.1 ± 0.2	53.5 ± 0.3	0

[a] n = number of times the test was repeated.
[b] Mean ± standard error.

Table 2
Duck anti-AIV (H5N1) HI antibody response induced by rduIL-18 ($n = 6$)[a]

Group	Animal no.	rduIL-18 dose (ng/duck)	Serum HI titer (log_2)[b]			
			First week	Second week	Third week	Fourth week
Vaccines	6	100	0	6.3 ± 0.6	7.1 ± 0.7	8.7 ± 0.5
	6	150	0	7.9 ± 0.3[c]	8.9 ± 0.2[c]	9.9 ± 0.4[c]
	6	200	0	8.1 ± 0.4[c]	9.6 ± 0.5[c]	10.6 ± 0.6[c]
	6	–	0	6.1 ± 0.5	6.8 ± 0.4	8.7 ± 0.6
Control	6	–	0	0	0	0

[a] n = number of times the test was repeated.
[b] Mean HI titer ± standard error.
[c] Significant differences between dog groups ($P \leq 0.05$).

magglutinin have been shown to release lymphokines, which could also provoke the proliferation of duck and chicken lymphoblasts, but not mouse lymphoblasts (Bertram et al., 1997). In our experiment, based on pure denatured rduIL-18 as a reference antigen, the concentration of rduIL-18 in the protein preparation was determined using an Ag-capture ELISA developed by our group. It was found that 3.12 ng rduIL-18/well showed significant in vitro proliferation of duck lymphocytes, while 25 ng rduIL-18/well was needed for chicken lymphocytes ($P < 0.05$). After each duck was subcutaneously vaccinated with a dose of 150–200 ng rduIL-18, the vaccinated ducks showed strengthened immune responses to AIV vaccine immunization. The data indicate that rduIL-18 has biological effects, though the effect on chicken lymphocytes is relatively weak. These results imply that rduIL-18 is an important immunoregulatory molecule, which has been well-studied in chicken and mammalian counterparts.

In earlier studies, it was reported that the lymphocyte proliferation effect could be affected by factors such as the IL-2 preparation process, SMC viability as well as IL-2 concentration (Stepaniak et al., 1999; Thiagarajan et al., 1999). In our study, to overcome these factors, the rduIL-18 was first quantified by the Ag-capture ELISA and SMC concentrations were also assessed according to Thiagarajan et al. (1999) with modifications. To assure that all proliferation induced by the recombinant protein preparations was attributable to duIL-18, the protein preparation from *E. coli* M15 strain transfected with empty pQE30 vector and SMC were used as negative controls. Additionally, we used polymixinB Sepharose to remove LPS contaminants (Issekutz, 1983; Molvig and Baek, 1987). The results (Fig. 5) clearly show that there is no LPS effect in all lymphocyte proliferation assays.

The anti-duIL-18 mAb generated in this study could specifically recognize both rduIL-18 and native duIL-18 in both ELISA and Western blot. In the neutralizing assay, the mAb and pAb against rduIL-18 can neutralize the biological activities of both rduIL-18 and endogenous duIL-18. The mAb with neutralization functionality will be a powerful tool in further investigations of the active site of duck IL-18 as well as in the study on its basic immunobiological responses in either normal or infection conditions.

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