ELSEVIER

CYTOKINE

www.elsevier.com/locate/issn/10434666

Cytokine 30 (2005) 328–338

cDNA cloning and functional analysis of goose interleukin-2

Ji-Yong Zhou*,[1], Ji-Gang Chen[1], Jin-Yong Wang, Jian-Xiang Wu, Hui Gong

Laboratory of Virology and Immunology, Institute of Preventive Veterinary Medicine, College of Animal Sciences, Zhejiang University, 268 Kaixuan Road, Hangzhou, Zhejiang Province 310029, PR China

Received 16 November 2004; received in revised form 16 December 2004; accepted 21 December 2004

Abstract

cDNA encoding goose IL-2 (GoIL-2) was cloned from Con A-stimulated goose splenic mononuclear cells (SMC) using oligonucleotide primers based on the conserved sequence of duck (DuIL-2), chicken (ChIL-2) and turkey IL-2s (TuIL-2). The GoIL-2 cDNA is 718 nt long, which contains an open reading frame (ORF) of 423 base pairs encoding a protein of 141 aa. The GoIL-2 shows, respectively, 79%, 82–85%, and 91–92% identities with TuIL-2, ChIL-2 and DuIL-2 in cDNA, and also shows, respectively, 63%, 63–64%, and 82–85% identities with TuIL-2, ChIL-2 and DuIL-2 in amino acid sequence. Recombinant GoIL-2 (rGoIL-2) protein expressed in *Escherichia coli* has an approximate molecular weight of 18 kDa. The rGoIL-2 has biological effect on goose and duck as well as chicken lymphocytes in a dose-dependent manner, though the effect on duck and chicken lymphocytes has been found to be relatively weak. In addition, rGoIL-2 also strengthens goose immune responses induced by vaccinating the inactivated oil emulsion vaccine against avian influenza virus. The monoclonal antibodies (mAb) to rGoIL-2 recognized the binding epitopes of nature GoIL-2 protein expressed in vero cells. Antiserum and mAb 5B10 to rGoIL-2 can inhibit the biological activity of rGoIL-2 and endogenous GoIL-2. The results, at the first time, indicated that goose IL-2 reserves species-specialties in the biological functions and can be used as a potential immunoadjuvant for goose vaccination and immunotherapeutic purposes. Finally, the mAbs to rGoIL-2 also provide a useful tool for further immunobiological studies of IL-2 in avian immune systems.

Keywords: Goose; Interleukin-2; Biological activity; Monoclonal antibody; Epitope

1. Introduction

IL-2 is a glycoprotein produced principally by activated T cells and some B cells and a cytokine that regulates lymphocyte function. IL-2 controls the amplification of native T cells by initially stimulating growth following antigen activation, and also has effects on NK cells, monocytes/macrophages and neutrophils [1–4]. IL-2 has been developed as an immunotherapeutic agent in chronic microbial infection and cancer, and as an enhancing agent in vaccine [5–7]. Previous reports also found that IL-2 exerts its effects by binding to the high affinity IL-2 receptors (IL-2R) present on those cells [8]. IL-2R is composed of three distinct subunits: IL-2Rα, IL-2Rβ and IL-2Rγ which are encoded, respectively, by distinct and structurally unrelated genes [9]. Resting T cells express low levels of IL-2Rα-subunit (Tac antigen) that binds to IL-2 with a low affinity. IL-2Rβ-subunit alone or in combination with α or γ produce a receptor with intermediate affinity, and the γ-subunit alone does not bind IL-2, while a receptor comprised of three subunits offers the highest affinity for IL-2 [8].

Up to now, in addition to human IL-2, IL-2 genes for more than 30 animal species also have been cloned and identified [10]. Recently, IL-2 gene for chicken (ChIL-2) and turkey (TuIL-2) have been cloned and sequenced

* Corresponding author. Tel.: +86 571 8697 1698; fax: +86 571 8697 1821.

E-mail address: jyzhou@zju.edu.cn (J.-Y. Zhou).

[1] The first two authors contributed equally to this work.

1043-4666/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2004.12.015

successfully [11–13]. The ChIL-2 gene is mapped to chromosome 4, linked to annexin V with synteny with mouse chromosome 3 and human chromosome 4 [12]. Our previous study revealed that some variations exist in the open reading frame (ORF) of ChIL-2 from different chicken breeds [14]. The cDNA coding for ChIL-2 protein was introduced into several prokaryotic and eukaryotic expression systems to produce biologically active recombinant cytokines [13,15,16]. ChIL-2 plays an important role as an immunoregulatory molecule identical to mammalian IL-2 and has been shown to stimulate T lymphoblast cells, increase the proportion of both $CD4^+$ and $CD8^+$ peripheral blood T cells [17], enhance NK cells activity and vaccine response to *Eimeria* parasites [18], and strengthen the protective immunity against infectious bursal disease virus [19], indicating that ChIL-2 has practical importance in potentially enhancing immune responses to vaccines and infectious agents in poultry. Furthermore, mAbs to ChIL-2 have also been produced, and used to study the biological activities of ChIL-2 [20,21]. Comparatively, little work has been performed on avian IL-2s and their receptors. Only a mAb designated INN-CH-16 can react with a 48–50 kDa antigen expressed on activated chicken T lymphocytes and inhibited the proliferation of Con A-stimulated blasts in response to supernatants of stimulated chicken T cells. These features led to the hypothesis that INN-CH-16 may recognize the ChIL-2R [22,23].

As for waterfowl cytokines in 1997, Bertram et al. [24] found lymphokines released by duck peripheral blood lymphocytes and splenocytes stimulated with PHA, which was of similar functional homology to ChIL-2 in maintaining the lymphocyte proliferation. Recently, the cloning and functional characterization of duck IL-2 have been reported [25–27]. However, no information was available on the goose cytokines. Hereby, the goal of the present study was to molecularly clone the cDNA fragment of goose IL-2 (GoIL-2), to express the GoIL-2 open reading frame in vitro expression system, to prepare mAbs to GoIL-2, and to detect the bioactivity of the recombinant GoIL-2 (rGoIL-2) protein in vitro and in vivo.

2. Results

2.1. Molecular cloning and analysis of goose IL-2 cDNA

The mRNA was isolated from goose SMC stimulated with 10 μg/ml Con A at 0, 1, 2, 4, 8, 12, 14, 16, 18, 24, 48 and 72 h. GoIL-2 cDNA fragment was amplified by RT-PCR using these mRNAs as templates. An approximately 0.8 kb DNA fragment, in size, was amplified from nine of the 12 mRNAs (Fig. 1). The sequencing results revealed that cDNA fragment of GoIL-2 was

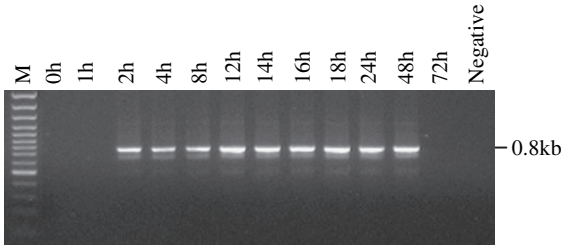


Fig. 1. RT-PCR amplification of GoIL-2 mRNA extracted from goose SMC harvested 0, 1, 2, 4, 8, 12, 14, 16, 18, 24, 48 and 72 h after Con A stimulation.

718 nt in length (GenBank accession no. AY392557). GoIL-2 cDNA fragment encodes a polypeptide of 141 amino acid residues with a predicted molecular mass of 16.29 kDa. Analysis of the signal peptide revealed that there was a potential cleavage site between amino acid residues 21 and 22 (arrow in Fig. 2). The predicted signal peptide of GoIL-2 was similar in size to that of mammalian animals (20 aa) and other birds (22 aa).

The deduced amino acid sequence of GoIL-2 was compared with those of the several avian and mammalian species. The alignment analysis showed that GoIL-2 shared 85% identity to DuIL-2 (GenBank accession no. AY193713), 63% to ChIL-2 (GenBank accession no. AF294321), 64% to TuIL-2 (GenBank accession no. AJ007463), 9% to human IL-2 (GenBank accession no. U25676), and 7–20% to other mammalian IL-2s. As shown in Fig. 2, in IL-2 sequences of the compared avian species, there are 78 conserved amino acid residues including three cysteine residues (position 63, 70 and 119), indicating that there is at least one di-sulfate bond in the molecules. However, Lys^{48} and Asn^{80} are present only in GoIL-2 amino acid sequence. $Thr^{34,45,114}$, $Lys^{73,105}$, $Ile^{75,110}$, $Asp^{86,90}$, Glu^{104} and Arg^{132} are the conserved amino acid residues in goose and duck IL-2s, but are variable in chicken and turkey IL-2s. On the contrary, four amino acid residues (Ala^{14}, Ile^{42}, His^{51} and Leu^{131}) were conserved in chicken and turkey IL-2s, but were variable in goose and duck IL-2s. Phylogenetic analysis (Fig. 3) shows further that the avian IL-2s were subdivided into two monophyletic lineages. The goose and duck IL-2s formed a monophyletic group distinct from ChIL-2 and TuIL-2, confirming the similarity between duck and goose IL-2s.

2.2. Expression and purification of the His-GoIL-2 fusion protein

The gene fragment encoding GoIL-2 protein (without signal peptide) was cloned into the pBAD/His B expression vector, where the araB promoter is controlled by arabinose. After induction with 0.2% L-(+)-arabinose and resolved in a SDS-PAGE, the expressed recombinant GoIL-2 with an approximate molecular weight of 18 kDa

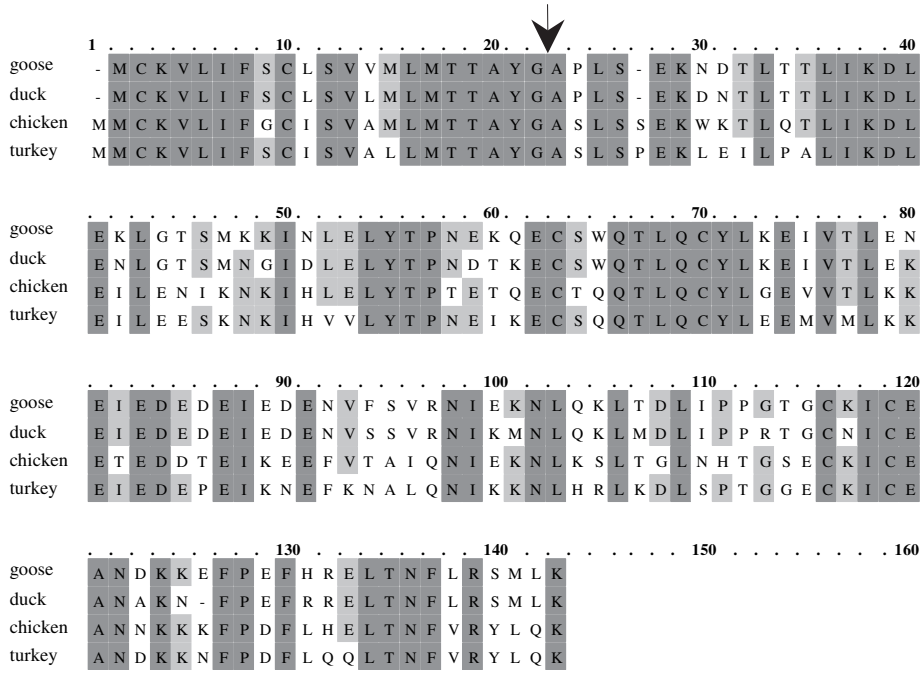


Fig. 2. Comparison of the predicted amino acid sequences of goose, chicken, duck and turkey IL-2s. The arrow indicates the predicted cleavage site of the signal peptide sequence. The dark shadow reveals the conserved amino acid residues in all the four poultry IL-2s. The lighter shadow shows the semi-conserved amino acid residues.

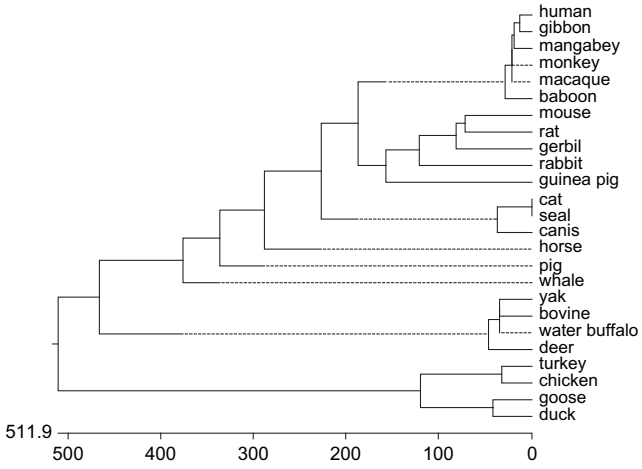


Fig. 3. Phylogenetic tree of IL-2 amino acid sequences of several mammalian and non-mammalian species. The numbers of the staff gauge indicate the evolution distance of different species. GenBank accession numbers of the compared sequences are as follows: human IL-2 (U25676); gibbon IL-2 (K02292); mangabey IL-2 (U19846); monkey IL-2 (D63352); macaque IL-2 (U19852); baboon IL-2 (U88365); mouse IL-2 (K02292); rat IL-2 (M22899); gerbil IL-2 (X68779); rabbit IL-2 (AF068057); guinea pig IL-2 (AB010093); cat IL-2 (L19402); seal IL-2 (AF072871); canis IL-2 (D30710); horse IL-2 (X69393); pig IL-2 (X58428); whale IL-2 (AF009570); yak IL-2 (AY294019); bovine IL-2 (M13204); buffalo IL-2 (AF363786); deer IL-2 (U14682); turkey IL-2 (AJ007463); chicken IL-2 (AF483600); duck IL-2 (AY193713).

was readily observed (Fig. 4, lane 3). In Western blot analysis, this band could also be recognized by anti-X-press™ mAb, mAbs (5H6 and 5F6) to DuIL-2 (Fig. 4, lanes 6–8), but was not recognized by mAbs (2B3, 4G12 and 1H4) to DuIL-2 (Fig. 4, lanes 9–11), mAbs (1A12, 4A3, 3E7 and 4E1) to ChIL-2 (Fig. 4, lanes 12–15), mAb to mouse (Fig. 4, lane 16) and human (Fig. 4, lane 17) IL-2s. In contrast, no such band was detected in the lysates of the bacterial cells containing only pBAD/His B (Fig. 4, lane 2). In addition, the band of His-GoIL-2 protein

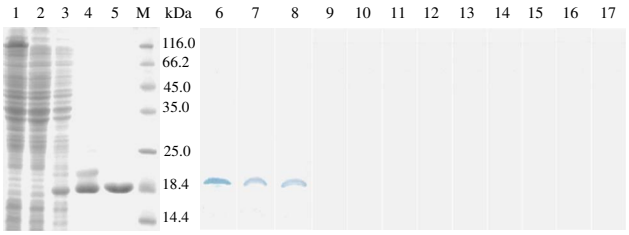


Fig. 4. Analysis of His-GoIL-2 fusion protein expressed in *E. coli* LMG 194 strain. Lane 1 is extracts of positive control bacteria transfected with pBAD/His/LacZ. Lane 2 shows extracts of negative control bacteria containing pBAD/His B vector. Lane 3 indicates extracts of bacteria containing pBAD-GoIL-2. Lane 4 represents His-GoIL-2 fusion protein purified under native conditions. Lane 5 reveals His-GoIL-2 fusion protein purified under denaturing conditions. Lane M is a molecular weight standard. Lanes 6–8 represent rGoIL-2 protein recognized by anti-X-press mAb, anti-DuIL-2 mAbs 5H6 and 5F6 to DuIL-2. Lanes 9–17 are rGoIL-2 protein which cannot react with mAbs 2B3, 4G12 and 1H4 to DuIL-2, anti-ChIL-2 mAbs (1A12, 4A3, 3E7 and 4E1), anti-mouse IL-2 mAb and anti-human IL-2 mAb.

purified from the sonicated bacterial cells under native (Fig. 4, lane 4) and denaturing (Fig. 4, lane 5) conditions also can be observed.

2.3. Production and identification of mAbs specific for GoIL-2

Following immunization with rGoIL-2 protein purified under denaturing conditions as an immunogen, five murine hybridoma clones were identified to produce mAbs (3B2, 5B10, 3C4, 5D4 and 2F7). Data in Table 1 showed that all these mAbs recognized rGoIL-2 by ELISA and Western blot assays. The mAbs did not react with 6 × Histamine, rChIL-2 or with rDuIL-2. Isotype of the five mAbs was found to be all IgG1 and κ chain. Rabbit antiserum against rGoIL-2 produced as described in Section 4.4 could also specifically react with rGoIL-2, but do not react with any protein in the cell lysate of *Escherichia coli* LMG194 strain transformed with empty pBAD/His B (data not shown).

2.4. Eukaryotic expression of GoIL-2

In order to express the GoIL-2 protein in the eukaryotic cells, pEGFP-GoIL-2 plasmid was transfected into vero cells. As shown in Fig. 5b, the EGFP-GoIL-2 expressed in vero cells as early as 3 h after transfection. In immunocytochemical staining, the acetone-fixed pEGFP-GoIL-2-transfected vero cells were detected, respectively, by five mAbs to rGoIL-2 (Fig. 5d–h), while no signal was observed in vero cells transfected with pEGFP-c2 vector (Fig. 5c), demonstrating that these mAbs hold the epitopes of GoIL-2 protein expressed in vero cells.

2.5. In vitro bioactivity of goose IL-2

The concentration of the *E. coli*-derived soluble rGoIL-2 in the protein preparation was 0.656 mg/ml. The biological activity of *E. coli*-derived rGoIL-2 and endogenous GoIL-2 was tested by in vitro proliferation of goose, duck and chicken spleen cells. Goose (5×10^6 cells/well), duck (5×10^6 cells/well) and chicken (5×10^6 cells/well) spleen cells were cultured in the presence of either rGoIL-2, irrelevant protein control, RPMI-1640–FCS or the supernatants from Con A-stimulated SMC in a series of 2-fold dilutions. Data in Fig. 6A showed that 1.6 ng rGoIL-2/well can significantly stimulate in vitro proliferation of goose lymphocytes, while rGoIL-2 was 6.3 ng/well for duck lymphocytes and 25 ng/well for chicken lymphocytes. These results indicated that the stimulation effect of rGoIL-2 on both chicken and duck lymphocytes was weak. On the other hand, 0.8 ng/well rChIL-2 was needed in the proliferation assay of chicken lymphocytes and 12.5 ng rChIL-2/well for duck lymphocytes and 6.3 ng rChIL-2/well for goose lymphocytes (Fig. 6B). 6.3 ng/well rDuIL-2 induces significantly in vitro proliferation of duck lymphocytes

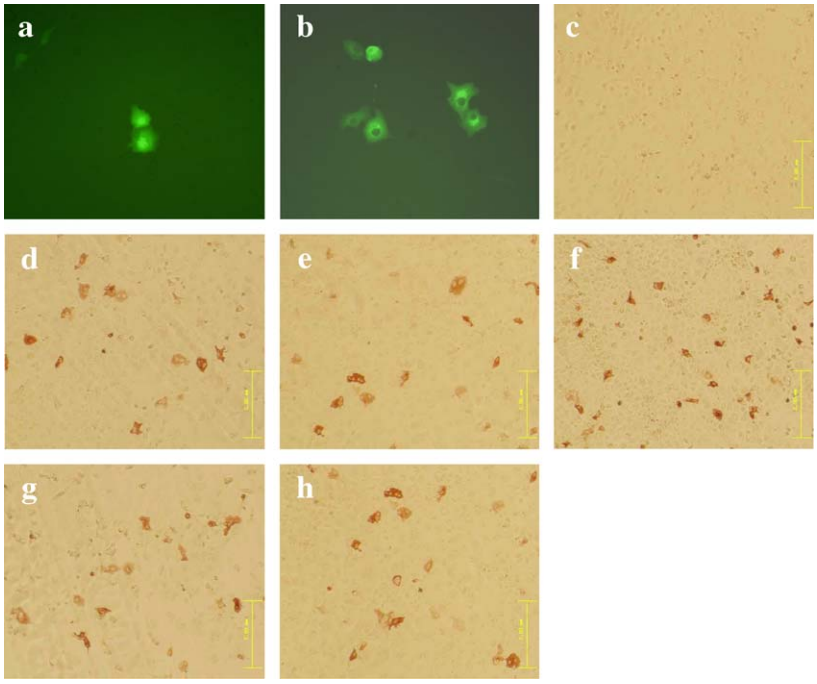


Fig. 5. GoIL-2 expressed in vero cells (×110). (a) (fluorescence) and (c) (immunocytochemical staining) are vero cells transfected with pEGFP-c2 vector alone. (b) Indicates GFP-GoIL-2 expressed in vero cells 3 h after transfection (fluorescence). (d)–(h) (immunocytochemical staining) are GoIL-2 protein in vero cells recognized by mAbs 3B2, 5B10, 3C4, 5D4 and 2F7 to rGoIL-2, respectively.

Table 1
Characteritics of anti-rGoIL-2 mAbs

MAbs	Isotype	Indirect ELISA				Immunoblotting			Neutralization
		6 × His	rChIL-2	rDuIL-2	rGoIL-2	rChIL-2	rDuIL-2	rGoIL-2	
3B2	IgG1, κ	−	−	−	+	−	−	+	−
5B10	IgG1, κ	−	−	−	+	−	−	+	+
3C4	IgG1, κ	−	−	−	+	−	−	+	−
5D4	IgG1, κ	−	−	−	+	−	−	+	−
2F7	IgG1, κ	−	−	−	+	−	−	+	−

"+" indicates positive; "−" shows negative.

while 12.5 ng rDuIL-2/well is needed for goose lymphocytes and 12.5 ng rDuIL-2/well for chicken lymphocytes (Fig. 6C). Neither the protein control from *E. coli* LMG194 strain transfected with pBAD/His B vector alone, nor the RPMI-1640−FCS could stimulate the proliferation of activated goose, duck and chicken lymphocytes.

2.6. Ability of mAbs to neutralize the biological activity of recombinant and endogenous IL-2

Five mAbs and one pAb to rGoIL-2 were tested for their ability to neutralize the biological function of the recombinant and endogenous GoIL-2 as measured by the inhibition of Con A-stimulated lymphocyte proliferation. Only mAb 5B10 and pAb exhibited neutralizing activity in a dose-dependent manner (Fig. 7). mAb 5B10 neutralized 85% of the lymphocyte-stimulating activity of rGoIL-2 and 80% for endogenous GoIL-2. The inhibiting effect of pAb on the activity of rGoIL-2 and endogenous GoIL-2 was found to be 100%.

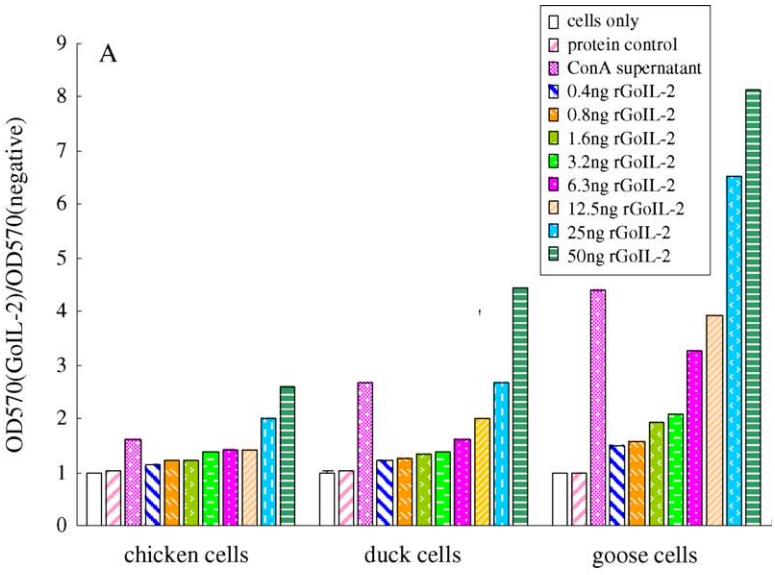
2.7. In vivo immune response to AIV vaccine induced by rGoIL-2

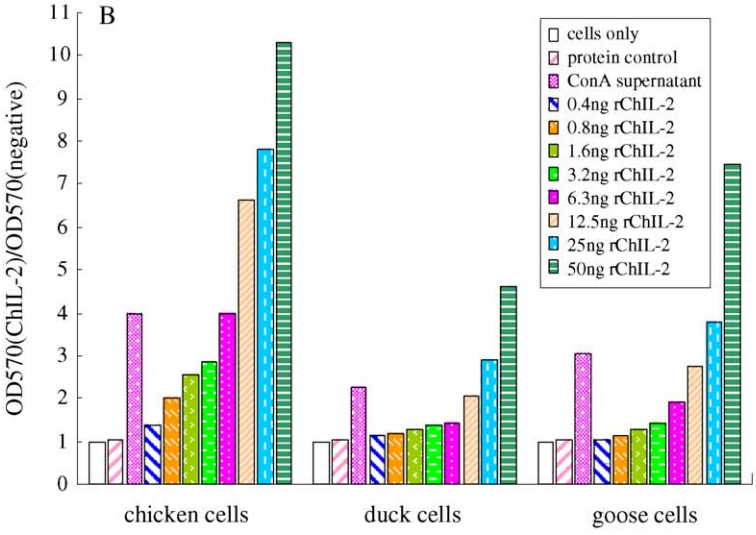
Following the intramuscular inoculations with the rGoIL-2 and the inactivated oil emulsion AIV vaccine, blood samples were collected at specified times and the serum HI antibody to AIV was measured. As shown in Table 2, in gooses injected intramuscularly with both rGoIL-2 protein (300 ng or 350 ng per goose, respectively) and AIV vaccine 2 weeks after immunization, the average titers of HI antibodies to AIV reached 6.3−6.4 log 2, while the average titers of HI antibody to AIV were 5.2−5.3 log 2 in gooses only vaccinated with AIV vaccine or with both 250 ng rGoIL-2 and AIV vaccine. The results clearly showed that 300 ng rGoIL-2 per goose strengthened in vivo immune responses induced by the inactivated oil emulsion AIV vaccine.

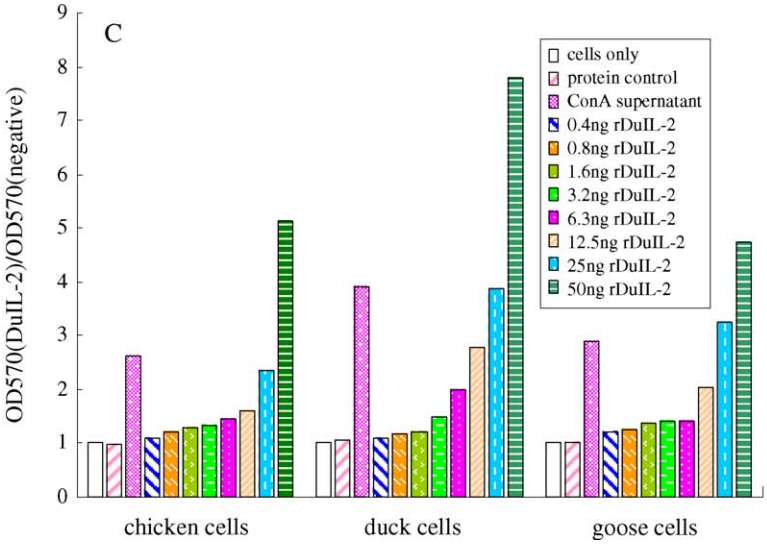
3. Discussion

Up to date, information about goose IL-2 has not been available. Recently, the cloning and functional characterization of chicken, turkey and duck IL-2s have been reported [11−15,17,25−27]. The amino acid identity amongst these avian IL-2s was up to 58−73%. In this report, the goose IL-2 cDNA was cloned by RT-PCR using primers based on the highly conserved regions among chicken, turkey and duck IL-2s. We found that GoIL-2 mRNA was undetectable using RT-PCR in goose SMC 1 h and 72 h after Con A stimulation, but was detected in all samples from 2 h to 48 h after stimulation, showing that GoIL-2 expression began 1 h after stimulation and stopped 72 h after stimulation. Sundick et al. [11] detected the ChIL-2 mRNA 5 h after stimulation with Con A. In mitogen-stimulated mouse spleen cells, the IL-2 gene is only transiently expressed with maximal mRNA steady state levels between 6−14 h post-stimulation [28]. IL-2 mRNA expression of northern elephant seal peaked in the first 8 h following Con A stimulation [29].

Whether rGoIL-2 protein expressed in *E. coli* possessed a biological function, we measured the bioactivity of GoIL-2 protein using in vitro lymphocyte proliferation and in vivo immune responses of AIV vaccine vaccination. As little as 1.6 ng/well of rGoIL-2 could significantly stimulate in vitro proliferation of goose lymphocytes while 6.3 ng rGoIL-2/well was needed for duck lymphocytes and 25 ng rGoIL-2/well for chicken lymphocytes were need (Fig. 6A). In addition, after each goose was intramuscularly vaccinated with a dose of 300−350 ng rGoIL-2, the vaccinated gooses reveal strengthening immune responses to AIV vaccine immunization. These data suggested that rGoIL-2 had biological effects, though the effect on duck and chicken lymphocytes was relatively weak. These evidences mentioned above led to sufficient support that functional similarity existed between goose and duck as well as chicken IL-2 molecules. Furthermore, these results implied that the rGoIL-2 was an important immunoregulatory molecule well-studied in chicken and mammalian counterparts [30−33]. Similarly, an in vitro proliferation of chicken, duck and goose lymphocytes induced by ChIL-2 as well as DuIL-2 was similar to that of GoIL-2 (Fig. 6B and C). The result further demonstrated that the effect of rGoIL-2, rChIL-2 and rDuIL-2 was dose dependent and certain species-specialties existed. Previous reports showed that there was certain species-characteristic


A
OD570(GoIL-2)/OD570(negative)
cells only
protein control
ConA supernatant
0.4ng rGoIL-2
0.8ng rGoIL-2
1.6ng rGoIL-2
3.2ng rGoIL-2
6.3ng rGoIL-2
12.5ng rGoIL-2
25ng rGoIL-2
50ng rGoIL-2
chicken cells
duck cells
goose cells


B
OD570(ChIL-2)/OD570(negative)
cells only
protein control
ConA supernatant
0.4ng rChIL-2
0.8ng rChIL-2
1.6ng rChIL-2
3.2ng rChIL-2
6.3ng rChIL-2
12.5ng rChIL-2
25ng rChIL-2
50ng rChIL-2
chicken cells
duck cells
goose cells


C
OD570(DuIL-2)/OD570(negative)
cells only
protein control
ConA supernatant
0.4ng rDuIL-2
0.8ng rDuIL-2
1.6ng rDuIL-2
3.2ng rDuIL-2
6.3ng rDuIL-2
12.5ng rDuIL-2
25ng rDuIL-2
50ng rDuIL-2
chicken cells
duck cells
goose cells

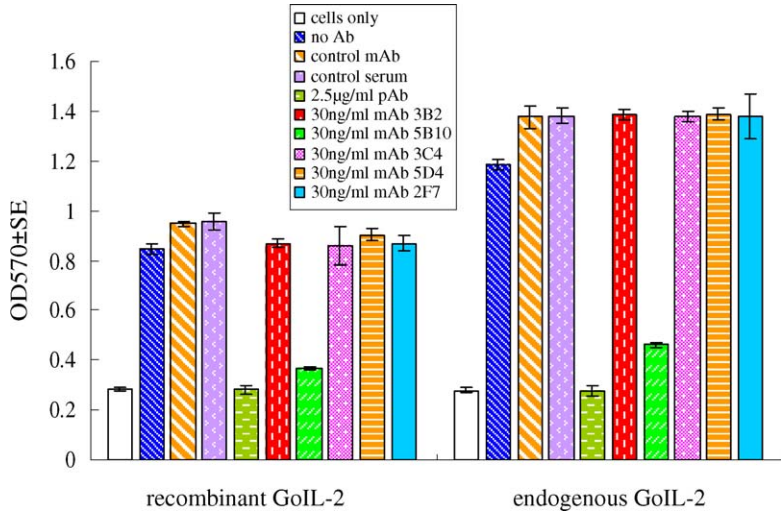


Fig. 7. Neutralizing activity of anti-IL-2 Abs in goose IL-2 lymphocyte proliferation assay induced by rGoIL-2 and supernatant of 24 h Con A-stimulated goose SMC. Concentration of mAb 5B10 used was 30 ng/ml, and that of polyclonal antibody was 3 μg/ml. Non-immunized rabbit serum and mAb to spike protein of IBV [40] were used as negative controls. Cells without addition of any antibody were used as background control. Values are expressed as mean counts ± standard error.

between IL-2 from cells of various species [34–38]. The proliferative response of human lymphocytes to recombinant porcine IL-2 was only 50% of that seen with recombinant HuIL-2 [38]. Recombinant ovine IL-2 was able to stimulate the proliferation of bovine lymphocytes but not porcine lymphocytes. It was not surprising that there was 97% amino acid identity between the ovine and bovine proteins while the amino acid identity between ovine and porcine IL-2 was 73%. Both ovine and bovine IL-2 amino acid residues involved in the binding to the human equivalents of the IL-2Rα (p55) and IL-2Rβ (p75) subunits showed further that both these two domains of the ligand were entirely conserved [34,38]. Comparison of the ovine and porcine IL-2 amino acid sequences revealed that six of the 25 residues involved in binding to the IL-2Rα (p55) were different. As for the IL-2Rβ (p75) binding domain, five of the 10 amino acid residues were different. Hence, the amino acid residues in the two receptor binding domains were less conserved between ovine and porcine IL-2 compared with the ovine and bovine IL-2 [39].

In our study, five mAbs specific for rGoIL-2 were produced. Anti-GoIL-2 mAbs do not recognize rDuIL-2 and rChIL-2 proteins. While the *E. coli*-derived rGoIL-2 could be recognized by mAbs 5H6 and 5F6 to rDuIL-2, indicating that five anti-GoIL-2 mAbs recognized a conserved epitope in the duck and chicken molecules. And mAbs 5H6 and 5F6 to rDuIL-2 recognized conserved epitopes in duck and goose molecules. Furthermore, all mAb to *E. coli*-derived GoIL-2 reacted specifically with EGFP-GoIL-2 protein expressed in vero cells, evincing that the five mAbs remained the binding epitopes of nature GoIL-2. However, in neutralizing assay, only one of the five mAbs (mAb 5B10) was able to neutralize the activity of rGoIL-2 and endogenous GoIL-2 in Con A-induced lymphocyte proliferation assay, indicating that only mAb 5B10 reacts with the functional domain of GoIL-2 protein.

4. Materials and methods

4.1. Animals

The embryonated eggs of Eastern Zhejiang White goose (Xiangshan institute of Eastern Zhejiang White goose, China) and Muscovy duck (Yuyao Shennong poultry Co. Ltd., Zhejiang, China) were hatched at our laboratory, and reared in a special room with unlimited access to feed and water and used at 30 days. Thirty day-old specific-pathogen-free (SPF) Leghorn chickens were obtained from Beijing Merial Vital Laboratory Animal Technology Co. Ltd., Beijing, China. Four week-old BALB/c mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China. New Zealand white rabbits (NZW

Fig. 6. Lymphocyte proliferation was induced by goose, chicken and duck IL-2s. (A) Effect of rGoIL-2 on chicken, duck and goose lymphocytes under different concentrations. (B) Effect of rChIL-2 on chicken, duck and goose lymphocytes under different concentrations. (C) Effect of rDuIL-2 on chicken, duck and goose lymphocytes under different concentrations. Cells without addition of any protein and protein preparation from bacteria transformed with empty pBAD/His B vector were used as negative controls. Supernatants from chicken, duck and goose splenocytes stimulated for 24 h with Con A were used as positive controls. The proliferation values are dose-responsive to rChIL-2, rDuIL-2 or rGoIL-2 produced from prokaryotic system.

Table 2
Goose anti-AIV (H9N2) HI antibody response induced by rGoIL-2

Group	Animal No.	rGoIL-2 dose (ng/goose)	Serum HI titer (log 2)[a]			
			1st week	2nd week	3rd week	4th week
Vaccine	10	250	0	5.2	5.6	6.6
	10	300	0	6.3	6.7	7.7
	10	350	0	6.4	6.9	8.2
	10	×	0	5.3	5.8	6.5
Control	10	×	0	0	0	0

[a] HI titer was geometry mean titer.

rabbit) were purchased from Laboratory Animal Center of Zhejiang Academy of Medicine, Zhejiang, China. All laboratory animals and animal subjects used in this study have been approved by the scientific ethical committee of the Zhejiang University.

4.2. RNA isolation, RT-PCR and sequencing

Goose splenic mononuclear cells (SMC) were prepared as previously described by Zhou et al. [14]. SMC stimulated with Con A for 0, 1, 2, 4, 8, 12, 14, 16, 18, 24, 48 and 72 h were harvested, respectively. Total cellular mRNA was extracted with Trizol reagent (Gibco BRL, Gaithersburg, MD). Primers were designed according to the conserved cDNA sequence for DuIL-2, ChIL-2 and TuIL-2 (GenBank accession no. AF294323, AF017645 and AJ007463, respectively). Sense primer (5′-AATACTAGCACAGACAACCAG-3′) corresponded nt positions 59–81. Antisense primer (5′-TTACTGAAAGATTTATTAAATATCATCTA-3′) was complementary to nt positions 798–824. The cDNAs were synthesized from total cellular RNA using the antisense primer. PCR was conducted with sense and antisense primers under the conditions consisting of 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 45 °C for 45 s and DNA extension at 72 °C for 2 min. The final DNA extension was carried out at 72 °C for 10 min. RT-PCR products were detected in 1% agarose gel. RT-PCR product was sequenced. The predicted amino acid sequence of GoIL-2 protein was compared with that of avian and mammalian IL-2s from GenBank using DNASTAR 5.0 software (DNASTAR Inc., Madison, WI, USA). Putative Signal peptide of GoIL-2 protein was determined using prediction servers at http://www.cbs.dtu.dk./services/.

4.3. Expression and purification of recombinant GoIL-2

Based on the cDNA sequence of the above-mentioned GoIL-2, the GoIL-2 ORF without the putative signal peptide sequence was obtained by PCR using the upstream primer 5′-GC*GAATTC*GCACCTCTATCAGAGAA-3′ containing the *Eco*RI site and the downstream primer 5′-GC*AAGCTT*TTATTTTAGCATAGATCT-3′ containing the *Hin*dIII site. Following digestion with *Eco*RI and *Hin*dIII, the PCR product was subcloned into the vector pBAD/His B (Invitrogen, Carlsbad, CA) for expression as a His-tagged protein, named as pBAD-GoIL-2. The pBAD-GoIL-2 plasmid was transformed into competent *E. coli* LMG194 cells (Invitrogen) and the transformants were screened on LB plates for ampicillin resistance. Plasmid DNA from resistant colonies was identified and sequenced to determine the size and orientation of the insert. His-GoIL-2 expression was induced by the manufacturer's protocols, and checked by SDS-PAGE as described previously by Zhou et al. [40]. For the purification of the soluble recombinant His-GoIL-2 (rGoIL-2), the bacterial pellet was suspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea, pH 8.0) and lysed thoroughly by sonication on ice. The lysate was centrifuged at 10,000 × *g* for 20 min at room temperature (RT). Then the supernatant was collected and applied to a nickel column (Qiagen) to purify histidine-tagged proteins according to the manufacturer's instructions. The concentration of the purified rGoIL-2 was determined spectroscopically at 595 nm using a protein assay kit based on the Bradford reagent (Biorad, Hercules, CA). Western blot analysis of the expressed rGoIL-2 protein was checked as previously described by Zhou et al. [40] with anti-X-press™ mAb (Invitrogen), anti-human IL-2 mAb (Bender Medsystem Gmbh, Austria), and anti-DuIL-2 mAbs [26], anti-mouse IL-2 mAb (R&D systems Inc., U.S.A), anti-ChIL-2 mAbs [41].

To prepare native soluble His-GoIL-2 for proliferation assay, the bacterial pellet was washed with PBS (pH 7.2) for three times and resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole) at 2–5 ml per gram wet weight with 0.01% Triton-X 100, frozen in liquid nitrogen, thawed entirely under 37 °C water bath and sonicated on ice for three 30 s pulses with intervening 20 s pauses. The lysed cells were centrifuged at 10,000 × *g* for 20 min at 4 °C. The rGoIL-2 was purified under native conditions from the resulting supernatant by the above-mentioned nickel column. The purified rGoIL-2 was dialyzed against PBS, concentrated using the PEG 6000. Polymixin B Sepharose (Sigma, St. Louis, MO) was used to remove LPS from the purified proteins. The bacterial protein from *E. coli* LMG194 strain transformed with empty pBAD/His B vector were prepared and used as protein control.

4.4. Production and characterization of monoclonal and polyclonal antibodies to GoIL-2

MAbs specific for rGoIL-2 were obtained by immunizing mice with purified rGoIL-2 as described previously

[26,40]. Briefly, the spleen cells isolated from immunized mice were fused with the sp2/0 myeloma cells by standard methods. Hybridoma supernatants were screened for specific binding to rGoIL-2 by the indirect ELISA as described earlier [19], using 6 × His, cell lysate of *E. coli* LMG194, rDuIL-2 [26], rChIL-2 [41] and rGoIL-2 as antigens. Hybridomas secreting mAbs to GoIL-2 were isolated and cloned by limiting dilution for acites production. The isotypes of the mAbs were determined using a commercial kit (Southern Biotechnology Associates, Inc., Birmingham, AL 35260, USA). Western blot analysis of mAbs to rGoIL-2 was screened as the above-mentioned method.

Anti-rGoIL-2 rabbit polyclonal antibody (pAb) was generated by bi-weekly immunization of NZW rabbits with rGoIL-2-CFA emulsion (100 μg of rGoIL-2 per rabbit). Immunized rabbits were bled 14 days after last injection and the serum was collected.

4.5. Preparation of endogenous GoIL-2 protein

The suspensions of the goose SMC were prepared as the method mentioned above. Goose SMC were diluted to 1×10^7 cells/ml in RPMI-1640 containing 10% FCS and 10 μg/ml Con A, and incubated at 40 °C in 5% CO_2 for 0, 4, 8, 12, 24, 48,72 and 96 h. At each time point, the residual Con A was moved by incubating with 0.1 M a-methyl-D-mannoside (Sigma Chemical Co., St. Louis, USA) for 30 min and the supernatant was harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C.

4.6. Antigen (Ag)-capture ELISA for GoIL-2

For quantification of rGoIL-2 expressed in *E. coli* and endogenous GoIL-2 in supernatants from Con A-stimulated SMC, an Ag-capture ELISA was developed with certain modifications as described previously [20]. Briefly mAbs and pAb to GoIL-2 were purified by fast protein liquid chromatography (Amersham–Pharmacia Biotech, Piscataway, NJ), and rGoIL-2 purified under denaturing conditions was used as a reference protein. Ninety-six-well flat-bottom plates (NUNC, Denmark) were coated with mAb (10 μg/ml, 100 μl/well) to GoIL-2 in 0.1 M carbonate buffer, pH 9.6 at 37 °C for 1 h followed at 4 °C overnight. Each well was blocked with 200 μl PBS containing 3% BSA for 1 h at RT followed by three washes with 0.05 M PBST (PBS containing 0.01% Tween 20). One hundred microlitres of culture supernatant from Con A-stimulated SMC or protein preparations containing rGoIL-2 was added to each well and incubated for 4 h at RT. pAb to rGoIL-2 (100 μl/well) in PBS–0.1% BSA was added and incubated for 1 h at RT after five washes. After incubation with HRP-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Inc., Maryland, USA) for 30 min at RT. Peroxidase activity was detected by adding 100 μl/well of *o*-phenylenediamine (0.4 mg/ml in 0.05 M PBS, pH 5.0) substrate (Sigma) incubated for 15 min, and reaction was stopped by the addition of 50 μl of 2 M sulfate acid. Reactions were monitored by an automated microtiter plate reader at 490 nm.

4.7. Construction of eukaryotic expression vector, transfection and immunocytochemical staining

The GoIL-2 ORF with the signal peptide sequence was amplified by PCR using the upstream primer 5′-GC*GAATTC*ATGATGTGCAAAGTACTGATC3-′ containing the *Eco*RI site and downstream primer 5′-TT*GGATCC*TTTTTGCAGATATCTCAC-3′ containing the *Bam*HI site. The PCR product was subcloned into the pEGFP-c2 plasmid (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. The resulting plasmid pEGFP-GoIL-2 was transfected into the vero cells which had been seeded into 24-well plates (1×10^5 cells/well) using a commercially available lipofectamine 2000 (2.5 μg/ml, Invitrogen, Carlsbad, CA) in Opti-MEM medium (Gibco). The cells were examined under an inverted fluorescence microscope 3 h post-transfection. Then the cells were fixed with 80% cold acetone at −20 °C. After washing three times with PBS, the cells were blocked for 1 h at 37 °C with 5% skim milk and 0.1% Triton-X 100 in PBST. The cells were then incubated with the mAbs to rGoIL-2 diluted in PBST containing 2% skim milk for 1 h at 37 °C. Washed three times with PBST, the cells were incubated for 1 h at 37 °C with HRP-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories). Color development was carried out with 9-aminoethyl carbazole (Sigma) and hydrogen peroxide in 0.05 M sodium acetate buffer (pH 5.0). The cell expressed GoIL-2 was stained reddish-brown in this assay.

4.8. Bioassay and neutralization

Lymphocyte proliferation induced by rGoIL-2 was detected by MTT test [42]. Briefly, SMC from the chickens, ducks and gooses were, respectively, suspended at 1×10^7 cells/ml in RPMI-1640–FCS containing 10 μg/ml Con A, and incubated at 40 °C in 5% CO_2 for 24 h. SMC stimulated by Con A for 24 h were incubated in RPMI-1640 medium containing 0.1 M a-methyl-D-mannoside (Sigma) at 40 °C for 30 min. The cells were applied to Histopaque-1077 (Sigma) and live cells were selected after centrifugation. The enriched viable cell population was resuspended in RPMI-1640 containing 10% FCS and cultured at 5×10^6 cells/ml with the supernatant containing endogenous GoIL-2 as well as rGoIL-2 proteins in triplicates in 96-well microtiter plates (100 μl/well, Costar, Corning, NY, USA). The cells containing RPMI-1640–FCS and protein control from *E. coli* LMG194 strain transformed with

pBAD/His B vector alone were used as negative controls. After incubation at 40 °C for 48 h, 20 μl MTT (5 mg/ml) was added to each well and reacted for 4 h. One hundred microlitres of lysis buffer (10% SDS–0.01 mol/l HCl) was added to each well. Plates were incubated for 20 h at 40 °C under 5% CO_2 and OD value was measured at 570 nm. $OD_{GoIL\text{-}2}/OD_{negative}$ value of 1.5 or above was defined as the positive criteria for the biological activity of rGoIL-2.

Neutralizing capacity of mAbs and pAb to rGoIL-2 was screened in 96-well plates as described previously with modifications [43]. Briefly, by fixing rGoIL-2 with antibodies in different concentrations, His-GoIL-2 (1.6 ng/well) and supernatant of Con A-stimulated goose SMC (100 μl/well) was mixed, respectively, with an equal volume of serial 10-fold dilutions of mAbs or pAb and incubated for 1 h at 37 °C. One hundred microlitres of cells (5×10^6 cells/ml) stimulated by Con A for 24 h were added to each well. Non-immunized rabbit serum and mAb to spike protein of IBV [40] were used as negative controls. Cultures were incubated for 48 h at 40 °C and cell proliferation was measured as above. The percent neutralizing ability of monoclonal and polyclonal antibodies against rGoIL-2 was calculated with the formula as described by Zhou et al. [27].

4.9. Goose immunization and antibody detection

Forty-five day-old Eastern Zhejiang white gooses were divided into five groups, with 10 gooses per group. After Group 1, Group 2 and Group 3 gooses were each intramuscularly and, respectively, injected with a dose of 250 ng, 300 ng and 350 ng rGoIL-2, all gooses were immunized intramuscularly with the commercial inactivated oil emulsion vaccine against avian influenza virus according to the manufacture's instruction at once [AIV (H9N2), Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China]. Gooses in Group 4 only were each intramuscularly vaccinated with the inactivated AIV oil emulsion vaccine (H9N2), while gooses in the 5th Group were each intramuscularly immunized with the oil adjuvant as a control. Under the same feeding condition, blood samples were collected via wing vein puncture at 1, 2, 3 and 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).

Acknowledgements

This work was funded by Zhejiang Provincial Nature Science Foundation of China (Grant No. R303145) and National Natural Science Foundation of China (Grant No. 30230270). We thank Dr. Qi-Jun Chen (Microbiology and Tumor Biology Center, Karolinska Institute, Sweden) for critical suggestions and comments.

References

[1] Swain SL. Lymphokines and the immune response: the central role of interleukin-2. Curr Opin Immunol 1991;3:304–10.
[2] Ferrante A. Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors. Immunol Ser 1992;57:417–36.
[3] Whittington R, Faulds D. Interleukin-2. A review of its pharmacological properties and therapeutic use in patients with cancer. Drugs 1993;46:446–514.
[4] Espinoza-Delgado I, Bosco MC, Musso T, Gusella GL, Longo DL, Varesio L. Interleukin-2 and human monocyte activation. J Leukoc Biol 1995;57:13–9.
[5] Hazama M, Mayumi-Aono A, Asakawa N, Kuroda S, Hinuma S, Fujisawa Y. Adjuvant-independent enhanced immune responses to recombinant herpes simplex virus type 1 glycoprotein D by fusion with biologically active interleukin-2. Vaccine 1993;11:629–36.
[6] Rosenberg SA. Progress in human tumour immunology and immunotherapy. Nature 2001;411:380–4.
[7] Nakao M, Hazama M, Mayumi-Aono A, Hinuma S, Fujisawa Y. Immunotherapy of acute and recurrent herpes simplex virus type 2 infection with an adjuvant-free form of recombinant glycoprotein D-interleukin-2 fusion protein. J Infect Dis 1994;169:787–91.
[8] Waldmann TA. The IL-2/IL-2 receptor system: a target for rational immune intervention. Immunol Today 1993;14:264–70.
[9] Eicher DM, Damjanovich S, Waldmann TA. Oligomerization of IL-2Ralpha. Cytokine 2002;17:82–90.
[10] Zelus D, Robinson-Rechavi M, Delacre M, Auriault C, Laudet V. Fast evolution of interleukin-2 in mammals and positive selection in ruminants. J Mol Evol 2000;51:234–44.
[11] Sundick RS, Gill-Dixon C. A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. J Immunol 1997;159:720–5.
[12] Kaiser P, Mariani P. Promoter sequence, exon: intron structure, and synteny of genetic location show that a chicken cytokine with T-cell proliferative activity is IL2 and not IL15. Immunogenetics 1999;49:26–35.
[13] Lawson S, Rothwell L, Kaiser P. Turkey and chicken interleukin-2 cross-react in vitro proliferation assays despite limited amino acid sequence identity. J Interferon Cytokine Res 2000;20:161–70.
[14] Zhou JY, Chen JG, Wang JY, Kwang J. Cloning and genetic evolution analysis of chIL-2 gene of Chinese local breeds. Prog Biochem Biophys 2003;30:384–9.
[15] Stepaniak JA, Shuster JE, Hu W, Sundick RS. Production and in vitro characterization of recombinant chicken interleukin-2. J Interferon Cytokine Res 1999;19:515–26.
[16] Zhou JY, Chen JG, Wang JY, Zhang DY, Zheng XJ. Expression of chicken interleukin-2 gene in *Pichia methanolica*. J Zhejiang Univ (Agric Life Sci) 2003;29:419–23.
[17] Hilton LS, Bean AG, Kimpton WG, Lowenthal JW. Interleukin-2 directly induces activation and proliferation of chicken T cells in vivo. J Interferon Cytokin Res 2002;22:755–63.
[18] Lillehoj HS, Choi KD, Jenkins MC, Vakharia VN, Song KD, Han JY, et al. A recombinant *Eimeria* protein inducing interferon-gamma production: comparison of different gene expression systems and immunization strategies for vaccination against coccidiosis. Avian Dis 2000;44:379–89.
[19] Hulse DJ, Romero CH. Partial protection against infectious bursal disease virus through DNA-mediated vaccination with the

VP2 capsid protein and chicken IL-2 genes. Vaccine 2004;22: 1249–59.
[20] Rothwell L, Hamblin A, Kaiser P. Production and characterisation of monoclonal antibodies specific for chicken interleukin-2. Vet Immunol Immunopathol 2001;83:149–60.
[21] Miyamoto T, Lillehoj HS, Sohn EJ, Min W. Production and characterization of monoclonal antibodies detecting chicken interleukin-2 and the development of an antigen capture enzyme-linked immunosorbent assay. Vet Immunol Immunopathol 2001;80:245–57.
[22] Hala K, Schauenstein K, Neu N, Kromer G, Wolf H, Bock G, et al. A monoclonal antibody reacting with a membrane determinant expressed on activated chicken T lymphocytes. Eur J Immunol 1986;16:1331–6.
[23] Schauenstein K, Kromer G, Hala K, Bock G, Wick G. Chicken-activated-T-lymphocyte- antigen (CATLA) recognized by monoclonal antibody INN-CH 16 represents the IL-2 receptor. Dev Comp Immunol 1988;12:823–31.
[24] Bertram EM, Jilbert AR, Kotlarski I. An homologous in vitro assay to detect lymphokines released by PHA-activated duck peripheral blood lymphocytes and spleen cells. Vet Immunol Immunopathol 1997;56:163–74.
[25] Wang JY, Zhou JY, Chen JG, Zhang ZH. Cloning and evolution analysis of a duck lymphokine gene homologous to chicken IL-2. Chin J Vet Sci 2003;23:545–7.
[26] Gong H, Wu JX, Wang JY, Chen JG, Zhou JY. Production and characterization of monoclonal antibody to duck IL-2. J Zhejiang Univ (Agric Life Sci) 2004;30:169–72.
[27] Zhou JY, Wang JY, Chen JG, Wu JX, Gong H, Teng QY, et al. Cloning, in vitro expression and bioactivity of duck interleukin-2. Mol Immunol 2005;42:589–98.
[28] Swoboda R, Wecker E, Schimpl A. Regulation of IL 2 expression in mitogen-activated murine T lymphocytes. Immunobiology 1987;174:300–12.
[29] Shoda LK, Brown WC, Rice-Ficht AC. Sequence and characterization of phocine interleukin 2. J Wildl Dis 1998;34:81–90.
[30] Schat KA, Calnek BW, Weinstock D. Cultured avian lymphocytes with natural killer cell activity. Prog Clin Biol Res 1987;238:157–69.
[31] Lillehoj HS, Kang SY, Keller L, Sevoian M. *Eimeria tenella* and *E. acervulina*: lymphokines secreted by an avian T cell lymphoma or by sporozoite-stimulated immune T lymphocytes protect chickens against avian coccidiosis. Exp Parasitol 1989;69:54–64.
[32] Corbel C, Thomas JL. Establishment of an IL-2-dependent, antigen nonspecific chicken T-cell line. Dev Comp Immunol 1990;14:439–46.
[33] Choi KD, Lillehoj HS. Role of chicken IL-2 on gammadelta T-cells and *Eimeria acervulina*-induced changes in intestinal IL-2 mRNA expression and gammadelta T-cells. Vet Immunol Immunopathol 2000;73:309–21.
[34] Goodall JC, Emery DC, Bailey M, English LS, Hall L. cDNA cloning of porcine interleukin 2 by polymerase chain reaction. Biochim Biophys Acta 1991;1089:257–8.
[35] Taniguchi T, Matsui H, Fujita T, Takaoka C, Kashima N, Yoshimoto R, et al. Structure and expression of a cloned cDNA for human interleukin-2. Nature 1983;302:305–10.
[36] English LS, Binns RM, Licence ST. Characterization of pig T cell growth factor and its species-restricted activity on human, mouse and sheep cells. Vet Immunol Immunopathol 1985;9:59–69.
[37] Denyer MS, Martin S, Wardley RC. Cross reactions between porcine, bovine and ovine interleukin-2 preparations. Res Vet Sci 1987;42:252–4.
[38] Seow HF, Mucha MJ, Hurst L, Rothel JS, Wood PR. Expression of ovine interleukin-2 cDNA in *Escherichia coli*. Vet Immunol Immunopathol 1997;56:107–17.
[39] Collins RA, Tayton HK, Gelder KI, Britton P, Oldham G. Cloning and expression of bovine and porcine interleukin-2 in baculovirus and analysis of species cross-reactivity. Vet Immunol Immunopathol 1994;40:313–24.
[40] Zhou JY, Cheng LQ, Zheng XJ, Wu JX, Shang SB, Wang JY, et al. Generation of the transgenic potato expressing full-length spike protein of infectious bronchitis virus. J Biotechnol 2004;111:121–30.
[41] Li ZK, Gong H, Chen JG, Zhou JY. Production and characterization of monoclonal antibody to recombinant chicken IL-2. J Zhejiang Univ (Agric Life Sci) 2004;30:541–4.
[42] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[43] Zhou JY, Wu JX, Cheng LQ, Zheng XJ, Gong H, Shang SB, et al. Expression of immunogenic S1 glycoprotein of infectious bronchitis virus in transgenic potatoes. J Virol 2003;77:9090–3.