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Porcine lactoferrin as feedstuff additive elevates avian immunity and potentiates vaccination

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Abstract In this study, recombinant porcine lactoferrin (PLF) was used as feedstuff additive to investigate the effects of peripheral lymphocyte proliferation and serum antibody titers in chickens vaccinated against the infectious bursal disease (IBD) virus. Treatment groups were fed three doses of PLF powder in their diet (0.5, 1.0, and 2.0% w/w), and the IBD vaccine was administrated at 1 and 3 weeks of age. At 8, 12, and 16 weeks after vaccination, serum IBD antibody titers were measured via the micro-method and T cell proliferation rates were evaluated. The results revealed that a high dose of PLF led to significant increases in serum IgA, IgG and IBD-specific antibody titers ($P < 0.05$). PLF administration, at either low or high doses, enhanced the expression of IFN-γ and IL-12 in chicken T lymphocytes. These results suggest that PLF enhances cell-mediated immunity and augment the ability of IBD vaccination to strengthen subsequent anti-viral responses.

Keywords Porcine lactoferrin · Lymphocyte proliferation · Serum antibody titer · Cytokine · Infectious bursal disease (IBD) · Vaccination

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Introduction

Infectious bursal disease (IBD) has a sudden onset with a short incubation period (2–3 days). Typically 100% morbidity is expected, but the mortality rate varies depending on the virus strain (Nagarajan and Kibenge 1997; Saif 1998). The IBD virus has a worldwide distribution is resistant to a variety of disinfectants, and is environmentally stable. Current world-wide exposure of chickens to the IBD virus is responsible for multi-billion US dollar losses in the poultry industry (Liu et al. 2005). Serologic

prevalence of natural infection with the virus in chickens was reported to be 58.6% in Taiwan (Wu et al. 1983). Strategies to control IBD are largely based on vaccination programs.

Lactoferrin (LF) is a 75–80 kDa multi-functional, iron-binding glycoprotein that is present in external secretions of mammals, such as breast milk, tears, saliva, and mucous (Brock 2002). LF is thought to play an important role in the non-specific immune response, since the protein is primarily found in external secretions and mucosa. LF has been demonstrated to have antimicrobial properties, function as an antioxidant, promote growth of lymphocytes, regulate iron absorption, and act as a transcription activator (Mulder et al. 2008; Oh et al. 2004). Porcine lactoferrin (PLF) is secreted in the colostrum at a higher level than other mammalian lactoferrins (Chu et al. 1993; Yang et al. 2000). Piglets that consume sufficient colostral milk have been demonstrated to exhibit lower incidences of diarrhea, respiratory tract infections, infection-induced wheezing, and anemia after weaning (Lee et al. 1998a, b).

In previous studies, we generated a series of transgenic mice capable of expressing and secreting recombinant PLF in milk. Pups suckling PLF-enriched milk from transgenic female mice had significantly greater villous height in their duodenums and heavier body weight compared to pups suckling normal milk (Wu et al. 2007). Oral administration of PLF-enriched milk protects neonates from pathogenic challenges to the gastrointestinal tract, which indicates that PLF has a natural role in selective decontamination of the digestive tract (Yen et al. 2009). Following inoculation with enterovirus type 71 (EV71) at 4 days after birth, pups ingesting PLF-enriched milk exhibited a significantly higher survival rate and lower viral genomic RNA content, indicating that PLF had a blocking effect on EV71 viral infection (Chen et al. 2008a). Therefore, we hypothesize that PLF may play an important antiviral and immunomodulatory role in animals that had been raised in their natural environment.

The observed properties of LF may be related to its immunoregulatory effects on T-helper 1 (Th1) or T-helper 2 (Th2) cell activities. LF stimulates the differentiation of T cells from immature precursors (Li et al. 2006). Studies performed under non-pathogenic conditions have indicated that LF supports the proliferation and differentiation of Th cells into Th1 or Th2 cell types. In addition, LF plays different roles in disease by affecting the Th1/Th2 cytokine balance in a manner that is dependent on the immune status of the host (Fischer et al. 2006). Recent studies have indicated that LF is a useful and effective adjuvant that improves the immunogenicity of the Bacillus Calmette-Guerin (BCG) vaccine, with the potential to reduce related tissue damage (Hwang et al. 2007a, b).

In the present study, we investigated the potential of utilizing PLF as an immune stimulator to enhance peripheral lymphocyte proliferation and serum antibody titers in chicken vaccinated against IBD virus. Possible dose-dependent immune enhancement by PLF was also evaluated.

Materials and methods

Preparation of recombinant PLF

Single colony of the pPICZαC–rPLF transformed yeast was inoculated in a 500-ml fillister-type flask containing 200 ml BMGY broth and transferred into a 10 l fermenter for large-scale recombinant PLF production as described in our previous report (Chen et al. 2004). The concentrations of PLF protein present in *Pichia pastoris* culture media were measured by SDS-PAGE and western blot analyses. Culture medium was then concentrated and partially purified using a 30% saturated ammonium sulfate. The precipitate was resuspended in a 5 mM Tris buffer containing 50 mM NaCl and dialyzed against the same buffer. The desalted fractions were passed through a Hitrap heparin column and then separated by fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech., USA).

Reagents

RPMI-1640 (GIBCO Invitrogen, Carlsbad, CA) supplemented with benzylpenicillin (100 IU/ml) and streptomycin (100 IU/ml) was used for in vitro cell culture, washing and re-suspension of the cells, and dilution of the mitogen (Hung et al. 2009). Concanavalin A (ConA) (Sigma Chemicals, St. Louis, MO) was dissolved in RPMI-1640 medium to a final concentration of 0.025 mg/ml. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Amersco Inc., Solon, OH) was dissolved in calcium-

and magnesium-free (CMF) phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 5 mg/ml. These reagents were filtered through 0.22 μm filters (Chen et al. 2003, 2006a). The ConA solution was stored at −20°C and the MTT solution and RPMI-640 media were stored at 4°C. The lymphocyte separation medium (Ficoll-Hypaque) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals.

Animal trials

Forty chickens were randomly divided into four groups, each containing equal numbers of birds with similar body weights (BWs) (the average BW was 30 g). Group 1 was the PLF-free control group. Groups 2 to 4 were fed PLF powder at one of three concentrations: 0.5% (w/w; low dosage), 1% (middle dosage) and 2% (high dosage), respectively. Body weight and weight gain for each female and male bird were recorded every 4 weeks from birth to 16 weeks. The IBD vaccine was administered twice at 14 day intervals. The first vaccine (Bursine®-2, Fort Dodge Animal Health Inc., Charles, IA) was orally administered at 1 week of age and the second (IBD BLEN, Merial Select Inc., Gainesville, GA) at 3 weeks of age. Six chickens were sampled randomly from each group at 8, 12, and 16 weeks for examination of peripheral lymphocyte proliferation by the MTT assay. Eight chickens were sampled randomly from each group at 8 and 16 weeks for the determination of serum IBD antibody titers using an ELISA kit (Hung et al. 2009). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Livestock Research Institute. The animal trials were performed in duplicate.

Peripheral lymphocyte proliferation assay

Blood samples (5 ml per chicken) were collected and immediately transferred to aseptic capped tubes containing sodium heparin. The samples were diluted with an equal volume of Hanks' solution and carefully layered onto the surface of the lymphocyte separation medium. After centrifugation, the lymphocyte band was collected and washed twice with RPMI-1640 media without fetal bovine serum (FBS). 80 μl of the cell suspension were added per well of a 96-well tissue culture plate. Another 20 μl of ConA was added to each well. A working concentration of 5 μg/ml ConA was used for the stimulation of lymphocytes. After 44 h of incubation at 39°C in the 5% CO2 incubator, 20 μl of MTT (5 mg/ml) were added to each well. The plates were incubated for another 4 h, and then 100 μl of DMSO were added to each well. The plates were shaken for 5 min to completely dissolve the precipitate. Light absorbance at 570 nm was measured with an ELISA plate reader (Bio-Rad Laboratories Inc., Irvine, CA).

Measurement of IgA, IgG, and IBD antibodies in serum

Total IgA and IgG concentrations in the sera were measured with the Chicken IgA and IgG ELISA Kits (Bethyl Laboratories, Inc., Montgomery, TX), according to the manufacturer's instructions. The sera were diluted up to 4×10^3-fold for IgA detection and 8×10^4-fold for IgG detection in the sample diluent solution from the kit, and absorbance was recorded at 450 nm by use of an ELISA plate reader (Liu et al. 2008). The IBDV-specific antibody titer was determined by ELISA kit (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD), according to the manufacturer's instructions. For each chicken, absorbance of a 20 μl buffered serum sample was evaluated at 650 nm using an ELISA plate reader (Chen et al. 2008b).

Detection of cytokine mRNA by semi-quantitative RT-PCR

Peripheral lymphocytes were collected from the PLF-treated and untreated groups and were harvested at 24 h after ConA stimulation. Total RNA was isolated from the tissues using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. The RNA samples were subsequently treated with deoxyribonuclease I (MBI Fermentas Inc., Lithuania) to remove any contaminating genomic DNA (Chen et al. 2002). Approximately 900 ng of total RNA was reverse-transcribed by MuLV reverse transcriptase using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster, CA) and oligo d(T)16 primer. The PCR primer sequences and annealing conditions for IL-2, IL-4, IL-12, INF-γ, and GAPDH were shown in previous report (Hung et al. 2009). The amplified RT-PCR products were subjected to electrophoresis at 100 V through a 2% agarose gel (Invitrogen, Carlsbad, CA) for

approximately 30 min (Chen et al. 2006b). The DNA band intensities were quantified by use of a densitometer (Kodak Molecular Image System, Rochester, NY).

Statistical analysis

The experimental data were analyzed using the General Linear Model (GLM) procedure developed by SAS, as previously described (Chen et al. 2008c). The differences among the PLF-treated groups and the control group were compared by least squares means comparisons. Statements of statistical significance are based on * $P < 0.05$ and ** $P < 0.01$.

Results

Production and purification of recombinant PLF

For large-scale production of PLF powder as a feedstuff additive, an expression system was generated using recombinant methylotrophic yeast, *P. pastoris*, harboring the full-length *PLF* cDNA driven by the inducible promoter of the alcohol oxidase 1 gene (*AOX1*) and the yeast α-mating factor signal peptide. The PLF was purified by fast protein liquid chromatography (FPLC) system (Fig. 1a) and checked the molecular size by SDS-PAGE (Fig. 1b). A PLF powder was prepared by adsorption with wheat bran until frozen dry at a final concentration of 100 g PLF/kg. Control feedstuff additive lacking PLF was prepared according to the same procedure using culture medium from non-transformed yeast.

Effect of PLF on body weight gain

After feeding of chickens on a diet supplemented with PLF for 16 weeks, the average body weight gain of the four groups was determined (Fig. 2). During the brooding stage from birth to 4 weeks and the early growth stage from 5 to 8 weeks, the body weight gains slightly increased in PLF-treated groups in a dose-dependent manner, although the increase was not statistically significant. The high dose PLF group (2% PLF powder in diet; group D) exhibited significantly enhanced body weight gain at the later growth stages, from 9 to 12 weeks, and the low dose PLF group (0.5% PLF powder in diet; group B) exhibited increased body weight gain at the finishing stage, from 13 to 16 weeks, when compared to the control group (group A) ($P < 0.05$).

Effect of PLF on peripheral lymphocyte proliferation

To study the effects of PLF on peripheral lymphocyte proliferation, peripheral lymphocytes that had been collected from chickens that were fed different dosages of PLF were treated with or without ConA mitogen in vitro. Changes in the proliferation ratio (ConA + /ConA−) of peripheral lymphocytes are summarized in Table 1. Peripheral lymphocyte proliferation in the middle dosage of PLF (PLF_M)-treated

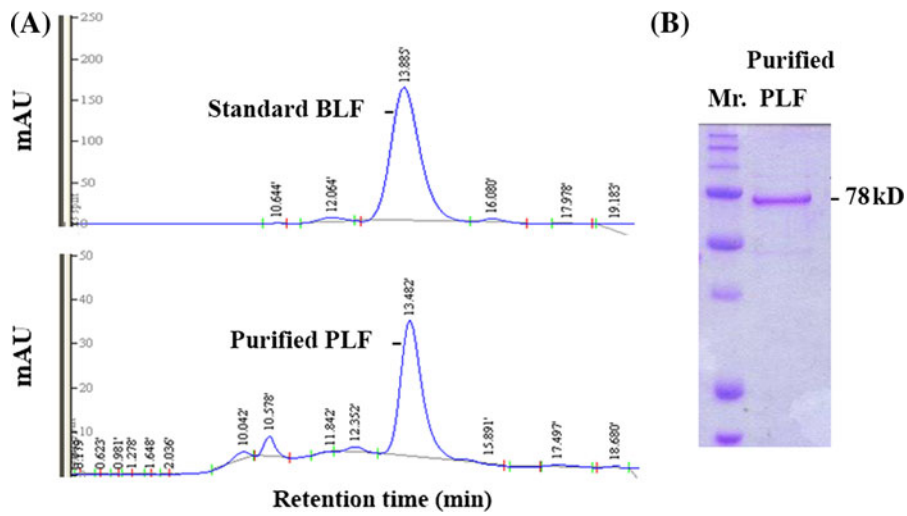

Fig. 1 Production and purification of recombinant PLF from transformed *P. pastoris* yeast. **a** Secreted recombinant PLF protein in yeast culture medium was purified by FPLC system. A commercial bovine lactoferrin (BLF) was used as a standard control in this assay. **b** The purity and molecular weight of purified PLF was checked by 12% SDS-PAGE

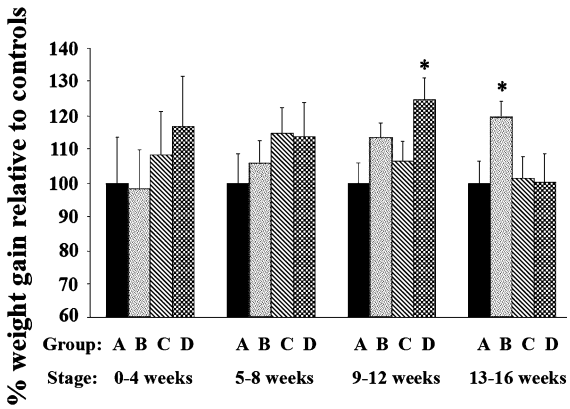

Fig. 2 Effects of PLF on avian body weight gain. Group A was fed a basal diet of 1% non-PLF powder and served as a control group. The weight percentages of recombinant yeast added to the chick feed in treatment groups B through D were 0.5, 1, and 2%, respectively. The animals were weighed every 4 weeks, according to the different growth periods, and plotted as percentages of weight gain relative to the control group. Data are presented as mean ± SD. The *asterisks* represent significant differences ($P < 0.05$) compared to the respective control group

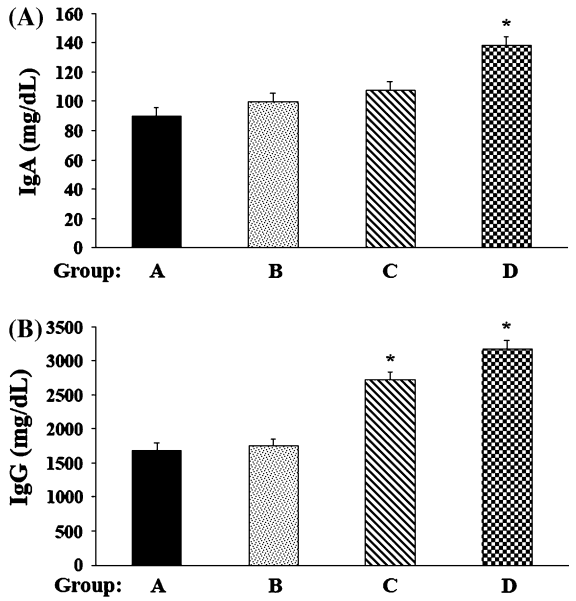

Fig. 3 Effects of PLF on avian serum IgA (**a**) and IgG (**b**). The control group was fed a basal diet lacking the PLF supplement (group A). The levels of serum IgA and IgG (mg/dl) in the 0.5, 1, and 2% PLF-treated chicken groups are reported as those for groups B–D, respectively. Blood samples were collected when the chickens were 16 weeks old. The *asterisks* represent significant differences ($P < 0.05$) compared to the respective control group

group were increased at 16 weeks relative to the control group ($P < 0.05$). Remarkably, the proliferation rate for the high dose of PLF (PLF_H)-treated group was significantly higher than that of the control group ($P < 0.05$) at 12 and 16 weeks.

Effect of PLF on the systemic antibody response

To evaluate the effects of PLF on serum antibody levels, the presence of IgA and IgG in sera of PLF-treated and untreated chickens was assessed. The sera from the PLF_H-treated chickens contained significantly higher levels of IgA than that from the untreated control group ($P < 0.05$) (Fig. 3a). The IgG levels were significantly higher in both the PLF_M- and PLF_H-treated groups compared to the control group ($P < 0.05$) (Fig. 3b).

Enhanced serum IBD antibody titer in response to PLF

Changes in the IBD antibody titers in response to PLF are listed in Table 2**.** In general, the antibody titers of all treatment groups at each time point were higher than those of the control group, and the serum IBD antibody

Table 1 The effects of supplemental PLF on peripheral lymphocyte proliferation of chickens at different ages

Group	Dose (%)	Lymphocyte proliferation ratio (ConA +/ConA−)		
		8 weeks	12 weeks	16 weeks
Control	0	1.90	2.13	1.87
PLF_L	0.5	1.63	2.39	1.75
PLF_M	1.0	2.35	2.83	2.80*
PLF_H	2.0	2.62	3.23*	3.09*
SE		0.29	0.26	0.25

PLF_L low dose of PLF, *PLF_M* middle dose of PLF, *PLF_H* high dose of PLF, *SE* standard error

* Significant difference ($P < 0.05$ versus control group)

titers in all groups at 16 weeks were lower than at 8 weeks. After vaccination, the serum antibody titers in the PLF_H-treated group were significantly higher than those of the control group ($P < 0.05$) and other treatment groups at 16 weeks.

Table 2 The effects of supplemental PLF on the IBDV antibody titer ($\times 10^3$) of chickens at different ages

Group	Dose (%)	IBDV antibody titer ($\times 10^3$)	
		8 weeks	16 weeks
Control	0	5.892	5.080
PLF_L	0.5	9.697	7.246
PLF_M	1.0	9.310	7.473
PLF_H	2.0	9.602	9.572*
SE		1.419	0.966

PLF_L low dose of PLF, *PLF_M* middle dose of PLF, *PLF_H* high dose of PLF, *SE* standard error

* Significant difference ($P < 0.05$ versus control group)

Cytokine gene expression in response to PLF

For evaluation of the effect of PLF on the immune system, changes in cytokine expression at 8 weeks were determined and are shown in Fig. 4. In the IL-2 and INF-γ networks, the IL-2 and INF-γ mRNA levels for the normal control group were low and their expressions were elevated following ConA stimulation (Fig. 4a). PLF administration suppressed IL-2 expression in both low- and high-dose groups, especially in the low-dose group ($P < 0.05$). However, PLF increased INF-γ expression in the PLF_L-treated group ($P < 0.05$) and PLF_H-treated group ($P < 0.01$) in a dose-dependent manner. In the IL-4 and IL-12 networks, the expression levels of IL-4 and IL-12 were

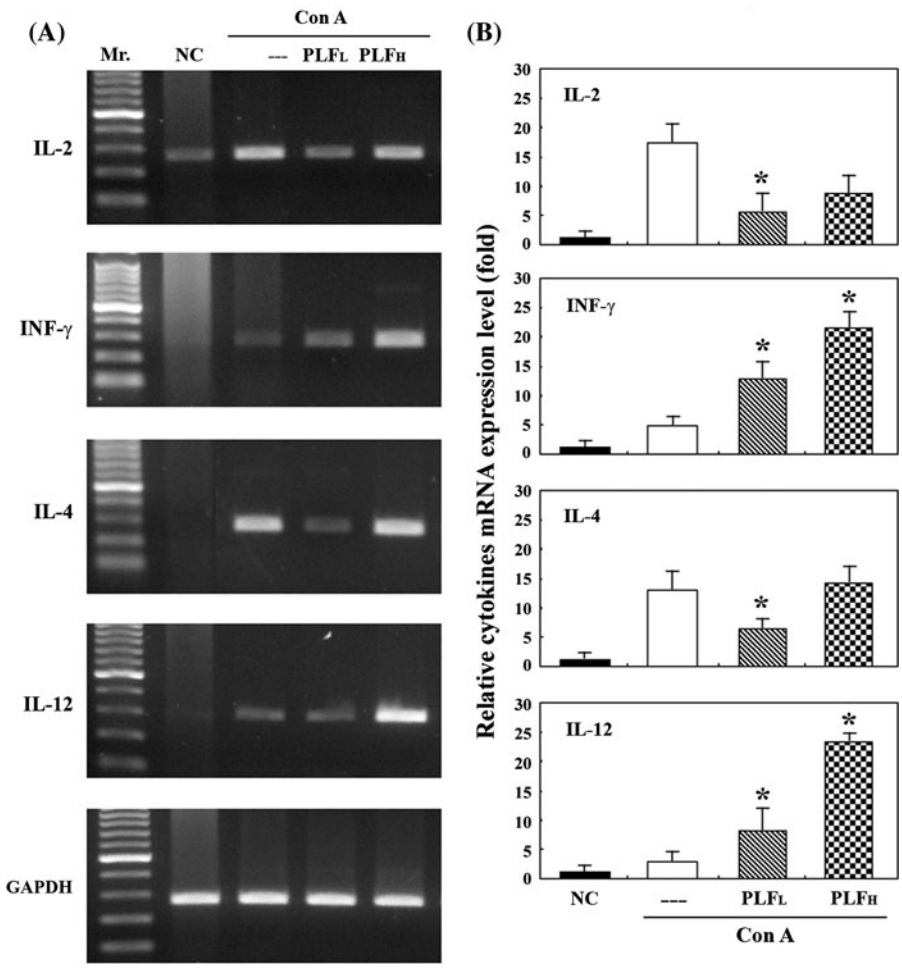

Fig. 4 Quantitative analysis of IL-2, INF-γ, IL-4, and IL-12 mRNA levels in peripheral lymphocytes treated with different PLF doses following ConA stimulation. **a** Representation of mRNA levels determined by semi-quantitative RT-PCR. *Mr.* 100-bp DNA ladder, *NC* normal cultured chicken peripheral lymphocytes (8 weeks) without ConA stimulation were used as a normal control, – no PLF added to the culture, *PLF_L* low-dose PLF treatment group, *PLF_H* high-dose PLF treatment group. Results are representative of three experiments. **b** Relative mRNA expression levels in control and PLF-treated groups were quantified by use of a densitometer. GAPDH was used as an internal control for each mRNA calculation. * $P < 0.05$; ** $P < 0.01$

relatively low in the normal control group. ConA increased the level of IL-4, but not IL-12. IL-4 was suppressed in the PLF_L-treated group ($P < 0.05$), and slightly increased in the PLF_H-treated group. In particular, the levels of IL-12 were significantly increased in the PLF_L-treated group ($P < 0.05$) and PLF_H-treated group ($P < 0.01$) (Fig. 4b).

Discussion

The traditional IBDV infection control strategy involves protection of chickens via passive immunization (Fussell 1998); however, traditional inactivated or attenuated vaccines do not provide sufficient protection against antigenic variants or highly virulent (vv) IBDV (Yu et al. 2001). Improving the effectiveness of IBDV vaccine is a critical area of research. A common goal for those improving the current vaccine is to increase antigen-specific cell mediated immunity (Vogel 2000). Lactoferrin is an excellent adjuvant candidate, as it has been shown to promote T-cell mediated delayed-type hypersensitivity against a variety of antigens (Zimecki and Kruzel 2000). Dietary LF has also been shown to enhance serum IgG and IgA levels (Shan et al. 2007). The high dosage of PLF administered in the PLF_H-treated group significantly enhanced IBDV-specific antibody titers at 16 weeks of age (Table 2). In addition, the high-dose of PLF enhanced serum IgA and IgG titers, and the middle-dose of PLF increased the serum IgG titer (Fig. 3). These results demonstrated that PLF is capable of significantly enhancing the effectiveness of the IBDV vaccine and generating host protection against IBDV infection. LF could enhance the protection of vaccinated chickens from IBDV infection.

Traditional IBDV vaccines were developed to stimulate B cells; however, recent studies have revealed the importance of T cells in IBDV pathogenesis. These studies have indicated that cell-mediated immunity may be more central to IBDV infection than previously assumed (Rautenschlein et al. 2002). Chicken IL-2 (ChIL-2) enhances protective immunity against avian pathogens and may serve as a new weapon for the control of infectious diseases in poultry. Recently, a report demonstrated that LF increased ConA-induced spleen lymphocyte proliferation by three times and enhanced IL-2 production (Shan et al. 2007). In this study, we demonstrated that a high-dosage of PLF enhanced lymphocyte proliferation (Table 1), and low-dosage PLF suppressed ChIL-2 expression (Fig. 4).

As it is a major inducer of macrophage antipathogenic activity, IFN-γ has been extensively studied in salmonellosis. Systemic administration of this cytokine during the first few days after challenge with a virulent strain reduced the severity of *Salmonella* infection in mice and rats (Lalmanach and Lantier 1999). IFN-γ severely restricts foot-and-mouth disease virus (FMDV) replication or even cured persistently infected bovine epithelial cells (Zhang et al. 2002). The study presented herein demonstrates that PLF increases the expression of IFN-γ in a dose-dependent manner (Fig. 4), and thereby strengthens the immune system.

Chicken Th2 cells are necessary for inducing the humoral response to combat parasite invasion. Similar to mammals, the chicken genome contains a cluster of Th2 cytokine genes, including IL-4 and other cytokines that are expressed in lymphoid tissues. This gene cluster is associated with down-regulation of the inflammatory (Th1) response and, consequently, with promotion of Th2 cell development (Avery et al. 2004).

In contrast to IL-4, IL-12 facilitates the proliferation and activation of Thl cells and increases IFN-γ production (Lee et al. 1998a, b). IL-12 induces IFN-γ synthesis by neonatal CD4 T-cells. In addition, IL-2 acts synergistically with IL-12 to trigger IFN-γ production (Fox et al. 1999). Recently, an in vitro study demonstrated that the addition of LF to activated macrophages leads to an increase in IL-12 and reduction in IL-10 (Hwang et al. 2007a, b). The results presented herein demonstrate that PLF-treated chickens produce more IL-12 than IL-4. In addition to a more rapid upregulation of the IFN-γ mRNA response, these results reveal that PLF can skew lymphocyte subset development towards Th1 rather than Th2 cells. Thus, it is proposed that PLF strengthened immunity via the mechanism outlined in Fig. 5.

In conclusion, the use of PLF as an immunologic enhancer and probe for immunologic quality control is a new strategy. The results of this study confirm that PLF is an effective immunopotentiator that may improve protection against IBD after inoculation with IBDV vaccine. PLF was also to possess synergistic

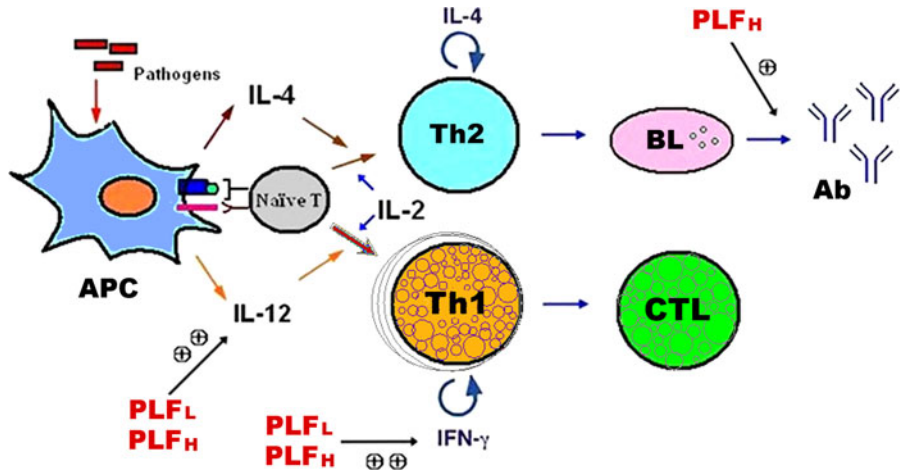

Fig. 5 Proposed mechanism of PLF functions in avian immunity enhancement. High-dose of PLF (PLF_H) and low-dose of PLF (PLF_L) administrations will increase IL-12 expression from antigen presenting cells (APC) to stimulate naïve T cells differentiation. They also enhance IFN-γ expression to stimulate T-help 1 (Th1) cells differentiation and cytotoxic T lymphocytes (CTL) function. The PLF_H administration will further increase B lymphocytes (BL) producing specific antibodies

immunologic capabilities with IL-12 and INF-γ. PLF was shown to induce production of sufficient quantities of IL-12 and INF-γ after antigen attacked, and could bias the lymphocyte subset development towards the Th1 lineage. PLF enhances cell-mediated immunity and increases IBDV vaccine effectiveness, which strengthens subsequent anti-viral responses.

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