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Technical report

Development and characterization of mouse monoclonal antibodies specific for chicken interleukin 18

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ABSTRACT

Four mouse monoclonal antibodies (mAbs) which are specific for chicken interleukin 18 (chIL18) were produced and characterized by enzyme-linked immunosorbent assay (ELISA), Western blotting, quantitative real-time PCR and neutralization assays. Using Western blot analysis, monoclonal antibodies specific for chIL18 identified a 23 kDa *Pichia pastoris*-expressed chIL18 and 66 kDa *E. coli*-derived MBP fusion protein of chIL18. Bioassays for chIL18 using primary chicken spleen cells showed dose-dependent IFN-γ mRNA expression and induction of IFN-γ from primary splenocytes, and triggered nitric oxide (NO) production in the HD11 macrophage cell line. These mAbs showed neutralizing chIL18 activity. Taken together, these mouse mAbs which detect chicken IL-18 will be significant new immune reagents and useful tools for basic and applied research in poultry.

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1. Introduction

Interleukin 18 (IL-18) was first identified as an interferon-γ (IFN-γ) inducing factor (IGIF) that is produced by macrophages and other cells, and shows synergistic action with IL-12 to induce strong cell-mediated immunity following infection with microbial products, such as lipopolysaccharide (LPS) (Gu et al., 1997; Nakamura et al., 1989). IL-18 stimulates natural killer (NK) cells and certain T cells to release interferon-γ (IFN-γ) or type II IFN which plays an important role in activating the macrophages or other cells (Dinarello, 1999). Chicken IL-18 (chIL18) was first identified from a chicken bursal EST database as a cytokine that exhibits approximately 30% sequence identity to the IL-18s of humans and other mammals with predicted protein of 198 amino acids (Schneider et al., 2000). Since then, IL-18 has been identified in two other avian species, turkey and duck. Recombinant chIL18 stimulated primary chicken splenocytes to induce a high level of IFN-γ secretion, especially by CD4+ T cells (Gobel et al., 2003; Puehler et al., 2003). In addition, IL-18 gene transcripts were highly up-regulated during experimental avian coccidiosis using chicken cDNA macrophage microarray and may play an important protective role against *Eimeria* infections (Dalloul et al., 2007; Hong et al., 2006b,c).

A major obstacle in poultry research is the lack of sufficient immunological reagents as well as low sequence homologies between avian and mammalian cytokines (Staeheli et al., 2001; Yoo et al., 2008). In an effort to facilitate the progress of veterinary immunology research

Abbreviations: PBL, peripheral blood lymphocytes; SPL, splenocytes; MBP, maltose binding protein.

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and to develop commercially available immune reagents tools, the U.S. Veterinary Immune Reagent Network (VIRN) was formed in 2007 (http://www.vetimm.org). This paper reports the development of mouse monoclonal antibodies (mAbs) that are capable of detecting chIL18, and can be used for basic and applied research in poultry.

2. Materials and methods

2.1. Production of chicken interleukin 18

The chicken IL-18 gene was initially identified from a bursal EST, and recombinant chicken IL-18 was produced in an *E. coli* expression system as described (Li et al., 2001). Briefly, IL-18 cDNA was cloned into pMAL-c2 (GenBank No. NM_204608; New England Biolabs, Ipswich, MA) vector to produce a maltose binding protein (MBP) fusion protein containing IL-18. This protein was purified on an amylase affinity column (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. *Pichia pastoris*-expressed recombinant chIL18 was obtained from Kingfisher Biotech, Inc. (www.kingfisherbiotech.com).

2.2. Monoclonal antibody production and validation of antigen specificity by ELISA

Mouse mAbs were produced using the previously described protocol (Yun et al., 2000). BALB/c mice (National Cancer Institute, Frederick, MD) were immunized biweekly by intraperitoneal and subcutaneous injections with 50 μg of recombinant chIL18-MBP or *P. pastoris*-expressed recombinant chIL18 in Freud's adjuvant (Sigma, St. Louis, MO), and the final boost injection was given intravenously with 25 μg of chIL18 without adjuvant 3 days prior to fusion. Mice producing high serum antibody titers were selected by IL-18-specific ELISA, their splenic lymphocytes fused with SP2/0 cells (ATCC), and hybridomas selected in medium supplemented with hypoxanthine, aminopterin, and thymidine (all from Sigma) with final confirmation of binding in ELISA as described (Min et al., 2002; Yun et al., 2000). Briefly, 96-well microtiter plates were coated overnight with 1 μg/well of MBP or purified recombinant IL-18 protein which was expressed in *E. coli* or *P. pastoris*. The plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with PBS containing 1% BSA. Mouse antibodies K55 (pan chicken lymphocyte) and anti-IFN-γ (Yun et al., 2000) were used as negative or positive controls (100 μl/well), incubated with agitation for 1 h at room temperature, washed with PBS-T, and bound antibody detected with peroxidase-conjugated rabbit anti-mouse IgG (Sigma) and peroxidase-reactive substrates in phosphate citrate buffer (Sigma), 3,3′,5,5′-tetramethylbenzidine (Sigma), and hydrogen peroxide (Sigma). Optical density at 450 nm (OD_{450}) was determined with a microplate reader (Bio-Rad, Richmond, CA). The immunoglobulin isotypes for the chIL18-reactive mAbs E1, E3, E17, or E24 were determined by an IsoQuick™ Strips and Kits (Sigma) according to the manufacturer's instructions.

2.3. Western blot analysis of chIL18 mAbs

To determine the binding activity of IL-18 mAbs using a Western blot, *E. coli*-expressed recombinant chicken IL-18-MBP (4 μg/lane) and *P. pastoris*-expressed chIL18 (4 μg/lane) were mixed with equal volumes of sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.004% bromophenol blue). The samples were than heated for 5 min at 95 °C, resolved on 15% SDS polyacrylamide gels, and electroblotted to nitrocellulose (Immobilon-P, Millipore, Bedford, MA). Mouse mAbs E1, E3, E7, and E24 were used to react with recombinant chIL18 expressed in *E. coli* (chIL18-MBP) in lane 1 and *P. pastoris* in lane 2 as shown in Figure 2. The membrane reacted with monoclonal anti-rabbit anti-polyhistidine antibody conjugated with peroxidase as described (Hong et al., 2006a).

2.4. Bioassay of recombinant chicken IL-18

In order to analyze the biological function and optimal concentration of IL-18 on the induction of IFN-γ, normal spleen lymphocytes (SPL) (5×10^6/ml) isolated from 3-week-old SPAFAS chickens (Charles River Laboratories, Preston, CT) were incubated in Iscove's Modified Dulbecco's Media (IMDM, Sigma), supplemented with 10% FBS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin with several concentrations of recombinant chicken IL-18 (0, 30, 60, 120, 250, and 500 ng/ml) in 96-well or 24-well plates at 41 °C in a 5% CO_2 incubator. mRNA expression levels of IFN-γ were deterined using quantitative real-time PCR as described (Hong et al., 2006b).

After 24, 48, and 72 h incubation with *E. coli*- and *P. pastoris*-expressed recombinant chicken IL-18 or COS7 cell-expressed chicken IFN-γ protein on spleen, the culture supernatants from these plates were added to HD11 (5×10^5/well), an established chicken macrophage cell line, in 96-well plates and incubated for 24, 48, or 72 h at room temperature. The control medium (plain IMDM) served as the negative control and COS7 cell-expressed IFN-γ served as the positive control. Culture supernatants were tested for nitric oxide (NO) as described (Lee et al., 2009). Culture supernatants (100 μl) were transferred to clean 96-well plates (triplicate), mixed with 100 μl of Griess reagent (Sigma), incubated for 15 min at room temperature, and optical density at 540 nm (OD_{540}) was measured. IL-18 (1000 ng/ml) was also used as the negative control. The nitrite concentration was determined using a standard curve generated with known concentrations of sodium nitrite.

2.5. Neutralizing activity of chIL18 mAbs

To assess the neutralization activity of chIL18 mAbs, normal spleen lymphocytes (5×10^6/ml) were incubated with 500 μl of 120 or 240 ng/ml recombinant chicken IL-18 (ChIL18-MBP) which was pre-incubated with 500 μl of mAbs (E1, E3, or E17) in a 24-well plate for 1 h, at 41 °C in a 5% CO_2 incubator for 24, 48, or 72 h. One hundred micro-

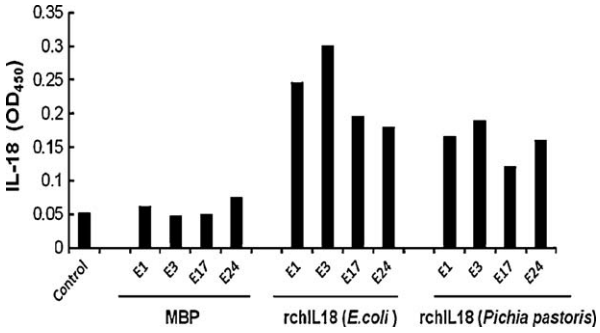

Fig. 1. ELISA to detect chIL18 mAbs. The 96-well microtiter plates were coated overnight with 1 μg/well of MBP or purified recombinant chIL18 protein which was expressed in *E. coli* or *P. pastoris*. Four different mAbs (E1, E3, E17, E24) were added (100 μl/well), and the bound antibodies were detected with peroxidase-conjugated rabbit anti-mouse IgG as described in Section 2. K55 (pan chicken lymphocyte) mAb was used as the negative control.

liters of each serially diluted (1:1 to 1:128) supernatant was then added to HD11 cells (5×10^5/well) in a 96-well plate and incubated for 24, 48, or 72 h. HD11's supernatants were then tested for NO production to measure the neutralization effect of chIL18 mAbs.

2.6. Statistical analysis

Statistical analyses were performed using SPSS 12.0K software for Windows (NUIT, Evanston, IL). Mean ± S.D. values for each group ($N = 3$) were calculated and differences between groups were analyzed by the Student's *t*-test, or the Tukey's HSD test. Differences were considered significant at $p < 0.05$ or $p < 0.01$.

3. Results and discussion

3.1. Production of mouse mAbs to chIL18

From two successful fusions, 18 hybridomas for *E. coli* and 109 hybridomas for *P. pastoris* were cloned and selected for further characterization based on their strong ELISA reactivity to recombinant chIL18 expressed in *E. coli* and yeast. Four mAbs (E1, E3, E17 and E24) were selected for further characterization based on their strong reactivity with recombinant IL-18-MBP fusion protein and *P. pastoris*-expressed chIL18 (Fig. 1). However, these antibodies did not bind to MBP protein alone and showed their specific reactivity only with chIL18 protein expressed in *E. coli* and yeast. K55 (pan chicken lymphocyte) mAb was used as the negative control, and it did not bind to chIL18. Antibody isotypes of these mAbs as determined by the IsoQuick strip kits are as follows: E1 and E3 are IgG2a-κ, E17 is IgG1-κ, and E24 is IgG2b-κ.

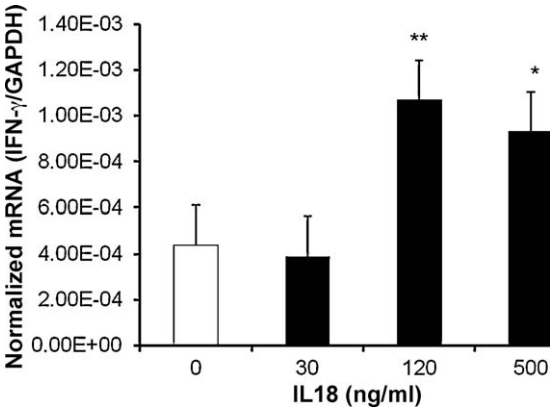

Fig. 3. Induction of IFN-γ transcripts by recombinant *P. pastoris*-expressed chIL18. Primary splenocytes were cultured with 30, 120 and 500 ng/ml of chIL18 for 24 h and IFN-γ transcripts were measured using quantitative real-time PCR normalized with GAPDH. $^{*}p < 0.05$, $^{**}p < 0.01$.

3.2. Western blotting

Fig. 2 shows the molecular weights 66 and 23 kDa of *E. coli* (chIL18-MBP)- and *P. pastoris*-expressed chIL18 proteins, respectively as determined by Western blot analysis using chIL18 mAbs E1, E3, E17, and E24 (Fig. 2A–D). The 66 kDa band includes the predicted molecular weight of 43 kDa for the maltose binding protein (MBP) derived from the pMAL-c2 vector and 23 kDa chIL-18 (lane 1), and lane 2 shows 23 kDa with intact *P. pastoris*-expressed chIL18 protein.

3.3. Bioassay of recombinant chicken IL-18

IL-18 is a cytokine with pleiotropic properties, such as proinflammatory properties, and can induce the increase of

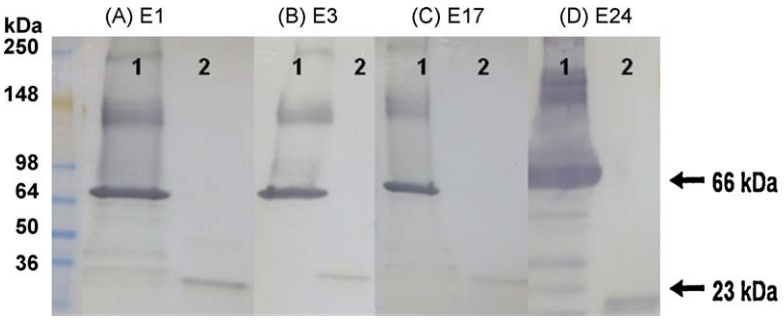

Fig. 2. Western blot analysis of chIL18 mAbs. Mouse mAbs: (A) E1, (B) E3, (C) E17, and (D) E24 were used to react with recombinant chIL18 expressed in *E. coli* (chIL18-MBP) in lane 1 and *P. pastoris* in lane 2. Bacterially expressed chIL18-MBP (4 μg/lane) and yeast-expressed, HPLC-purified chIL18 (4 μg/lane) were resolved on a SDS-PAGE gel, and reacted with peroxidase-conjugated rabbit anti-mouse IgG as described in Section 2.

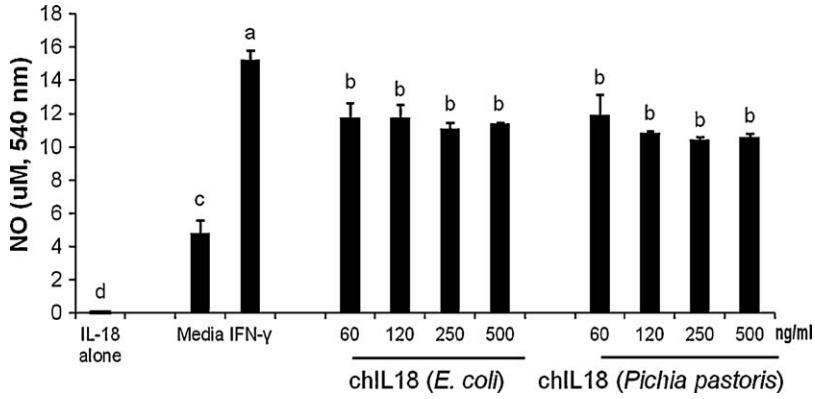

Fig. 4. Induction of nitric oxide production by chIL18-induced IFN-γ. The biological activity of recombinant chIL18 was assayed by stimulating primary splenocytes with chIFN-γ (control), or *E. coli*- or *P. pastoris*-expressed chIL18 for 72 h. Supernatants from these cultures were added to HD11 culture and NO production was measured after 48 h of incubation. The control media (plain IMDM) served as a negative control and COS7 cell-expressed IFN-γ as a positive control. IL-18 alone (1000 ng/ml) was also used as a negative control. Doses of recombinant chIL18 tested were 60, 120, 250 and 500 ng/ml. Values with different superscripts denote significant difference at $p < 0.05$.

interferon-gamma (IFN-γ) or type II interferon that plays an important role in activating macrophages or other cells (Okamura et al., 1995; Puehler et al., 2003; Schneider et al., 2000). Two different bioassays were used to show the biological function of chIL18. Induction of IFN-γ by chIL18 in primary splenocytes or HD11 was shown by measuring IFN-γ transcripts after stimulation with recombinant chIL18. To confirm the activity of recombinant IL-18 protein in inducing IFN-γ, mRNA expression levels on the fresh splenocytes were examined using quantitative real-time

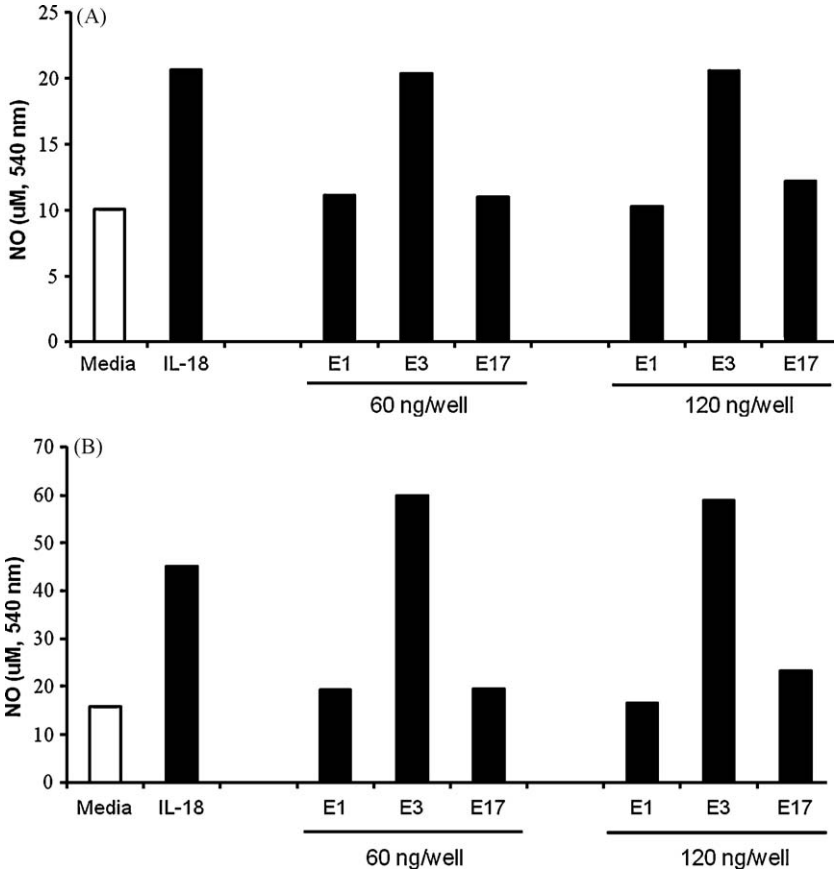

Fig. 5. Neutralization of IL-18 bioactivity by chIL18 mAbs. Recombinant *P. pastoris*-expressed chIL18 (60 and 120 ng per well) and mAbs (1:1) were incubated for 30 min and then cultured with primary splenocytes for 72 h. Supernatants were then added to HD11 cells and NO production was measured from HD11 supernatants at 24 h (A) and 72 h (B) post-culture.

PCR (Fig. 3). The transcriptional level of IFN-γ on the spleen cells was highly expressed when cells were stimulated with 120 ng/ml of recombinant chicken IL-18 ($p < 0.01$) as reported by Puehler et al. (2003).

The biological activity of chIL18 was also shown by its ability to induce IFN-γ-mediated upregulation of nitric oxide synthase gene expression in the HD11 chicken macrophage cell line. When the supernatants of spleen cells, which were stimulated with recombinant IL-18 for 24–72 h were added to HD11 cells and incubated for 24–72 h, the production of NO was significantly raised at doses between 60 and 120 ng/ml of recombinant *E. coli*- or *P. pastoris*-expressed IL-18 protein (Fig. 4). IL-18 alone, which was used as the negative control, did not induce NO. These results indicated that rchIL18 protein effectively induced IFN-γ from splenocytes and triggered nitric oxide production in the HD11 macrophage cell line.

3.4. Neutralization activity of chIL18 mAbs

The neutralizing activity of chIL18 mAbs was tested at different time intervals (24, 48, 72, and 96 h) using splenocytes with rchIL-18 and mAbs. In addition, culture with supernatants on HD11 cells was tested at various time points (24, 48, 72, 96, and 120 h) to measure NO production. Higher levels of NO production were observed when splenocytes were cultured with recombinant chIL18 for 96 h compared to 24 h. Out of three mAbs tested, E1 and E17 mAbs showed a significant inhibition of chIL18 to induce IFN-γ release in primary splenocytes at both 60 and 120 ng per well chIL18 concentrations (Fig. 5A and B). Interestingly, even though E3 mAb showed strong binding activity against the chIL18 antigen, it did not show any neutralization activity. Both E1 and E17 mAbs effectively reduced IL-18-induced IFN-γ production by splenocytes at either incubation times. Three separate trials were carried out with consistent results. In summary, this report documents the generation of four new mAbs which specifically recognize chicken IL-18 in ELISA and Western blots. Furthermore, two out of three mAbs successfully neutralize the biological function of chIL18 and reduced IL-18-induced IFN-γ production by primary splenocytes. These mAbs will be useful immune reagent tools that can be utilized in basic and applied research for poultry immunity research.

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References

Dalloul, R.A., Bliss, T.W., Hong, Y.H., Ben-Chouikha, I., Park, D.W., Keeler, C.L., Lillehoj, H.S., 2007. Unique responses of the avian macrophage to different species of Eimeria. Mol. Immunol. 44, 558–566.

Dinarello, C.A., 1999. Interleukin-18. Methods 19, 121–132.

Gobel, T.W., Schneider, K., Schaerer, B., Mejri, I., Puehler, F., Weigend, S., Staeheli, P., Kaspers, B., 2003. IL-18 stimulates the proliferation and IFN-gamma release of CD4+ T cells in the chicken: conservation of a Th1-like system in a nonmammalian species. J. Immunol. 171, 1809–1815.

Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M.A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., Kurimoto, M., Tanimoto, T., Flavell, R.A., Sato, V., Harding, M.W., Livingston, D.J., Su, M.S., 1997. Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. Science 275, 206–209.

Hong, Y.H., Lillehoj, H.S., Dalloul, R.A., Min, W., Miska, K.B., Tuo, W., Lee, S.H., Han, J.Y., Lillehoj, E.P., 2006a. Molecular cloning and characterization of chicken NK-lysin. Vet. Immunol. Immunopathol. 110, 339–347.

Hong, Y.H., Lillehoj, H.S., Lee, S.H., Dalloul, R.A., Lillehoj, E.P., 2006b. Analysis of chicken cytokine and chemokine gene expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet. Immunol. Immunopathol. 114, 209–223.

Hong, Y.H., Lillehoj, H.S., Lillehoj, E.P., Lee, S.H., 2006c. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following Eimeria maxima infection of chickens. Vet. Immunol. Immunopathol. 114, 259–272.

Lee, S.H., Lillehoj, H.S., Cho, S.M., Chun, H.K., Park, H.J., Lim, C.I., Lillehoj, E.P., 2009. Immunostimulatory effects of oriental plum (*Prunus salicina* Lindl.). Comp. Immunol. Microbiol. Infect. Dis. 32, 407–417.

Li, G., Lillehoj, H.S., Min, W., 2001. Production and characterization of monoclonal antibodies reactive with the chicken interleukin-15 receptor alpha chain. Vet. Immunol. Immunopathol. 82, 215–227.

Min, W., Lillehoj, H.S., Li, G., Sohn, E.J., Miyamoto, T., 2002. Development and characterization of monoclonal antibodies to chicken interleukin-15. Vet. Immunol. Immunopathol. 88, 49–56.

Nakamura, K., Okamura, H., Wada, M., Nagata, K., Tamura, T., 1989. Endotoxin-induced serum factor that stimulates gamma interferon production. Infect. Immun. 57, 590–595.

Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., et al., 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature 378, 88–91.

Puehler, F., Gobel, T., Breyer, U., Ohnemus, A., Staeheli, P., Kaspers, B., 2003. A sensitive bioassay for chicken interleukin-18 based on the inducible release of preformed interferon-gamma. J. Immunol. Methods 274, 229–232.

Schneider, K., Puehler, F., Baeuerle, D., Elvers, S., Staeheli, P., Kaspers, B., Weining, K.C., 2000. cDNA cloning of biologically active chicken interleukin-18. J. Interferon Cytokine Res. 20, 879–883.

Staeheli, P., Puehler, F., Schneider, K., Gobel, T.W., Kaspers, B., 2001. Cytokines of birds: conserved functions—a largely different look. J. Interferon Cytokine Res. 21, 993–1010.

Yoo, J., Chang, H.H., Bae, Y.H., Seong, C.N., Choe, N.H., Lillehoj, H.S., Park, J.H., Min, W., 2008. Monoclonal antibodies reactive with chicken interleukin-17. Vet. Immunol. Immunopathol. 121, 359–363.

Yun, C.H., Lillehoj, H.S., Choi, K.D., 2000. Chicken IFN-gamma monoclonal antibodies and their application in enzyme-linked immunosorbent assay. Vet. Immunol. Immunopathol. 73, 297–308.