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Cloning and functional characterization of chicken interleukin-17D

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Abstract

The chicken interleukin-17D was cloned from a testis cDNA library prepared from the Korean native chicken. The full-length chicken IL-17D (chIL-17D) cDNA consisted of a 348 nucleotide sequence encoding an open reading frame of 116 amino acids with a predicted molecular mass of 13.3 kDa. Comparison of the deduced amino acid sequence of chIL-17D with homologous proteins from human, mouse and opossum revealed 64%, 53% and 76% identity, respectively, including six conserved cysteine residues present in the mammalian polypeptides. The chIL-17D gene transcript was expressed in a wide range of tissues, and highest levels were in pancreas, thymus and lung. Following *Eimeria maxima* infection, levels of the chIL-17D mRNA were up-regulated in the intestinal jejunum, bursa, lung, and spleen but decreased in the thymus. Infected chickens also expressed greater levels of chIL-17D mRNA in CD4⁺, CD8⁺ and TCR1⁺ intestinal intraepithelial lymphocytes while decreased expression was seen in TCR2⁺ cells. Treatment of CHCC-OU2 fibroblasts with chIL-17D recombinant protein induced the expression of IL-6 and IL-8. Collectively, these results suggest that chIL-17D has structural and functional similarities to mammalian IL-17Ds and that it plays an important role in local gut innate immune responses during experimental coccidiosis.

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Keywords: IL-17D; Chicken; Cytokine; Local immune response

1. Introduction

Interleukin-17 (IL-17) was originally described as a cytokine secreted exclusively by activated memory T

cells that induced fibroblasts to secrete other cytokines involved in proinflammatory or hematopoietic processes, such as IL-6, IL-8 and granulocyte-colony stimulating factor (G-CSF) (Yao et al., 1995a,b; Broxmeyer, 1996; Fossiez et al., 1996). With the completion of human genome sequences and the availability of public genomic databases, a number of homologous proteins comprising an IL-17 family have been identified, including IL-17A (original IL-17), -17B, -17C, -17D, -17E and -17F (Li et al., 2000; Lee et al., 2001; Starnes et al., 2001, 2002). The IL-17 families, as well as their cognate receptors, have no sequence similarity to any other known cytokines

Abbreviations: EST, expressed sequence tag; IELs, intraepithelial lymphocytes; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-CSF.

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or receptors and thus appear to represent a distinct ligand-receptor signaling system (Yao et al., 1995a; Kawaguchi et al., 2004). Three members of this family, IL-17A, -17E (IL-25), and -17F, have been the most characterized and have been shown to be proinflammatory in nature (Kawaguchi et al., 2004).

Functional studies of IL-17 cytokines have described a broad range of effects on cells and tissues. IL-17A was expressed by activated CD4⁺ and CD8⁺ T cells, but not resting T cells (Yao et al., 1995a), neutrophils and eosinophils (Teunissen et al., 1998; Chakir et al., 2003; Ferretti et al., 2003). Similarly, IL-17F was produced primarily by activated T cells and monocytes and stimulated the production of IL-6, IL-8 and G-CSF (Hymowitz et al., 2001; Starnes et al., 2001). CD4⁺ helper T cells that produce IL-17A and IL-17F are referred to as T_H-17 cells. T_H-17 cells also produce IL-22, IL-26, interferon- γ , chemokine CCL20 and transcription factor ROR γ t, but their function in immunity remains to be fully determined (Wilson et al., 2007). IL-17B mRNA was identified in adult pancreas, small intestine and stomach, whereas IL-17C mRNA was not detected by blot hybridization in several adult tissues examined (Li et al., 2000). Neither of these transcripts was found in activated T cells. The IL-17D gene appeared to be most homologous to IL-17B and was preferentially expressed in resting CD4⁺ T cells, skeletal muscle, brain, pancreas, heart, lung and adipose tissue (Starnes et al., 2002). IL-17E was restricted to T_H2 cells and over-expression of this cytokine resulted in the production of IL-4, IL-5, IL-13, and IgE during airway eosinophilia, suggesting that it might be involved in the allergic response (Hurst et al., 2002). Clinically, IL-17 family members have been linked to many disease processes such as rheumatoid arthritis (Kotake et al., 1999; Bush et al., 2001; Chabaud et al., 2001), chronic obstructive pulmonary disease (Linden et al., 2000), psoriasis (Teunissen et al., 1998), and allograft rejection (Van Kooten et al., 1998; Antonysamy et al., 1999).

Nucleotide sequences homologous to human IL-17A, -17B, -17D and -17F have been identified in the chicken genome (Min and Lillehoj, 2002; Kaiser et al., 2005). Among these, only IL-17A has been cloned and characterized (Min and Lillehoj, 2002). In that study, a cDNA encoding chIL-17A was isolated from an expressed sequence tag (EST) library prepared from intestinal intraepithelial lymphocytes (IELs) of chickens infected with *Eimeria* parasites, the etiologic agent of avian coccidiosis. ChIL-17A mRNA was expressed by activated T cells and its recombinant protein induced chicken embryonic fibroblasts to secrete IL-6. In the

current study, we have extended these findings by the identification and characterization of chIL-17D from a testis cDNA library.

2. Materials and methods

2.1. Animals and experimental *Eimeria* infection

Broiler chickens were purchased from Longenecker's Hatchery (Elizabethtown, PA, USA) and housed in wire cages with feed and water provided *ad libitum*. At 3 weeks of age, the chickens were orally inoculated with 1.0×10^4 sporulated oocysts of *Eimeria maxima* (Tyson strain) or PBS as a negative control as described (Hong et al., 2006c). All protocols were approved by the Institutional Animal Care and Use Committee of the Beltsville Agricultural Research Institute.

2.2. Cloning of chIL-17D cDNA

Construction of a 26-week-old Korean native chicken testis EST library in the Uni-ZAP XR cloning vector was described (Shin et al., 2005). The chIL-17D EST (GenBank accession number CO771003) was identified based on nucleotide sequence homology to mammalian IL-17D sequences in GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Predicted amino acids sequences of chicken and mammalian IL-17Ds were compared using CLUSTAL W (1.83) (<http://www.ebi.ac.uk/Tools/clustalw/>). The complete chIL-17D coding sequence was isolated by 5'-RACE (Clontech, Mountain View, CA, USA) and RT-PCR. Briefly, total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA) from testis, bursa, spleen, thymus, lung, jejunum and muscle tissues of *E. maxima*-infected 2-week-old broiler chickens. Total RNA (5.0 μ g) from pooled tissue RNA was treated with 1.0 U of TURBO DNase (Ambion, Austin, TX, USA) and incubated at 70 °C for 10 min. Using this total RNA, 5'-RACE was performed according to the manufacturer's instructions. DNase I treated total RNA reverse-transcribed at 42 °C for 1 h using the StrataScript first strand synthesis system (Stratagene, La Jolla, CA, USA). PCR was performed using restriction endonuclease-anchored primers (Bam HI, Hind III) and cDNA as template. PCR products were digested with Bam HI and Hind III (Roche, Indianapolis, IN, USA), ligated into the corresponding restriction endonuclease sites of pET32a (+) (Novagen, Madison, WI, USA) and transformed into BL21 (DE3) competent cells (Invitrogen).

2.3. Tissue distribution of *chIL-17D* mRNA

Tissue samples were obtained from 2-week-old broiler chickens as described (Hong et al., 2006c). Quantitative RT-PCR oligonucleotide primers for *chIL-17D* and *GAPDH* are listed in Table 1. Reverse transcription of total RNA was performed as described above. Amplification and detection were carried out using equivalent amounts of total RNA from each tissue with the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) for 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min (Hong et al., 2006a). Each RT-PCR experiment contained triplicates of test samples and 2-fold dilutions of standard RNA. Mean threshold cycle values (C_t) for the *chIL-17D* and *GAPDH* products were calculated for each experiment and the levels of *chIL-17D* transcripts were normalized to those of *GAPDH* using the Q-gene program (Muller et al., 2002).

2.4. Magnetic cell separation

Intestinal IELs were isolated from *E. maxima*-infected chickens at 72 h post-infection and sorted into marker-specific fractions ($CD4^+$, $CD8^+$, $TCR1^+$ and $TCR2^+$) using the magnetic BD IMag Cell Separation System (BD Biosciences Pharmingen, San Jose, CA, USA) as described (Hong et al., 2006a). Magnetic particles were isolated, the positive cell fractions were resuspended in serum-free Hanks' balanced salt solution and *chIL-17D* transcripts were analyzed by quantitative RT-PCR as described above.

2.5. Expression of *chIL-17D* recombinant protein

E. coli BL21(DE3) transformed with the pET32a(+)-*IL-17D* plasmid was induced with 1.0 mM isopropyl β -D-galactopyranoside (IPTG, Amersham Biosciences, Piscataway, NJ, USA) for 4 h at 26 °C and the soluble polyhistidine-tagged *chIL-17D* recombinant protein was purified on a Ni^{2+} -NTA His-bind resin column (Novagen) according to the manufacturer's protocol. A

mock preparation was made from *E. coli* transformed with pET32a(+) empty vector and fractionated in an identical manner on the Ni^{2+} -NTA His-bind resin. Purified proteins were heated at 95 °C for 5 min in SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue), resolved on 15% SDS-PAGE gels and analyzed by Western blotting with anti-polyhistidine antibody as described (Hong et al., 2006a). *ChIL-17D* recombinant protein was dialyzed in PBS before testing for its biological activity.

2.6. Biological activity of *chIL-17D* recombinant protein

CHCC-OU2, an embryonic fibroblast cell line (Ogura and Fujiwara, 1987), was cultured in 6-well plates at 1.0×10^6 cells/well in Dulbecco's Modified Eagle's medium (Sigma) supplemented with 10% FBS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin at 41 °C. The cells were untreated or treated with 1.0 or 4.0 μ g of *chIL-17D* recombinant protein or an equivalent amount of the mock preparation for 24 h and *IL-6*, *IL-8/CAF* [CXCLi2] and *GM-CSF* gene expression determined by quantitative RT-PCR using the primers listed in Table 1.

2.7. Statistical analysis

Mean \pm S.D. values for each group ($N = 3$) were calculated and differences between groups were analyzed by the Student's *t*-test, Dunnet multiple comparison test or the Tukey–Kramer multiple comparison test using InStat[®] software (Graphpad, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Isolation of *chIL-17D* cDNA

Computer-assisted alignment of translated cDNA sequences indicated that a 923 nucleotide chicken

Table 1

Sequences of primer sets used for quantitative RT-PCR

RNA target	Primer sequences		Product size (bp)	Accession number
	Forward	Reverse		
<i>GAPDH</i>	5'-GGTGGTGCTAAGCGTGTAT-3'	5'-ACCTCTGTCATCTCTCCACA-3'	264	K01458
<i>IL-17D</i>	5'-GCTGCCATCATGGGATCTTTGGTG-3'	5'-CGATGACGGCTTGTCTGGTTGAC-3'	248	EF570583
<i>IL-6</i>	5'-CAAGGTGACGGAGGAGGAC-3'	5'-TGGCGAGGAGGGATTCT-3'	254	AJ309540
<i>IL-8/CAF</i> [CXCLi2]	5'-GGCTTGCTAGGGAAATGA-3'	5'-AGCTGACTCTGACTAGGAACTGT-3'	200	AJ009800
<i>GM-CSF</i>	5'-CGCCACCACAACATACTC-3'	5'-ACGATCCGCTTTCTTCT-3'	202	AJ621740

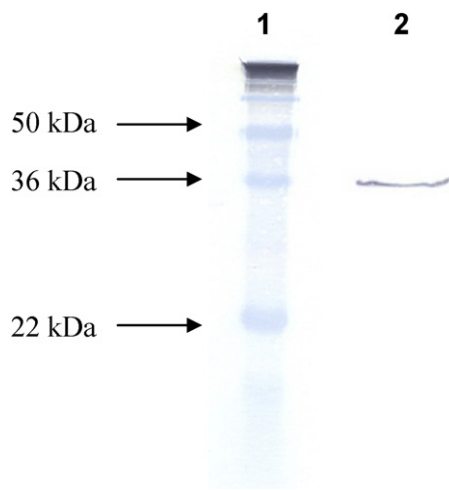


Fig. 2. Western blot analysis of chIL-17D recombinant protein. *E. coli* BL21(DE3) transformed with the pET32a(+)-IL-17D plasmid was induced with 1.0 mM IPTG, purified with a Ni²⁺-NTA His-bind resin column and analyzed by Western blotting using anti-polyhistidine antibody. Lane 1, Prestained protein markers with sizes indicated on the left in kilodaltons (kDa). Lane 2, chIL-17D.

or $p < 0.0001$) but down-regulated in the thymus ($p < 0.0001$) (Fig. 4A). The net effect of *Eimeria* infection was to produce relatively uniform expression levels in the organs examined. Next, we quantified the levels of the chIL-17D mRNA in jejunum IELs following separation into CD4⁺, CD8⁺, TCR1⁺ and TCR2⁺ subpopulations with over 98% purity. As shown in Fig. 4B, mRNA levels were increased in CD4⁺, CD8⁺ and TCR1⁺ cells but decreased in TCR2⁺ cells. The greatest increase was noted in CD4⁺ IELs where levels of the chIL-17D transcript were augmented approximately 1100-fold compared with uninfected controls.

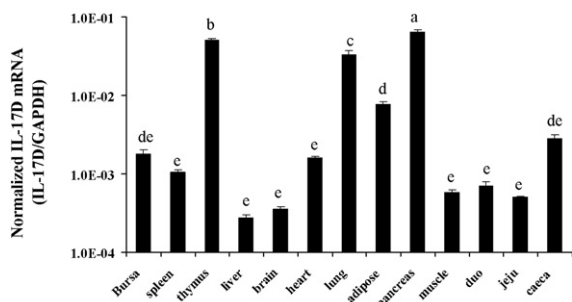


Fig. 3. Tissue distribution of chIL-17D mRNA. Total RNA was isolated from the indicated sources, analyzed for IL-17D mRNA by quantitative RT-PCR and normalized to GAPDH mRNA. Each bar represents the mean \pm S.D. of triplicate determinations. Bars with different letters are significantly different ($p < 0.05$). ChIL-17D regression equation: $C_t = 3.05x + 32.6$, $r^2 = 0.99$; GAPDH regression equation: $C_t = 3.42x + 32.2$, $r^2 = 0.99$.

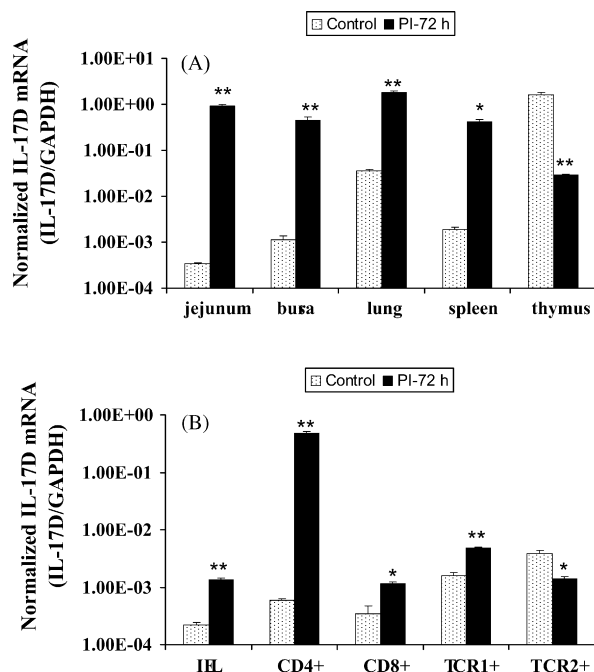


Fig. 4. Tissue and T cell distribution of chIL-17D mRNA following *E. maxima* infection. Chickens were uninfected or infected with *E. maxima* at 3 weeks of age, tissues (A) or T cell subpopulation from intestinal jejunum IELs (B) were prepared at 72 h post-infection (PI), analyzed for IL-17D mRNA by quantitative RT-PCR and normalized to GAPDH mRNA. Each bar represents the mean \pm S.D. of triplicate determinations. * $p < 0.001$; ** $p < 0.0001$ comparing *E. maxima* infected chickens with uninfected controls.

3.4. Cytokine/chemokine induction by chIL-17D recombinant protein

Our laboratory previously reported that chIL-17A recombinant protein, purified as a polyhistidine tagged fusion protein, stimulated the production of the proinflammatory cytokine IL-6 by chicken embryonic fibroblasts, suggesting a functional role for the cytokine in avian immunity (Min and Lillehoj, 2002). Therefore, it was of interest to determine the effects of chIL-17D protein on induction of proinflammatory cytokines and chemokines. Compared with fibroblasts that were either untreated or treated with a mock control preparation from empty vector transformed *E. coli*, IL-6 and IL-8 transcript levels were significantly up-regulated following treatment with purified chIL-17 recombinant protein (Fig. 5). By contrast, expression of GM-CSF was unaffected (data not shown).

4. Discussion

This report documents the molecular cloning of a chicken cDNA homologous to mammalian IL-17D,

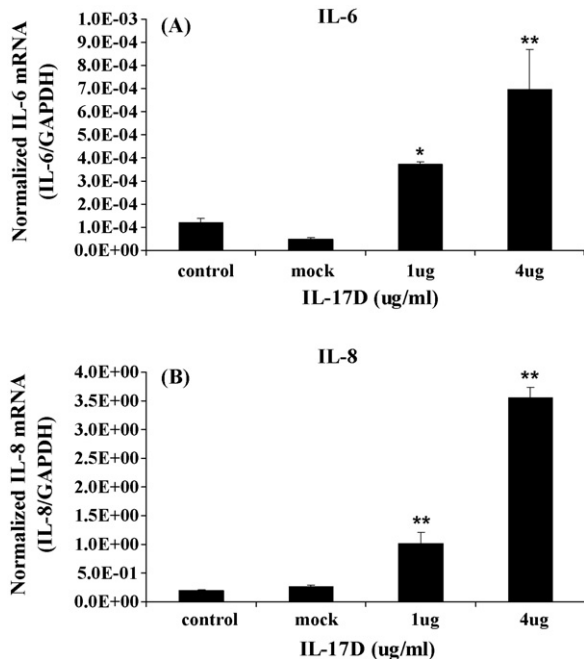


Fig. 5. IL-6 and IL-8/CAF [CXCL12] mRNA levels following chIL-17D recombinant protein treatment. CHCC-OU2 chicken embryonic fibroblasts in six-well plates were untreated or treated for 24 h with a mock preparation or the indicated amounts of purified chIL-17D recombinant protein. IL-6 (A) and IL-8 (B) mRNA levels were determined by quantitative RT-PCR and normalized to GAPDH mRNA. Each bar represents the mean \pm S.D. of triplicate determinations. Control, untreated fibroblasts. Mock, a preparation from pET32a(+) empty vector transformed *E. coli* corresponding to 1.0 μ g of purified chIL-17D recombinant protein. IL-6 regression equation: $C_t = 2.97x + 40.4$, $r^2 = 0.95$; IL-8 regression equation: $C_t = 3.73x + 39.1$, $r^2 = 0.99$. * $p < 0.05$; ** $p < 0.01$ comparing chIL-17D-treated cells with mock-treated cells.

analysis of the expression of its corresponding gene transcript and characterization of its encoded protein. Endogenous chIL-17D mRNA was present in all organs examined with highest levels in pancreas, thymus and lung. Following *E. maxima* infection, chIL-17D transcript levels were increased in intestinal IELs, bursa, lung, and spleen but decreased in the thymus. Among IEL subpopulations, infected chickens expressed greater levels of the chIL-17D mRNA in CD4⁺, CD8⁺ and TCR1⁺ cells but decreased expression in TCR2⁺ cells. Unlike mammals, chicken T cells are divided into three separate subpopulations on the basis of their cell surface antigen expression and their biological function ($\alpha\beta$ -T cell receptors, TCR2 and 3, and $\gamma\delta$ -TCR, TCR1) (Davidson and Boyd, 1992). The $\alpha\beta$ -TCRs (TCR2 and 3) are known to mediate MHC-restricted antigen recognition by T-cells, whereas the physiological role of T cells expressing TCR1 ($\gamma\delta$ -TCR) is not well defined (Gobel, 1996). Treatment of

chicken fibroblasts with chIL-17D recombinant protein induced the expression of IL-6 and IL-8 but not GM-CSF. Taken together, these results suggest that chIL-17D plays an important role in innate immunity in the intestine during experimental coccidiosis.

Based on its sequence homology to mammalian IL-17Ds, and in particular the presence of six conserved cysteine residues, it is strongly suggested that this cDNA encodes chIL-17D. The degree of sequence identity shared by chIL-17D with the mammalian proteins (53–76%) is somewhat higher than that between chicken and mammalian IL-17As (37–46%) (Min and Lillehoj, 2002). When compared among them, the mammalian proteins exhibit the greatest homology is in their C-termini, suggesting that the N-terminus may be involved in receptor specificity in IL-17 families in human (Starnes et al., 2002). It is of interest to note, however, that when aligned with the mammalian proteins, chIL-17D appears to be shorter than mammalian peptides at its N-terminus, with homology only evident beginning with Arg-12 in the chicken protein corresponding to Arg-97, Ser-95 and Arg-98 in the human, mouse and opossum sequences. Several attempts to sequence the entire 5'-truncated or spliced region using several tissue RNA samples from bursa, lung, spleen, thymus, testis, IELs and muscle, failed to demonstrate any existence of alternative spliced form of chicken IL-17D. These results indicate that chicken IL-17D has only single exon gene.

Sequence homologies between members of chIL-17s range from 17% (chIL-17A vs. chIL-17B), 19% (chIL-17A vs. chIL-17D) and 24% (chIL-17B vs. chIL-17D). These values are generally lower than those between human IL-17s (20–50%) (Moseley et al., 2003). This difference may be due, in part, to the fact that the genes for the chicken IL-17s are located on different chromosomes, chIL-17A on chromosome 3, chIL-17B on chromosome 13, and the compiled chIL-17D cDNA which was mapped to chromosome 1 genomic contig (Kaiser et al., 2005) as described in human (Moseley et al., 2003).

The pattern of tissue and organ chIL-17D expression was similar to human IL-17D, both of which are unusual for IL-17 family members (Starnes et al., 2002). For example, human IL-17A and -17F are mainly expressed by activated CD4⁺ T cells and monocytes (Yao et al., 1995a; Starnes et al., 2001). On the other hand, chIL-17A expression was only observed by Con A-activated splenic lymphocytes and a reticuloendotheliosis virus-transformed chicken lymphoblast cell line (CU205), but not in normal tissues (Min and Lillehoj, 2002). Interestingly, while chIL-17A mRNA levels did not change following *E. tenella* infection, they were highly up-regulated following primary infection with

Eimeria acervulina or *E. maxima* (Hong et al., 2006b,c). The ability of the latter species of *Eimeria* to stimulate the expression of chIL-17 family members now appears also to apply to chIL-17D (Fig. 4). However, it is not clear why *E. maxima* infection led to decreased chIL-17D transcript levels in the thymus or TCR2⁺ IELs, particularly since Starnes et al. (2002) reported that human IL-17D was expressed by resting CD4⁺ cells. One possibility is that chIL-17D plays a role in local immune responses that might occur in these tissues after structural damage, such as occurs in the intestine during avian coccidiosis as well as myocardial infarction or stroke in humans (Starnes et al., 2002).

Human IL-17D stimulated vascular endothelial cells to secrete IL-6, IL-8 and GM-CSF (Starnes et al., 2002). This effect has been suggested to be mediated through NF- κ B activation, as was shown for IL-17A and -17E (Yao et al., 1995a; Jovanovic et al., 1998; Laan et al., 1999; Lee et al., 2001; Jones and Chan, 2002; Starnes et al., 2002; Moseley et al., 2003). Despite its ability to induce GM-CSF, IL-17D suppressed the proliferation of myeloid progenitors in colony formation assays. Unlike human IL-17D, we were unable to demonstrate chicken GM-CSF expression in response to chIL-17D. However, the ability of chIL-17D to stimulate proinflammatory cytokine and chemokine expression suggests that it, as well as chIL-17A which also induced IL-6 production (Min and Lillehoj, 2002), may be important soluble mediators in the initiation, sustenance and control of avian inflammatory responses. In summary, although the role of chIL-17D requires further characterization especially with respect to its potential action during protective immunity against economically important pathogens, its similarity with mammalian IL-17D indicates that it may become an attractive target for mitigating excessive inflammatory pathology or enhancing protective immunity when used as a vaccine adjuvant.

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