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Molecular cloning and characterization of chicken lipopolysaccharide-induced TNF-α factor (LITAF)

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Abstract

The inflammatory response to parasites, bacteria, and viruses is mediated by multiple host factors. TNF-α is one of the most pleiotropic cytokines in mammals, but has yet to be identified in avian species. In the current study, we isolated a full-length cDNA encoding the chicken homologue of LPS-induced TNF-α factor (LITAF), transcription factor, with an open reading frame of 148 amino acids and a predicted molecular mass of 16.0 kDa. Quantitative RT-PCR analysis showed that chicken LITAF mRNA was predominantly expressed in spleen and intestinal intraepithelial lymphocytes (IELs). LITAF mRNA levels were up-regulated following in vitro stimulation of macrophages for 4 h with *Escherichia coli* or *Salmonella typhimurium* endotoxin, and 18–48 h after treatment with *Eimeria acervulina*, *E. maxima*, or *E. tenella*, three causative agents of avian coccidiosis. LITAF mRNA was up-regulated by more than 700-fold in IELs isolated from *E. maxima*-infected birds. Purified recombinant LITAF protein expressed in *E. coli* or COS7 cells exhibited cytotoxic activity against chicken tumor cell lines in vitro, presumably through autocrine activation of TNF-α or its chicken homologue. This supposition was strengthened by the fact that treatment of chicken macrophages with recombinant LITAF induced the expression of TL1A (TNFSF 15), a member of the TNF ligand super family. We conclude that chicken LITAF may play an important role in the regulation of TNF-α gene expression during the course of coccidiosis or tumorigenesis.
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1. Introduction

Lipopolysaccharide (LPS) is a major component of the Gram-negative bacterial outer membrane and one of the most potent stimulators of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-12, by monocytes and macrophages [1,2]. TNF-α exhibits multiple effects on different tissues and cells in vivo as well as in vitro,

which may be either beneficial or detrimental to the host depending on the context of its production [1,3]. Transcription of the TNF-α gene is driven by a variety of regulatory factors [4]. While nuclear factor (NF)-κB was suggested to be necessary for TNF-α production by LPS-stimulated monocytes and macrophage [5], evidence for the involvement of NF-κB in TNF-α gene transcription is controversial [6].

Recently, a novel transcription factor, named LPS-induced TNF-α factor (LITAF), was cloned and characterized [7,8]. LITAF mRNA was mostly expressed in lymphoid tissues including peripheral blood leukocytes, lymph nodes, and spleen and non-lymphoid organs, especially placenta and liver in human and mouse. Chromosomal localization mapped the LITAF gene to 16p12–16p13.3 in human and 16B1–16B3 in mouse. LPS stimulation of monocytes and macrophages induced LITAF production followed by LITAF binding to the human TNF-α gene promoter and transcription of the TNF-α gene. LITAF exerts its effects on TNF-α expression through formation of a complex with the signal transducer and activator of transcription (STAT6(B)) [9].

In contrast to mammals, relatively little is known about chicken TNF-α. Chicken TL1A which was recently designated as the TNF superfamily 15 (TNFSF 15) is the homologue of human TL1A and belongs to the TNF ligand superfamily [10]. TL1A was induced in vivo following LPS injection and showed cytotoxic activity against the L929 cell line and cultured chicken fibroblast cells, suggesting that it may function as a substitute for TNF-α [11]. Our prior studies [12], as well as those of Rautenschlein et al. [13], identified similar TNF-α-like factors in chicken macrophages stimulated with LPS or *Eimeria*, an intestinal protozoan parasite responsible for avian coccidiosis. To further characterize anti-microbial proteins expressed by chickens during coccidiosis, we constructed an expressed sequence tag (EST) library from *Eimeria*-infected intestinal intraepithelial lymphocytes (IELs) [14]. Among the 125 novel genes identified, one cDNA displayed nucleotide sequence homology to mammalian LITAFs. This report describes the isolation of the chicken LITAF cDNA and characterization of its encoded transcript and polypeptide.

2. Materials and methods

2.1. Cloning of LITAF

Construction of the intestinal IEL EST library in the pCMV-SPORT 6 vector was described previously [14]. The LITAF EST was identified based on sequence homology to mammalian LITAF sequences available in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The complete LITAF coding sequence was isolated by RT-PCR using restriction endonuclease-anchored primers and cDNA as template. Briefly, 5.0 µg of total RNA was prepared from intestinal IELs using TRIzol (Invitrogen, Carlsbad, CA) [15], treated with 1.0 U of DNase I (Sigma, St. Louis, MO), heated at 70 °C for 10 min, and reverse-transcribed at 42 °C for 1 h using the StrataScript first strand synthesis system (Stratagene, La Jolla, CA) with 5.0 µg of oligo(dT) primer, 25 mM of dNTPs, and 50 U of reverse transcriptase in a total volume of 19 µl. *Nco* I forward primer (5′-CCCCCATGGGGAGCTCTTTTGTGCCTGT-TTATTTC-3′) and *Not* I reverse primer (5′-CGC-GGCCGCGAAGCTTCAGTATGGGTTTAGAG-A-3′) (restriction sites underlined) were added to the template and PCR performed for 1 cycle at 95 °C for 10 min, 35 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min using Platinum Taq DNA polymerase (Promega, Madison, WI). PCR products were digested with *Nco* I and *Not* I (Roche, Indianapolis, IN), ligated into the corresponding restriction endonuclease sites of pET32a(+) (Novagen, Madison, WI), and transformed into BL21(DE3) competent cells (Invitrogen). For expression in COS7 cells, pET32a(+)-LITAF plasmid DNA was digested with *Kpn* I and *Not* I, gel-purified using the QIAquick Gel extraction kit (Qiagen, Valencia, CA), and subcloned into the pTriEx-4 plasmid under control of the CMV immediate early promoter (Novagen).

2.2. Induction of chicken LITAF by LPS and Eimeria

Confluent HTC macrophages [16] were stimulated in 25 cm² culture flasks (Corning Costar, Corning, NY) with 4.0 µg/ml of LPS from *E. coli* 0111:B4, *Salmonella enteritidis*, or *S. typhimurium* (Sigma) or were infected in six well plates with 5 × 10⁶ sporozoites of *Eimeria acervulina*, *E. maxima*, or *E. tenella*. At 4, 18, and 48 h post-treatment, the cells were collected and LITAF mRNA levels determined by quantitative RT-PCR.

2.3. Eimeria infection

Fertilized eggs of specific pathogen-free chickens were obtained from SPAFAS (Charles River Laboratories, Wilmington, MA) and hatched at

the Animal and Natural Resources Institute, USDA (Beltsville, MD). Chickens were provided free access to feed and water, and inoculated with 1×10^4 sporulated oocysts of *E. acevulina*, *E. maxima*, or *E. tenella* at 3 weeks of age as described [17] and secondary infection performed with 2×10^4 oocysts of the same parasites at day 14 post-primary infection. Tissue samples were collected from five birds at 1 day intervals post-inoculations. Briefly, IEL samples were collected from the duodenal region for *E. acervulina*, the Meckel's diverticulum to the ileac region for *E. maxima* and the caeca region for *E. tenella*. Gut tissues were cut longitudinally and washed three times with ice-cold HBSS containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma) to remove intestinal contents. The mucosa layer of intestine was carefully scraped using a surgical round-shaped scalpel. After several washing with HBSS, total RNA from pooled samples was prepared using TRIzol (Invitrogen) and LITAF mRNA levels determined by quantitative RT-PCR. All experiments were approved by the Animal and Natural Resources Institute IACUC.

2.4. Quantitative RT-PCR

Oligonucleotide primers for LITAF, TL1A, and GAPDH quantitative RT-PCR are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA isolated from bursa, thymus, spleen, peripheral blood, intestine, heart, brain, muscle and liver using TRIzol (Invitrogen) described [17,18] with the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene), and the following thermal profile: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. Each RT-PCR experiment contained test samples and 2-fold dilutions of standard RNA in triplicate. To normalize RNA levels between samples within individual experiments, the mean threshold cycle value (C_t) for the LITAF and GAPDH products were calculated by pooling values from all samples in that experiment. The levels of LITAF transcripts were normalized to those of GAPDH using the Q-gene program [19].

2.5. Expression of recombinant LITAF protein

E. coli BL21(DE3) transformed with the pET32a(+)-LITAF plasmid was induced with 1.0 mM isopropyl β-D-galactopyranoside (IPTG, Amersharm Biosciences, Piscataway, NJ) for 4 h at 26 °C, inclusion bodies were harvested and solubilized, and recombinant protein was purified on a Ni^{2+}-NTA His-bind resin column (Novagen) according to the manufacturer's protocol. COS7 cells (ATCC, Manassas, VA) were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in 75 cm^2 culture flasks at 37 °C in 5% CO_2 incubator and transiently transfected at 80–90% confluence with the pTriEx4-LITAF plasmid DNA using lipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. At 18 h post-transfection, the cells were cultured in IMDM containing 10 mM insulin–transferrin–sodium selenite supplement (Sigma) and incubated for 24 h. Recombinant histidine-tagged LITAF protein was purified from cell-conditioned culture medium on a Ni^{2+}-NTA His-bind resin column as above. 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

Table 1
Quantitative RT-PCR primers

Target	Primer sequence	Product size (bp)	Accession number
LITAF	5′-TGTGTATGTGCAGCAACCCGTAGT-3′ 5′-GGCATTGCAATTTGGACAGAAGT-3′	229	AY765397
TL1A[a]	5′-CCTGAGTATTCCAGCAACGCA-3′ 5′-ATCCACCAGCTTGATGTCACTAAC-3′	292	AB194710
GAPDH	5′-GGTGGTGCTAAGCGTGTTAT-3′ 5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458

[a] From Takimoto et al. [11].

blue), resolved on 15% SDS-PAGE gels, and transferred to nitrocellulose (Immobilon-P, Millipore). Membranes were blocked with SuperBlock T20 blocking buffer (Pierce, Rockford, IL) at 4 °C for 16 h, incubated with peroxidase-labeled anti-polyhistidine antibody (Sigma) for 1 h at room temperature, washed three times with PBS containing 0.1% Tween 20, and developed with 4-chloro-1-naphthol substrate (Sigma).

2.6. LITAF-induced cytotoxicity

HTC and HD11 macrophage cell lines [16,20], CHCC OU-2, an embryonic fibroblast cell line [21], and LCSS-RP9, a virally transformed B-cell line [22] were cultured at 41 °C in RPMI-1640 (Sigma) supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were washed with HBSS, resuspended to 1.0×10^5 (HTC, HD11) or 1.25×10^4 (CHCC OU-2, LCSS-RP9) cells/ml, 100 μl placed in flat-bottomed 96 well microtiter plates (Corning Costar), and incubated for 48 h with purified LITAF protein or serial dilutions of purified protein from COS7 cells transfected with the pTriEx4-LITAF plasmid. Cell numbers as a measure of viability were determined using 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium (WST-8, Cell-Counting Kit-8[®], Dojindo Molecular Technologies, Gaithersburg, MD) as described [23,24] and optical density (OD) measured at 450 nm.

2.7. LITAF-induced TL1A mRNA expression

Purified recombinant LITAF protein (0.1, 0.5, or 2.5 μg/ml) was added to HD11 macrophage cell cultures for 24 h and TL1A mRNA levels were quantified by RT-PCR using published primer sequences [11].

2.8. Statistical analysis

Mean ± S.D. values for each group ($N = 3$ or 5) were calculated, differences between groups were analyzed by the Dunnet multiple comparison test or the Tukey–Kramer multiple comparison test using InStat[®] software (Graphpad, San Diego, CA), and considered significant at $p < 0.05$.

3. Results

3.1. Isolation of chicken LITAF

An EST clone (GenBank accession No. CD75928) encoding LITAF was identified from a chicken intestinal IEL cDNA library [14] based on nucleotide sequence homology to mammalian LITAFs. The full-length cDNA was isolated by RT-PCR and consisted of a 740 nucleotide sequence containing an open reading frame of 444 nucleotides and predicted to encode a 148 amino acid polypeptide of 16.0 kDa (GenBank accession No. AY765397). Alignment of amino acid sequences from the chicken, human, horse, rat, and mouse proteins identified seven conserved cysteine residues (except for the absence of cysteines 4–7 in the human sequence) (Fig. 1A). Comparison of these sequences using the CLUSTAL W (1.82) program revealed chicken LITAF to be 48% identical to human, 59% to horse, and 64% to rat and mouse LITAFs. By contrast, inter-mammalian LITAF comparisons revealed greater levels of homology, ranging from 66% (human vs. horse, rat, and mouse) to 96% (rat vs. mouse). By evolutionary distance analysis, chicken LITAF appeared most closely related to the rodent proteins (Fig. 1B).

3.2. LITAF tissue expression

LITAF transcripts were expressed primarily in placenta, peripheral blood leukocytes, lymph node, and spleen in human [7] and in liver, heart, kidney, spleen, lung and testis in mice [8]. In the chicken, LITAF mRNA was expressed predominantly in the spleen and intestinal IELs, with intermediate levels in the bursa, peripheral blood leukocytes (PBL), liver, thymus, and lowest levels in heart, brain, and muscle (Fig. 2).

3.3. Chicken LITAF mRNA is induced by LPS and Eimeria parasites

Several studies reported that LPS induced human and mouse LITAF expression in monocytes and macrophages [8,9], although no reports have examined the stimulatory activity of non-bacterial mediators. Following the treatment of chicken HTC macrophages with LPS from E. coli or S. enteritidis, expression of LITAF mRNA was up-regulated at 4 h post-stimulation and down-regulated at 18 and 48 h (Fig. 3A) compared with

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LITAF,human    MSVPGPYQAATGPSSAPSAPPSYEETVAVNSYYPTPPAPMPGPTTGLVTGPDGKGMNPPS 60
LITAF,horse    MSAPGSYQASAGPSSVPTAPPSYEETVGVHSYFPTPPAPVPGPTTGLVTGPDGKGMNPPA 60
LITAF,rat      MSAPGPYQAAAGPSVMPTAPPTYEETVGVNSYYPTPPAPQPGPATGLITGPDGKGMNPPS 60
LITAF,mouse    MSAPGPYQAAAGPSVVPTAPPTYEETVGVNSYYPTPPAPMPGPATGLITGPDGKGMNPPS 60
LITAF,chicken  -------MSAPSGFPAPSAPPSYEETVGINVNYPHP-YPVPQPG---LR-PDGKGMNPPQ 48
                 ::.. *:***:*****.:: :* * * * :  ********

LITAF,human    YYTQPAPIPNNNPITVQTVYVQHPIT-FLDRPIQMCCPSCNKMIVSQLSYNAGALTWLSC 119
LITAF,horse    YYTQPVPVPNANAIAVQTVYVQQPVS-FFDRPVQMCCPSCNKMIVTQLSYTAGALTWLSC 119
LITAF,rat      YYTQPVPVPNANAIAVQTVYVQQPIS-FYDRPIQMCCPSCNKMIVTQLSYNAGALTWLSC 119
LITAF,mouse    YYTQPVPVPNANAIAVQTVYVQQPVS-FYDRPVQMCCPSCSKMIVTQLSYNAGALTWLSC 119
LITAF,chicken  YSGQ--PMPTSTPVTVQTVYVQQPVVLFYDRPVQMSCPSCNQMIVTRLCYESGALTWLSC 106
               *   *  *:.*. ..::*******:*:  *  ***:**.****.:***::*.* :********

LITAF,human    GSLCLLGVHSGLLLHPLLRGCPAGRGPLLSQLQSSPGHLQAFVGLSQTWREPGAAG-SPF 178
LITAF,horse    GSLCLLGCIAGCCFIPFCVDALQDVDHYCTQLQSSPGHLQAFERLGQTRREPCAAERSFP 179
LITAF,rat      GSLCLLGCVAGCCFIPFCVDALQDVDHYCPNCKALLGTYKRL------------------ 161
LITAF,mouse    GSLCLLGCVAGCCFIPFCVDALQDVDHYCPNCKALLGTYKRL------------------ 161
LITAF,chicken  GGLFLLGCIAGCCLIPFCVDALKDVEHFCPNCNAHVGSYKRL------------------ 148
               *.* *** :*  : *: .. .  .: :: *  : :
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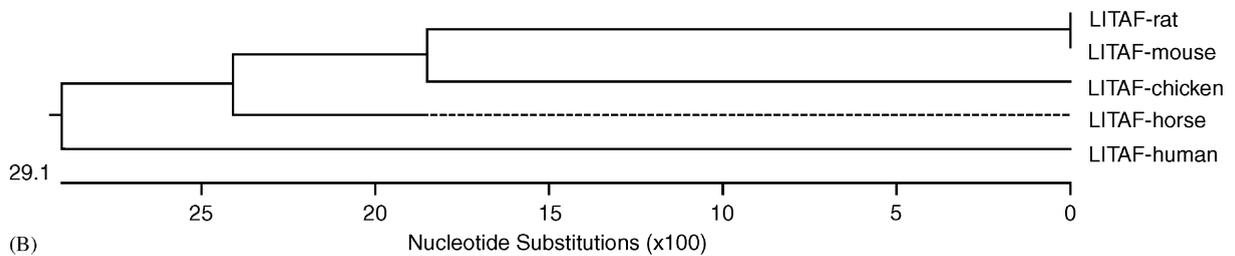
(A)

(B)

Fig. 1. Sequence comparison of proteins encoded by mammalian and chicken LITAFs. (A) Sequences were aligned using the CLUSTAL W (1.82) program (www.ebi.ac.uk/clustalw/). The seven conserved cysteine residues are highlighted by black boxes. Asterisks (*) indicate identical amino acid residues. Human LITAF, accession No. NP_004853; horse LITAF, accession No. AAM34206; rat LITAF, accession No. NM_019980; mouse LITAF, accession No. AAH18559; chicken LITAF, accession No. AY765397. Single dots (.) indicate homologous amino acid changes. Double dots (:) indicate conserved amino acid changes. (B) Phylogenetic relationship of mammalian and chicken LITAFs constructed based on amino acid sequences aligned using CLUSTAL W.
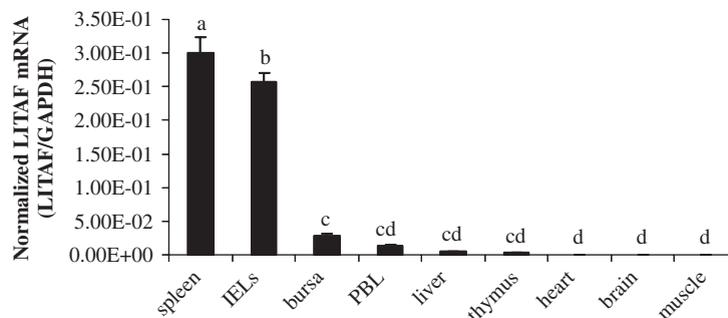

Fig. 2. Tissue distribution of LITAF mRNA. Total RNA was isolated from the indicated sources of 3 week of age, analyzed for LITAF mRNA by quantitative RT-PCR, and normalized to GAPDH mRNA. Each bar represents the mean $\pm$ S.D. of five birds. Significant level was performed at $p < 0.05$. LITAF regression equation: $C_t = 3.20x + 28.5$, $R^2 = 0.99$; GAPDH regression equation: $C_t = 3.42x + 32.2$, $R^2 = 0.99$.
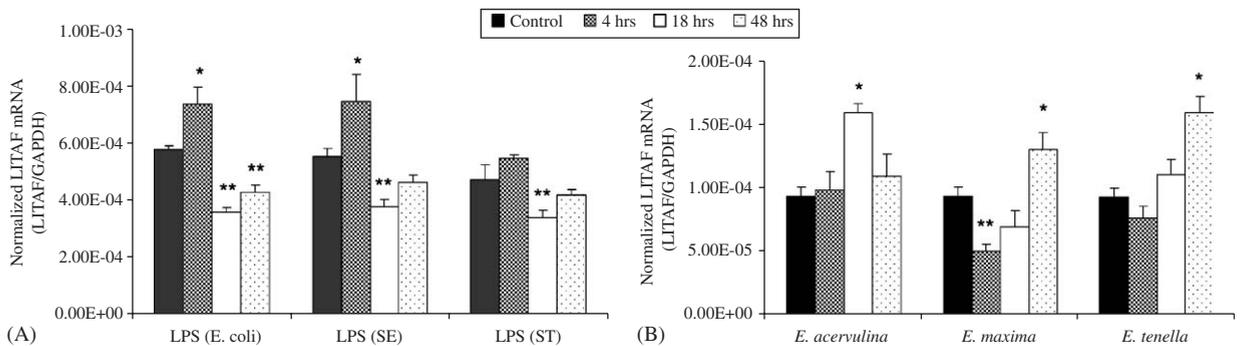
Fig. 3. Chicken LITAF mRNA is induced by LPS and *Eimeria* parasites in chicken macrophages. HTC macrophages were treated with LPS from *E. coli*, *S. enteritidis* (SE) or *S. typhimurium* (ST) (A) or *E. acervulina*, *E. maxima* and *E. tenella* parasites (B) for the indicated times. LITAF mRNA levels were determined by quantitative RT-PCR and normalized to GAPDH mRNA. Each bar represents the mean $\pm$ S.D. of triplicate samples. Asterisks indicate significant increase in LITAF mRNA expression when compared to its control group (*, $p < 0.05$; **, $p < 0.01$).
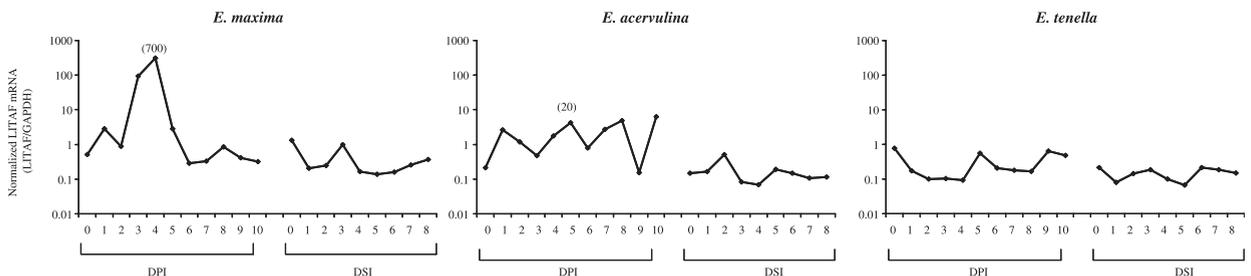

Fig. 4. Kinetics of LITAF mRNA expression following *Eimeria* infection. Chickens were non-infected (day 0) or orally infected with 1×10^4 oocysts of *E. maxima*, *E. acevulina*, or *E. tenella* on days 0 and reinfected with 2×10^4 oocysts of same species of *Eimeria* at 14 days post-primary infection. Intestinal IELs were isolated from the duodenum, jejunum, or caeca, respectively, at the indicated times post-infection, LITAF transcripts were measured by quantitative RT-PCR, and normalized to GAPDH mRNA. Each data point represents the mean value of five birds. DPI, days post-primary infection; DSI, days post-secondary infection. Ratio between gene expressions at 4, 5 days and 0 are indicated in parentheses as a fold change.

non-treated cells. *S. typhimurium* LPS did not stimulate LITAF transcription at 4 h, although lower levels were seen at 18 h compared with controls. HTC cells treated with viable *Eimeria* parasites also induced LITAF transcripts. However, the kinetics of LITAF expression in *Eimeria*-treated HTC cells was delayed compared with LPS induction. Maximum mRNA levels after *E. acervulina* stimulation were observed at 18 h post-treatment, while highest levels following *E. maxima* and *E. tenella* were apparent after 48 h (Fig. 3B).

3.4. Chicken LITAF expression profiles during Eimeria infection

Different species of *Eimeria* infect separate regions of the intestinal tract, with *E. acervulina* mainly localizing to the duodenum, *E. maxima* to the jejunum, and *E. tenella* to the caeca [25]. To determine if LITAF transcripts were increased in these regions following infection with the various *Eimeria* species, chickens were orally infected with oocysts of *E. acervulina*, *E. maxima*, or *E. tenella* on day 0 and on day 14 after primary infection, and LITAF transcripts quantified in duodenum, jejunum, and caeca IELs on day 0 (non-infected), between days 1 and 10 post-primary infection, and between days 1 and 8 post-secondary infection. As illustrated in Fig. 4, infection with *E. maxima* drastically up-regulated LITAF mRNA expression at days 3 and 4 post-primary infection. In contrast, no consistent changes in LITAF transcript levels were seen following primary infection with *E. tenella*, or following secondary infection with all three parasites.

3.5. Cytotoxic effect of LITAF recombinant protein

Because mammalian LITAF has been shown to induce TNF-α [7], we predicted that the treatment of tumor cells with purified chicken LITAF protein would produce a cytotoxic effect through a TNF-α or TNF-α-like autocrine pathway. To test this hypothesis, chicken tumor cells were treated with purified LITAF protein, cultured for 48 h, and cell numbers determined as a measure of viability. To express LITAF protein, its cDNA was subcloned into prokaryotic and eukaryotic expression vectors containing a polyhistidine tag and introduced into *E. coli* and COS7 cells, respectively. Western blot analysis of LITAF purified from COS7 cells using anti-polyhistidine antibody revealed a prominent band at 16.0 kDa, indicative of LITAF expression (Fig. 5). The faster migrating band likely represented a minor proteolytic degradation fragment of the intact protein, as previously reported [26,27]. As shown in Fig. 6A, treatment of four different avian transformed cell lines with purified LITAF significantly decreased cell viability compared with media controls ($p < 0.05$). Furthermore, LITAF purified from pTriEx4-LITAF-transfected COS7 cells inhibited the viability of LCSS-RP9 avian leukosis virus-transformed cells in a dose-dependent manner (Fig. 6B).

3.6. LITAF stimulates TL1A gene expression

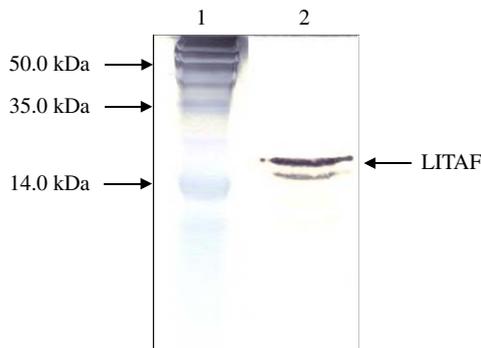Because a chicken homologue of mammalian TNF-α has yet to be identified, we investigated the

Fig. 5. Western blot analysis of recombinant chicken LITAF. COS7 cells were transfected with pTriEx4-LITAF plasmid DNA, recombinant LITAF was purified from cell-conditioned culture medium on a Ni^{2+}-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. Lane 2, polyhistidine-tagged LITAF.

ability of LITAF to stimulate the expression of TL1A, a member of the TNF ligand superfamily and suggested to function in part as a substitute for TNF-α in chicken [11]. As shown in Fig. 7, recombinant LITAF up-regulated TL1A mRNA levels in HD11 macrophages in a dose-dependent manner.

4. Discussion

In this paper, we report cloning of the chicken LITAF gene and preliminary characterization of its encoded mRNA and polypeptide. The cloning strategy was based on nucleotide sequence homology between the avian and mammalian genes. To further implicate its identity as LITAF, we showed that gene expression was stimulated by LPS treatment of macrophages, a hallmark of mammalian LITAF activity. Additional circumstantial evidence supporting our claim is provided by the expression pattern of the chicken gene in various tissues, which matches that previously reported for human and mouse LITAF [7,8] and correlates with the tissue distribution of mouse TNF-α biosynthesis during experimental endotoxemia [28]. Finally, recombinant LITAF stimulated expression of chicken TL1A.

Conclusive identification of the avian homologue of TNF-α remains elusive despite multiple reports describing TNF-like factors [12,13,29,30]. Recently, Takimoto et al. [11] reported the molecular cloning and functional characterization of chicken TL1A which is known as VEGI/TNFSF15 [10]. TL1A expression was induced by LPS and the recombinant protein exhibited cytotoxic activity against L292 cells, a TNF-α-sensitive murine fibrosarcoma, and chicken fibroblasts. In support of their suggestion, our results indicated that recombinant LITAF induced the expression of TL1A mRNA in chicken macrophages. While mammalian LITAF might require the participation of an additional transcription factor such as STAT6(B) to induce TNF-α gene expression, over-expression of LITAF alone also up-regulates TNF-α production, although less dramatically than LPS treatment [7,9]. Interestingly, a similar pattern of chicken TL1A expression is observed when comparing LITAF over-expression and LPS treatment of avian cells (Fig. 7 and unpublished observations).

In addition to bacterial endotoxin, our results are the first to indicate that non-LPS microbial components are capable of inducing LITAF expression.
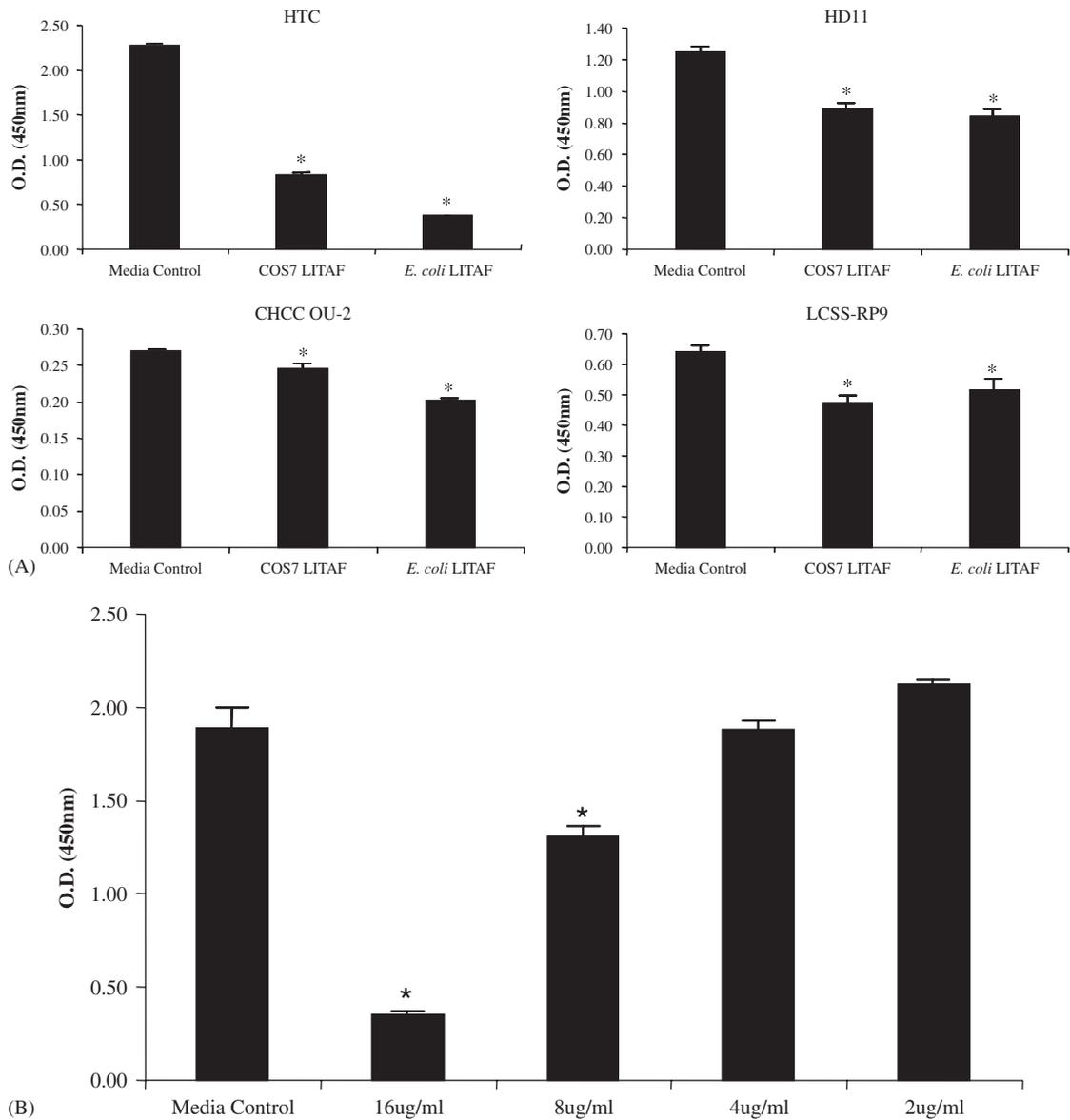
Fig. 6. Cytotoxic activity of recombinant LITAF. (A) HTC, HD11, CHCC OU-2, and LSCC-RP9 cells were treated for 48 h with recombinant LITAF purified from *E. coli* (16 μg/ml) or COS7 cells (27 μg/ml) and cell viability was determined using WST-8. (B) LSCC-RP9 cells were treated for 48 h with various dilutions of cell-conditioned medium from COS7 cells transfected with the pTriEx4-LITAF expression plasmid and cell viability was determined. The concentration of recombinant LITAF protein in the undiluted (1:1) cell culture medium was 16 μg/ml. Control groups were treated with complete media alone. Each bar represents the mean ± S.D. of triplicate samples. Asterisks (*) indicate significantly decreased cell viability compared with the medium-only control ($p < 0.05$).

Different *Eimeria* species induced different expression patterns of LITAF mRNA in HTC macrophages (Fig. 3B). For example, maximum mRNA level of LITAF was observed after 18 h post-treatment with *E. acervulina* sporozoites, whereas *E. maxima* and *E. tenella* sporozoites induced maximal LITAF expression at 48 h post-infection.

These results may be caused by the inherent difference in their pathogenicity.

After primary infection with *E. maxima*, LITAF mRNA was dramatically up-regulated at 3–4 days post-infection (700-fold). The peak in LITAF activity was correlated with the maximum production of a TNF-like factor by chicken macrophages
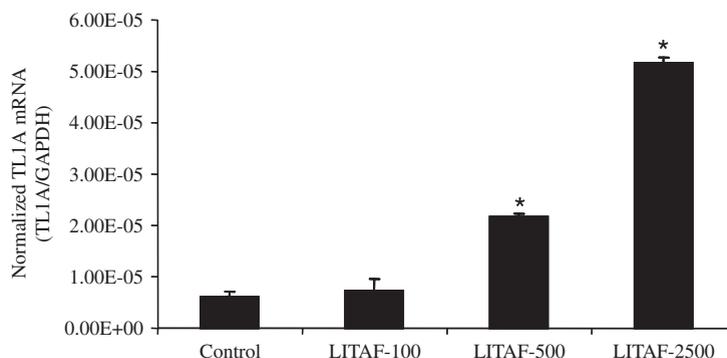
Fig. 7. Induction of TL1A mRNA expression by LITAF. Recombinant LITAF purified from *E. coli* was added to HD11 macrophage cells at the indicated concentrations, the cells were incubated for 24 h, TL1A mRNA levels were determined by quantitative RT-PCR, and normalized to GAPDH mRNA. Each bar represents the mean $\pm$ S.D. of triplicate samples. Asterisks (*) indicate significantly up-regulated TL1A mRNA compared with non-treated media controls ($p < 0.01$). TL1A regression equation: $C_t = 3.33x + 36.4$, $R^2 = 0.99$; GAPDH regression equation: $C_t = 3.42x + 32.2$, $R^2 = 0.99$.

from spleens of *Eimeria*-infected chickens observed by Byrnes et al. [30]. It also corresponds with the appearance of the most characteristic local and systemic pathophysiological changes in the chicken intestine induced by *Eimeria* parasites.

Although it is not clear why LITAF mRNA expression in *E. tenella* infection was lower than those of *E. maxima* and *E. acervulina* infections, we can postulate that the differences in the LITAF mRNA expression levels among three *Eimeria* species are influenced by the immunogenicity of *Eimeria* species. While a large number of oocysts are generally required to generate a good immune response against *Eimeria*, some exception has been noted, e.g., *E. maxima* is highly immunogenic and requires only a small number of oocysts to induce almost complete immunity [31,32].

Thus, the robust production of LITAF mRNA in response to *Eimeria* infection, for example, with *E. maxima* and *E. acervulina* infections may directly account not only for the production of chicken TNF-α/TL1A, but also many of the pathological features observed with avian coccidiosis during the early course of infection.

Mammals respond to LPS using Toll-like receptor (TLR) 4. To the best of our knowledge, however, no reports have linked LPS binding to TLR4 with LITAF activation, although it is not unreasonable to suggest that the LPS-TLR4 pathway initiates a signal transduction cascade leading to TNF-α production through LITAF. Our observations that diverse microbial agonists (LPS, *Eimeria*) induced chicken LITAF expression suggests that more than one TLR may be involved in

this response. Yarovinsky et al. [33] identified TLR11 as a receptor on murine dendritic cells for a profilin-like molecule from *Toxoplasma gondii*, an apicomplexan parasite phylogenetically related to *Eimeria*. Our own studies have shown that a homologous profilin from *E. acervulina* (3-1E) also activated murine dendritic cells through TLR11 (unpublished observations). Thus, at least two TLRs (TLR4, TLR11) are candidate receptors that mediate avian innate immune responses through a LITAF/TNF-α/TL1A pathway.

In addition to its involvement in innate immunity, other studies have indicated the involvement of LITAF in apoptosis. This could explain the observation that recombinant chicken LITAF protein killed CHCC OU-2 embryonic fibroblast cell line as well as other leukocyte tumor cell lines (Fig. 6). For example, LITAF was shown to be identical to the p53-inducible gene 7 (PIG7) and was up-regulated during p53-mediated apoptosis [34,35]. Moriwaki et al. [36] have suggested that SIMPLE (small integral membrane protein of the lysosome/late endosome) which is a lysosomal membrane protein identical to LITAF/PIG7 at the nucleotide level, could be involved in host cell apoptosis as a mean to limit the spread of intracellular bacteria such as *Mycobacterium tuberculosis*. The present study indicates this mechanism also may be applicable in the case of intracellular infection by *Eimeria*.

In summary, molecular cloning and functional characterization of chicken LITAF indicates that it may play an important role in the regulation of TNF-α/TL1A gene expression during the course of bacterial and parasite infection as well as

tumorigenesis. Further characterization of the LITAF regulatory network will shed light on new therapeutic approaches to combat pathogen infections and tumor development in avian species.

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