Functional characterization of tumor necrosis factor superfamily 15 (TNFSF15) induced by lipopolysaccharides and *Eimeria* infection

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Abstract

A full-length cDNA encoding chicken tumor necrosis factor superfamily 15 (TNFSF15) was isolated and its functional role was investigated. TNFSF15 transcripts were primarily expressed in spleen, liver, intestinal intraepithelial lymphocytes (IEL), peripheral blood lymphocytes and bursa. *In vitro* infection of HTC macrophages with three species of *Eimeria* sporozoites induced TNFSF15 gene expression. *In vivo* experiments revealed that TNFSF15 gene was highly increased following primary infections with *Eimeria acervulina* or *Eimeria maxima*. In contrast, no consistent changes in transcript levels were seen following primary infection with *Eimeria tenella*, or following secondary infection with any of the three *Eimeria* species. Following infection with *E. acervulina* and *E. maxima*, TNFSF15 transcripts were primarily expressed in intestinal $CD4^+$ and $TCR2^+$ IEL, respectively. A dose-dependent cytotoxic effect of recombinant TNFSF15 protein was observed on HTC and LSCC-RP9 tumor cells. These results indicate that TNFSF15 plays an important role in local inflammatory response to *Eimeria*.

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Keywords: TNFSF15; Chicken; LPS; TNF-α; *Eimeria*; Lipopolysaccharide; Cytotoxicity; Coccidiosis

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1. Introduction

Extensive research on members of tumor necrosis factor superfamily (TNFSF) and TNFSF receptor (TNFSFR) has revealed a diverse array of biological functions, including immune cell homeostasis, development, and organogenesis [1,2]. TNFSF and TNFSFR proteins are abundantly expressed in the immune system, and are critically involved in the differentiation, proliferation, and apoptosis of immune cells. TNF-like activities were described in chicken peripheral blood-derived macrophages following *in vivo Eimeria* infection, in chicken

alimentary secretions, or in macrophages following *in vitro* stimulation with bacterial lipopolysaccharide (LPS) [3–6]. Administration of exogenous chicken TNF-like factor (TNLF) induced body weight loss indicating its role in a pathological effect [3].

Several avian TNFSF members have been isolated and characterized. These include TNFSF8 (CD30 ligand, or CD30L), TNFSF10 (TNF-related apoptosis-inducing ligand, or TRAIL), TNFSF13B (B cell activating factor of TNF superfamily, or BAFF), TNFSF15, and LPS-induced TNF-α factor (LITAF) [7–11]. In addition, with the recent availability of the genomic sequence and annotated genomic analysis in chicken, additional TNFSF members have been described, including TNFSF4 (OX40L), TNFSF5 (CD40L), and TNFSF6 (FasL) [12]. Several of these chicken TNFSF members are homologous to their mammalian counterparts. In particular, the nucleotide sequence of the chicken TNFSF15 gene is similar to mammalian TL1A and the long form of mammalian vascular endothelial growth inhibitor (VEGI) [10,13,14]. Takimoto et al. [10] showed that chicken TNFSF15 decreased feed intake, and increased rectal temperature, and mediated cytotoxicity *in vitro* against murine fibroblasts or primary chicken cell cultures. However, the immunological studies of chicken TNFSF15 have been absent.

Avian coccidiosis is caused by multiple species of *Eimeria* spp., which develop within chicken intestinal epithelial cells resulting in malabsorption of nutrients, inefficient feed utilization, and impairment of animal growth [15–17]. *Eimeria* undergoes a complex life cycle, composed of intracellular, extracellular, asexual, and sexual stages. Host immune responses to coccidia infection are complex and involve many different types of effector cells and molecules [16]. Recently, we reported that the expression of a chicken TNFSF member, LITAF, was significantly upregulated in *Eimeria*-infected chickens [11]. Furthermore, purified recombinant LITAF protein expressed in *Escherichia coli* or COS7 cells mediated cytotoxic activity against chicken tumor cell lines. The present study was undertaken to determine the immunological properties of TNFSF15 and its roles in host responses to *Eimeria*. We found that TNFSF15 transcripts were significantly increased following infection with *Eimeria* in the gut or in the macrophages following stimulation with bacterial LPS. These results indicate that TNFSF15 and LITAF

play an important role in the host response to coccidiosis.

2. Materials and methods

2.1. Animals, Eimeria infection, and preparation of IEL

Fertilized eggs of specific pathogen-free SPAFAS chickens were obtained from Charles River Laboratories (Wilmington, MA) and hatched at the Animal and Natural Resources Institute of the Beltsville Agricultural Research Center. Chickens were provided free access to feed and water and experimentally infected with *Eimeria* as described [18,19]. Primary oral inoculations were made with 1×10^4 sporulated oocysts of *Eimeria acervulina* (strain #12), *Eimeria maxima* (Tysons) and *Eimeria tenella* (WLR-1) at 3 weeks of age, and secondary inoculations were made with 2×10^4 oocysts of the same parasites at day 14 post-primary infection. IEL were collected from the duodenal region of *E. acervulina* infected chickens, from the Meckel's diverticulum to the ileac region of *E. maxima* infected birds, and the caeca region of *E. tenella* inoculated chickens as described [18,19]. Briefly, the dissected intestinal tissues were cut longitudinally and washed three times with ice-cold Hank's balanced salt solution (HBSS) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma, St. Louis, MO). The mucosal layer was scraped away with a surgical round-shaped scalpel, the remaining tissue was washed with HBSS, and IEL were isolated from homogenized tissues by Percoll gradient centrifugation. Animal experiments were performed according to the approved guidelines established by the Beltsville Area Institutional Animal Care and Use Committee (IACUC).

2.2. Cloning of TNFSF15

The full-length TNFSF15 gene was isolated by RT-PCR as described [20] using primers based on the published sequence [10]. Briefly, 5.0 μg of total RNA was prepared from intestinal IEL using TRIzol (Invitrogen, Carlsbad, CA), treated with 1.0 U of DNase I (Sigma), 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. PCR was

performed using the TNFSF15 forward primer (5′-GCTCGTACGCCAATTGCC-3′) and the reverse primer (5′-CAGTAAAAAGGCACCGAAGAA-3′) for 1 cycle at 95 °C for 15 min, 35 cycles at 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min using HotStarTag DNA polymerase (Qiagen, Valencia, CA). Amplicons were cloned into pCR2.1-TOPO (Invitrogen), isolated by EcoRI/HindIII digestion, ligated into the corresponding restriction endonuclease sites of pET32a (+) (Novagen, Madison, WI), and transformed into Rosetta 2 (DE3) competent cells (Novagen). Recombinant TNFSF15 protein was induced with 1 mM isopropyl beta-D-galactopyranoside (IPTG, Amersham Biosciences, Piscataway, NJ) at 25 °C for 5 h. TNFSF15 protein expressing clones were selected and DNA sequences confirmed by sequence analysis (Applied Biosystems, Foster City, CA) at the Center for Biosystems Research (University of Maryland, College Park, MD).

2.3. Induction of TNFSF15 by LPS and Eimeria

Confluent HTC macrophages [21] grown in 6-well plates were stimulated with 4.0 μg/ml of LPS from *E. coli* 0111:B4, *Salmonella enteritidis*, or *S. typhimurium* (Sigma), or were infected with 5.0×10^6 sporozoites of *E. acervulina*, *E. maxima*, or *E. tenella* as described [11]. At 4, 18, and 48 h post-treatment, the cells were harvested and TNFSF15 mRNA levels were determined by quantitative real time RT-PCR.

2.4. Magnetic cell separation of IEL

Intestinal IEL were collected at peak TNFSF15 expression time (5 days post-primary infection (DPI)) following infection with 1.0×10^4 sporulated oocysts (per bird) of *E. acervulina* or *E. maxima.* The collected IEL were separated into CD4$^+$, CD8$^+$, TCR1$^+$, or TCR2$^+$ subpopulations using the corresponding antibodies and the magnetic BD IMag Cell Separation System according to the

manufacturer's instructions (BD Biosciences Pharmingen, San Jose, CA). Total cell numbers were adjusted to 2.0×10^7 cells/ml and incubated on ice for 45 min with pre-titrated amounts of anti-CD4, anti-CD8, anti-TCR1, or anti-TCR2 monoclonal antibodies [22,23]. The cells were washed twice with HBSS containing 3% FBS, incubated on ice for 15 min with 20 μg/ml of biotinylated goat anti-mouse IgG secondary antibody (BD Biosciences), washed with 1X BD IMag buffer, and incubated on ice for 30 min with 40 μl/ml of streptavidin-labeled magnetic particles (BD IMag Streptavidin Particles Plus—DM). Magnetic particles were isolated, the positive and negative cell fractions were resuspended in HBSS, and TNFSF15 mRNA levels were quantified by quantitative RT-PCR.

2.5. Quantitative RT-PCR

Oligonucleotide primers for TNFSF15 and GAPDH quantitative RT-PCR are listed in Table 1. Amplification and detection of target genes were carried out using equivalent amounts of cDNA prepared from total RNA of each sample as described [11,18] with the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). The thermal profile was 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. Each reaction was performed in triplicate. To normalize mRNA expression levels between samples within individual experiments, the mean threshold cycle value (C_t) for the TNFSF15 and GAPDH products were calculated by pooling values from all samples in that experiment. The levels of TNFSF15 transcripts were normalized to those of GAPDH transcripts using the Q-gene program [24].

2.6. Expression of recombinant TNFSF15 protein

E. coli Rosetta 2(DE3) cells transformed with the TNFSF15-pET32a(+) plasmid were induced with

Table 1
Primer sets for quantitative RT-PCR

Target gene	Primer sequence	Product size (bp)	Accession number
TNFSF15[a]	5′-CCTGAGTTATTCCAGCAACGCA-3′ 5′-ATCCACCAGCTTGATGTCACTAAC-3′	292	NM_001024578
GAPDH	5′-GGTGGTGCTAAGCGTGTTAT-3′ 5′-ACCTCTGTCATCTCTCCACA-3′	264	NM_204305

[a]From Takimoto et al. [10].

1.0 mM IPTG for 5 h at 25 °C, inclusion bodies were harvested and solubilized, and the polyhistidine-tagged recombinant TNFSF15 protein was purified using Ni^{2+}-NTA His-bind resin (Qiagen) according to the manufacturer's protocol. The purified protein was resolved on a 15% SDS-PAGE gel, transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA), the membrane was blocked for 2 h at room temperature with SuperBlock T20 (Pierce, Rockford, IL), incubated for 1 h at room temperature with peroxidase-labeled anti-polyhistidine antibody (Sigma), washed with PBS containing 0.05% Tween 20, and developed with 4-chloro-1-naphthol substrate (Sigma).

2.7. Determination of TNFSF15 cytotoxic activity

Chicken HTC macrophages and the retrovirus-transformed LCSS-RP9 B cell line 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 chicken embryonic fibroblast cell line CHCC-OU2 [4] was cultured at 37 °C in Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. One hundred microliter of cell suspensions (5.0×10^3 or 1.0×10^4 cells/ml) were cultured in flat-bottom 96-well microtiter plates (Corning Costar) for 48 h with
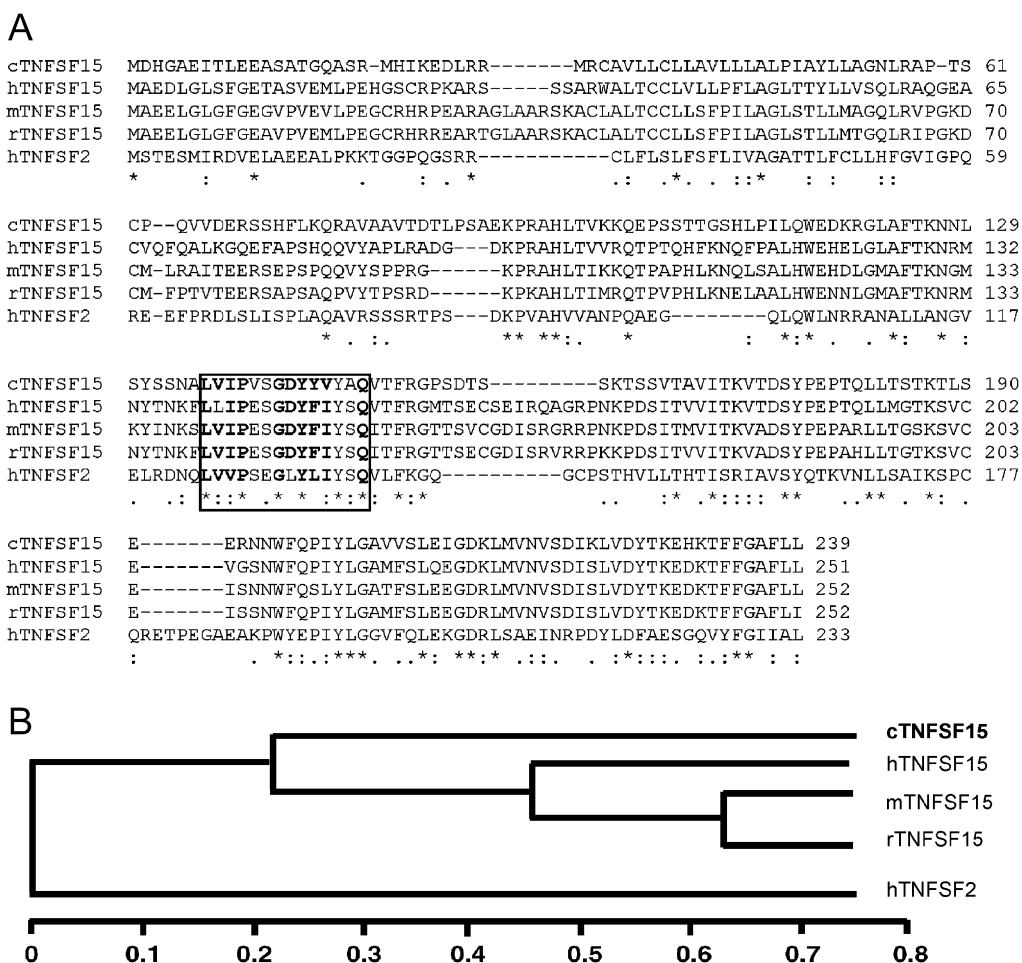

Fig. 1. Sequence comparison of proteins encoded by mammalian and chicken TNFSF15s. (A) Sequences aligned using the CLUSTAL W (1.83) program (http://www.ebi.ac.uk/clustalw/). The TNF superfamily ligand signature sequence is indicated by the box and critical residues conforming to the signature are in bold. cTNFSF15, chicken TNFSF15, accession no. NP_001019749; hTNFSF15, human TNFSF15, accession no. NP_005109; mTNFSF15, mouse TNFSF15, accession no. NP_796345; rTNFSF15, rat TNFSF15, accession no. NP_665708; hTNFSF2, human TNF-α, accession no. NP_000585. Asterisks (*) indicate identical amino acid residues. Single dots (.) indicate homologous amino acid changes. Double dots (:) indicate conserved amino acid changes. (B) Phylogenetic relationships of mammalian and chicken TNFSF15s and human TNF-α were constructed based on amino acid sequences aligned using CLUSTAL W.

serial dilutions of recombinant TNFSF15 protein or control protein (polyhistidine) from the empty expression vector. Cell numbers were determined using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8, Dojindo Molecular Technologies, Gaithersburg, MD) as described in [25] and OD_{450} was measured according to the manufacturer's protocol.

2.8. Statistical analysis

Mean $\pm$ 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 Graphpad Prism 4 software (Graphpad, San Diego, CA), and considered significant at $p < 0.05$.

3. Results

3.1. Analysis of chicken TNFSF15

A full-length cDNA of chicken TNFSF15 (accession number NP_001019749) consisted of a 717 bp open reading frame predicted to encode a 239 amino acid polypeptide with a molecular weight of 26 kDa. Comparison of the predicted chicken TNFSF15 protein sequence with mammalian TNFSF members using CLUSATAL W (1.83) revealed 51% identity to human TNFSF15, 44% to mouse and rat TNFSF15, and 21% to human TNFSF2 (TNF-α) (Fig. 1A). Importantly, eight of the 10 critical residues comprising the signature sequence of

TNFSF members in chicken TNFSF15 were well conserved with those of the mammalian proteins (boldface residues within box). Not unexpectedly, inter-mammalian TNFSF15 comparisons revealed greater levels of identity compared with chicken–mammalian comparisons, ranging from 62% (human vs. mouse) to 84% (mouse vs. rat). By evolutionary distance analysis, chicken TNFSF15 appeared most closely related to the mammalian TNFSF15s compared with human TNFSF2 (Fig. 1B).

3.2. Tissue distribution of TNFSF15

Expression of mammalian TNFSF15 was reported to be largely restricted to endothelial cells of diverse lymphoid organs [14,26]. As shown in Fig. 2, chicken TNFSF15 transcripts were highly expressed in spleen and liver, with intermediate levels in intestinal IEL, peripheral blood leukocytes (PBL) and bursa, and lower levels in heart, muscle and thymus.

3.3. Stimulation of TNFSF15 expression by LPS and Eimeria

We previously reported that bacterial LPS and Eimeria infection induced expression of chicken LITAF, another TNFSF member [11]. To determine whether expression of TNFSF15 was modulated in a similar manner, HTC macrophages were treated with 4.0 μg/ml of purified LPS from E. coli, S. enteritidis, or S. typhimurium, or 5×10^6 viable
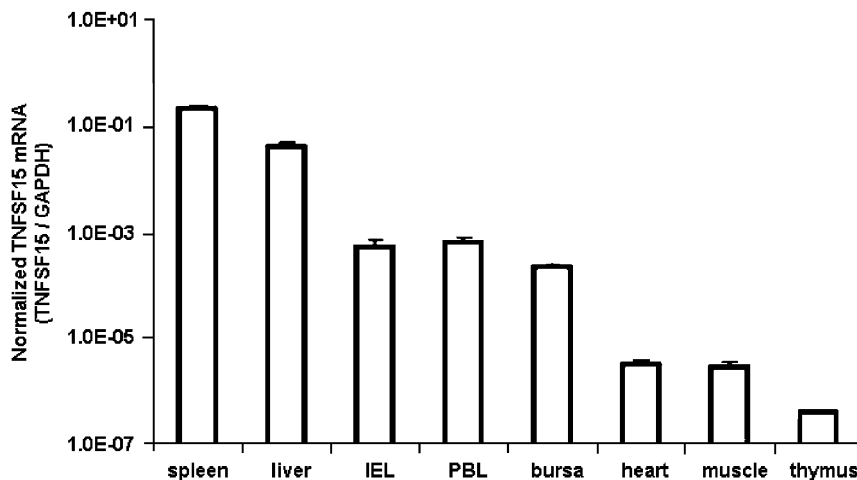

Fig. 2. Tissue distribution of chicken TNFSF15 mRNA. Total RNA was isolated from the indicated organs and cells, TNFSF15 transcripts were analyzed by quantitative RT-PCR, and normalized to GAPDH mRNA. Each bar represents the mean $\pm$ S.D. of 5 birds.

E. acervulina, *E. maxima* and *E. tenella* sporozoites for 4, 18, or 48 h and TNFSF15 transcript levels were measured by quantitative RT-PCR. TNFSF15 mRNA levels were significantly increased at 4 and 18 h post-stimulation with *E. coli* LPS compared with the PBS vehicle control, but returned to basal levels at 48 h (Fig. 3A). Treatment with LPS from *Salmonella* significantly increased levels of TNFSF15 expression at all times examined, although that levels at 48 h were less than those levels at 4 or 18 h. The kinetics of TNFSF15 mRNA levels following treatment of HTC cells with

Eimeria parasites were similar to those observed after LPS, i.e. significantly increased levels at 4 and/ or 18 h with return to basal levels by 48 h (Fig. 3B).

3.4. Kinetics of TNFSF15 mRNA expression during Eimeria infection in vivo

TNFSF15 transcripts were quantified in intestinal IEL from uninfected chickens (day 0), and from 1 to 9 days post-primary infection, and 1–7 days post-secondary infection (DSI) with *E. maxima*, *E. acervulina*, and *E. tenella*. Interestingly, as depicted
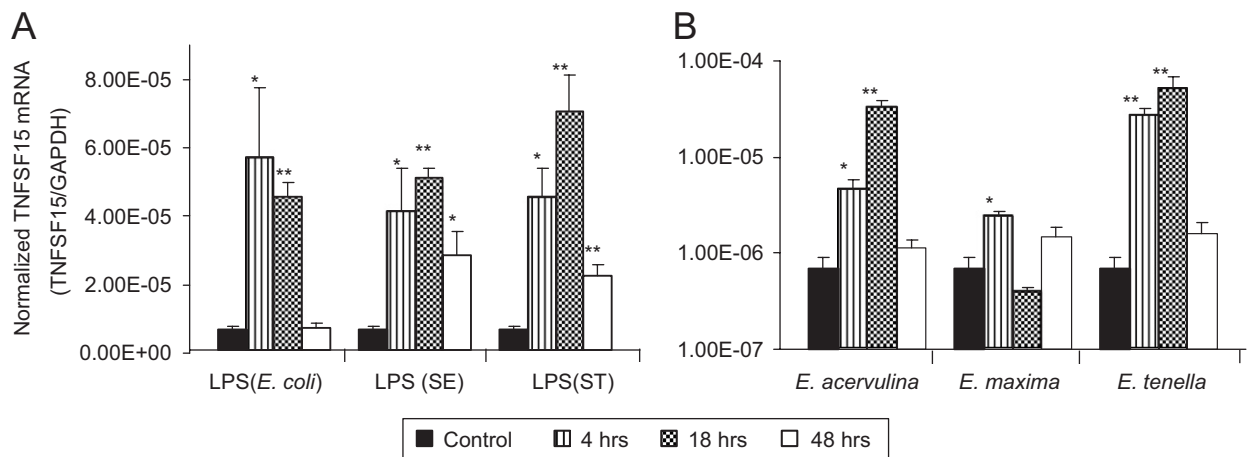

Fig. 3. Induction of chicken TNFSF15 by LPS and *Eimeria* parasites in HTC macrophages. Cells were non-treated or treated with LPS from *E. coli*, *S. enteritidis* (SE) or *S. typhimurium* (ST) (A) or *E. acervulina, E. maxima* and *E. tenella* sporozoites (B) for the indicated times. TNFSF15 mRNA levels were determined by quantitative RT-PCR and normalized to GAPDH mRNA. Each bar represents the mean ± S.D. of triplicate samples. Asterisks indicate significantly increased TNFSF15 mRNA levels compared with non-treated control groups (*$p < 0.05$; **$p < 0.01$).
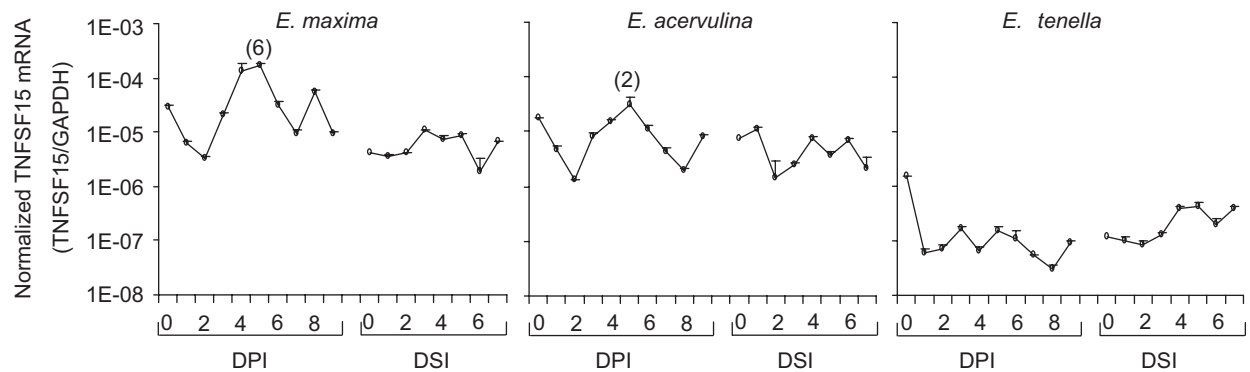

Fig. 4. Kinetics of chicken TNFSF15 mRNA expression following *Eimeria* infection. Chickens were uninfected (day 0) or orally infected with 1×10^4 oocysts of *E. maxima*, *E. acervulina*, or *E. tenella* on day 0 and reinfected with 2×10^4 oocysts of same species of *Eimeria* on day 14 post-primary infection. Intestinal IEL were isolated from the duodenum, jejunum, or caeca, respectively, at the indicated times post-infection, TNFSF15 transcripts were measured by quantitative RT-PCR, and normalized to GAPDH mRNA. Each data point represents the mean ± S.D. of triplicate samples. Ratio between TNFSF15 gene expressions at 5DPI and uninfected controls are indicated in parentheses. DPI, days post-primary infection; DSI, days post-secondary infection.

in Fig. 4, transcript levels of TNFSF15 were consistently down-regulated at 1–2 DPI following all 3 infections. With *E. maxima* infection, however, the levels of TNFSF15 were increased 5- and 6-fold at 4DPI and 5DPI, respectively, but TNFSF15 level returned to pre-infection level afterwards. Following *E. acervulina* infection, TNFSF15 levels exhibited increase up to 2-fold compared to that of pre-infection level. Following *E. tenella* infection, however, TNFSF15 levels consistently remained low. Interestingly, following secondary *Eimeria* infections, the levels of TNFSF15 transcript were consistently lower than those of uninfected controls (0 DPI). Taken together, these results show that the expression of TNFSF15 transcripts was increased with *E. maxima* following primary but not secondary infection.

3.5. TNFSF15 expression in IEL T cell subpopulations

To identify the T cell subpopulation that was responsible for TNFSF15 expression, we collected duodenum and jejunum IEL at 5 DPI following *E. acervulina* or *E. maxima* infections, respectively as described in the above experiment. IEL samples were collected at 5DPI and separated into four subsets, $CD4^+$, $CD8^+$, $TCR1^+$, and $TCR2^+$ using magnetic cell separation system using subpopulation-specific monoclonal antibodies as described in the Section 2. Interestingly, following *E. acervulina* infection, TNFSF15 transcripts were primarily expressed in the $CD4^+$ IEL subpopulation in the duodenum, whereas its highest transcript levels were found in the $TCR2^+$ IEL cells in the jejunum following *E. maxima* infection when the same number of cells (2.0×10^7 cells/ml) were used for TNFSF15 mRNA (Fig. 5).

3.6. Cytotoxic activity of recombinant TNFSF15 protein

Human TNFSF15 possesses cytotoxic activity against tumor cells [13]. Therefore, we explored the possibility that chicken TNFSF15 has the ability to inhibit the growth of chicken tumor cells. Initially, the TNSF15 gene was expressed in *E. coli* as a 47 kDa polyhistidine-tagged recombinant protein consisting of the 26 kDa TNFSF15 plus 21 kDa tags (Fig. 6). Purified TNFSF15 protein inhibited the growth of HTC macrophage and LSCC-RP9 B cell tumors in a dose-dependent manner (Fig. 7). The cytotoxic activity of TNFSF15 was similar to that observed using LITAF, a TNFSF member with known anti-tumor effects [11]. The anti-tumor property of TNFSF15 was also evident by microscopic visualization of cell membrane blebbing and cell shrinking. In contrast, recombinant chicken TNFSF15 did not exhibit a detectable cytotoxic effect against CHCC OU-2 cells at levels as high as 50 μg/ml (data not shown).
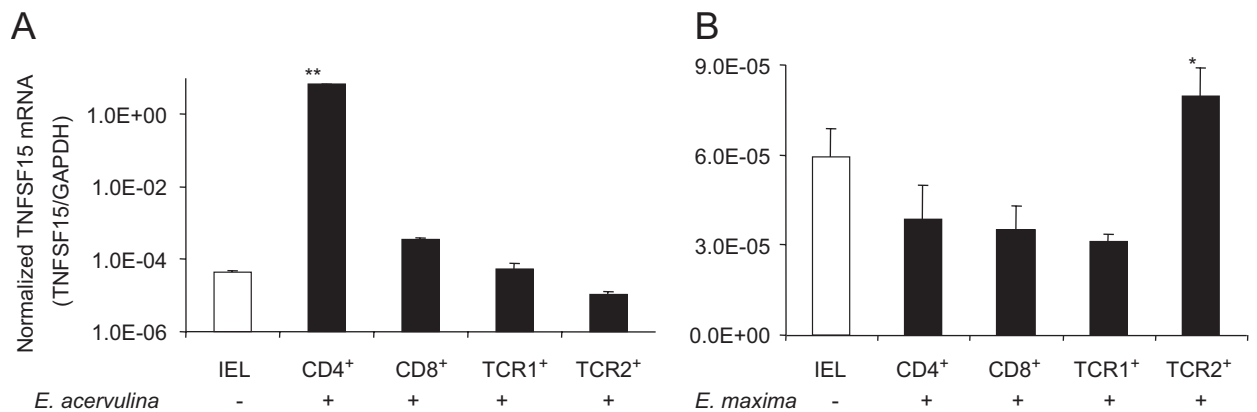

Fig. 5. Chicken TNFSF15 mRNA expression in intestinal IEL. Chickens were non-infected or orally infected with 1×10^4 oocysts of *E. acervulina* or *E. maxima*, IEL were isolated from the duodenum (A) or jejunum (B) on day 4 post-infection, and separated into $CD4^+$, $CD8^+$, $TCR1^+$ and $TCR2^+$ subpopulations. TNFSF15 transcripts were analyzed by quantitative RT-PCR and normalized to GAPDH mRNA. Each bar represents the mean±S.D. of 5 birds. Asterisks indicate significantly increased expression of TNFSF15 mRNA compared with uninfected control groups (*$p < 0.05$; **$p < 0.01$). TNFSF15 regression equation: $C_t = -3.24x + 36.2$, $r^2 = 0.97$; GAPDH regression equation: $C_t = -3.42x + 32.2$, $r^2 = 0.99$.
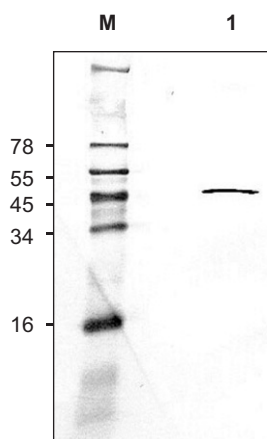
Fig. 6. Western blot analysis of recombinant chicken TNFSF15 protein. TNFSF15 was purified from *E. coli* lysates on a Ni^{2+}–NTA His-bind resin column, and analyzed by Western blotting using anti-polyhistidine antibody. Lane 1, polyhistidine-tagged TNFSF15. M, prestained protein markers with sizes indicated on the left in kDa.

4. Discussion

This report demonstrates the biological and functional characterizations of chicken TNFSF15. Chicken TNFSF15 exhibited a high level of amino acid sequence identity with mammalian TNFSF15, including a well-conserved TNF ligand superfamily signature sequence. TNFSF15 gene expression was observed in diverse lymphoid organs, and was induced in macrophages treated with LPS or *Eimeria* parasites *in vitro* as well as in the intestinal IEL from *Eimeria*-infected chickens. Finally, TNFSF15 protein was cytotoxic for avian tumor cells, but not for non-cancer fibroblasts. These results corroborate previous reports describing TNF-like or TNF-α activities in chickens [3,4,6,11] as well as the ability of infectious microorganisms and their exoproducts to stimulate its production [27,28].

Mammalian TNFSF15 expression is largely limited to endothelial cells of diverse tissues and organs [13,26]. For example, Zhai et al. [26] reported that human TNFSF15 was predominantly expressed in well-vascularized organs such as placenta, lung, kidney, pancreas, spleen, prostate, small intestine and colon. In our study, TNFSF15 was constitutively expressed in the spleen, liver, intestinal IEL, bursa, and PBL whereas its levels in the heart and thymus were relatively low. When expression levels of TNFSF15 were investigated following infections with three major *Eimeria*

parasites, TNFSF15 gene expression was down-regulated at the beginning of infections (up to 2DPI) but increased by 2- to 6-fold following primary infections with *E. acervulina* and *E. maxima* but not with *E. tenella*. In all three *Eimeria* infections, the level of TNFSF15 mRNA returned to the pre-infection level. In contrast, chicken LITAF transcripts which were previously shown to regulate the expression of TNFSF15 increased 20–700-fold following infections with *E. acervulina* and *E. maxima* [11]. These results suggest that both LITAF and TNFSF15 play roles in innate immune response to *Eimeria* infections.

Eimeria sporozoites preferentially invade specific regions of the gut with *E. acervulina* mainly localizing to the duodenum, *E. maxima* to the jejunum, and *E. tenella* to the caeca. Intestinal IEL are the major effector cells that mediate host immunity to coccidia infection [16,17,23]. Interestingly, we found that chicken TNFSF15 was primarily expressed in certain intestinal IEL subsets depending on the infecting *Eimeria* species, specifically in the duodenum $CD4^+$ subpopulation of *E. acervulina*-infected birds, and in the jejunum $TCR2^+$ subpopulation of *E. maxima*-infected chickens. In inflammatory bowel disease (IBD) patients, TNFSF15 was produced by $CD4^+$ T cell subsets in the intestinal lamina propria with increasing TNFSF15-positive cells associated with the severity of inflammation [30,31]. This finding suggests that its expression may be related to the production of Th1 polarizing cytokines. A similar function of TNFSF15 may be operative in local host response to *E. acervulina* infection in chickens.

Because human TNFSF15 primarily inhibited the proliferation of 2 types of endothelial cells (umbilical and venus cells) out of 5 different kinds of endothelial cells [13,26], the differences in its expression levels between chicken and human may be related to the endothelial cell types of each organ. The remarkable reduction of vascularization of tumors by mouse TNFSF15 suggested that it might be useful as an angiogenesis-based anti-cancer therapeutic [26]. Indeed, Zilberberg et al. [29] reported a 17 amino acid cyclopeptidic VEGI that is capable of inhibiting not only the binding of TNFSF15 to endothelial cells, but also VEGI-induced endothelial cell migration.

Apoptosis is an important component of the host immune response against microbial infection and the apoptotic pathways constitute critical processes during cellular homeostasis and disease
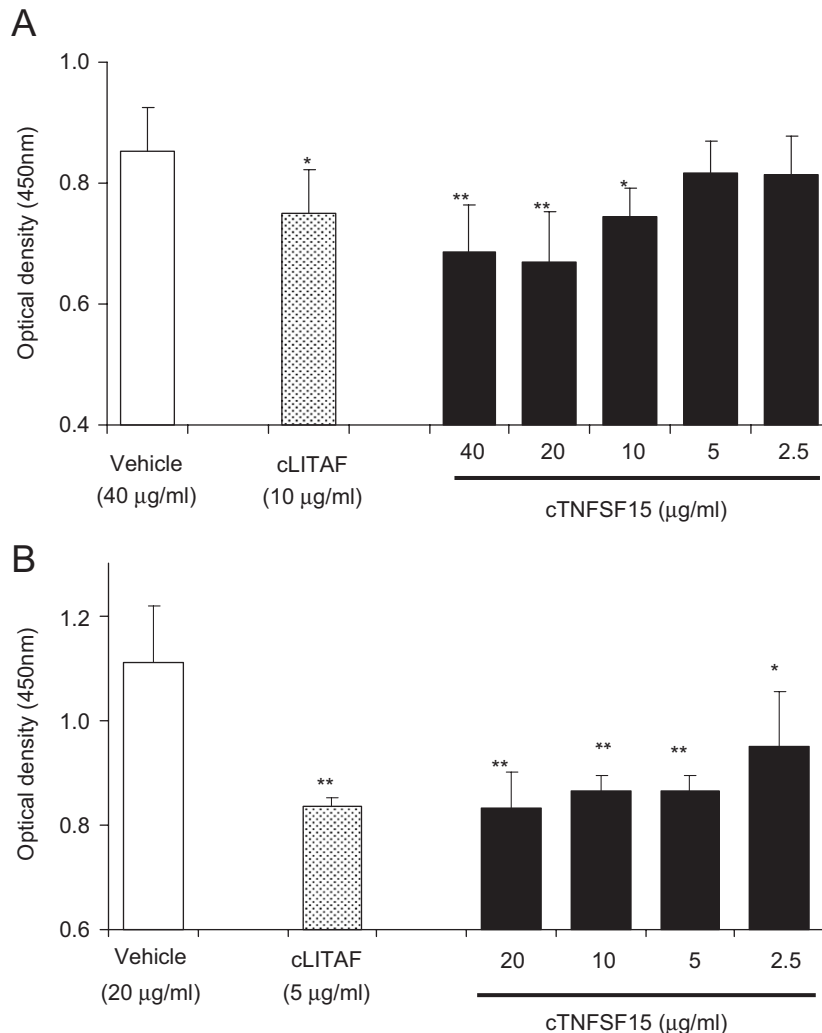
Fig. 7. Dose–response cytotoxic activity of recombinant TNFSF15 protein. (A) HTC macrophages and (B) LCSS-RP9 retrovirus-transformed B cells were treated for 48 h with serial dilutions of purified recombinant TNFSF15, LITAF, or vehicle control (empty vector). Cell viability was determined using WST-8 as described in the Section 2. Each bar represents the mean $\pm$ S.D. of triplicate samples. Asterisks indicate significantly decreased cell viability compared with the vehicle control (*$p < 0.05$; **$p < 0.01$).

states within the intestinal epithelium [32]. Mammalian TNFSF15s have been shown to exert inhibition of endothelial cell proliferation, T cell costimulation, and angiogenesis [13,14,26]. Recently, Takimoto et al. [10] showed a cytotoxic effect of chicken TNFSF15 against the murine fibroblast cell line L929 and non-tumorized primary chicken fibroblasts. However, their study did not investigate cytotoxicity of chicken TNFSF15 against chicken tumor cells. In a previous report [11], we showed that LITAF upregulated TNFSF15 gene expression in HD11 macrophages. Our current results support an apoptotic or necrotic role for TNFSF15 as previously suggested for the homo-

logous mammalian TNFSF15 proteins [33]. Kim et al. [34] demonstrated that TNFSF15 expression was induced in human umbilical vein endothelial cells by IL-1α, IL-1β or TNF-α, suggesting its crucial role in balancing between apoptotic and anti-apoptotic pathways. Based on this suggestion, we can speculate that chicken TNFSF15 might function to protect against intestinal spread of *Eimeria* infection, either alone or synergistically with LITAF and/or other inflammatory factors by apoptosis of infected cells.

In acute and chronic inflammatory responses, there are increases in circulating monokines (IL-1 and IL-6) and altering hormonal milieu to cause an

altered nutrient requirements, and reduced growth rate [35]. These array of metabolic changes include reduced skeletal muscle accretion, increased energy utilization and redistribution of cartilage and bone. Many of these effects are due to enhanced secretion of metalloproteinases, impaired chondrocyte proliferation, and increased use of dietary nutrients as energy sources in lieu of body growth [35]. During immune responses to infections, wasting of lean tissues and loss of nitrogen by increased protein breakdown in skeletal muscle resulted in body weight loss or growth retardation [36]. Infusion of TNF-α enhance muscle proteolysis synergistically with IL-1 by promotion of muscle catabolism in rats [36]. Furthermore, human TNF-α inhibited myogenesis by reversible decrease of muscle specific α cardiac actin gene expression and lipid mobilization in adipocyte cell cultures [37]. In addition, exogenous TNF-α administration stimulated hepatic fatty acid and cholesterol synthesis resulting in hyperlipidemia in rats [38], anorexic body weight loss in mice [36]. When birds are infected with *Eimeria*, they exhibit reduced feed intake resulting in immediate onset of body weight loss [3,35]. In our previous study, the highest level of chicken TNLF was detected at 4 DPI, and body weight loss occurred in 6 days post-infection by administration of chicken TNLF produced in chicken monocyte cultures by stimulation of *E. tenella* [3]. Interestingly, the peak expression period of TNFSF15 and LITAF following *Eimeria* infection *in vivo* would primarily coincide with body weight loss [11]. The rapid increases of LITAF and TNFSF15 gene expression seen in the previous and current studies might be associated with enhanced body weight loss seen in *Eimeria*-infected birds, especially in *E. maxima* and *E. acervulina* infections [39]. Human muscle cells are sensitive to human TNF, which reversibly inhibit muscle specific α-cardiac actin gene, resulting in reduced muscle growth [37]. Because chicken muscle represents larger portion of whole body, it is worthwhile to investigate the effects of chicken TNFSF15 and LITAF on chicken muscle cell growth that will influence overall growth rate and body weight gain of chickens during inflammatory responses.

Acknowledgments

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