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Role of Chicken TL1A on Inflammatory Responses and Partial Characterization of Its Receptor

Tetsuya Takimoto,* Kan Sato,† Yukio Akiba,* and Kazuaki Takahashi1*  

The role of chicken TNF-like ligand 1A (ChTL1A) on inflammation and its receptor candidates was investigated to further understand its function as a proinflammatory cytokine. ChTL1A increased the viability of CHO-K1 cells transfected with chicken TNFR2 or decoy receptor 3 and bound to TNFR2 and decoy receptor 3. ChTL1A was detected in chicken blood samples taken 4 h after LPS injection. Increased mRNA for inflammatory response-related factors such as IL-1β, IL-6, ChTL1A, IFN-γ, inducible NO synthase, and cyclooxygenase 2 were found in spleen samples following LPS injection. Ceruloplasmin and α1 acid glycoprotein (as positive acute phase proteins) were increased in chicken plasma 12 h after ChTL1A injection. The injection of anti-ChTL1A Ab was able to prevent typical increases in plasma nitrite plus nitrate, ceruloplasmin, and α1 acid glycoprotein concentrations following LPS injection. These results indicate that ChTL1A is a proinflammatory cytokine in chickens, animals that do not have TNF-α and lymphotixin α orthologous genes, and that its proinflammatory action is, at least in part, expressed through binding to TNFR2.  


Abbreviations used in this paper: iNOS, inducible NO synthase; AG, acid glycoprotein; Cer, ceruloplasmin; ChTL1A, chicken tumor necrosis factor-like ligand 1A; COX-2, cyclooxygenase 2; Dr, decoy receptor; Dr, death receptor; TACE, TNF-α-converting enzyme; ORF, open reading frame.

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Overexpression of chicken TNFRs in CHO-K1 cells and cytotoxic assay of recombinant ChTL1A

The full-length ORF of each chicken TNF receptor was amplified by PCR using the following primers: chicken TNF-R2 forward primer 5'-CTC GAG ATG GGG CCG CGC TGG GTG CTG C-3' and reverse primer 5'-GAA TTC AAC AGT TTT CAT CCC CAT ATC T-3', and reverse primer 5'-GAA TTC AAC AGT TTT CAT CCC CAT ATC T-3'. The primers were synthesized by Qiagen.

CHO-K1 cells (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Aobaku, Japan) were propagated in RPMI 1640 (Invitrogen) containing 10% FBS, 100 U/ml penicillin, and 100 µl/ml streptomycin at 37°C in 5% CO2. Cells cultured to subconfluence were washed with PBS. Liposomes of 10 µl/ml were added. To immunoprecipitate ChTL1A, anti-ChTL1A IgG was added and the solution was mixed gently at 4°C for 4 h. Following that, a bed volume of plasmid DNA and 4 µl/ml Lipofectamine 2000 (Invitrogen) were added to the cells, which were then incubated for 24 h at 37°C in 5% CO2. The cells were selected by G418 (0.4 mg/ml) and TNF receptor expression was checked by Western blot using HRP-labeled anti-V5 (Invitrogen). Unmanipulated pIND/V5-His vector was used as a negative control.

To measure the biological activities of ChTL1A on TNFRs, the standard TNF-α bioassay was performed (10). Cells overexpressing each type of receptor were seeded in triplicate into flat-bottom 96-well microplates (2 µersham Biosciences) at 4°C overnight. After centrifugation at 500 g for 5 min, the supernatant was removed and the Sepharose beads were washed three times with 10-bed volumes of PBS. One hundred fifty microliters of lystate containing TNFR2/FLAG or DcR3/FLAG was added to the Sepharose. The suspension was incubated with gentle agitation at 4°C for 4 h. After centrifugation at 500 x g for 5 min, the supernatant was removed and the Sepharose was washed as above. Bound protein was eluted with 200 µl of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) at 4°C for 15 min. After centrifugation at 500 x g for 5 min, the supernatant was collected. Elution and centrifugation were repeated twice more and the three eluates were pooled. The sample was desalinated by using a PD-10 column (Amersham Biosciences) and lyophilized.

The lyophilized samples were resuspended with 20 µl of PBS and added to 20 µl of 2 X electrophoresis sample buffer (200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol in 100 mM Tris-HCl, pH 6.8). Samples were boiled for 5 min before being applied to SDS-PAGE gel electrophoresis (12% acrylamide) using the MiniPROTEAN 3 Electrophoresis System (Bio-Rad). Each lane was loaded with either 25 µl of sample or 5 µl of crude lystate. After electrophoresis, proteins were transferred to Hybond-C (Amersham Biosciences) by wet blotting using the Mini Trans-Blot Cell (Bio-Rad). The membrane was incubated for 1 h in blocking solution (7% skim milk; Difco) in PBS-T (PBS and 0.05% Tween 20), then overnight at 4°C with the mouse anti-FLAG M2 IgG (1/5000 dilution in PBS-T; Sigma-Aldrich), washed three times with PBS-T; then incubated for 1 h at room temperature with anti-mouse IgG conjugated to HRP (1/5000 dilution in PBS-T). Immunoblots were visualized on Hyperfilm (Amersham Biosciences) with ECL Western Blotting Substrate (Pierce). The lystate containing TNFR2/FLAG or DcR3/FLAG was used as a positive control.

Time course of appearance of ChTL1A in blood following LPS stimulation

Chickens were injected with LPS (1.5 mg/kg body weight) and venous blood was sampled 0-24 h later. Blood was centrifuged (1500 x g, 10 min) to prepare plasma and this was stored at −20°C. Five hundred microliters of plasma was diluted 1/3 by PBS and 30 µl of anti-ChTL1A serum was added. To immunoprecipitate ChTL1A, anti-ChTL1A IgG was added and the solution was mixed gently at 4°C for 4 h. Following that, a bed volume of 50 µl of protein A-Sepharose CL-4B (Amersham Biosciences) was added and mixing was continued overnight. After the wash, the bound protein was eluted by 40 µl of 1 X electrophoresis sample buffer. Electrophoresis, transfer to membrane, and blocking were conducted as described above. The membrane was incubated as above with rabbit anti-ChTL1A Ab (1/200 dilution in PBS-T, washed, and incubated as above with anti-rabbit IgG conjugated to HRP (1/5000 dilution in PBS-T) and developed with ECL.

Real-time quantitative RT-PCR

To study expression of factors related to inflammation (IL-1β, IL-6, ChTL1A, IFN-γ, iNOS, and COX-2), four chickens were administered recombinant ChTL1A protein (200 µg/kg body weight). Total RNA was prepared by TRIzol reagent (Invitrogen) from liver, spleen, and abdominal adipose tissue. First-strand cDNA was synthesized as previously described (7). Real-time quantitative RT-PCR analysis was performed using the Cycler iQ Real Time Detection System (Bio-Rad). Each cDNA preparation served as a template in a 20 µl PCR containing 2 nM MgCl2, 0.5 mM of each primer, and 0.5 X SYBR Green master mix (BioWhittaker). Cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for

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**Table I. Primer set for quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Set</th>
<th>PCR Product Size (bp)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>5'-AGT GGG CCG TTC GGG CTG 3'</td>
<td>795</td>
<td>Y15006</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-AGT CCA GGA GAT TGC 3'</td>
<td>238</td>
<td>AJ305450</td>
</tr>
<tr>
<td>iNOS</td>
<td>5'-AGT CCA GGA GAT TGC 3'</td>
<td>288</td>
<td>X99774</td>
</tr>
<tr>
<td>COX-2</td>
<td>5'-AGT CCA GGA GAT TGC 3'</td>
<td>292</td>
<td>AB194710</td>
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<td>225</td>
<td>M64990</td>
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<td>GAPDH</td>
<td>5'-AGT CCA GGA GAT TGC 3'</td>
<td>543</td>
<td>AF047874</td>
</tr>
</tbody>
</table>
30 s, 60–65°C for 1 min, and 72°C for 1 min. SYBR Green fluorescence was detected at the end of each cycle. At the end of each run, melting curve profiles were also recorded. Sense and antisense primers are shown in Table I.

The specificity of amplification was further verified by electrophoresis of product on a 0.8% agarose gel, to check size, followed by DNA sequencing. Results are expressed as the ratio of each inflammatory factor to GAPDH.

Changes in plasma ceruloplasmin (Cer) and α1 acid glycoprotein (α1 AG) concentration following injection of recombinant ChTL1A

To investigate inflammatory activity of recombinant ChTL1A, changes in Cer and α1 AG were determined following injection of ChTL1A in male 3-wk-old broiler chickens, weighing ~1 kg. Recombinant ChTL1A was dissolved in sterilized PBS. ChTL1A (0.5 ml, 200 μg/kg body weight) and GST (200 μg/kg body weight) were injected i.v. into four birds each. Venous blood was sampled and plasma prepared as described above. Cer concentration was measured by the p-phenylenediamine colorimetric method (11). Plasma (0.1 ml) was diluted in 2 ml of 0.1 M acetic acid buffer (pH 6.4) and incubated at 37°C for 5 min. p-Phenylenediamine (27 mM) was added to the mixture and incubated at 37°C for 30 min. The reaction was stopped by addition of 50 μl of 1.5 M sodium azide and A530 was measured using a 0-min incubation as a blank. Concentration was calculated as: Cer concentration (mg/L) = 752×A530.

Plasma α1 AG concentration was measured by using an ECOS check chicken α1 AG quantitative assay kit (Laboratory of Metabolic Eco System) based on the method of single radial immunodiffusion.

FIGURE 1. Changes in viability of CHO-K1 cells that overexpress full-length chicken TNFRs (TNFR2 or DcR3) following addition of ChTL1A. pIND vector was used as negative control. The effect was quantified by measuring absorbance (OD = main wavelength: 455 nm; reference wavelength: 655 nm) using a cell counting kit 8. The cell viability was calculated at each ChTL1A concentration/ChTL1A-free viability. **, p < 0.01 compared with values of the pIND vector transfection.

FIGURE 2. ChTL1A interacts with chicken TNFR2 (A) and chicken DcR3 (B). ChTL1A/GST or GST was purified by glutathione-Sepharose, and binding to FLAG/TNF receptors was studied by pull-down assay and Western blotting. The lysate containing TNFR2/FLAG or DcR3/FLAG was used as positive control.

Effect of anti-ChTL1A antiserum on LPS-injected chickens

Fifteen male broiler chicks (Ross), 13 days old, were randomly assigned to one of three experimental groups. Chicks were housed in a temperature-controlled room (25°C) and fed ad libitum on a commercial diet. In each group, the mean bird weight was 480 g.

Five chicks were injected i.v. with protein A-purified rabbit anti-ChTL1A IgG and five with polyclonal rabbit IgG (0.5 mg/kg body weight). After 30 min, all 10 birds were injected i.p. with LPS (1.5 mg/kg body weight) and, after another hour, were injected i.v. with the appropriate IgG as above. Another five chicks received only the LPS injection.

Feed intake was recorded every 2 h until 16 h after treatment. Also, to determine nitrite plus nitrate, Cer and α1 AG, blood was taken from a wing vein before treatment and then after 8 and 24 h.

Statistical analysis

We used Student’s t test to compare means. The threshold of significance was 0.05.

FIGURE 3. Changes in plasma ChTL1A protein following i.p. injection of LPS (1.5 mg/kg body weight). The protein immunoprecipitated by rabbit anti-ChTL1A Ab was analyzed by Western blot. ChTL1A was detected with rabbit anti-ChTL1A serum used as first Ab and HRP-labeled anti-rabbit Ab and visualized by ECL.

FIGURE 4. mRNA expression of IL-1β, IL-6, ChTL1A, IFN-γ, iNOS, and COX-2 in various tissues following i.v. injection of ChTL1A. ChTL1A (0.5 ml; 200 μg/kg body weight, □) or GST (200 μg/kg body weight, □) was i.v. injected and each tissue was taken after 2 h. GST was used as a negative control. Each bar represents the mean and SE from four chickens. Arbitrary units (AU) are the ratio of each mRNA expression to the GAPDH mRNA expression. Expression of mRNA was measured by real-time PCR. **, p < 0.01 and *, p < 0.05 compared with control.
proinflammatory effect of ChTL1A recombinant protein in vivo

Fig. 4 shows the effect of injecting ChTL1A (200 μg/kg body weight) on proinflammatory factor mRNA levels. Expression of IL-1β and IL-6, two well-known proinflammatory cytokines, was significantly increased compared with the GST-negative control (IL-1β: 172-fold increase in spleen, 7-fold in adipose tissue; IL-6: undetected came to be detectable in liver, 59-fold increase in spleen, undetected came to be detectable in adipose tissue). Moreover, TNF-α and TL1A also induced IFN-γ expression, a cytokine involved in the differentiation of helper T cells in humans (9, 12). ChTL1A increased IFN-γ mRNA expression 12-fold in chicken spleens, similar to that reported in humans following TNF-α administration (Fig. 4). The highest level of increased expression of these cytokines was observed in chicken spleens (Fig. 4). The expression of iNOS (predominantly expressed by activated macrophages and catalyzes NO production) was also significantly increased by ChTL1A injection 7-fold in liver and 9-fold in spleen (Fig. 4). ChTL1A injection raised splenic COX-2, which is rate limiting in the production of PGE2, one of the acute phase reactants, mRNA levels 5-fold (Fig. 4). In addition, proof of inflammation was observed not only through altered mRNA expression, but also via plasma parameters. Cer and α1 AG concentrations were increased (Fig. 5, A and B); these changes being similar to those seen after LPS injection (13–15). ChTL1A fusion protein and GST preparations did not contain detectable levels of LPS (<100 ng/mg protein) using an Endospecy ES-50M kit (Seikagaku). Since the recombinant protein was injected at 200 μg/kg body weight, LPS contamination was presumably <20 ng/kg body weight. There were no significant differences in plasma inflammatory parameters between an i.v. LPS-injected control group (50 ng/kg body weight) and a PBS-injected group (data not shown). This implies that ChTL1A is a proinflammatory cytokine in chickens.

Effect of anti-ChTL1A antiserum on LPS-injected chickens

The injection of anti-ChTL1A IgG (total 1.0 mg/kg body weight) blocked the LPS-induced changes in plasma Cer at 24 h, α1 AG at 24 h, and nitrate plus nitrate at 8 h. The Cer, α1 AG, and nitrate plus nitrate plasma concentrations in chickens injected with rabbit IgG (control) did not differ from those of chickens injected with LPS (Fig. 6).

Discussion

Binding with receptor and signal transduction

In mammals, TL1A is reported to bind to DcR3 and DR3 (16) and its signals are transmitted through DR3, but not DcR3 (9). It is well known that TNF-α binds to two receptors, TNFR1 and TNFR2, and can result in apoptosis (1, 17). In birds, TNFR2 and DcR3 cDNA have been reported (18, 19), but the TNFR1 and DR3 homologs are yet to be identified. We therefore investigated whether ChTL1A binds to chicken TNFRs, TNFR2 or DcR3.

In this study, ChTL1A could bind to both chicken TNFR2 and chicken DcR3 (Fig. 2). A single band was detected by anti-FLAG Ab although there was any weakly signal at other molecular mass analogs are yet to be identified. We therefore investigated whether ChTL1A binds to chicken TNFRs, TNFR2 or DcR3.

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specific binding of ChTL1A to these receptors. In addition, recombinant ChTL1A has marked cytotoxic activity against cells over-expressing chicken TNFRs TNFR2 and DcR3. Thus, we have demonstrated for the first time that in birds, the toxic activity of ChTL1A involves chicken TNFR2 and chicken DcR3. This suggests that the mechanisms that induce cell death in chickens and mammals are different. In mammals, DcR3 is secreted into the blood, binds with the ligand for DR3 and, consequently, inhibits DR3 signal transduction (20). In contrast, our results demonstrated that the CHO-K1 cells transfected with chicken DcR3 still had high cytoxic sensitivity induced by ChTL1A in a dose-dependent manner, whereas cells transfected with control pIND only showed very low cytoxic sensitivity (Fig. 1). This suggests that DcR3 might induce cell death in chickens, but further investigation is needed. A predicted sequence for TNFR1 can be found in the chicken genome database (21); however, the connection between ChTL1A and these receptors remains to be elucidated.

These receptors were expressed in different tissues in chicken. TNFR2 was reported to be mainly expressed in spleen and bursa of Fabricius, but little expressed in liver (18). In contrast, DcR3 was reported to be mainly expressed in oviduct but little expressed in spleen (19). Our study showed that the response to ChTL1A in the spleen was higher than in other tissues (Fig. 4). Therefore in spleen, ChTL1A might interact with TNFR2 rather than DcR3 and promote inflammation. In vivo injection of chicken TNFR2 and/or DcR3 Abs may elucidate the proinflammatory mechanism.

ChTL1A protein secretion into plasma during inflammation

We previously reported that in chicken spleens, ChTL1A mRNA increased following LPS injection, with peak expression 2 h after injection (7). In the present study, proteins of ~17 kDa were detected in plasma 4 h after LPS injection (Fig. 3). In mammals, TNF-α binds to membranes as a 26-kDa precursor protein, where it is cleaved sequentially by TNF-α-converting enzyme (TACE), releasing into the blood the 17-kDa form (22). Human TL1A is also a secretion protein that has a similar transmembrane domain to TNF-α (9). Our previous study predicted that ChTL1A might have transmembrane domain and TACE cleavage site (7). It is likely, therefore, that ChTL1A is cleaved by TACE in a similar manner to TNF-α in mammals because TACE has been identified in the chicken genome (21). Thus, in chickens, ChTL1A can be secreted into plasma, suggesting it may both play a local autocrine and/or paracrine role and also be circulated. In humans, plasma TNF-α concentrations peak 2–4 h after LPS injection (23). Thus, our findings in chickens are similar to those in mammals. In addition, a 17-kDa protein was detected using anti-human TNF-α Ab in chicken macrophage culture medium (6). Taken together, the current results suggest that ChTL1A is a secreted molecule with TNF-α-like activity.

The importance of ChTL1A in inflammation

In the first stage of infection, an inflammatory factor is expressed that subsequently influences both the immune and metabolic responses. In mammals, TNF-α promotes IL-1β and IL-6 production, i.e., it is a significant inflammatory cytokine (3, 24). This similarity to ChTL1A was confirmed when increased expression of IL-1β and IL-6 was observed following ChTL1A injection (Fig. 4). ChTL1A injection also increased the expression of IFN-γ and ChTL1A itself. ChTL1A, therefore, induces inflammation through the same cytokines.

Because ChTL1A has similar proinflammatory actions to mammalian TNF-α (Fig. 5), we wondered whether it might also activate NF-κB signaling through TNFR2. Unfortunately, we were unable to test this because the function of chicken TNFR2 has not been completed to date. ChTL1A injection also increased the expression of iNOS and COX-2. The effect of ChTL1A to increase plasma NO concentration confirmed the results of our previous study (7) and could be due to an increase in iNOS expression by the liver and spleen.

In mammals, TNF-α is an important proinflammatory cytokine that regulates a host’s response to infection as well as inflammation and trauma. Acute phase protein production is induced mainly by proinflammatory cytokines such as IL-1, IL-6, and/or TNF-α, while NO production is induced by IL-1, TNF-α, and/or IFN-γ. Thus, proinflammatory cytokines are also important regulators of metabolic responses in the acute phase of inflammation. In chickens, α1 AG, Cer, and NO are acute phase substances released following LPS injection (13, 14, 25). Our results demonstrate that ChTL1A promotes inflammation by raising these. For TNF-α, this effect results from IL-6 induction (26). In our study, the peak of acute phase protein occurs after the peak of NO production; hence ChTL1A may indirectly induce acute phase reactants through IL-6 induction rather than directly as mammalian TNF-α functions. Therefore, we suggest that, in chickens, the mode of action of ChTL1A is similar to that of TNF-α in mammals in acute phase protein induction.

Finally, we observed that anti-ChTL1A IgG down-regulated inflammation. We previously ascertained, that following LPS injection under similar conditions, plasma nitrate plus nitrite become maximal after 9–14 h, and plasma Cer and α1 AG become maximal after 24 h (13–15). We therefore measured the concentration of Cer at 24 h, α1 AG, at 24 h and nitrate plus nitrate at 8 h. This suggests that ChTL1A is important in the inflammatory response. ChTL1A plays a significant role in the initiation of inflammation, because its Ab suppressed NO production and suppressed acute phase protein production. The results show that ChTL1A is an important regulating factor for both immune and inflammation responses in chickens and may do the same job as mammalian TNF-α functions in chickens, animals that lack genes orthologous to TNF-α and lymphotoxin-α.

In conclusion, we demonstrated that ChTL1A binds to TNFR2, the receptor for TNF-α in mammals, and its signals are transmitted via TNFR2. ChTL1A proteins were detected in plasma 4 h after LPS injection, suggesting that ChTL1A is secreted into the blood, like mammalian TNF-α, and subsequently influences the immune responses of other tissues. ChTL1A plays an important role as a proinflammatory cytokine, as recombinant ChTL1A increased the expression of several inflammation-related factors such as iNOS and COX-2 in chicken spleen. In plasma, it increased the concentrations of acute phase proteins. Anti-ChTL1A IgG abolished the increase in the acute phase reactants following LPS injection.

These results indicate that ChTL1A is a key proinflammatory cytokine in chickens, animals that do not have TNF-α and lymphotoxin-α orthologous genes, and that the proinflammatory actions of ChTL1A occur, at least in part, through binding to TNFR2.

Disclosures

The authors have no financial conflict of interest.

References


