**The identification, characterization, and function of two TREMs genes in
Chinese Yangzhou goose (*Anas cygnoides*)**

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ABSTRACT

Triggering receptor expressed on myeloid cells (TREM) is a cell-surface receptor primarily expressed on macrophages. Here, two novel TREM genes, *AcTREM1* and *AcTREM2*, were identified from *Anas cygnoides*. *AcTREM1* cDNA contained a putative signal peptide, two IG domains, and a transmembrane domain. The deduced *AcTREM2* sequence also contained a signal peptide, an IG domain, and a transmembrane domain. qRT-PCR, fluorescence in situ hybridization, and immunofluorescence experiments showed that *AcTREM1* and *AcTREM2* were mainly expressed in the liver and spleen, and both genes and proteins were mainly distributed in cytoplasm. *AcTREM1* expression in the liver and spleen was significantly upregulated following lipopolysaccharide (LPS) challenge at an early stage of infection and then decreased at a later stage. Changes in *AcTREM2* expression were reciprocal to those of *AcTREM1* in the liver and spleen after LPS challenge. Our results indicate that *AcTREM1* and *AcTREM2* participate in the antibacterial immunity of *A. cygnoides*.

Keywords: *Anas cygnoides*; TREM gene; Innate immunity; Pattern recognition receptor

1. Introduction

The Yangzhou goose (*Anas cygnoides*) is a commercially important food source widely cultivated in China; geese have long been supplying meat, fatty liver, and byproducts, such as down, to numerous parts of the world (Shi et al., 2008; Xu et al., 2016). However, with the rapid expansion of the goose industry, water pollution has increased. Bacterial diseases can have serious implications and can lead to a sharp decline in both egg fertility and hatchability (Jiang et al., 2011). Bacterial pollution and loss of performance were found to be associated with the presence of lipopolysaccharide (LPS), a product of Gram-negative bacteria. LPS can initiate transmembrane and intracellular signal transduction through a combination with pattern recognition receptors (PRRs) on the surface of macrophages.

Previous studies have identified many cell-surface receptors, such as members of the Toll-like receptor (TLR) (Gerondakis et al., 2007; Krishnan et al., 2007), Siglec

(Crocker et al., 2007), and C-type lectin families (Robinson et al., 2006); others are intracellular sensing mechanisms such as nucleotide-binding oligomerization domain (Nod)-like receptors (NLR) (Kanneganti et al., 2007). The triggering receptor expressed on the myeloid cell (TREM) family is a novel PRR family and was discovered in 2000 (Bouchon et al., 2000). Members of the TREM family are expressed mainly by the multiple myeloid cell lineage (Ford and Mcvicar, 2009). The genes encoding TREM-1 and -2 are located on human chromosome 6p21. In addition, there are homologous genes called TREM-1 like (TREM-like transcript [TLT]-1) and TREM-2 like transcript (TLT-2), which, similar to TREM-1 and TREM-2, encode a single variable-type immunoglobulin (IG) domain (Sharif and Knapp, 2008). Reverse transcription polymerase chain reaction (RT-PCR) analysis of mammal TREM-1 has shown that there is high expression in the bone marrow, thymus, and spleen (Ramanathan et al., 2004; Ramanathan et al., 2005).

TREM-1 is a receptor that activates polymorphonuclear neutrophils (PMN) and monocytes, and has an important role in pro-inflammatory responses in bacterial disease (Bouchon et al., 2000). TREM-1 associates with the DAP12 (DNAX activation protein 12) signaling adaptor protein via a positive charge in its transmembrane domain (Bouchon et al., 2000; Colonna and Facchetti, 2003). DAP12 is an immunoreceptor tyrosine-based activation motif-bearing a transmembrane adapter molecule, and transmit signals into the cell upon engagement of a group of DAP 12-associating cell surface molecules, including TREM-1 and -2 (Klesney-Tait and Colonna, 2007; Tessarz and Cerwenka, 2008). There appears to be molecular cross-talk between the TREM-1 signaling cascade and the intracellular signaling cascades initiated by TLR2 and -4, and possibly TLR3, -5, -7/8, and -9, as well as some of the NLR receptors (Bleharski et al., 2003; Netea et al., 2006; Radsak et al., 2004). Previous studies have shown that TREM-1 expression is strongly upregulated following exposure to extracellular bacteria or cell-wall components such as LPS or lipoteichoic acid (Bouchon et al., 2000; Colonna and Facchetti, 2003). Activation of monocytes by LPS induces proinflammatory cytokine production (Bleharski et al., 2003; Bouchon et al., 2000; Bouchon et al., 2001; Colonna and Facchetti, 2003).

TREM-2 is another member of the TREM family, which inhibits the release of

inflammatory mediators; therefore, it is an important *in vivo* anti-inflammatory receptor (Chen et al., 2013; Hamerman et al., 2006; Turnbull et al., 2006). TREM-2 has two main functions in macrophages: it acts as a phagocytic receptor and as an immune modulatory receptor. With regard to its function as a phagocytic receptor, previous studies have demonstrated that TREM-2 expression promotes macrophage-mediated bacterial killing (Charles et al., 2008; N'Diaye et al., 2009). After TREM-2 binds a ligand, activation of the TREM-2/dead cell activated receptor-associated protein (DAP12)-mediated signal transduction pathway induces a series of intracellular protein tyrosine phosphorylation reactions and enzymatic reactions, which then activate the myeloid cells and participate in T-cell responses. This is the major *in vivo* pathway that regulates inflammation within the central nervous system (Ford and Mcvicar, 2009; Ito and Hamerman, 2011; Lanier, 2009; Turnbull et al., 2006). The TREM-2 receptor may exhibit its anti-inflammatory effects in lung tissue and moderate the inflammatory response, which is beneficial for the body in the prevention of infectious diseases while preventing damage to tissues and organs.

Previous studies have shown that TREM proteins are highly conserved, as indicated by their presence not only in mice and humans, but also in pigs and cows (Ramanathan et al., 2004; Ramanathan et al., 2005). However, in avian species, TREM proteins are only found in chicken (Kogut et al., 2012; Viertlboeck et al., 2013; Viertlboeck et al., 2006). To further understand the molecular functions of TREM proteins in the innate immune system of *A. cygnoides*, the present study identified two novel TREMs, designated *AcTREM1* and *AcTREM2*, from *A. cygnoides*. Full-length cDNA sequences of *AcTREM1* and *AcTREM2* were cloned and characterized. Expression profiles and distribution in spleen, liver, lung, intestine, and bursin challenged with LPS were analyzed. The present study aimed to identify a TREM signaling pathway in geese, which will contribute to further understanding of the potential role of TREMs in the goose immune response. The results of our study indicate that TREM proteins have important functions in the innate immune response against LPS challenge in *A. cygnoides*.

2. Materials and methods

2.1. Animals

Healthy Yangzhou geese were purchased from Sunlake Swan Farm (119°58'E, 31°48'N), in Henglin Township, Changzhou, Jiangsu Province, China. The birds were fed ad libitum with a mixed feed containing 12.5% crude protein, and the diet was supplemented with green grass. Feed was given during the daytime, with ganders having free access to drinking water. LPS (*Escherichia coli* Serotype 055:B5) was purchased from Sigma (St. Louis, MO, USA). pRSET vector and *E. coli* BL21 (DE3) were produced by the TransGen Biotech Company (Beijing, China). *E. coli* was grown in Luria-Bertani (LB) broth (3 g tryptone, 3 g NaCl, 1.5 g yeast extract, 300 mL double distilled water) at 37°C.

2.2. LPS challenge and sample preparation

During the experiment, 200 geese were randomly divided into four groups of 50 individuals. Geese in the three experimental groups were injected with 0.5, 1.0, and 2.0 mg/kg LPS respectively. For the control group, 50 geese were injected with 50 µL PBS (0.14 M NaCl, 3 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.3). At 3, 6, 12, 24, 36, and 48 h post-injection, eight individuals from each group were sampled. All these samples were stored at -80°C for subsequent RNA extraction.

To investigate *AcTREMI* and *AcTREM2* mRNA tissue distribution, total RNA from eight tissues, including heart, liver, spleen, lung, kidney, chest muscle, intestine, and bursin of unchallenged geese was extracted from eight animals as parallel samples. All samples were stored for RNA preparation as previously described.

2.3. Total RNA isolation and cDNA synthesis

Total RNA from different tissues was extracted using TRIzol reagent following the manufacturer's instructions. RNA quality was assessed by electrophoresis on a 1.2% agarose gel, and the total RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer (NanoDrop 2000c Thermo Scientific). First-strand cDNA for quantitative real-time (qRT) PCR analysis was obtained using the PrimeScript® 1st Strand cDNA synthesis kit with the Oligo dT Primer from Takara (Dalian, China). The reaction was incubated at 30°C for 10 min

and 42°C for 1 h. The reaction was terminated by heating at 72°C for 15 min. The cDNA mix was diluted 10 times by PCR-grade water.

2.4. Gene cloning of full-length AcTREM1 and AcTREM2 cDNA and sequence analysis

Fragments (5' and 3') of *AcTREM1* and *AcTREM2* were obtained using the 5' and 3' RACE System for Rapid Amplification of cDNA Ends amplification kit (Invitrogen, Shanghai, China), according to the manufacturer's instructions. The specific RACE-PCR primers designed using the *AcTREM1* and *AcTREM2* genes sequences and Primer 3 software (<http://frodo.wi.mit.edu/primer3/>) are listed in Table S1.

Homology searches for nucleotide and amino acid sequence similarities were conducted with BLAST programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>). The SignalP 4.1 program was utilized to predict the presence and location of the signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>). Domain predictions were conducted with the Simple Modular Architecture Research Tool (<http://smart.emblheidelberg.de/>). Multiple sequence alignment was performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). A cladogram was constructed based on amino sequence alignment by the neighbor-joining (NJ) algorithm embedded in the MEGA 5 program.

2.5. Real-time PCR analysis of mRNA expression

Tissue distribution and temporal expression of *AcTREM1* and *AcTREM2* were determined by SYBR Green fluorescent RT-PCR. Two primers for *AcTREM1*, namely, a sense primer AcTREM-1-RT-F and a reverse primer AcTREM-1-RT-R (Table S1), were used to amplify a 151-bp fragment. A further two primers for *AcTREM2*, namely, a sense primer AcTREM-2-RT-F and a reverse primer AcTREM-2-RT-R (Table S1), were used to amplify a 167-bp fragment. In addition, PCR products were sequenced to verify RT-PCR specificity. The gene encoding β -actin was used as a reference gene. Each PCR well (20 μ L) contained 10 μ L of SYBR

Premix Ex Taq II (Takara, Japan), 1 μ L of complementary DNA, 10 pmole of each forward and reverse primer (Table. S1), and 7 μ L ultrapure water. The thermal cycling profile used was 95°C for 30 s, 40 cycles of 94°C for 5 s, and 60°C for 30 s. Fluorescence yields obtained from three replicate reactions of each complementary DNA sample were analyzed using the Mastercycler® ep realplex (Eppendorf, Germany); eight biological replicate experiments were also used to ensure the validity and accuracy of experimental results. Gene expression levels were expressed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6. AcTREM1 and AcTREM2 protein expression, purification, antibody preparation, and enzyme-linked immunosorbent assay (ELISA)

The sequence that encodes mature AcTREM1 and AcTREM2 peptides was amplified using the specific primers AcTREM-1-ex-F and AcTREM-1-ex-R, AcTREM-2-ex-F, and AcTREM-2-ex-R (Table S1). PCR products and pRSET were digested with the corresponding restriction enzymes and subsequently ligated. cDNA fragments were inserted into a pRSET plasmid. Next, recombinant plasmids pRSET-AcTREM-1 and pRSET-AcTREM-2 were transformed into *E. coli* BL21 (DE3) cells for isopropyl-b-D-1-thiogalactopyranoside (IPTG)-induced recombinant expression. rAcTREM1 and rAcTREM2 were expressed as inclusion bodies. Recombinant protein was purified according to a previously described method (Ying et al., 2015). Purified recombinant protein was dialyzed using Tris-HCl buffer (50 mM, pH 8.0) to remove imidazole in the elution buffer.

New Zealand white rabbits were immunized with 100 μ g of purified proteins homogenized in complete Freund's adjuvant once every 2 weeks for a total of three times. A boost injection in incomplete Freund's adjuvant was given for a further week. Rabbit serum was collected 7 days after the last immunization. Antiserum was stored at -80°C. Antibody titers of the immunized rabbits were determined by ELISA.

2.7. Western blotting analysis and identification of AcTREM1 and AcTREM2 in spleen by peptide mass fingerprint (PMF)

After treatment with LPS, liver and spleen samples were briefly washed with

cold PBS, and then were placed on ice and lysed in RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 1% Triton X-100; 10 mM NaF; 1 mM Na₃VO₄; protease inhibitor cocktail [1:1000]). Lysates were sonicated for 10 s and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected. Protein concentration was measured by BCA assay (Applygen Technologies, Inc.; Beijing, China). Thirty-micrograms of protein-lysate from each sample were subjected to 12% SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto polyvinylidene fluoride membranes. The membranes were then blocked using 5% (w/v) fat-free dry milk/Tris-buffered saline (TBS) at 4°C overnight, and then washed with TBS/0.05% Tween-20 three times for 20 min each. Membranes were then incubated with a primary antibody against phosphorylated forms of AcTREM1 and AcTREM2, or β -actin (13E5) (Cell Signaling Technology, Inc.; Boston, MA) for 12 h at 4 °C, and subsequently washed three times for 20 min each. The membranes were incubated for 1 h at room temperature with a goat anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) diluted 1:2000 in 5% (w/v) fat-free dry milk/TBS. Membranes were washed three times, and signals were visualized using a SuperSignal WestPico kit (Thermo Fisher Scientific; Waltham, MA, USA). Band intensity was normalized to that of β -actin. The final result was calculated as the mean of three independent repetitions.

The major AcTREM1 and AcTREM2 proteins of band spleen, with molecular weights of 35 and 25 kDa, respectively, were extracted and separated. Then, the molecular identity of the proteins was determined by MALDI-TOF-MS, and their peptide mass fingerprints (PMF) were obtained. Finally, the figures obtained were identified by comparing with the amino acid sequences of AcTREM1 and AcTREM2.

2.8. Immunofluorescence and fluorescence in situ hybridization (FISH) to detect AcTREM1 and AcTREM2 gene and protein expression in spleen

For FISH, the spleen was fixed in 4% paraformaldehyde for 2 h and operated as described by Sunde (Sunde et al., 2003). Primers with Cry3 probes were designed according to the AcTREM1 and AcTREM2 open reading frame sequences (ORF)

(Table S1). In sections, the cells with fragmented and condensed nuclei were determined using 4',6-diamidino-2-phenylindole (DAPI) staining. Stained cells were examined using a Ti-s inverted phase-contrast microscope. A Nikon Intensilight C-HGFI (Nikon, Japan) UV fiber optic illuminator was used to obtain fluorescence microscopy data.

In immunofluorescence experiments, 4- μ m serial sections were cut from formalin-fixed paraffin-embedded blocks and mounted on poly-L-lysine-coated glass slides. Immunostaining was performed using the linked streptavidin-biotin horseradish peroxidase (LSAB-HRP) technique. After sequential deparaffinization and rehydration, slides were treated with 0.3% H₂O₂ for 15 min to quench endogenous peroxidase. Antigen retrieval was performed by microwave heating of the slides, which had been immersed in 10 mM citrate buffer pH 6 (3 min at 650 W, and then two $\times$ 3 min at 350 W). After microwaving, the sections were blocked for 60 min with 1.5% normal horse serum (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS buffer before incubation with the primary antibody. Primary anti-AcTREM1 and anti-AcTREM2 antibodies were diluted 1: 150 and 1:250 with 0.05 M Tris-HCl buffer pH 7.4 containing 1% bovine serum albumin and then incubated overnight. After two washes with PBS, slides were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:2000) (Invitrogen, USA). Negative control slides, which were not incubated with a primary antibody, were included for each staining experiment. Immunofluorescence was evaluated separately by two investigators. Stained cells were also examined using a Ti-s inverted phase-contrast microscope and Nikon Intensilight C-HGFI (Nikon, Japan) UV fiber optic illuminator was used to obtain fluorescence microscopic data.

2.9. Statistical analysis

Differences between the LPS challenged and control groups were analyzed by one-way analysis of variance (ANOVA). Differences between treatment means were compared by the mean $\pm$ standard error ($n = 8$) and were considered significant at $P < 0.05$. Statistical analyses were performed using SPSS software (Version 11.0).

3. Results and discussion

3.1. Sequence characteristics of *AcTREM1* and *AcTREM2*

The complete nucleotide sequence of *AcTREM1* cDNA was 1441-bp long and consisted of an ORF of 930 bps encoding a 309-amino acid peptide, a 5' terminal untranslated region (UTR) of 32 bp, and a 3' UTR of 479 bp with a poly (A) tail (Fig. S1A). SMART analysis showed that the *AcTREM1* protein contained a putative signal peptide of 18 amino acids at the N-terminus and had two immunoglobulin (Ig) domains. TMHMM analysis showed that *AcTREM1* possesses a transmembrane domain at positions 837 to 906 (Fig. S1A). The full-length cDNA of *AcTREM2* was 1269 bp and consisted of a 582-bp ORF encoding a 193-amino acid polypeptide, a 54-bp 5' UTR, and a 633-bp 3' UTR with a poly-(A) tail (Fig. S1B). A signal peptide was present in the amino acid sequence between residues 1 and 18. The deduced *AcTREM2* protein also contained an Ig domain and a transmembrane domain of 23 amino acids (Fig. S1B). The results indicate that TREM-1 is a member of the Ig superfamily. It is a 30-kDa glycoprotein receptor containing an ectodomain of the Ig-like V-type, a transmembrane region, and a short intracytoplasmic domain (Arts et al., 2013; Kelker et al., 2004) .

BLASTP analysis showed that *AcTREM1* shared 58% identity with *AftREM1* from *Aptenodytes forsteri*, 54% identity with *PcTREM1* from *Pelecanus crispus*, 53% identity with *GgTREM1* from *Gallus gallus* and *BrsTREM1* from *Buceros rhinoceros silvestris*, and 52% identity with *LdTREM1* from *Leptosomus discolor*. Multiple sequence alignment revealed that *AcTREM1* and the aforementioned amino acid sequences (*GgTREM1*, *PcTREM1*, *AftREM1*, *BrsTREM1*, and *LdTREM1*) all contained a signal peptide domain, two Ig domains, and a transmembrane region domain, except for *PcTREM1* (Fig. S2A). Using the same method, we found that the amino acid sequence of *AcTREM2* is homologous to that of *Anser cygnoides domesticus* *AcdTREM2*-like (identity = 93%), *Anas platyrhynchos* *ApTREM2* (identity = 89%), *Haliaeetus albicilla* *HaTREM2* (identity = 82%), *Charadrius vociferus* *CvTREM2*-like (identity = 82%), *Egretta garzetta* *EgTREM2* (identity = 80%), and *Melopsittacus undulatus* *MuTREM2* (identity = 77%). *AcTREM2* and the other proteins (*AcdTREM2*-like, *ApTREM2*, *HaTREM2*, *CvTREM2*-like, *EgTREM2*

and MuTREM2) possess a signal peptide domain, an Ig domain, and a transmembrane region domain (Fig. S2B). For Fig. S3A, a phylogenetic tree was constructed using the amino acid sequences of selected PIRIX1s, TREM1s, and TREM1s. The first group was composed of PIRIX1s and TREM1s, and the second group was composed of TREM1s and TREM1s. AcTREM1 grouped with GgTREM1, which belongs to the TREM1 group. In Fig. S3B, the phylogenetic tree was clustered into two distinct clades. One clade included three sequences (AcTREM2, ApTREM2, and AcdTREM2-like) belonging to the family Anatidae group.

3.2. Expression, purification, antibody preparation, antigen identification, tissue expression, and cellular localization of AcTREM1 and AcTREM2

For further functional analyses, pRSET-AcTREM-1 and pRSET-AcTREM-2 vectors were used to express recombinant *AcTREM1* and *AcTREM2* with a His tag using *E. coli* BL21 (DE3) strain. Following expression, proteins with an apparent molecular mass of 35 kDa and 25 kDa were detected in positive transformants containing pRSET-AcTREM-1 and pRSET-AcTREM-2 by SDS-PAGE (Fig. S4A) and western blotting (Fig. S4B). Western blotting analysis of AcTREM1 and AcTREM2 expression in the spleen and liver of geese, revealed bands corresponding to the size of the AcTREM1 (35 kDa) and AcTREM2 (25 kDa) proteins (Fig. S4C). To determine which bands related to AcTREM1 and AcTREM2 in spleen samples, the PMF results showed that a 35-kDa protein band represented AcTREM1 and a 25-kDa protein band represented AcTREM2 (Fig. S5).

The spleen is the largest immune organ in avian species, and plays an important role in humoral and cellular immunity. The liver is an important detoxification organ in animals. qRT-PCR analyses were employed to quantify mRNA expression of *AcTREM1* and *AcTREM2* in the heart, liver, spleen, lung, kidney, chest muscle, intestine, and bursin. As shown in Fig. 1.iA, *AcTREM1* was detected in some tissue samples. The *AcTREM1* transcript was predominantly found in the liver and spleen. In the kidney, chest muscle, intestine, and bursin, *AcTREM1* expression was relatively low. *AcTREM2* was detected in all examined tissues (Fig. 1.iB). Within tested tissues, *AcTREM2* was mainly expressed in liver, spleen, and bursin, with a relatively lower

expression level in the heart, lung, kidney, chest muscle, and intestine.

FISH analysis (Fig. 1.iC, D and E) showed that these two genes were located in the spleen, and were mainly distributed in the cytoplasm. Furthermore, immunofluorescence analysis revealed that two proteins were abundant in the spleen of geese and that these were mainly distributed in cytoplasm (Fig. 1.iiA, B and C). In vertebrates, the spleen and liver contain a large number of macrophages. Previous studies have investigated TREM-1 expression in monocytes/macrophages (Sharif and Knapp, 2008). TREMs are important inflammatory mediators and have been detected in liver endothelial cells and hepatic stellate cells (Duan et al., 2015). In mammals, RT-PCR analysis of bovine TREM-1 revealed high expression in the spleen, and a similar analysis of pig TREM-1 revealed high expression in the bone marrow, lung, and colon (Ramanathan et al., 2004; Ramanathan et al., 2005).

3.3. Gene and protein expression of *AcTREM1* and *AcTREM2* post-LPS challenge

LPS has been shown to severely impair extensive physiological activities in both avian species and mammals. LPS can induce sickness, reduce immune function (Star et al., 2008), cause embryo mortality (Smith and Thomas, 1956), and even result in death (Galanos et al., 1979; Nikami et al., 2008). To study the functions of *AcTREM1* and *AcTREM2* in the immune response of Yangzhou geese, the levels of *AcTREM1* and *AcTREM2* expression in the spleen and liver following LPS challenge were measured using qRT-PCR.

The temporal expression profiles of the *AcTREM1* gene in the control and LPS-challenged samples are shown in Fig. 2. Following the injection of geese with 1.0 or 2.0 mg/kg LPS, *AcTREM1* was upregulated in the spleen (Fig. 2A) at 3 h, reaching the highest level at 6 h, decreasing at 12 h, and then dropping to nadir level at 48 h. This change in splenic *AcTREM1* gene expression was similar to that in the liver: hepatic *AcTREM1* gene expression was significantly increased (about 6-fold) at 3, 6, and 12 h (Fig. 2B). Our results further showed that LPS regulated the levels of *AcTREM1* in the spleen and liver both dose- and time-dependently (Fig. 3). As shown in Fig. 3 (A–C), levels of AcTREM1 protein first increased (at 3 and 6 h) and then decreased (at 12 h, 24 h and 48 h) in the spleen of individuals injected with 1.0 mg/kg

LPS. We also found that, at high concentrations, LPS increased the protein levels of AcTREM1 in advance, an effect that persisted for a long time. In the liver (Fig. 3 [D–F]), the change in AcTREM1 protein levels was similar to that in the spleen; however, little effect was observed with 0.5 mg/kg LPS. This result showed that in mammals, TREM-1 expression is strongly upregulated upon exposure to extracellular bacteria or cell-wall components such as LPS or lipoteichoic acid (Bouchon et al., 2000; Colonna and Facchetti, 2003). Furthermore, LPS synergistically enhances the production of cytokines by monocytes in response to the agonistic monoclonal antibody, indicating that TREM-1 amplifies inflammatory responses initiated by TLRs (Bleharski et al., 2003; Bouchon et al., 2000; Bouchon et al., 2001; Colonna and Facchetti, 2003). TREM-1 expression is also increased on human monocytes following LPS exposure in vivo and in vitro (Knapp et al., 2005). In vitro exposure to LPS, lipoteichoic acid, and *Streptococcus pneumoniae* induced a similar upregulation, which was not associated with increased Tumor necrosis factor (TNF)- or Interleukin (IL)-10, but was PI3K-dependent (Knapp et al., 2005).

Unlike TREM-1, TREM-2 is not expressed on peripheral blood monocytes, but is expressed on immature dendritic cells, macrophages that have been newly recruited into the peritoneum or air spaces, bone marrow derived macrophage (BMDM), microglia, and a variety of macrophage cell lines (Sharif and Knapp, 2008). The results of in vitro studies discussed herein indicate that TREM-2 is a negative regulator of the inflammatory response. Negative regulation of inflammation is important as it acts to ensure that the production of pro-inflammatory cytokines is kept in check, as overproduction can be detrimental to the host. However, at the onset of infection, low expression levels of negative regulators are required to ensure an adequate immune response is initiated. Many negative regulators, including those which modulate TLR responses, have been shown to be important in bacterial infection (Krishnan et al., 2007; Lang and Mansell, 2007). In this study, the changes observed in *AcTREM2* gene expression exhibited patterns that were reciprocal to those of *AcTREM1* in the liver and spleen after LPS challenge (Fig. 2). Changes in AcTREM2 protein levels were negatively correlated with changes in AcTREM1 expression. The results of this study showed that *AcTREM2* gene expression was

significantly decreased in 1.0 and 2.0 mg/kg LPS-treated spleens and livers 3 and 6 h after exposure (Fig. 2 [C and D]). At 12 and 24 h, *AcTREM2* gene expression was increased in 2.0 mg/kg LPS-treated spleens and livers (Fig. 2 [C and D]). These results suggest that the TREM-2 level dropped in the early stage of inflammation, weakening the inhibition of proinflammatory cytokine secretion, and enhancing the body's immune response. Using the aforementioned methods to detect the levels of AcTREM2 protein in the spleen and liver, we found that the higher the concentration of LPS, the more obvious the effect on AcTREM2 protein expression. In the spleen (Fig. 3 [G-I]), we observed that with increasing time, protein levels of AcTREM2 first increased and then decreased, and were finally upregulated. In contrast, the protein levels of *AcTREM2* in the liver first decreased and then increased (Fig. 3 [J-L]).

In conclusion, this study is the first to identify two novel TREMs (AcTREM1 and AcTREM2) from *A. cygnoides*. Our results indicate that these two genes may participate in the antibacterial immunity of *A. cygnoides*. The results of the current study provide valuable information that may yield insights into the molecular mechanism of the innate immune system, and the evolution of avian TREMs.

Acknowledgments

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References

- Arts, R.J., Joosten, L.A., Jw, V.D.M., Netea, M.G., 2013. TREM-1: intracellular signaling pathways and interaction with pattern recognition receptors. *J. Leukoc. Biol.* 93, 209–215.

- Bleharski, J.R., Kiessler, V., Buonsanti, C., Sieling, P.A., Stenger, S., Colonna, M., Modlin, R.L., 2003. A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J. Immunol.* 170, 3812–3818.
- Bouchon, A., Dietrich, J., Colonna, M., 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164, 4991–4995.
- Bouchon, A., Facchetti, F., Weigand, M.A., Colonna, M., 2001. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410, 1103–1107.
- Charles, J.F., Humphrey, M.B., Zhao, X., Quarles, E., Nakamura, M.C., Aderem, A., 2008. The innate immune response to *Salmonella enterica* serovar Typhimurium by macrophages is dependent on TREM2-DAP12. *Infect. Immun.* 76, 2439–2447.
- Chen, Q., Zhang, K., Jin, Y., Zhu, T., Cheng, B., Shu, Q., Fang, X., 2013. Triggering receptor expressed on myeloid cells-2 protects against polymicrobial sepsis by enhancing bacterial clearance. *Am. J. Resp. Crit. Care Med.* 188, 201–212.
- Colonna, M., Facchetti, F., 2003. TREM-1 (triggering receptor expressed on myeloid cells): a new player in acute inflammatory responses. *J. Infect. Dis.* 187 Suppl 2, S397–S401.
- Crocker, P.R., Paulson, J.C., Varki, A., 2007. Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* 7, 255–266.
- Duan, M., Wang, Z.C., Wang, X.Y., Shi, J.Y., Yang, L.X., Ding, Z.B., Gao, Q., Zhou, J., Fan, J., 2015. TREM-1, an inflammatory modulator, is expressed in hepatocellular carcinoma cells and significantly promotes tumor progression. *Ann. Surg. Oncol.* 22, 3121–3129.
- Ford, J.W., Mcvicar, D.W., 2009. TREM and TREM-like receptors in inflammation and disease. *Curr. Opin. Immunol.* 21, 38–46.
- Galanos, C., Freudenberg, M.A., Reutter, W., 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc. Natl. Acad. Sci. U.S.A.* 76, 5939–5943.
- Gerondakis, S., Grumont, R.J., Banerjee, A., 2007. Regulating B-cell activation and

- survival in response to TLR signals. *Immunol. Cell Biol.* 85, 471–475.
- Hamerman, J.A., Jarjoura, J.R., Humphrey, M.B., Nakamura, M.C., Seaman, W.E., Lanier, L.L., 2006. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J. Immunol.* 177, 2051–2055.
- Ito, H., Hamerman, J.A., 2011. TREM-2, triggering receptor expressed on myeloid cell-2, negatively regulates TLR responses in dendritic cells. *Eur. J. Immunol.* 42, 176–185.
- Jiang, D.L., Liu, L., Wang, C.L., Chen, F., Sun, A.D., Shi, Z.D., Jiang, D.L., Liu, L., Wang, C.L., Chen, F., 2011. Raising on water stocking density reduces geese reproductive performances via water bacteria and lipopolysaccharide contaminations in “Geese-Fish” production system. *Agr. Sci. China* 10, 1459–1466.
- Kanneganti, T.D., Lamkanfi, M., Núñez, G., 2007. Intracellular NOD-like receptors in host defense and disease. *Immunity* 27, 549–559.
- Kelker, M.S., Foss, T.R., Peti, W., Teyton, L., Kelly, J.W., Wüthrich, K., Wilson, I.A., 2004. Crystal structure of human triggering receptor expressed on myeloid cells 1 (TREM-1) at 1.47 Å. *J. Mol. Biol.* 342, 1237–1248.
- Klesney-Tait, J., Colonna, M., 2007. Uncovering the TREM-1-TLR connection. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 293, 1374–1376.
- Knapp, S., Gibot, S., De, V.A., Versteeg, H.H., Colonna, M., Van, d.P.T., 2005. Cutting edge: expression patterns of surface and soluble triggering receptor expressed on myeloid cells-1 in human endotoxemia. *J. Immunol.* 173, 7131–7134.
- Kogut, M.H., Genovese, K.J., Nerren, J.R., He, H., 2012. Effects of avian triggering receptor expressed on myeloid cells (TREM-A1) activation on heterophil functional activities. *Dev. Comp. Immunol.* 36, 157–165.
- Krishnan, J., Selvarajoo, K., Tsuchiya, M., Lee, G., Choi, S., 2007. Toll-like receptor signal transduction. *Exp. Mol. Med.* 39, 421–438.
- Lang, T., Mansell, A., 2007. The negative regulation of Toll-like receptor and associated pathways. *Immunol. Cell Biol.* 85, 425–434.

- Lanier, L.L., 2009. DAP10- and DAP12-associated receptors in innate immunity. *Immunol. Rev.* 227, 150–160.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402–408.
- N'Diaye, E.N., Branda, C.S., Branda, S.S., Nevarez, L., Colonna, M., Lowell, C., Hamerman, J.A., Seaman, W.E., 2009. TREM-2 (triggering receptor expressed on myeloid cells 2) is a phagocytic receptor for bacteria. *J. Cell Biol.* 184, 215–223.
- Netea, M.G., Azam, T., Ferwerda, G., Girardin, S.E., Kim, S.H., Dinarello, C.A., 2006. Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. *J. Leukoc. Biol.* 80, 1454–1461.
- Nikami, H., Mahmoud, M., Shimizu, Y., Shiina, T., Hirayama, H., Iwami, M., Dosoky, R., Ahmed, M., Takewaki, T., 2008. Capsaicin pretreatment attenuates LPS-induced hypothermia through TRPV1-independent mechanisms in chicken. *Life Sci.* 82, 1191–1195.
- Radsak, M.P., Salih, H.R., Rammensee, H.G., Schild, H., 2004. Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. *J. Immunol.* 172, 4956–4963.
- Ramanathan, B., Minton, J.E., Ross, C.R., Blecha, F., 2004. Characterization of bovine cDNA encoding triggering receptor expressed on myeloid cells 1 (TREM-1) *Vet. Immunol. Immunopathol.* 102, 85–89.
- Ramanathan, B., Minton, J.E., Ross, C.R., Blecha, F., 2005. Cloning of porcine triggering receptor expressed on myeloid cells-1 (TREM-1) and its induction by lipopolysaccharide, peptidoglycan, and *Salmonella enterica* serovar Typhimurium infection. *Dev. Comp. Immunol.* 29, 1–7.
- Robinson, M.J., Sancho, D., Slack, E.C., Leibundgutlandmann, S., Reis, e.S.C., 2006. Myeloid C-type lectins in innate immunity. *Nat. Immunol.* 7, 1258–1265.
- Sharif, O., Knapp, S., 2008. From expression to signaling: Roles of TREM-1 and TREM-2 in innate immunity and bacterial infection. *Immunobiol.* 213, 701–713.
- Shi, Z.D., Tian, Y.B., Wu, W., Wang, Z.Y., 2008. Controlling reproductive seasonality

- in the geese: a review. *Worlds Poult. Sci. J.* 64, 343–355.
- Smith, R.T., Thomas, L., 1956. The lethal effect of endotoxins on the chick embryo. *J. Exp. Med.* 104, 217–231.
- Star, L., Kemp, B., Van, d.A.I., Parmentier, H.K., 2008. Effect of single or combined climatic and hygienic stress in four layer lines: 1. Performance. *Poult. Sci.* 87, 1022–1030.
- Sunde, P.T., Olsen, I., Göbel, U.B., Theegarten, D., Winter, S., Debelian, G.J., Tronstad, L., Moter, A., 2003. Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth. *Microbiology* 149, 1095–1102.
- Tessarz, A.S., Cerwenka, A., 2008. The TREM-1/DAP12 pathway. *Immunol. Lett.* 116, 111–116.
- Turnbull, I.R., Gilfillan, S., Cella, M., Aoshi, T., Miller, M., Piccio, L., Hernandez, M., Colonna, M., 2006. Cutting edge: TREM-2 attenuates macrophage activation. *J. Immunol.* 177, 3520–3524.
- Viertlboeck, B.C., Hanczaruk, M.A., Amann, B., Bader, S.R., Schmitt, R., Sperling, B., Schwarz, S.C., Schmahl, W., Deeg, C.A., Göbel, T.W., 2013. Chicken immunoregulatory Ig-like receptor families: An overview and expression details on ggTREM-A1. *Dev. Comp. Immunol.* 41, 403–412.
- Viertlboeck, B.C., Schmitt, R., Göbel, T.W., 2006. The chicken immunoregulatory receptor families SIRP, TREM, and CMRF35/CD300L. *Immunogenetics* 58, 180–190.
- Xu, Q., Song, Y., Liu, R., Chen, Y., Zhang, Y., Li, Y., Zhao, W., Chang, G., Chen, G., 2016. The dopamine β -hydroxylase gene in Chinese goose (*Anas cygnoides*): cloning, characterization, and expression during the reproductive cycle. *BMC Genet.* 17, 1.
- Ying, H., Zhao, L.L., Feng, J.L., Zhu, H.X., Xin, H., Qian, R., Wen, W., 2015. A novel integrin function in innate immunity from Chinese mitten crab (*Eriocheir sinensis*). *Dev. Comp. Immunol.* 52, 155–165.

Fig. 1. Tissue distribution and cellular localization of *AcTREM1* and *AcTREM2*. i. Tissue distribution of *AcTREM1* (A) and *AcTREM2* (B) in the heart, liver, spleen, lung, kidney, chest muscle, intestine, and bursin of the Yangzhou goose (*Anas cygnoides*). Expression of the gene encoding β -actin was used as an internal control. Error bars represent the mean $\pm$ standard deviation (S.D). of eight independent PCR amplifications and quantifications. (C, D, and E) Fluorescence in situ hybridization (FISH) was used to detect *AcTREM1* (D) and *AcTREM2* (E) expression in spleen. (C) Sections were incubated without Cy3 probe (control group); Bars indicate 20 μ m. ii. Immunofluorescence was used to detect AcTREM1 and AcTREM2 protein expression in the spleen. (B) Spleen samples were incubated with Anti-AcTREM1 antibody; (C) Spleen samples were incubated with Anti-AcTREM2 antibody; (A) Spleens were incubated with pre-immune serum (control group); Bars indicate 20 μ m.

Fig. 2. qRT-PCR analysis of *AcTREM1* and *AcTREM2* expression in the spleen and liver of the Yangzhou goose (*A. cygnoides*) challenged with lipopolysaccharide (LPS) at different time periods. qRT-PCR analysis of *AcTREM1* (A and B) and *AcTREM2* (C and D) in the spleen and liver of the Yangzhou goose (*A. cygnoides*) under LPS challenged at 3, 6, 12, 24, and 48 h. Error bars represent the mean $\pm$ S.D. of eight independent PCR amplifications and quantifications.

Fig. 3. Western blotting analysis of AcTREM1 and AcTREM2 in the spleen and liver of the Yangzhou goose (*A. cygnoides*) challenged with LPS at different time periods. Western blotting of AcTREM1 (A and B) and AcTREM2 (C and D) in spleen and liver samples from the Yangzhou goose, *A. cygnoides* under different concentrations of LPS and challenged at 3, 6, 12, 24, and 48 h. (A, B, and C) Western blotting of AcTREM1 protein expression under different concentrations of LPS in the spleen of *A. cygnoides*. (D, E, and F) Western blotting for AcTREM1 protein expression under different concentrations of LPS, in the liver of *A. cygnoides*. (G, H, and I) Western blotting for *AcTREM2* protein expression under different concentrations of LPS in the spleen of *A. cygnoides*. (J, K, and L) Western blotting for AcTREM2 protein expression under different concentrations of LPS in the liver of *A. cygnoides*. Western

blotting for β -actin protein was performed as a loading control. Error bars represent the mean $\pm$ S.D. of three independent samples.