

# Fusion protein of tapasin and hepatitis B core antigen 18-27 enhances T helper cell type 1/2 cytokine ratio and antiviral immunity by inhibiting suppressors of cytokine signaling family members 1/3 in hepatitis B virus transgenic mice

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**Abstract.** Persistent hepatitis B virus (HBV) infection is characterized by a weak adaptive immune response, which is considered to be due to an imbalance of T helper cell types 1 and 2 (Th1/Th2). Suppressors of cytokine signaling (SOCS) family members, particularly SOCS1 and SOCS3, have been demonstrated to be important in the regulation of T cell differentiation. Previous studies by our group showed that the expressed and purified fusion protein of cytoplasmic transduction peptide (CTP) and HBV core antigen 18-27 (HBcAg18-27)-tapasin was able to enter the cytoplasm of bone marrow-derived dendritic cells (BMDCs), promoting the maturation of BMDCs and efficiently enhancing T cell immune responses *in vitro*. In the present study, HBcAg-specific immune responses induced by CTP-HBcAg18-27-tapasin in HBV were assessed in transgenic mice, and SOCS1 and SOCS3 were identified as negative regulators of this response. The Th1/Th2 cytokine ratio was analyzed by ELISA. The expression of T cell-specific T-box transcription factor (T-bet) and GATA-binding protein 3 (GATA-3), SOCS1 and SOCS3 were detected by real-time quantitative polymerase chain reaction and western blot analysis. The results demonstrated that CTP-HBcAg18-27-tapasin significantly increased the Th1/Th2 cytokine ratio in HBV transgenic mice. CTP-HBcAg18-27-tapasin immunization more efficiently suppressed the expression of serum

hepatitis B surface antigen (HBsAg), HBV DNA as well as liver HBsAg and HBcAg in HBV transgenic mice. Furthermore, CTP-HBcAg18-27-tapasin promotes T-bet but reduces GATA-3 expression. In addition, the expression of SOCS1 and SOCS3 was significantly downregulated in the CTP-HBcAg18-27-tapasin group compared with the control groups. In conclusion, the present study demonstrated that CTP-HBcAg18-27-tapasin enhanced the Th1/Th2 cytokine ratio and antiviral immunity by suppressing SOCS1/3 in HBV transgenic mice.

## Introduction

Persistent hepatitis B virus (HBV) infection is characterized by a weak adaptive immune response, which is thought to be due to inefficient CD4<sup>+</sup> T cell priming early in the infection, and subsequent development of a quantitatively and qualitatively ineffective CD8<sup>+</sup> T cell response. The HBV core 18-27 antigen (HBcAg18-27) is recognized as the most efficient cytotoxic T lymphocyte (CTL) epitope that primes specific immune responses against HBV infection in acutely infected patients (1,2). During the assembly of the MHC class I molecules with peptides in the peptide-loading complex, a series of transient interactions are made with endoplasmic reticulum-resident chaperones. Within the peptide-loading complex, the glycoprotein tapasin stabilizes the peptide-receptive MHCI conformation, which enhances specific MHC class I-restricted CTL activity (3). Thus, combining the specificity of the HBcAg epitope, the cell-penetrating properties of the cytoplasmic transduction peptide (CTP) (4) and the chaperone tapasin may elicit robust specific HBV immune responses. Previous studies by our group showed that the expressed and purified fusion protein CTP-HBcAg18-27-tapasin was able to enter the cytoplasm of bone marrow-derived dendritic cells (BMDCs), promote the maturation and cytokine interleukin-12p70 (IL-12p70) secretion of BMDCs and enhance cellular immune responses efficiently *in vitro* (5,6); however the mechanism has yet to be elucidated.

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CD4<sup>+</sup> T cells are mainly classified into two types of T helper (Th) cells depending on the on the activation of a certain antigen: Th1 and Th2. Th1 and Th2 cells are two distinct T cell subsets, defined by different functional abilities and cytokine profiles (7,8). Interferon- $\gamma$  (IFN- $\gamma$ ) is the signature cytokine of Th1 cells and interleukin-4 (IL-4) is the corresponding signature cytokine of Th2 cells. GATA-binding protein 3 (GATA-3) is a Th2-specific transcription factor, which is upregulated during Th2 differentiation (9,10). The transcription factor T-box expressed in T cells (T-bet) controls the expression of the hallmark Th1 cytokine IFN- $\gamma$  (11). The suppressors of cytokine signaling (SOCS) are members of a family of intracellular proteins that have emerged as key physiological regulators of cytokine-mediated homeostasis, including innate and adaptive immunity. Signal downregulation through SOCS members has been demonstrated to be important in the balance of cytokines that determines the onset of Th1 and Th2-mediated immune responses. In particular, for cytokine-induced SOCS1 and SOCS3, a role in the regulation of T cell differentiation has been discussed (12-14).

Therefore, the balance of Th1/Th2 is believed to be important for the direction of immune responses. Thus, in the present study, it was further shown that the CTP-HBcAg18-27-tapasin fusion protein was able to enhance the Th1/Th2 cytokine ratio and antiviral immune response in HBV transgenic mice, and it was demonstrated that this response was mediated by the suppression of SOCS1/SOCS3.

## Materials and methods

**Reagents, cells and fusion proteins.** All western blot antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and the fluorescent antibodies and isotype controls were purchased from eBioscience (San Diego, CA, USA). ELISA kits for IFN- $\gamma$ , IL-2, IL-4 and IL-10 were purchased from R&D Systems (Minneapolis, MN, USA). Hepatitis B surface antigen (HBsAg) was determined quantitatively using the IMX system (Abbott Laboratories, Chicago, IL USA) according to the manufacturer's instructions. The levels of HBV DNA were detected by fluorescent quantitative polymerase chain reaction (qPCR) assay kits (Qiagen, Hilden, Germany). Phorbol 12-myristate 13-acetate (PMA), ionomycin and monensin were obtained from Sigma Aldrich (St Louis, MO, USA). Soluble fusion proteins CTP-HBcAg18-27-tapasin, CTP-HBcAg18-27, HBcAg18-27-tapasin and HBcAg18-27 were purified and had undetectable endotoxin levels according to previous studies (6).

**Animals and immunization schedule.** The HBV transgenic mouse lineage, which was initially produced on a C57BL/6 background and the transgene consisted of 1.3 copies of the complete genome of HBV (subtype ayw), were obtained from the Key Liver Army Laboratory (458 Hospital, Guangzhou, Guangdong, China). A high level of HBsAg and HBV DNA in the sera was able to be detected in the HBV transgenic mice (15-16) and maintained in the experimental animal centre of the Shanghai No. 6 Hospital (Shanghai, China) under specific pathogen-free conditions. All experiments were approved by the laboratory animal ethical commission of Shanghai Jiao Tong University (Shanghai, China).

Mice were divided into five groups, with six mice in each group. Mice were immunized intramuscularly into the left tibialis anterior muscle three times at 1-week intervals with PBS, CTP-HBcAg18-27-tapasin (50  $\mu$ g), CTP-HBcAg18-27 (50  $\mu$ g), HBcAg18-27-tapasin (50  $\mu$ g) and HBcAg18-27 (50  $\mu$ g). Mice were sacrificed and serum samples, splenocytes and livers were collected at day seven following the third immunization.

**T lymphocyte isolation.** HBV transgenic mice spleens were dissociated on a 200-gauge nylon mesh. Splenocytes were collected and treated with lysis buffer to eliminate red cells, washed, and resuspended in culture medium consisting of RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal calf serum (Gibco-BRL) in six-well plates (Corning Inc., Corning, NY, USA). Mixed lymphocytes were derived from splenocytes using lymphocyte separation liquid (Beijing Combi Source Technology Co., Ltd., Beijing, China). T lymphocytes were derived from the mixed lymphocytes using nylon wool columns (Wako, Tokyo, Japan). Single-cell suspensions of lymphocytes ( $2 \times 10^6$  cells/well) were grown in six-well plates. The purities of the isolated T cells were determined by flow cytometry analysis following staining with anti-CD3-PE-Cy5 (eBioscience) and the samples with >80% purity were used for this experiment.

**Measurement of cytokine secretion.** The cells previously described ( $2 \times 10^6$  cells/ml) from spleens harvested from immunized HBV transgenic mice were cultured in 24-well plates at 37°C in the presence of 10  $\mu$ g/ml HBcAg18-27. Following 72 h of incubation, the supernatants were harvested in the presence of IFN- $\gamma$ , IL-2, IL-4 and IL-10 were detected by commercial mouse cytokine immunoassay ELISA kits according to the manufacturer's instructions. The concentrations of cytokines in the samples were determined from the standard curves. Data are expressed as pg/ml.

**Detection of HBV-associated markers in the serum of HBV transgenic mice.** HBsAg levels in sera were estimated with Abbott kits (Abbott laboratories). Sera from HBV transgenic mice were subjected to detection of HBV DNA by the fluorescent qPCR method using a commercial PCR kit (Qiagen) according to the manufacturer's instructions.

**Immunohistochemical analysis of the livers.** For histological analysis, liver tissue was fixed in 10% formalin, embedded in paraffin, sectioned (3  $\mu$ m) and stained with hematoxylin and eosin. Briefly, paraffin-embedded sections in PBS, pH 7.4, were treated for 10 min at 37°C with 3% hydrogen peroxide and washed with PBS. The sections were then blocked with 1% goat serum in PBS for 30 min at room temperature. Following washing with PBS, a goat anti-HBsAg polyclonal antibody and a goat anti-HBcAg polyclonal antibody (Novus Biologicals, Littleton, CO, USA) was applied overnight at 4°C following three rounds of washing in PBS. Sections were incubated for 30 min with biotinylated secondary antibody (Wuhan Boster Biological Technology, Ltd., Wuhan, Hubei, China) at 37°C, then for 30 min with streptavidin-biotin-peroxidase complex prior to being visualized with diaminobenzidine (DAB) and counterstained with hematoxylin.

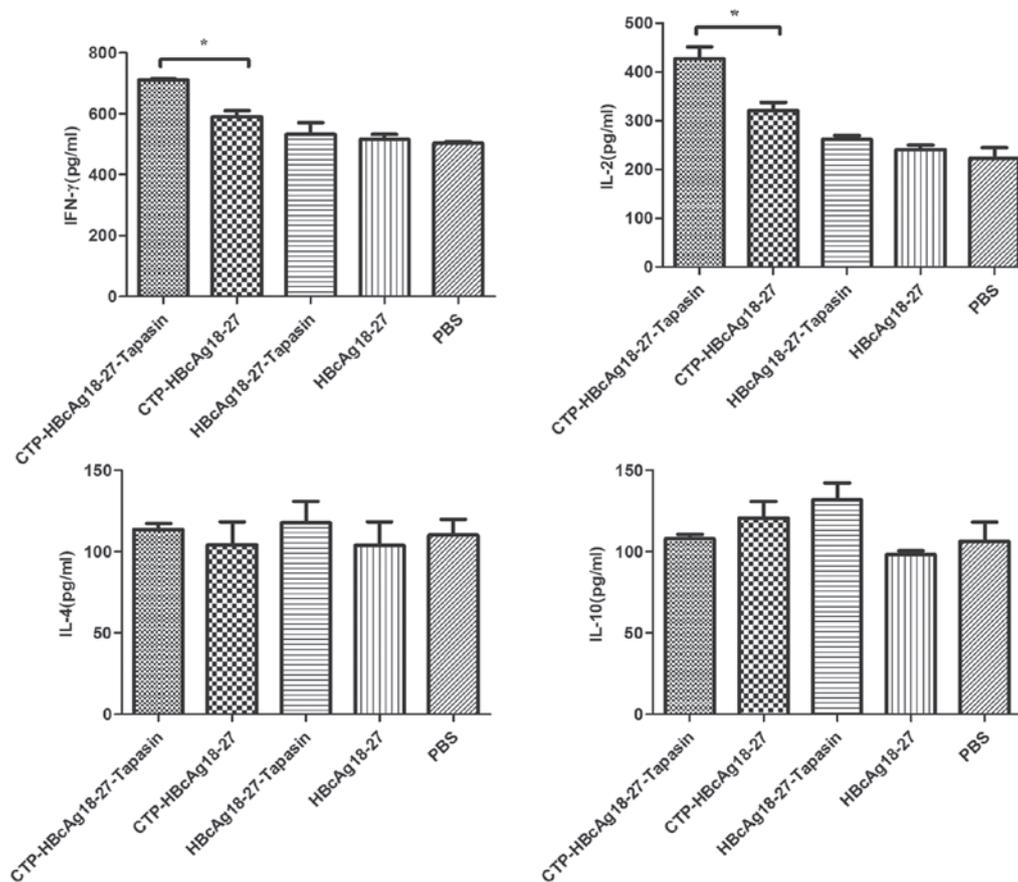


Figure 1. Cytokine production in the supernatant of T cells. Secretion of IFN- $\gamma$  and IL-2 (Th1-like) in mice immunized with CTP-HBcAg18-27-tapasin were significantly higher than in the CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 or PBS groups ( $P < 0.05$  by analysis of variance,  $n = 6$  for each group). However, the production of cytokines IL-4 and IL-10 (Th2-like) were extremely low and there was no significant difference in mice immunized with all the fusion proteins or PBS. IFN- $\gamma$ , interferon- $\gamma$ ; IL-2, interleukin-2; CTP, cytoplasmic transduction peptide; HBcAg18-27-tapasin, HBV core antigen 18-27-tapasin; IL-4, interleukin-4; IL-10, interleukin-10; PBS, phosphate-buffered saline.

**Analysis of mRNA.** T cells ( $2 \times 10^6$  cells/well) from spleens harvested from immunized mice were cultured in six-well plates at  $37^\circ\text{C}$ . Cells were then collected for total RNA isolation with Trizol<sup>®</sup> (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was generated using a PrimeScript 1st Strand cDNA Synthesis kit (Takara Bio, Inc., Shiga, Japan). Primers were designed by Primer Premier 5.0 according to the mRNA sequences of T-bet, GATA-3, SOCS1 and SOCS3 genes retrieved from GenBank, and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Primer sequences were as follows: T-bet forward primer 5'GTGGAGGTGAATGATGGAG3', reverse primer 5'AAGCAGTTGACAGTTGGGT3', 142 bp; GATA-3 forward primer 5'TTACCACCTATCCGCCCTAT3', reverse primer 5'CGGTCTGCCATTCATT3', 129 bp; SOCS1 forward primer 5'TCCGTGACTACCTGAGTTCCT3', reverse primer 5'ATCTCACCTCCACAACCACT3', 197 bp; SOCS3 forward primer 5'GCGGATTCTACTGGAGCG3', reverse primer 5'GGATGCGTAGGTTCTTGGTC3', 199 bp;  $\beta$ -actin forward primer 5'CTCCATCCTGGCCTCGCTCG3', reverse primer 5'GCTGTCACCTTCACCGTTCC3', 268 bp. Real-time PCR was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> reagents (Takara) on a LightCycler (Roche Diagnostics, Mannheim, Germany). PCR conditions were as follows: The thermal cycle parameters were 30 sec at  $95^\circ\text{C}$  followed by 40 cycles

of  $95^\circ\text{C}$  for 5 sec and  $60^\circ\text{C}$  for 20 sec. The amount of target was calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method. Three parallel reactions of each sample and internal control were run.

**Western blot analysis.** The T cells were washed twice with PBS, gently dispersed into a single-cell suspension and homogenised using radioactive immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China). Protein concentrations were determined using the BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA). Homogenates were diluted to the desired protein concentration with 2X SDS-PAGE loading buffer (Invitrogen). Samples were boiled and loaded onto polyacrylamide mini-gels (Invitrogen) for electrophoresis. Proteins from the gels were transferred to immobilon-polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA) using a semi-dry apparatus (Bio-Rad, Hercules, CA, USA). A rabbit anti-mouse T-bet (1:250), GATA-3 (1:250), SOCS1 (1:1,000) and SOCS3 (1:1,000) monoclonal antibody was used as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin-G antibody was used as the secondary antibody.

**Statistical analysis.** Data are expressed as the mean values  $\pm$  standard deviation and were analyzed by the SPSS

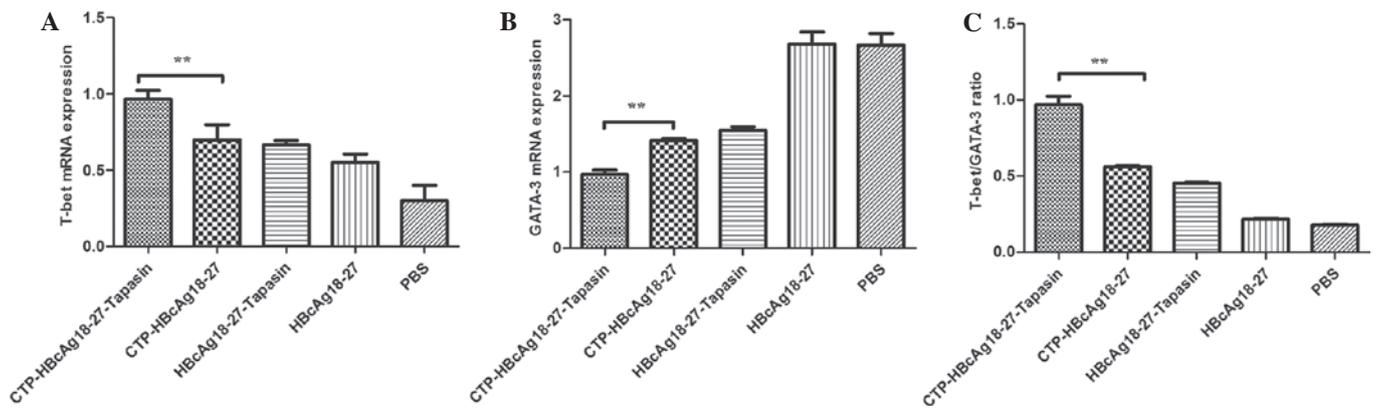


Figure 2. Expression of T-bet and GATA-3 mRNA. (A) The expression of T-bet mRNA was significantly upregulated in the CTP-HBcAg18-27-tapasin group compared with the PBS, CTP-HBcAg18-27, HBcAg18-27-tapasin and HBcAg18-27 groups. (B) In the CTP-HBcAg18-27-tapasin group, the expression of GATA-3 was lower than in the control group. (C) The ratio of T-bet/GATA-3 mRNA expression was calculated as the index of Th1/Th2 immune balance. Data are presented as the mean  $\pm$  standard deviation (n=6; \*\*P<0.01 by analysis of variance). T-bet, T-box expressed in T cells; GATA-3, GATA-binding protein 3; CTP, cytoplasmic transduction peptide; HBcAg18-27-Tapasin, HBV core 18-27 peptide-tapasin; Th, T helper cell; PBS, phosphate-buffered saline.

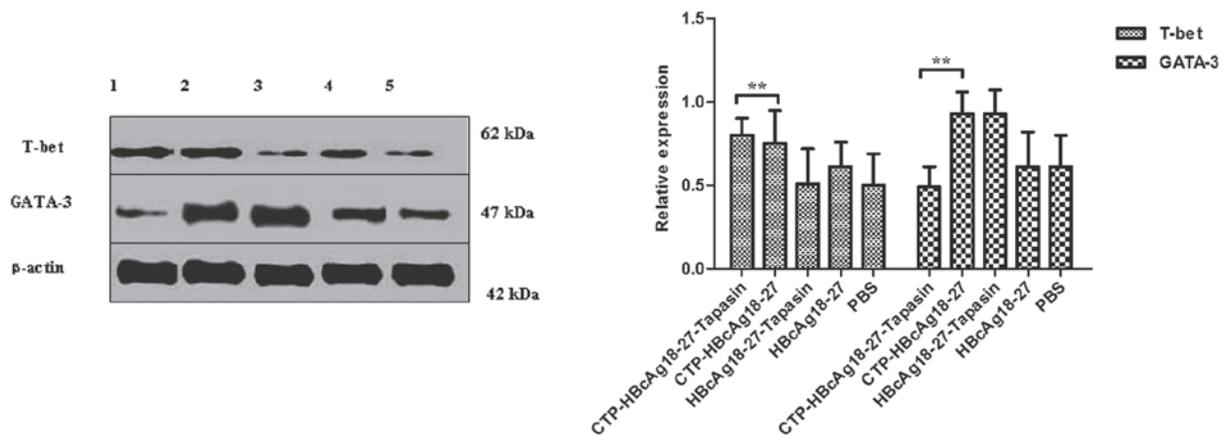


Figure 3. Expression of T-bet and GATA-3. The expression of T-bet was significantly upregulated in the CTP-HBcAg18-27-tapasin group compared with the PBS, CTP-HBcAg18-27, HBcAg18-27-tapasin and HBcAg18-27 groups. However, in the CTP-HBcAg18-27-tapasin group, the expression of GATA-3 was lower than in the control groups. 1, CTP-HBcAg18-27-tapasin; 2, CTP-HBcAg18-27; 3, HBcAg18-27-tapasin; 4, HBcAg18-27; 5, PBS. Data are presented as the mean  $\pm$  standard deviation (n=6; \*\*P<0.01). T-bet, T-box expressed in T cells; GATA-3, GATA-binding protein 3; CTP, cytoplasmic transduction peptide; HBcAg18-27-tapasin, HBV core 18-27 peptide-tapasin; PBS, phosphate-buffered saline.

16.0 software (SPSS, Inc., Chicago, IL). One-way analysis of variance and post-hoc least significant difference test were used to determine the statistical significance in comparison with the control. P<0.05 was considered to indicate a statistically significant difference.

## Results

*CTP-HBcAg18-27-tapasin stimulates the secretion of cytokines.* T cells from immunized animals were assayed for the secretion of the cytokines IFN- $\gamma$ , IL-2 (Th1-like), IL-4 and IL-10 (Th2-like) upon re-stimulation with HBcAg18-27. As displayed in Fig. 1, T cells from the CTP-HBcAg18-27-tapasin group produced higher levels of IFN- $\gamma$  (709.76 pg/ml) and IL-2 (410.42 pg/ml) than the other groups. However, the production of these cytokines was extremely low and there was no significant difference between mice immunized with CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 or PBS (P<0.05). However, there was no significant difference in the

production of cytokines IL-4 and IL-10 (Th2-like) between the groups of mice immunized with any of the fusion proteins or PBS. These findings suggest that CTP-HBcAg18-27-tapasin may enhance the secretion of cytokines IFN- $\gamma$  and IL-2, which regulate Th1 differentiation and promote antiviral immunity.

*CTP-HBcAg18-27-tapasin promotes T-bet but reduces GATA-3, resulting in an increased ratio of T-bet/GATA-3.* To further confirm the ratio between Th1 and Th2 in HBV transgenic mice, real-time PCR and western blot analysis was performed in T cells to analyze the expression of T-bet and GATA-3 in the various treatment groups. As displayed in Figs 2A and 3, the expression of T-bet was significantly upregulated in the CTP-HBcAg18-27-tapasin group compared with the CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 and PBS groups. However, in the CTP-HBcAg18-27-tapasin group, the expression of GATA-3 was lower than in the control groups (Figs 2B and 3). Furthermore, the alteration in the T-bet/GATA-3 ratio, which indexes the condition of Th1/Th2 differentiation, was increased in CTP-HBcAg18-27-tapasin

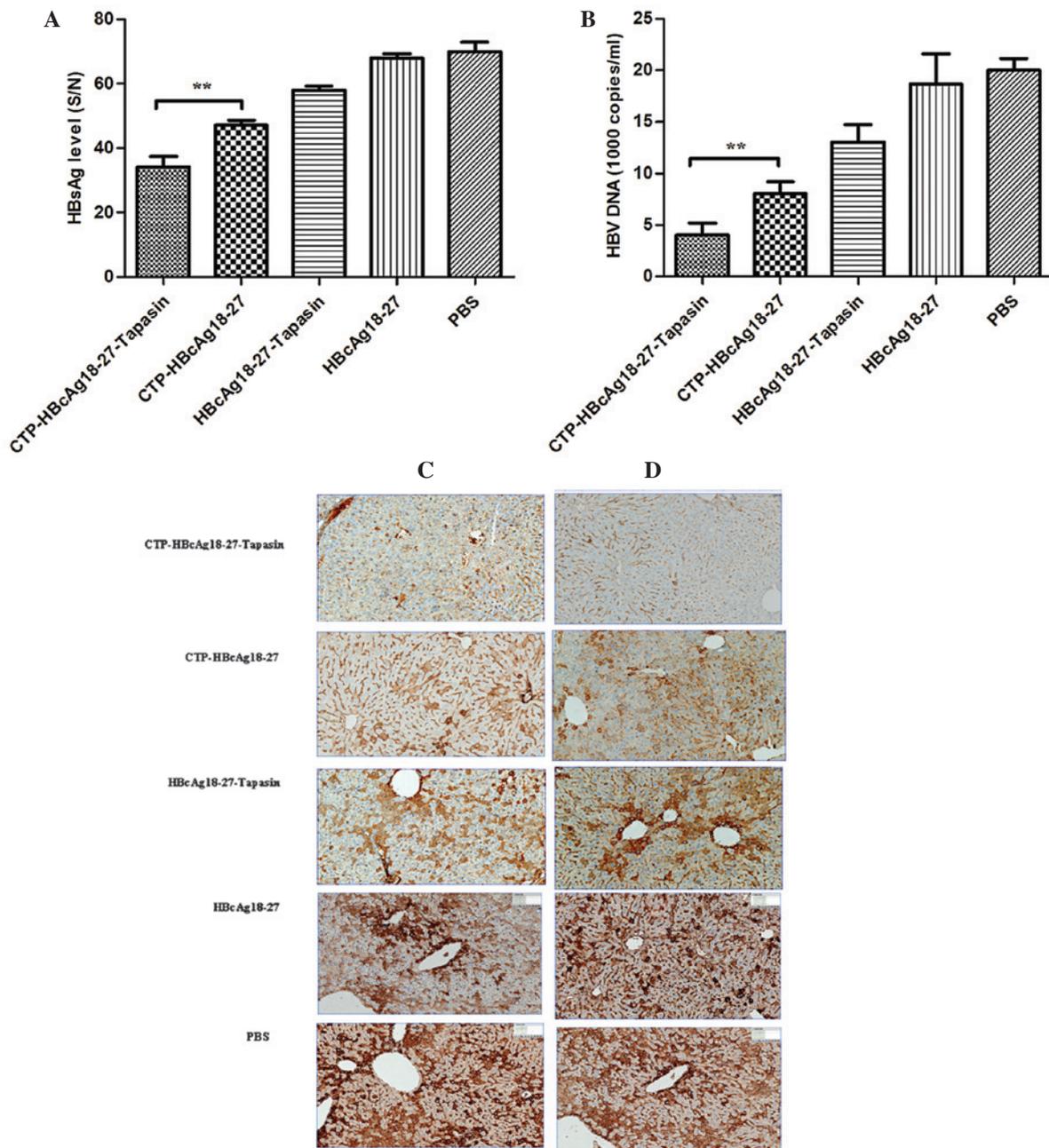


Figure 4. Detection of HBV-associated markers in the serum of HBV transgenic mice. The serum HBsAg and HBV DNA were quantitated by ELISA and fluorescent quantitative polymerase chain reaction, respectively. The (A) HBsAg and (B) HBV DNA levels in the sera of mice treated with CTP-HBcAg18-27-tapasin were significantly lower than in the other groups. Data are presented as the mean  $\pm$  standard deviation (n=6; \*\*P<0.01). (C and D) Immunohistological analysis of HBsAg and HBcAg in HBV in the livers of transgenic mice. Mice were treated with PBS, HBcAg18-27, CTP-HBcAg18-27, HBcAg18-27-tapasin and CTP-HBcAg18-27-tapasin. Liver sections were subjected to immunohistological analysis of (C) HBsAg and (D) HBcAg. Representative images are presented (original magnifications, x200). HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus; CTP, cytoplasmic transduction peptide; HBcAg18-27-tapasin, HBV core 18-27 peptide-tapasin.

group as compared with the other groups (Fig. 2C). These findings suggested that CTP-HBcAg18-27-tapasin may be important in the Th1/Th2 imbalance in HBV transgenic mice by regulating the expression of the transcription factors T-bet and GATA-3, which are able to affect Th1-type and Th2-type cytokine production in accordance with previous studies (17).

*CTP-HBcAg18-27-tapasin efficiently reduces the titers of serum HBsAg and HBV DNA as well as the HBsAg and HBcAg expression in liver tissue.* It was evaluated whether CTP-HBcAg18-27-tapasin immunization was able to

reduce HBsAg expression and the viral load in the serum of HBV transgenic mice. As displayed in Figs 4A and B, the inhibition of serum HBsAg or viral DNA in HBV transgenic mice immunized by CTP-HBcAg18-27-tapasin, CTP-HBcAg18-27, HBcAg18-27-tapasin or HBcAg18-27 demonstrated a significant difference. These results indicated that CTP-HBcAg18-27-tapasin immunization suppresses the expression of serum HBsAg and HBV DNA more efficiently than the other treatments in HBV transgenic mice. Serum HBsAg levels and titer of HBV DNA from the mice immunized with CTP-HBcAg18-27-tapasin

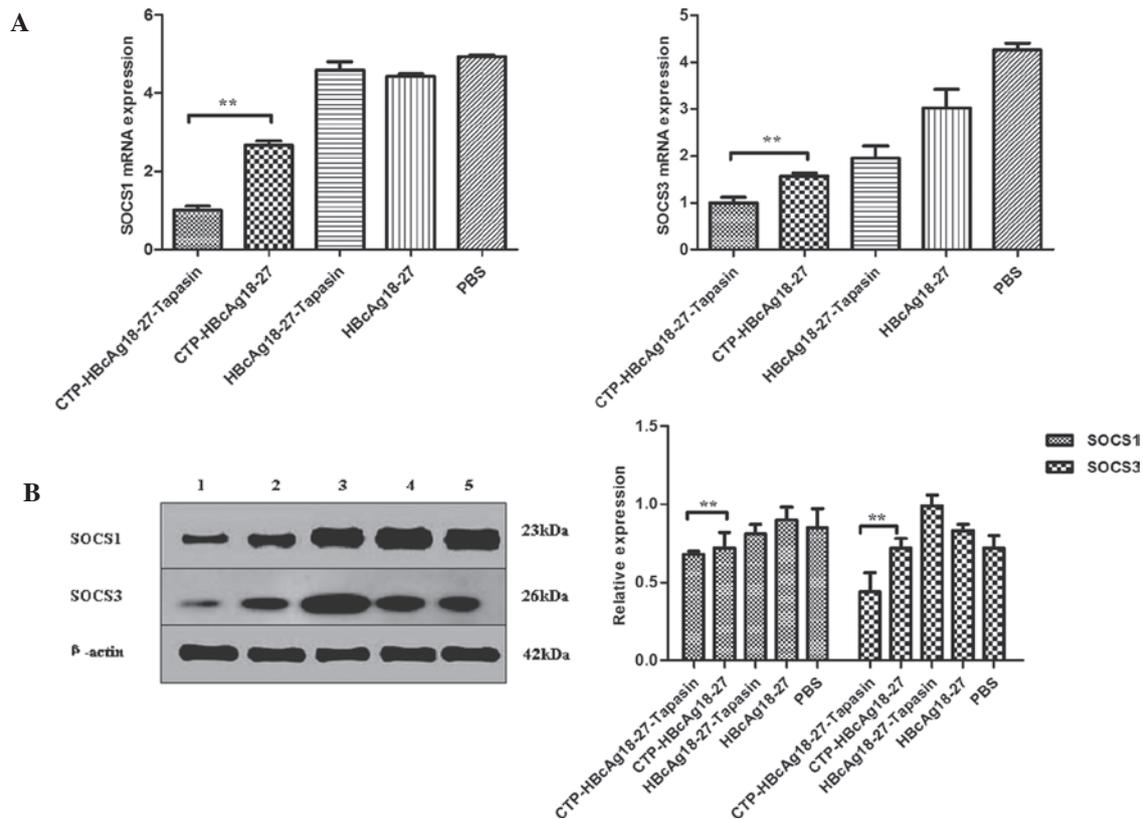


Figure 5. Expression of SOCS1 and SOCS3. (A) Expression of SOCS1 and SOCS3 mRNA was examined by real-time quantitative polymerase chain reaction. The expression of SOCS1 and SOCS3 mRNA was significantly downregulated in the CTP-HBcAg18-27-tapasin group compared with the PBS, CTP-HBcAg18-27, HBcAg18-27-tapasin and HBcAg18-27 groups. (B) Expression of SOCS1 and SOCS3 was assessed by western blot analysis. The expression of SOCS1 and SOCS3 was significantly downregulated in the CTP-HBcAg18-27-tapasin group compared with the PBS, CTP-HBcAg18-27, HBcAg18-27-tapasin and HBcAg18-27 groups. 1, CTP-HBcAg18-27-tapasin; 2, CTP-HBcAg18-27; 3, HBcAg18-27-tapasin; 4, HBcAg18-27; 5, PBS. Data are presented as the mean  $\pm$  standard deviation (n=6; \*P<0.05, \*\*P<0.01). SOCS, suppressors of cytokine signaling; CTP, cytoplasmic transduction peptide; HBcAg18-27-tapasin, HBV core 18-27 peptide-tapasin; PBS, phosphate-buffered saline.

decreased markedly compared with CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 or PBS (P<0.01). To further confirm the *in vivo* anti-HBV activity of fusion proteins in transgenic mice, immunohistological analysis was performed in livers from the various treatment groups. A large amount of HBsAg and HBcAg was detected (stained brownish yellow) in the cytoplasm of hepatocytes in mice treated with CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 or PBS. CTP-HBcAg18-27-tapasin immunization not only reduced the HBsAg and HBcAg levels, but also reduced the HBsAg and HBcAg expression in liver tissue (Fig. 4C and D).

*CTP-HBcAg18-27-tapasin enhances the Th1/Th2 cytokine ratio and antiviral immunity by targeting SOCS1/SOCS3.* To investigate whether the delivery of tapasin via CTP-HBcAg18-27 enhances specific immune responses and inhibits hepatitis B virus replication in transgenic mice through targeting SOCS1/SOCS3, the SOCS1 and SOCS3 expression in different groups was analyzed *in vitro*. The expression of SOCS1 and SOCS3 mRNA was detected by real-time PCR and the proteins were detected by western blot analysis. The expression of SOCS1 and SOCS3 was significantly downregulated in the CTP-HBcAg18-27-tapasin group compared with the CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 and PBS groups (Fig. 5).

## Discussion

Persistent HBV infection is commonly considered to be due to an inadequate host immune response. It is generally acknowledged that the cellular immune response contributes to viral clearance, particularly T-cell immunity to HBV (18). The correlation between viral spread and CD4<sup>+</sup>T cell priming determines the outcome of HBV infection (19). CD4<sup>+</sup>T cells are classified into two types of T helper cells depending on the activation of a certain antigen: Th1 and Th2. These cells differ in their pattern of secreted cytokines. Th1 cells secrete IFN- $\gamma$  and IL-2, which aid in the clearance of intracellular pathogens, while Th2 cells secrete IL-4 and IL-10, which alleviate extracellular infections (20,21). Subsequently, Th1-type (IFN- $\gamma$  and IL-2) and Th2-type (IL-4 and IL-10) cytokines were assessed as an index of the Th1/Th2 immune balance. The levels of IFN- $\gamma$  and IL-2 were significantly increased in mice immunized with CTP-HBcAg18-27-tapasin, while there was no significant difference in the production of cytokines IL-4 and IL-10 (Th2-like) in mice immunized with all the fusion proteins or PBS. Previous studies by our group showed that the expressed and purified fusion protein CTP-HBcAg18-27-tapasin was able to promote the maturation of BMDCs, increase IL-12p70 production and enhance cellular immune responses (5,6). Certain studies have demonstrated that the complete response to antiviral treatment predominantly correlated with Th1

responses accompanied with enhanced CTL activity in patients with chronic hepatitis B (22), implying that activation of Th1 immunity may be important for the successful treatment of HBV infection (23,24). There are several signaling pathways that are required for Th1 cell differentiation (25). IFN- $\gamma$  signaling activates signal transducer and activator of transcription protein 1 (STAT1) and reinforces the Th1 phenotype in a positive feedback loop (26,27). IL-12 signaling induces STAT4, which positively regulates numerous aspects of the Th1 genetic program. STAT1 and 4 also contribute to the regulation of T-box transcription factor Tbx21, (the gene that encodes T-bet) expression (28,29). Thus, CTP-HBcAg18-27-tapasin may increase IL-12p70 and IFN $\gamma$  production, which may mediate the IL-12/STAT4 and IFN $\gamma$ /STAT1 signaling pathways. These are required for Th1 cell differentiation and indirectly mediate CTL activity. This indicated that the effect of the molecular chaperone tapasin on intracellular antigen peptides via CTP transduction is able to mediate cellular immune responses by promoting dendritic cell maturation and the secretion of the cytokines IFN $\gamma$  and IL-2.

Furthermore, naïve T cells differentiate toward different T cell subtypes based on the expression of certain transcription factors. T-bet, a member of the T-box family of transcription factors, has been demonstrated to be involved in polarization toward Th1 cells, while GATA-3 has been demonstrated to be involved in Th2 differentiation (30,31). To further confirm the association between Th1 and Th2 in HBV transgenic mice, real-time PCR and western blot analysis were performed on T cells to assess the expression of T-bet and GATA-3 in the various treatment groups. The results revealed that the expression of T-bet was significantly upregulated in the CTP-HBcAg18-27-tapasin group compared with the CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 and PBS groups. However, in the CTP-HBcAg18-27-tapasin group, the expression of GATA-3 was lower than in the control groups. These findings suggested that CTP-HBcAg18-27-tapasin may be important in the Th1/Th2 imbalance in HBV transgenic mice by regulating the expression of the transcription factors T-bet and GATA-3, which are able to affect Th1-type and Th2-type cytokine production, as demonstrated in previous studies (17).

We evaluated whether CTP-HBcAg18-27-tapasin immunization was able to reduce HBsAg expression and the viral load in the serum of HBV transgenic mice. The results indicated that CTP-HBcAg18-27-tapasin immunization more efficiently suppresses the expression of serum HBsAg and HBV DNA than CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 or PBS in HBV transgenic mice. To further confirm the *in vivo* anti-HBV activity of fusion proteins in transgenic mice, immunohistological analysis was performed in livers from the various treatment groups. A large number of HBsAg and HBcAg were detected (stained brownish yellow) in the cytoplasm of hepatocytes in mice in the control groups. However, HBsAg and HBcAg expression was nearly undetectable with CTP-HBcAg18-27-tapasin treatment.

Thus, the HBcAg18-27-tapasin fusion protein enhances the Th1/Th2 cytokine ratio and antiviral immunity in transgenic mice; however, the mechanisms involved are likely to be complex. SOCS are members of a family of intracellular proteins that have emerged as key physiological regulators of

cytokine-mediated homeostasis, including innate and adaptive immunity. Signal downregulation through SOCS members has been demonstrated to be important in the balance of cytokines that determine the onset of Th1 and Th2-mediated immune responses (32,33). In the present study, the expression of SOCS1 and SOCS3 in T cells was significantly reduced in the mice immunized with CTP-HBcAg18-27-tapasin compared with CTP-HBcAg18-27, HBcAg18-27-tapasin, HBcAg18-27 or PBS. CTP-HBcAg18-27-tapasin may thus be important in the secretion of Th1-type and Th2-type cytokines in HBV transgenic mice by targeting SOCS1 and SOCS3, which are able to affect the Th1/Th2 balance. In conclusion, the present study demonstrated that vaccination with soluble CTP-HBcAg18-27-tapasin fusion protein was able to enhance the Th1/Th2 cytokine ratio and antiviral immunity by suppressing SOCS1/SOCS3 in HBV transgenic mice, which contributed to HBV clearance.

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