N-Acetylglucosaminyltransferase V exacerbates concanavalin A-induced hepatitis in mice

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Abstract. *N*-Acetylglucosaminyltransferase V (GnT-V) catalyzes β1-6 branching in asparagine-linked oligosaccharides and is one of the most important glycosyltransferases involved in carcinogenesis, cancer metastasis and immunity. To investigate the biological functions of GnT-V, the present study developed GnT-V transgenic (Tg) mice and the role of GnT-V in experimental immune-mediated hepatitis, induced by concanavalin A (ConA), were investigated. It was found that the aberrant expression of GnT-V exacerbated ConA-induced hepatitis in the Tg mice compared with the wild-type (WT) mice. The survival rate of the ConA-induced hepatitis at a high-dose of ConA was significantly lower in the Tg mice. Intravenously injected ConA is known to initially bind predominantly to the mannose gland of the liver sinusoidal endothelial cell (LSEC) surface and to leads to the activation of various immune cells. In the present study, the binding affinity of ConA to the LSECs did not differ between the WT and Tg mice. In addition, T cell receptor stimulation by anti-cluster of differentiation (CD)3/CD28 antibodies produced lower levels of T helper (Th)1 cytokine (interferon-γ) and higher levels of Th2 cytokine (interleukin-10) in the Tg mouse splenic lymphocytes compared with WT mice. The composition of the hepatic mononuclear cells revealed that CD11b-positive cells were significantly increased in the GnT-V Tg mice. In addition, F4/80-positive cells were significantly increased in the Tg mouse liver and the depletion of macrophages reduced the difference in the severity of ConA-induced hepatitis between the WT and Tg mice. In conclusion, the present findings indicated that the aberrant expression of GnT-V led to an increase in hepatic macrophage infiltration and enhanced ConA-induced hepatitis. Modulation of glycosylation may be a novel therapeutic target for immunity-associated acute hepatitis.

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Abbreviations: CCL2, C-C chemokine ligand 2; ConA, concanavalin A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GnT-V, *N*-acetylglucosaminyltransferase V; IFN-γ, interferon-γ; IL, interleukin; LSEC, liver sinusoidal endothelial cell; NK cell, natural killer cell; TCR, T cell receptor; TNF-α, tumor necrosis factor-α

Key words: glycosylation, *N*-glycan, macrophage, galectin-3, T cell receptor

Introduction

Concanavalin A (ConA) is a lectin that induces hepatitis through the modulation of various immune cells, including macrophages, T cells, natural killer (NK) cells and liver sinusoidal endothelial cells (LSECs) (1-4). Initially, intravenously injected ConA binds predominantly to the mannose gland of the LSEC surface and subsequently leads to the breakdown of the LSECs (5-7). Damaged LSECs produce inflammatory cytokines and chemokines, and LSEC detachment facilitates the binding of ConA to macrophages. T cells recognize the ConA-modified major histocompatibility complex class II and T cell receptor (TCR) of macrophages and are subsequently activated (4,8,9). In addition, these immune cells produce various inflammatory cytokines and chemokines, including interferon-γ (IFN-γ) (10,11), interleukin-2 (IL)-2 (11), IL-4 (12), IL-6 (12), C-C chemokine ligand 2 (CCL2) (13) and CXC-motif chemokine ligand 12 (9). Anti-inflammatory cytokines, including IL-10, are also involved in ConA-induced hepatitis. Among these biologically active substances, IL-10 has been reported to protect the liver from ConA-induced hepatitis (14,15).

Previous findings in glycobiology have provided direct evidence of the involvement of oligosaccharide changes in human diseases (16). Oligosaccharide modification of glycoproteins is predominantly divided into two types: *N*-glycans, attached to the asparagine residues, and *O*-glycans, attached to the serine/threonine residues (17) The branching formation on *N*-glycans is one of the most important factors regulating

the biological functions of oligosaccharides and terminal modifications, including sialylation and fucosylation (17). The branching formation on *N*-glycans is regulated by several types of *N*-acetylglucosaminyltransferases and the upregulation or downregulation of these glycosyltransferases may modify the biological functions of adhesion molecules and the signaling pathways of several growth factor receptors (18).

N-Acetylglucosaminyltransferase V (GnT-V) is involved in the synthesis of β1-6 GlcNAc branching formation on *N*-glycans (19). It is well-established that GnT-V is one of the most important glycosyltransferases involved in cancer metastasis (20), promoting cancer metastasis through the enhancement of growth factor signaling, integrin function and the expression of certain types of proteases (19-21). GnT-V also has important functions in the immune system. Deficiency of GnT-V in mice leads to an autoimmune disease phenotype and GnT-V-induced TCR oligosaccharide modification suppresses TCR signaling (22,23). These findings indicate that GnT-V decreases inflammatory responses through suppression of T cell activation. While the expression of GnT-V is low in the normal liver, it is increased during the progression of chronic disease and liver regeneration (24,25). Our previous study indicated that the expression of GnT-V in the normal mouse liver is higher in hepatic non-parenchymal cells, including immune cells, compared with hepatocytes (26). These findings indicate that GnT-V is important in the progression of liver diseases.

Considering these findings, GnT-V is expected to be important in the progression of liver disease through modulation of the immune system. However, the significance of changes in GnT-V-induced glycosylation in liver diseases remains to be elucidated. To address this issue, the present study investigated the role of GnT-V in experimental immune hepatitis using a mouse ConA hepatitis model.

Materials and methods

Mice. GnT-V (*Mgat5*) transgenic (Tg) mice (β-actin promoter; C57BL/6J background) were produced, as previously described (27). In the present study, wild type (WT) litter-mates were used as control mice. The animals were provided with unrestricted access to food and water, housed in temperature- and humidity-controlled rooms and maintained in a 12/12 h light/dark cycle. All experiments were performed using 8-12 week old male mice. At the end of each experimental period, blood was drawn aseptically from the inferior vena cava and centrifuged at 13,000 x g for 5 min at 4°C to collect the serum. Mice were anesthetized via intraperitoneal injection of pentobarbital (50 mg/kg), and the livers were subsequently removed and fixed with 10% buffered paraformaldehyde (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), flash frozen in liquid nitrogen for protein and mRNA extraction was performed as described previously (26). All experimental procedures described in the present study were approved by the Ethics Review Committee for Animal Experimentation of the Osaka University School of Medicine (Osaka, Japan).

ConA-induced hepatitis. ConA (Sigma-Aldrich, St. Louis, MO, USA) at 12.5 mg/kg body weight (BW) was dissolved in 200 µl phosphate-buffered saline (PBS; Sigma-Aldrich) and injected into WT and GnT-V Tg mice through the tail vein. Serum alanine aminotransferase (ALT) concentrations were measured using a Transaminase CII-test Wako kit (Wako Pure Chemical Industries, Tokyo, Japan). To examine the survival rate of the rats, a relatively high dose of ConA (20 mg/kg/BW) was injected intravenously.

Hematoxylin and eosin (H&E), terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL) and immunohistochemical staining*. Liver sections were stained with H&E, Apop Tag® Peroxidase *in Situ* Apoptosis Detection kit, according to the manufacturer's instructions (Merck Millipore, Darmstadt, Germany), or monoclonal human anti-F4/80 antibody (1:100; HCA154; Bio-Rad AbD Serotec, Oxford, UK).

Cytokine analysis. Splenic lymphocytes were collected, as previously described (28). The cells (1×10^6 cells) were cultured in flat-bottom 96-well culture plates for 48 h in RPMI-1640 (Sigma-Aldrich) with 10% fetal bovine serum (Sigma-Aldrich) and antibiotics/antimycotics in the presence of anti-mouse cluster of differentiation (CD)3 (5 µg/ml) and anti-mouse CD28 antibodies (5 µg/ml; BD Biosciences, San Jose, CA, USA). The culture supernatant was collected and production of the IFN-γ and IL-10 cytokines were determined by enzyme-linked immunosorbent assay (ELISA; eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The levels are expressed as the mean ± standard deviation of 1×10^6 cells. These cells (3×10^6 cells) were also cultured in flat-bottom 96-well culture plates for 24 h in RPMI-1640 with 10% fetal bovine serum and antibiotics/antimycotics in the presence of ConA (5 µg/ml). The culture supernatant was collected and the production of IFN-γ and IL-10 cytokines was determined by ELISA (eBioscience), according to the manufacturer's instructions. The levels are expressed as the mean ± standard deviation of 3×10^6 cells.

Quantification of gene expression levels. Total RNA was extracted from cells with a QIAshredder and an RNeasy Mini kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany) and transcribed into complementary DNA with a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Reverse transcription quantitative polymerase chain reaction was performed with a Thunderbird SYBR qPCR mix (Toyobo) using specific primers on a LightCycler according to the instructions provided by the manufacturer (Roche Diagnostics, Indianapolis, IN, USA). The cycling conditions were as follows: 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The primers used were as follows: *Ifn-γ* (cat. no. QT01038821), *Il-10* (cat. no. QT00106169), *Ccl2* (cat no. QT01747165), *Ccl5* (cat. no. QT00167832) and *18s* rRNA (cat. no. QT01036875; Qiagen). The primers for *T-bet*, *Gata-3* and *Galectin-3* were purchased from Sigma-Aldrich and the sequences were as follows: *T-bet*, sense 5'-GCC AGG GAA CCG CTT ATA TG-3' and antisense 5'-GAC GAT CAT CTG GGT CAC ATT GT-3'; *Gata-3*, sense 5'-TTA TCA AGC CCA AGC GAA G-3' and antisense 5'-TGG TGG TGG TCT GAC AGT TC-3' and *Galectin-3*, sense 5'-CAG GAT TGT TCT AGA TTT CAG G-3' and antisense 5'-TTG TCC TGC TTC

GTG TTA CAC-3'. The mRNA expression levels were normalized to the mRNA expression level of *18s* and expressed in arbitrary units.

Isolation of mouse LSECs. LSECs were isolated from WT and GnT-V Tg mice by performing a two-step collagenase-pronase perfusion of their livers, as described previously (29). Briefly, the livers were perfused for 3 min at room temperature at a flow rate of 4 ml/min with SC-1 solution containing 8,000 mg/l NaCl, 400 mg/l KCl, 78 mg/l NaH_2PO_4 $2H_2O$, 151 mg/l Na_2HPO_4 $12H_2O$, 2380 mg/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 350 mg/l $NaHCO_3$, 190 mg/l ethylene glycol tetraacetic acid and 900 mg/l glucose (pH 7.2), followed by digestion at 37°C for 3 min with 0.053% pronase and 0.027% collagenase dissolved in an SC-2 solution containing 8,000 mg/l NaCl, 400 mg/l KCl, 78 mg/l NaH_2PO_4 $2H_2O$, 151 mg/l Na_2HPO_4 $12H_2O$, 2,380 mg/l HEPES, 350 mg/l $NaHCO_3$ and 735 mg/l $CaCl_2$ $2H_2O$ (pH 7.5). Each digested liver was excised and cut into 2-mm sections. The resulting suspension was filtered through a 100 μm cell strainer and centrifuged at 50 x g for 1 min at 4°C to remove hepatocytes. This protocol was repeated three times and the supernatants were centrifuged at 300 x g for 10 min at 4°C. The pellet was washed and suspended in Dulbecco's modified Eagle's medium twice. Non-parenchymal cells were further separated from parenchymal cells by density-gradient centrifugation at 1,500 x g for 20 min at 4°C on a 30% Histodenz cushion (Sigma-Aldrich). The LSECs were then isolated by magnetic cell sorting using magnetic beads (MACS; Miltenyi Biotec, Gladbach, Germany) with rat anti-mouse CD31 antibody (11-0311-85; eBioscience), according to the manufacturer's instructions. The purity of the MACS-enriched LSECs was assessed by flow cytometric analysis with rat anti-mouse CD31 antibody (11-0311-85; eBioscience) using a FACS Canto II (BD Biosciences).

Western and lectin blot analyses. Immunoblotting was performed, as described previously (26). Briefly, ($2x10^6$) isolated mouse LSECs were lysed with 1% Triton X-100 and ~10 mg frozen liver tissue was then lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS). The samples were then subjected to heat denaturation at 98°C for 5 min, separated with SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was then blocked with either 5% skimmed milk for western blotting or 3% bovine serum albumin for lectin blotting. The following antibodies were used for immunodetection: Rabbit polyclonal anti-GnT-V (24D11; 1:3,000; Fujirebio, Tokyo, Japan), leukoagglutinating phytohemagglutinin (L_4-PHA) lectin (1:1,000; cat. no. J112; J-Oil Mills, Inc., Tokyo, Japan), ConA lectin (1:1,000; cat. no. J103; J-Oil Mills, Inc.) and rabbit polyclonal anti-GAPDH (1:3,000; cat. no. 2275-PC-1; Trevigen, Gaithersburg, MD, USA). Immunoreactive bands were visualized on an GE Healthcare film using Amersham Enhanced Chemiluminscence Western Blotting Detection reagents (GE Healthcare, Waukesha, WI, USA).

Isolation of mouse hepatocytes and liver mononuclear cells (MNCs). Mouse hepatocytes and MNCs from the liver were prepared, as previously described (30). Briefly, the mice were anesthetized and their abdomens were opened. The inferior vena cava and portal vein were cut to enable blood outflow. The liver was removed and gently passed through a stainless steel mesh. The liver cell suspension was collected and the hepatocytes were separated from the MNCs by centrifugation at 50 x g for 1 min. This procedure was repeated three times and the supernatants were centrifuged at 150 x g for 7 min at 4°C. The MNC populations were purified by centrifugation through a Percoll gradient. The cells were collected, washed in PBS and resuspended in 40% Percoll (Sigma-Aldrich). The cell suspension was gently overlaid onto 70% Percoll (Sigma-Aldrich) and centrifuged for 15 min at 1,400 x g. The MNCs were collected from the interface and were washed twice in PBS for use in subsequent analysis.

Flow cytometric analysis of hepatic MNCs and LSECs. ConA (12.5 mg/kg/BW; Sigma-Aldrich) dissolved in 200 μl PBS was injected into the WT and GnT-V Tg mice through the tail vein. After 2 h, the liver MNCs were isolated, as described above. The liver MNCs, stained with monoclonal rat anti-mouse CD4 (4800041-82), rat anti-mouse CD8α (11-0081-82), mouse anti-mouse CD19 (17-0191-829, rat anti-mouse CD11b (12-0112-82), hamster anti-mouse CD11c (48-0114-82), and mouse anti-mouse NK1.1 (17-5941-82) antibodies (eBioscience) and the LSECs, stained with anti-CD31 antibody, fluorescein isothiocyanate-labeled L_4-PHA (J512) and ConA (J503) (J-Oil Mills Inc.) were subjected to flow cytometric analysis using a FACS Canto II™ (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software version 7.6.1 (TreeStar Inc., Ashland, OR, USA). The detailed procedure has been described previously (31).

Depletion of macrophages. The suicidal liposome technique has been used previously to deplete macrophages (32). Clodronate-liposomes were purchased from Clodronate Liposomes, (VUmc FdG, Amsterdam, Netherlands). Briefly, the mice were injected with 200 μl clodronate-liposomes through the tail vein. The injection was performed 2 days prior to the ConA administration.

Statistical analysis. Statistical analysis was performed using JMP Pro 10.0 software (SAS Institute Inc., Cary, NC, USA). Kaplan-Meier curves were used to demonstrate the survival rates. The results are expressed as the mean ± standard deviation. Groups of data were compared by the Wilcoxon test for non-parametric data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ConA-induced hepatic injury is exacerbated in GnT-V Tg mice compared with WT mice in vivo. ConA (12.5 mg/kg/BW) was injected intravenously into WT and GnT-V Tg mice. Histological analyses of the liver tissue sections indicated that the GnT-V Tg mice were more sensitive to ConA-induced hepatic injury (Fig. 1A). Liver tissue sections in the GnT-V Tg mice exhibited more widespread necrotic areas compared with the WT mice. In addition, liver tissue sections from the GnT-V Tg mice exhibited increased numbers of
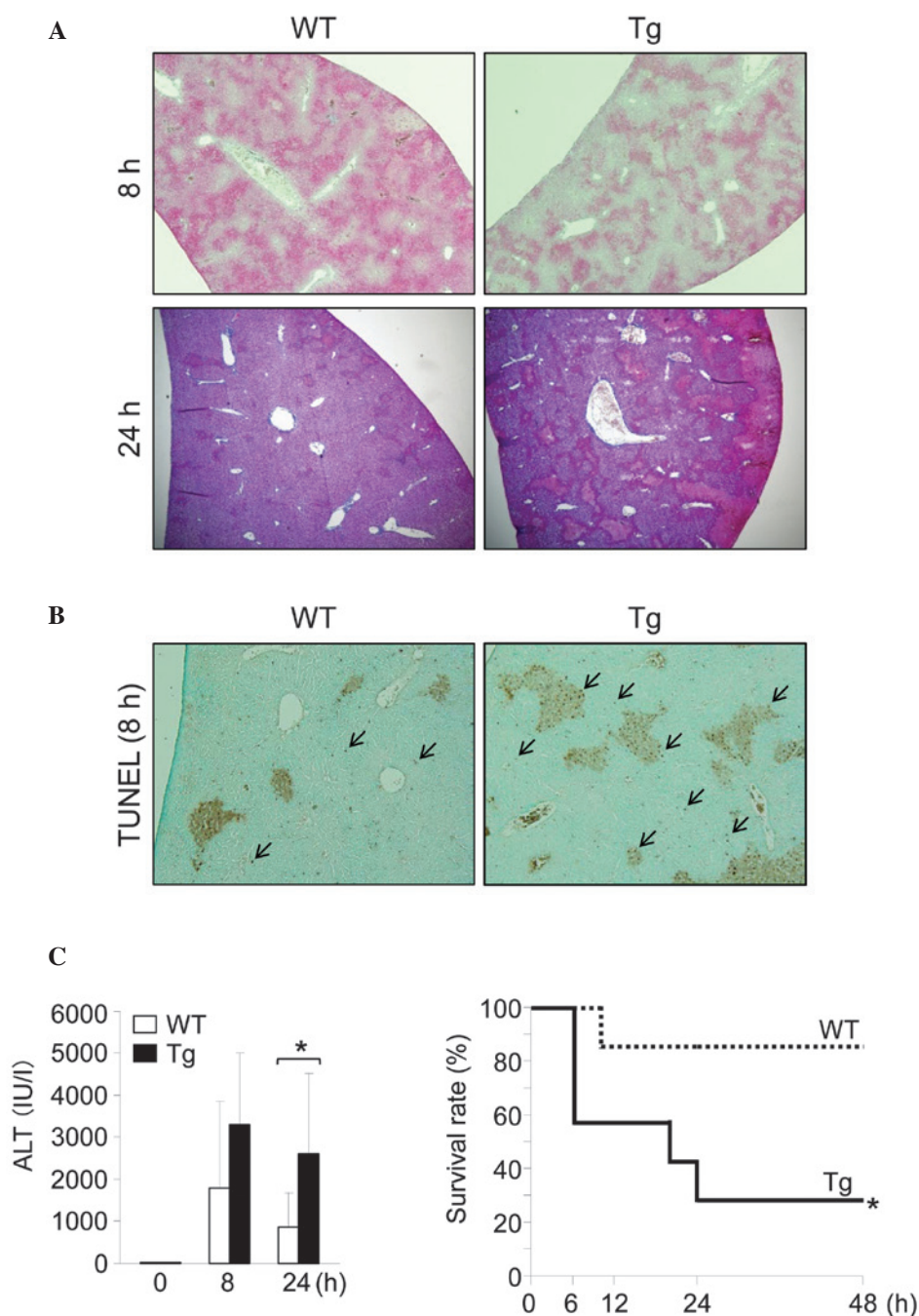
Figure 1. GnT-V Tg mice are sensitive to ConA-induced hepatic injury. (A) Photomicrographs of representative hematoxylin and eosin-stained mouse livers 8 or 24 h after the injection of ConA at 12.5 mg/kg BW (magnification, x40). (B) Photomicrographs of representative TUNEL-stained mouse livers 8 h after the injection of ConA at 12.5 mg/kg BW. Arrows indicate apoptotic hepatocytes (magnification, x40). (C) Serum ALT levels in mice 0 (n=3), 8 (n=8-9) and 24 (n=7-8) h after the injection of ConA at 12.5 mg/kg BW. Results are expressed as the mean ± standard deviation; *P<0.05. The survival rate following ConA injection. Mice were injected intravenously with ConA at 20 mg/kg BW and followed for 48 h (n=7); *P<0.05. GnT-V, *N*-acetylglucosaminyltransferase V; WT, wild-type; Tg, transgenic; ConA, concanavalin A; BW, body weight; ALT, alanine aminotransferase TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

TUNEL-positive cells (Fig. 1B). The serum ALT levels 24 h after ConA injection were significantly higher in the GnT-V Tg mice compared with those in the WT mice (Fig. 1C). To compare the survival rate of each mouse following ConA administration, a relatively high dose of ConA (20 mg/kg/BW) was injected into each mouse. A significantly higher mortality rate was identified in the GnT-V Tg mice compared with the WT mice (Fig. 1D). In total, >40% of the GnT-V Tg mice succumbed to mortality within 6 h following ConA

injection, with a survival rate of 14% observed 48 h after injection. By contrast, a survival rate of 86% was observed in the WT mice after 48 h.

Subsequently, the gene expression of inflammation-associated cytokines and transcription factors in the mouse livers were investigated. No significant differences were identified in the expression levels of the Th1-associated (*Ifn-γ* and *T-bet*) or Th2-associated (*Il-10* and *Gata-3*) genes in the mouse livers prior to or following injection of ConA (Fig. 2A). The serum
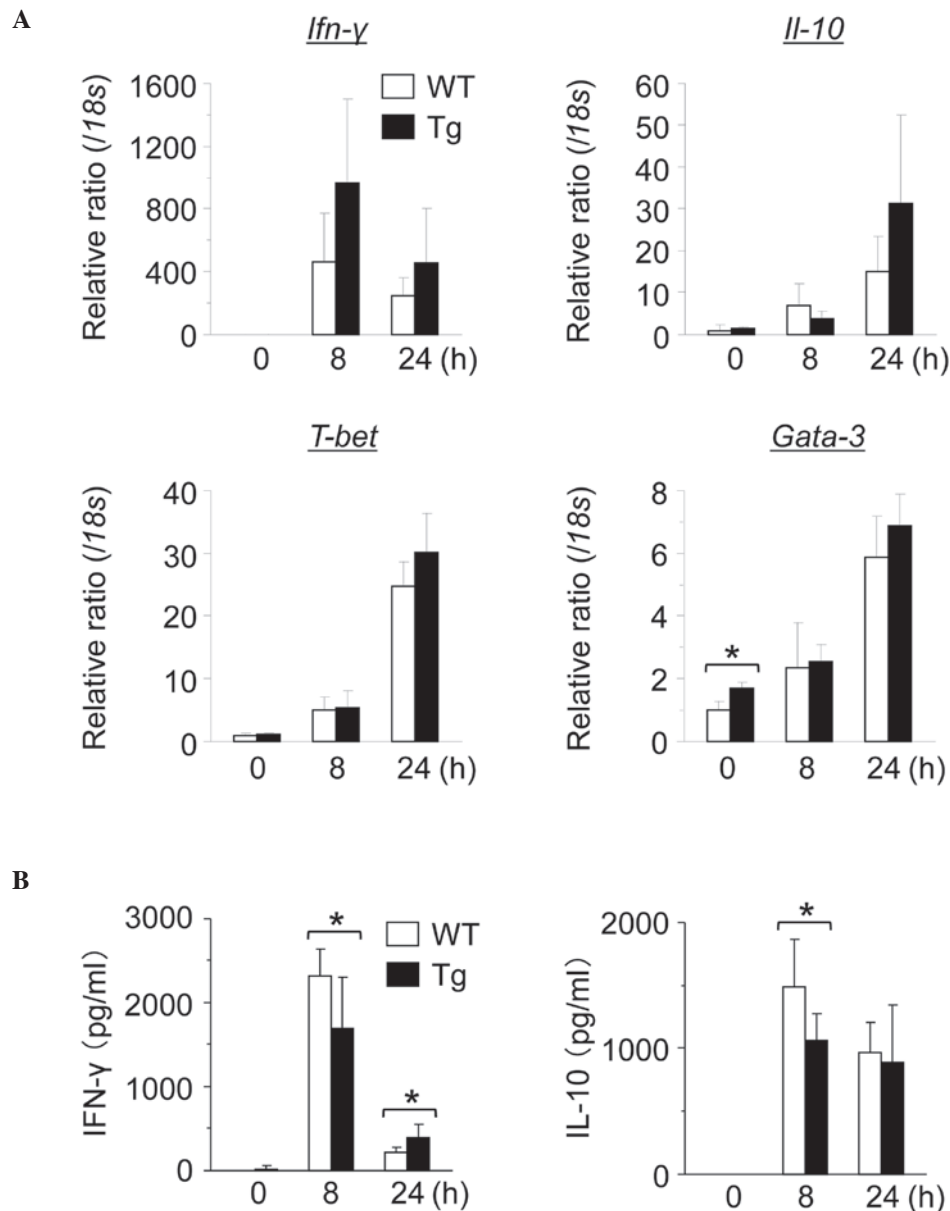
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Figure 2. Expression of *in vivo*-activated cytokines demonstrates no significant changes. Hepatic gene expression in WT and GnT-V Tg mice 0, 8, or 24 h after the injection of ConA at 12.5 mg/kg BW. Gene expression of (A) *Ifn-γ, Il-10, T-bet* and *Gata-3* in mouse livers was evaluated using reverse transcription quantitative polymerase chain reaction. The mRNA expression levels were normalized relative to the mRNA expression of *18s* and are expressed in arbitrary units. The results are expressed as the mean ± standard deviation; *P<0.05. (B) Serum cytokine levels in the WT and GnT-V Tg mice 0, 8, or 24 h after the injection of ConA at 12.5 mg/kg BW. Serum levels of IFN-γ and IL-10 were measured by ELISA at the indicated time points. Results are expressed as the mean ± standard deviation; *P<0.05. GnT-V, *N*-acetylglucosaminyltransferase V; WT, wild-type; Tg, transgenic; ConA, concanavalin A; BW, body weight; Ifn, interferon; IL, interleukin.

levels of IFN-γ and IL-10 in the GnT-V Tg mice 8 h after the injection of ConA were significantly lower compared with those in the WT mice (Fig. 2B).

Th1 to Th2 cytokine shift in GnT-V Tg mouse splenic lymphocytes in vitro. T cells are important in ConA-induced hepatitis (1,4,5). To investigate function of splenic lymphocytes from the WT and GnT-V Tg mice in cytokine production, the mouse splenic lymphocytes were stimulated with anti-CD3/CD28 antibodies *in vitro*. Levels of the IFN-γ Th1 cytokine were significantly lower in the GnT-V Tg compared with the WT mouse splenic lymphocytes (Fig. 3A), whereas levels of the IL-10 Th2 cytokine were higher in the

GnT-V Tg mouse splenic lymphocytes compared with the WT lymphocytes.

ConA activates lymphocyte function, including cytokine production (1,4,5). Subsequently, mouse splenic lymphocytes were stimulated with ConA *in vitro*. As was observed following stimulation with the anti-CD3/CD28 antibodies, the levels of gene expression and the production of IFN-γ in the GnT-V Tg mouse splenic lymphocytes were lower compared with those in the WT, whereas the levels of IL-10 were higher (Fig. 3B and C). IFN-γ is considered a typical pro-inflammatory cytokine (10,11) and IL-10 is known to protect the liver from ConA-induced hepatitis (14,15). Therefore, the exacerbated ConA-induced
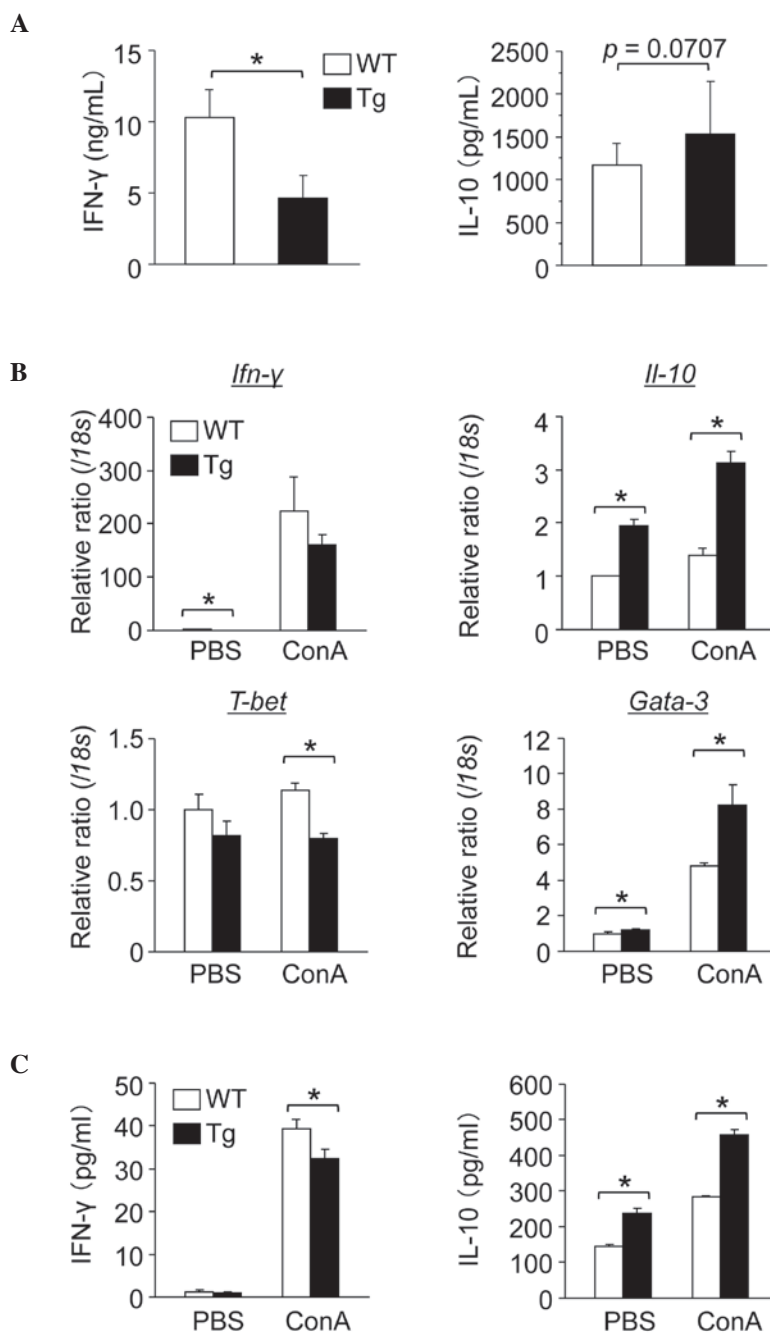
Figure 3. GnT-V Tg mouse splenic lymphocytes produce increased Th2 cytokines. (A) Anti-CD3/CD28 antibody-induced cytokine production levels secreted into the medium from splenic lymphocytes. Mononuclear cells of the spleen, isolated from WT and GnT-V Tg mice, were cultured for 48 h in the presence of anti-CD3 (5 µg/ml) and anti-CD28 (5 µg/ml) antibodies and levels of IFN-γ and IL-10 were measured using ELISA. The results are expressed as the mean ± standard deviation (n=3-8); *P<0.05. (B) Gene expression of cytokinines in the splenic lymphocytes following ConA stimulation is shown. Splenic lymphocytes from WT and GnT-V Tg mice were cultured for 24 h in the presence of ConA (5 µg/ml) and the gene expression levels of *Ifn-γ, Il-10, T-bet* and *Gata-3* were measured by reverse transcription quantitative polymerase chain reaction. The mRNA expression levels were normalized relative to that of *18s* and are expressed in arbitrary units. The results are expressed as the mean ± standard deviation (n=3); *P<0.05. (C) ConA-induced cytokine production levels secreted into the medium from splenic lymphocytes. Splenic lymphocytes from WT and GnT-V Tg mice were cultured for 24 h in the presence of ConA (5 µg/ml) and levels of IFN-γ and IL-10 were measured using ELISA. Results are expressed as the mean ± standard deviation (n=3); *P<0.05. WT, wild-type mouse splenic lymphocytes; GnT-V, *N*-acetylglucosaminyltransferase V; Tg, GnT-V transgenic mouse splenic lymphocytes; ConA, concanavalin A; BW, body weight; Ifn, interferon; IL, interleukin; PBS, phosphate-buffered saline; Th, T helper; CD, cluster of differentiation.

hepatitis in the GnT-V Tg mice *in vivo* was not explained by these *in vitro* results.

No difference is observed in the binding affinity of ConA to LSECs between WT and GnT-V Tg mice. The binding of ConA to the mannose gland of the LSEC surface, which is followed by LSEC damage, recruits T lymphocytes from the sinusoidal circulation and is an early event in T cell-mediated liver injury (5). The subsequent loss of function of the LSEC barrier exposes the underlying hepatocytes to further attack from activated T lymphocytes (5-7). To examine the binding affinity of ConA to LSECs from the WT and the GnT-V Tg mice, ConA lectin blotting and flow cytometric analysis were performed. The purity of the isolated LSECs was >75% (Fig. 4A).
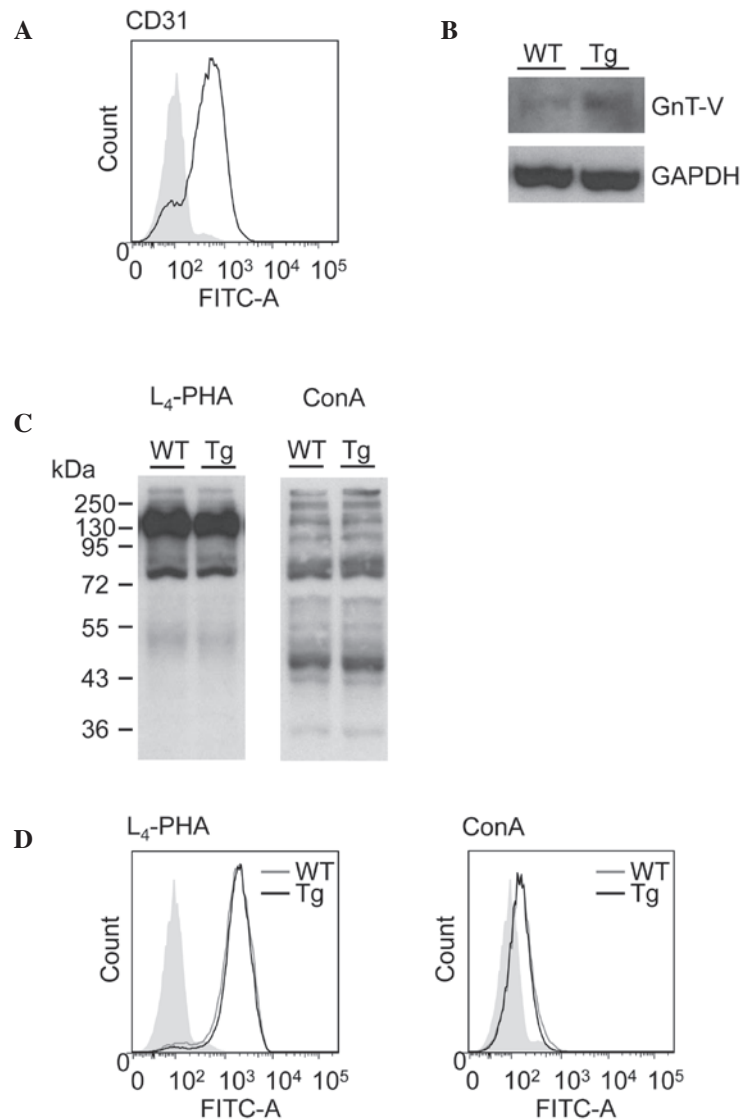
Figure 4. Binding affinity of ConA to LSECs does not differ between WT and GnT-V Tg mice. (A) Purity of the LSECs was analyzed by the expression of the cell surface marker, CD31. (B) Expression of GnT-V in LSECs was characterized by immunoblotting against GnT-V. Immunoblotting against GAPDH was performed as a loading control. (C) β1-6 N-acetylglucosamine branching (the product of GnT-V) and the binding affinity of ConA to LSECs were assessed by L_4-PHA and ConA by lectin blot analysis. (D) Results of the L_4-PHA and ConA lectin flow cytometric analysis. Data are representative of experiments repeated more than three times using three mice per experiment. WT, wild-type mouse LSECs; GnT-V, N-acetylglucosaminyltransferase V; Tg, GnT-V transgenic mouse LSECs; ConA, concanavalin A; LSEC, liver sinusoidal endothelial cell; CD, cluster of differentiation; L_4-PHA, leukoagglutinating phytohemagglutinin; FITC, fluorescein isothiocyanate.

To investigate the difference in the GnT-V and β1-6 GlcNAc branching of N-glycans expression in LSECs from the WT and GnT-V Tg mice, GnT-V immunoblotting, L_4-PHA lectin blotting and flow cytometric analysis were performed (Fig. 4B-D). L_4-PHA binds to the β1-6 GlcNAc branching of N-glycans, which is the product of GnT-V. Although the protein expression of GnT-V was increased in the GnT-V Tg mouse LSECs compared with the WT mouse LSECs (Fig. 4B), the expression of β1-6 GlcNAc branching of N-glycans, determined by L_4-PHA lectin blotting and flow cytometric analysis, did not differ between the WT and GnT-V Tg mouse LSECs (Fig. 4C, left panel and 4D, left panel). ConA lectin blotting and flow cytometric analysis also revealed no difference in the binding affinity of ConA to the LSECs between the WT and GnT-V Tg mouse LSECs (Fig. 4C and D).

Number of hepatic macrophages is significantly increased in GnT-V Tg compared with WT mice. Subsequently, the hepatic immune cells were examined and the liver MNC subset was investigated prior to or following the injection of ConA. Notably, the number of CD11b-positive cells was significantly increased in the GnT-V Tg liver compared with the WT mouse liver prior to and 2 h after ConA injection (Fig. 5A and B). In addition, the gene expression of the macrophage markers F4/80 and CD68 were significantly increased in the Tg mouse MNCs (Fig. 5C). F4/80 immunohistochemical staining of the livers also revealed that the number of hepatic F4/80-positive macrophages was significantly increased in the GnT-V Tg mice compared with the WT mice (Fig. 6A and B). These results indicated that the number of hepatic macrophages was higher in the GnT-V Tg mice than in the WT mice.
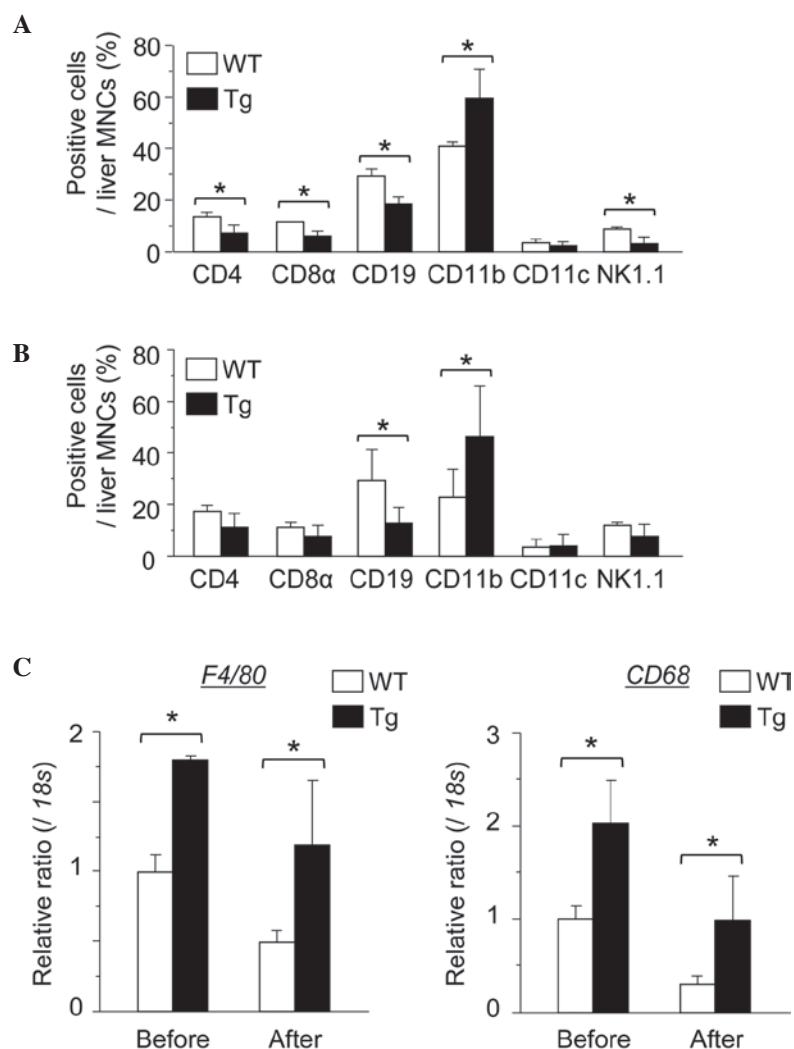
Figure 5. Hepatic macrophages are significantly increased in GnT-V Tg, vs. WT mice. Subset analyses of liver MNCs in the WT and GnT-V Tg mice (A) prior to and (B) 2 h after the injection of ConA at 12.5 mg/kg body weight were performed by flow cytometry. (C) Gene expression levels of the macrophage markers, F4/80 and CD68, in the MNCs before and 2 h after injection of ConA is shown. Results are expressed as the mean ± standard deviation (n=3); *P<0.05. WT, wild-type mouse liver MNCs; MNCs; ConA, concanavalin A; CD, cluster of differentiation; GnT-V, *N*-acetylglucosaminyltransferase V; Tg GnT-V, transgenic mouse liver; MNC, mononuclear cells.
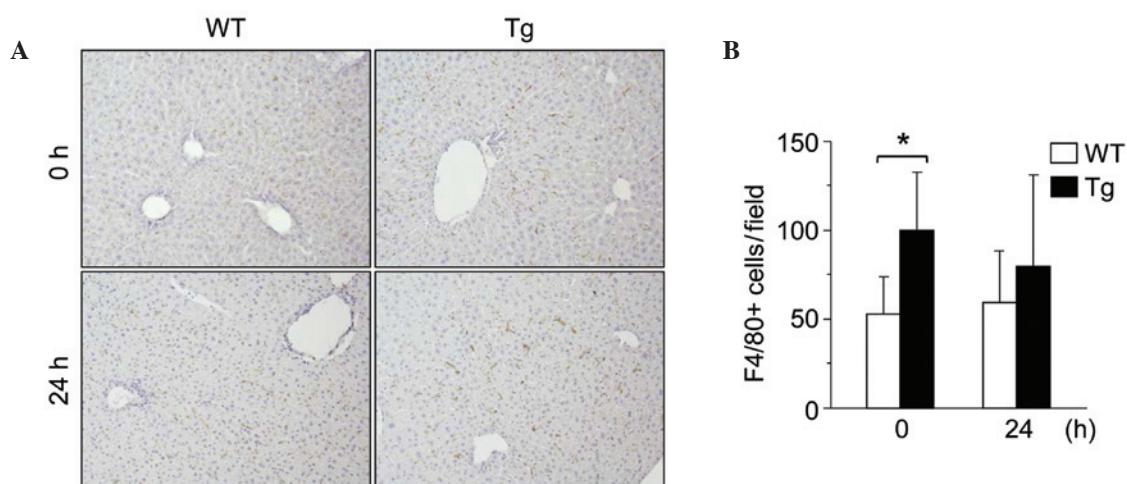

Figure 6. Number of hepatic F4/80-positive macrophages in GnT-V Tg mice is significantly increased compared with the number in WT mice. (A) Photomicrographs show representative mouse livers stained with anti-F4/80 antibody prior to (0 h) and 24 h after the injection of ConA at 12.5 mg/kg body weight (magnification, x200). (B) Quantification of the number of F4/80 positive cells/field is shown. Results are expressed as the mean ± standard deviation (0 h, n=9; 24 h, n=18); *P<0.05, compared with WT. WT, wild-type mouse liver; GnT-V, *N*-acetylglucosaminyltransferase V; Tg, GnT-V transgenic mouse liver; ConA, concanavalin A.
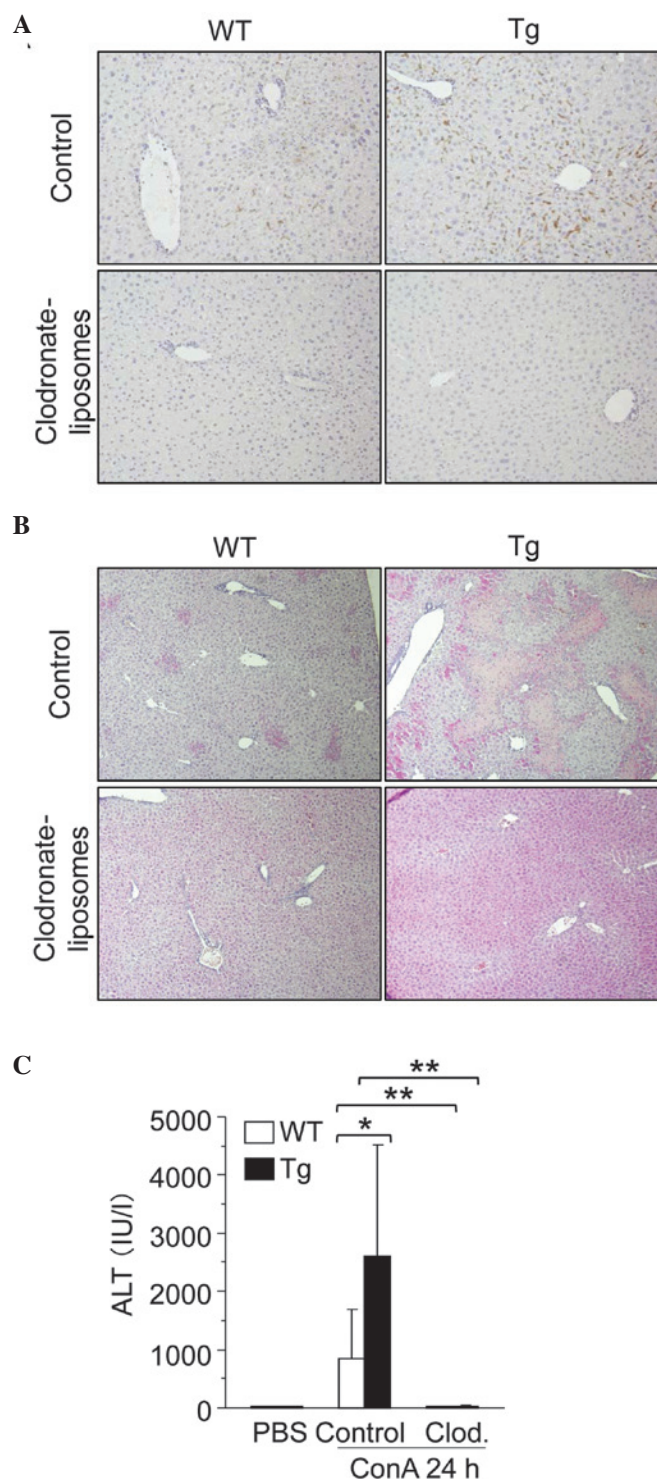
Figure 7. Hepatic macrophage depletion reduces the difference in the degree of hepatitis between WT and GnT-V Tg mice. (A) Representative photomicrographs mouse livers stained with anti-F4/80 antibody 24 h after the injection of ConA at 12.5 mg/kg BW (magnification, x200). Clodronate-liposomes were injected 2 days prior to ConA administration. (B) Representative photomicrographs of hematoxylin and eosin stained mouse livers 24 h after the injection of ConA at 12.5 mg/kg BW (magnification, x40). (C) Levels of serum ALT in mice (n=6) 24 h after the injection of ConA at 12.5 mg/kg BW. Results are expressed as the mean ± standard deviation; *P<0.05 and **P<0.01. WT, wild-type mice; GnT-V, N-acetylglucosaminyltransferase V; Tg, GnT-V transegenic mice; Clod, clodronate-liposomes; ALT, alanine aminotransferase; PBS, phosphate-buffered saline; ConA, concanavalin A; BW, body weight.

Depletion of hepatic macrophages reduces the degree of difference in ConA-induced hepatitis between WT and GnT-V Tg mice. To investigate the effect of hepatic macrophages in ConA-induced hepatitis in GnT-V Tg mice, macrophages were depleted from the WT and GnT-V Tg mice using clodronate-liposomes, and the severity of ConA-induced hepatitis was determined. The depletion of hepatic macrophages was confirmed by immunohistochemical staining with anti-F4/80 antibody (Fig. 7A). Macrophage depletion significantly suppressed ConA-induced liver injury in the WT and GnT-V Tg mice and it reduced the differences in liver injury between the WT and GnT-V Tg mice (Fig. 7B and C).
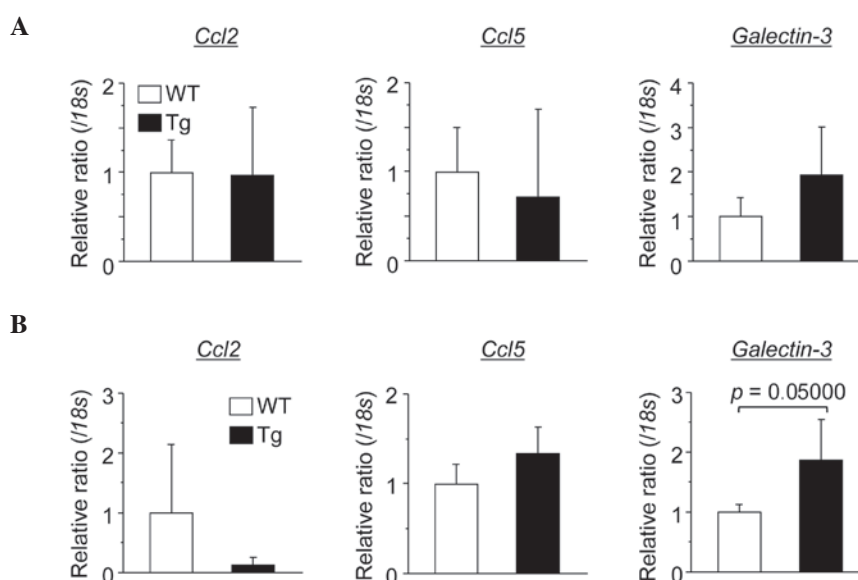
Figure 8. Gene expression of galectin-3 is elevated in Tg mouse hepatocytes. (A) Gene expression levels of macrophage chemoattractants in the mouse liver. (B) Gene expression levels of macrophage chemoattractants in the mouse hepatocytes. Results are expressed as the mean ± standard deviation. WT, wild-type mice; GnT-V, *N*-acetylglucosaminyltransferase V; Tg, GnT-V transgenic mice; Ccl, C-C chemokine ligand.

Expression of galectin-3 is elevated in Tg mouse hepatocytes. To elucidate the reason why the number of liver macrophages in the Tg mice was elevated compared with the number in the WT mice, the expression of macrophage chemoattractant genes (*Ccl2, Ccl5* and *Galectin-3*) were investigated in mouse hepatocytes (Fig. 8). The results demomnstrated that the gene expression of *galectin-3* was elevated in the Tg mouse hepatocytes compared with the WT mouse hepatocytes.

Discussion

In the present study, it was initially observed that ectopic expression of GnT-V exacerbated ConA-induced hepatitis despite the Th1 to Th2 cytokine shift observed in the GnT-V Tg mouse splenic lymphocytes. A relatively high dose of ConA induced a significantly higher mortality rate in the GnT-V Tg mice compared with the that in WT mice. The binding affinity of ConA to LSEC, which occurs in the first phase of ConA hepatitis, did not differ between the WT and GnT-V Tg mice. The results also revealed significant increases in hepatic macrophage infiltration in the GnT-V Tg mice liver compared with the WT mouse liver, prior to or following ConA injection. Notably, the gene expression of galectin-3, a hepatocyte and one of the major chemoattractants of macrophage, was increased in the Tg mice. These findings indicated that GnT-V-induced galectin-3 elevation recruited monocytes to the liver and resulted in an increased number of hepatic macrophages, ultimately leading to enhanced ConA-induced hepatitis in the Tg mice. The present study also observed that the depletion of macrophages inhibited and reduced the difference in the degree of ConA-induced hepatitis between the WT and GnT-V Tg mice. Considering these findings, the present study demonstrated that aberrant glycosylation, induced by GnT-V, increased hepatic macrophage infiltration and resulted in enhanced ConA-induced hepatitis.

In the liver, LSECs, Kupffer cells (hepatic macrophages), lymphocytes (T cells) and NK cells are involved in the immune response of ConA-induced hepatitis (5). Among these immune cells, T cells are critical in ConA-induced hepatitis (1,4,5). Therefore, to investigate the roles of GnT-V on T cell activation, splenic lymphocytes from WT and GnT-V Tg mice were stimulated with anti-CD3/CD28 antibodies or ConA *in vitro*. The GnT-V Tg mouse splenic lymphocytes produced lower levels of the IFN-γ Th1 cytokine and higher levels of the IL-10 Th2 cytokine compared with the WT mouse splenic lymphocytes. These findings were consistent with those of previous studies, which demonstrated that GnT-V-induced TCR oligosaccharide modification suppresses TCR signaling (22,23). However, these findings indicate that GnT-V tends to suppress inflammatory responses through suppression of T cell activation, which differ from the results of the present *in vivo* study.

ConA binds predominantly to LSECs within 15 min following intravenous injection. After 4 h, ConA begins to bind to hepatic macrophages (33), and activated lymphocytes are then trafficked towards hepatocytes, leading to inflammation (5,32). The present study hypothesized that the glycosylation differences between the LSECs of WT and GnT-V Tg mice may be important in the progression of ConA-induced hepatitis. To examine this hypothesis, differences in the lectin affinities of each mouse LSEC were investigated. It was found that ConA and L_4-PHA lectin bound equally to the WT and GnT-V Tg mouse LSECs, however, the expression of GnT-V was increased in the GnT-V Tg mice. Since levels of β1-6 GlcNAc branching are regulated by UDP-GlcNAc, a donor substrate of GnT-V (33), a change in the expression of GnT-V alone were insufficient to increase levels of β1-6 GlcNAc branching in the Tg mouse LSECs.

In response to these T cell and LSEC findings, the present study investigated hepatic macrophages as the main effector cell in the second phase of ConA-induced hepatitis (33,35). The hepatic macrophages were significantly increased in the

GnT-V Tg mice compared with the WT mice prior to and following ConA administration. It is understood that β1-6 GlcNAc branching extends repeated glycans of GlcNAc and galactose and results in a polylactosamine structure (36). Endogenous galectin-3 binds to this polylactosamine structure. Galectin-3 is a chemoattractant for monocytes and macrophages, which induces macrophage infiltration into the organs (35,37,38) and promotes inflammatory changes in various organs (35). Therefore, the polylactosamine structure induced in GnT-V Tg mice may increase the quantity of hepatic galectin-3. The present study also observed that the gene expression of galectin-3 was increased in the GnT-V Tg hepatocytes compared with the WT mouse hepatocytes. Therefore, GnT-V Tg mouse hepatocytes may produce higher quantities of galectin-3 than WT mouse hepatocytes. Increased hepatic galectin-3 in the GnT-V Tg mice may result in the elevated proportion of macrophages among the hepatic MNCs. In the present study, depletion of hepatic macrophages by clodronate-liposome infusion decreased the severity of ConA-induced hepatitis in the WT and GnT-V Tg mice and reduced the differences in liver injury between these mice. These results indicated that aberrant glycosylation by GnT-V elevated hepatic macrophage infiltration via an increase in hepatic galectin-3, exacerbating ConA-induced hepatitis. The reason for GnT-V-induced increases in hepatic galectin-3 and target glycoproteins for GnT-V in macrophages remains to be elucidated and its mechanisms require further investigation.

In conclusion, aberrant glycosylation by GnT-V led to increases in hepatic macrophage infiltration and enhanced ConA-induced hepatitis in mice. These findings indicate that the modulation of glycosylation may be a novel therapeutic target for immunity-associated acute hepatitis.

Acknowledgements

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