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Decreased β-catenin expression contributes to IFNγ-induced chemokine secretion and lymphocyte infiltration in Hashimoto's thyroiditis

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Abstract

Hashimoto's thyroiditis (HT) is a very common organ-specific autoimmune disease characterized by lymphocyte infiltration and the destruction of thyroid follicular cells (TFCs), in which IFN-γ and chemokines play pivotal roles. Moreover, β-catenin has been implicated in the regulation of T cell infiltration. However, whether β-catenin is involved in Hashimoto's thyroiditis is unknown. Here, we examined β-catenin expression in thyroid tissues and investigated its role in the pathogenesis of HT. The results showed that β-catenin expression was markedly reduced in the thyroid tissues of HT patients; more importantly, IFN-γ treatment markedly reduced the expression of β-catenin and was accompanied by the secretion of chemokines such as CCL5, CXCL16, GRO-β, and GRO-γ in TFCs *in vitro*, which was attributed to GSK-3β/β-catenin signaling pathway activation. Collectively, the decreased expression of β-catenin might contribute to IFNγ-induced chemokine secretion and lymphocyte infiltration in the development of HT.

Introduction

Hashimoto's thyroiditis (HT) is a very common organ-specific autoimmune disease in which the Th1-mediated immune response contributes to the destruction of thyroid follicular cells (TFCs) (1). In recent years, many studies have shown that HT development depends on an immune defect in an individual with genetic susceptibility together with environmental factors (2), such as iodine, selenium, smoking, and medications (e.g. amiodarone, alumtuzumab, iplimumab, interferon alpha, and other cytokines). However, the pathogenesis of HT is still not fully clear. One of the histopathological hallmarks of HT is lymphocyte infiltration into the thyroid gland. The recruitment of these lymphocytes into the site of

inflammation is a multistep event mediated by leukocytes, cytokines, and adhesion molecules (3).

IFN-γ, as an important Th1-type cytokine, is produced intrathyroidally by infiltrating inflammatory cells, acting in a paracrine manner (4, 5). IFN-γ can potentiate the expression of major histocompatibility complex class II molecules and adhesion molecules in TFCs (5). Importantly, a direct role of IFN-γ in autoimmune thyroid destruction has been documented in thyroid-specific IFN-γ transgenic mice (6). Furthermore, it has been reported that thyrocytes responding to IFN-γ are essential for developing lymphocytic spontaneous autoimmune thyroiditis and inhibition of thyrocyte proliferation in NOD.H-2h4 mice

(7). Recently, a group of low-molecular-weight peptides, namely, chemokines, were demonstrated to play a crucial role in leukocyte infiltration (8). Chemokines are small (~8–14 kDa), secreted proteins structurally similar to cytokines, responsible for attracting and recruiting different cell types during physiological processes of maturation and trafficking of immune cells throughout different lymphoid organs (9). Most of the attention has been devoted to understanding their activities as proinflammatory mediators (10, 11, 12), making them and their receptors classical targets for therapeutic interventions in inflammatory autoimmune diseases (9, 13, 14). The role of IFN-γ in directing chemokine production and leukocyte infiltration to the CNS in experimental autoimmune encephalomyelitis has been demonstrated. However, the relationship between IFN-γ and chemokines in HT is not clear.

A recent study demonstrated that tumor-intrinsic β-catenin signaling might result in the lack of T cell infiltration by decreasing chemokine CCL4 gene expression in melanoma models and patient-derived biopsies (15). As a central signaling molecule in the canonical Wnt pathway, β-catenin activates target genes in a complex with lymphoid enhancer factor (T cell factor transcription factors) in the nucleus. The regulation of β-catenin activity is thought to occur via a cytoplasmic multiprotein complex that includes the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β) that phosphorylates β-catenin, marking it for degradation by the proteasome (16). Lithium is known to prevent β-catenin degradation and, therefore, mimics canonical Wnt signaling (17).

The inverse relationship of active β-catenin signaling and T cell infiltration in both human melanoma samples and transgenic melanoma mouse models by Spranger and colleagues provides a first insight into a potential new mechanism of immune resistance. Until now, no research has been performed to determine whether β-catenin expression affects lymphocyte infiltration in HT. In the present study, we found that IFN-γ induced decreased expression of β-catenin, which contributed to the chemokine secretion of TFCs, and then affected the lymphocyte infiltration. In view of the potential importance of β-catenin in lymphocyte infiltration, β-catenin may have a key role in the autoimmune pathology of HT.

Materials and methods

Cell culture and samples

Human thyroid follicular epithelium (Nthy-ori 3-1) was purchased from the European Collection of Animal Cell

Cultures and cultivated in RPMI-1640 (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal bovine serum (Gibco) in the presence of 5% CO_2 and 37°C. Thyroid tissue samples were obtained from ten HT patients and five patients with a simple goiter. HT diagnosis was made based on clinical evaluations and Japanese guidelines as described previously (18, 19). All samples were obtained after obtaining written informed consent, in accordance with the regulations and approval of the Institutional Review Board of the Affiliated Hospital of Jiangsu University. This study was approved by the Ethics Committee of the Affiliated Hospital of Jiangsu University and conducted in accordance with the Declaration of Helsinki.

Immunohistochemistry detection

Thyroid tissues were obtained from the Department of Pathology of The Affiliated Hospital to Jiangsu University. Tissue sections were prepared as follows: samples were fixed in 10% neutralized formalin, embedded in paraffin, cut into 4-μm sections, and mounted on slides. After deparaffinization and rehydration, antigen retrieval was performed by boiling samples in 10 mmol/L citrate buffer (pH 6.0) for 10 min and then washing the slides with PBS. After inhibition of endogenous peroxidase activity for 10 min with methanol containing 3% H_2O_2, sections were blocked with 2% BSA in PBS for 30 min. It was then incubated at 4 °C (the corresponding isotype antibody was used as a negative control). After three washes with PBS, the sections were treated with horseradish peroxidase (HRP)-conjugated secondary antibody (Maixin Biotechnology Co., Ltd.). Tissue sections were then counterstained with 3,3'-diaminobenzidine and hematoxylin and observed under an optical microscope. HT showed effacement of thyroid architecture by diffuse lymphocyte infiltration and residual thyroid follicles. Then, the number of lymphocytes in each HT specimen was calculated. The results of quantitative analysis for all immunohistochemistry (IHC) samples by Image-Pro plus 6.0 software are presented graphically. The number of lymphocytes in the thyroid tissues per high-power field of β-catenin assessment was counted to evaluate the relationship between β-catenin expression and infiltrating lymphocytes (20). All statistics were based on data collected by independent pathologists blinded to the experimental conditions.

Reagents and antibodies

Nthy-ori 3-1 cells were treated with exogenous recombinant human IFN-γ (Peprotech, Rocky Hill, NJ, USA) in the culture

medium at a gradient concentration (0, 250, 500, 1000 U/mL). The control group included cells without any treatment. GSK-3β inhibition studies were performed by adding 10 mM lithium (Sigma-Aldrich) to the culture medium. Control cells were treated with the vehicle. The following primary antibodies and secondary antibodies were used: rabbit anti-human β-catenin, rabbit anti-human GSK-3β, rabbit anti-human p-GSK-3β, rabbit anti-β-actin (Cell Signaling Technology), and goat anti-rabbit IgG-HRP (Santa Cruz).

Western blot analysis

Briefly, whole-cell lysates, cytoplasm, and nuclear lysates were prepared with a protein extraction kit (Merck Millipore) as previously described by Dr Xu ([21]). Protein concentration was determined using a BCA protein concentration kit (Beyotime, Shanghai, China). First, 5 μg of protein was subjected to electrophoresis on a 10–15% acrylamide gel by SDS–PAGE and then transferred onto a polyvinylidenedifluoride membrane (Merck Millipore) by electrophoresis. After blocking for 1 h in 5% BSA, the membranes were incubated with antibodies against proteins or β-actin (standard controls), followed by HRP-conjugated secondary antibodies. The signals were detected using the Pierce ECL-plus substrate (Thermo Fisher Scientific) and scanned with a Fluor Chem FC3 camera system (ProteinSimple, San Jose, CA, USA). Images were analyzed using AlphaView software (ProteinSimple), and the results of quantitative analyses were presented graphically.

RT-PCR analysis

Total RNA was extracted from Nthy-ori 3-1 cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions after treatment with IFN-γ for 6 h. RNA was eluted with RNase-free water, and the RNA concentrations were determined using a BioMate 3S analyzer (Thermo Fisher Scientific). Reverse transcription–polymerase chain reaction (RT–PCR) was performed using the RNA PCR Kit ver. 3.0 (Takara Biotechnology, RR019B). One microgram of total RNA was reverse transcribed in a 20-μL volume with the PrimeScript® RT reagent Kit Perfect Real Time (Takara Biotechnology, Dalian, China) using random hexamers and oligo dT as primers. The qRT–PCR was performed with SYBR® Premix Ex Taq™ (TliRNaseH Plus) in a real-time PCR Mx3000PTM System (Genetimes Technology, China). For mRNA detection, the primers were as follows: β-catenin (forward: 5'-CAA CTA AAC AGG AAG GGA TGG A-3'; reverse: 5'-CTA TAC CAC CCA CTT GGC AGA C-3'); GRO-β (forward: 5'-CTG CGC CCA AAC CGA AGT

CAT A-3'; reverse: 5'-TTC AGG AAC AGC CAC CAA TAA GC-3'); GRO-γ (forward: 5'-GCA GGG AAT TCA CCT CAA GA-3'; reverse: 5'-GGT GCT CCC CTT GTT CAG TA-3'); CXCL16 (forward: 5'-GGG GGC AGT CAC CGC AGT CCT-3'; reverse: 5'-AAT AGC CGG GTG TGG TGG TGA GCA-3'); CCL5 (forward: 5'-CAC GCC TCG CTG TCA TCC TCA-3'; reverse: 5'-TTG GCG GTT CTT TCG GGT GAC-3'); GAPDH (forward: 5'-AGG TGA AGG TCG GAG TCA AC-3'; reverse: 5'-GGG TGG AAT CAT ATT GGA ACA-3'). GAPDH was used as the internal control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression levels.

Chemokine array

The levels of chemokines in the supernatants of the TFC cultures were determined using a RayBio Human Chemokine Antibody Array Kit (RayBiotech, Norcross, GA, USA). Briefly, TFCs were cultured in a six-well plate (2×10^5 cells/well) with 2 mL/well serum-free medium for 16 h and then treated with or without 500 U/ml recombinant human IFN-γ and 10 mM LiCl for 24 h. The treated and untreated supernatants were harvested and analyzed according to the manufacturer's instructions. The array was scanned by a laser scanner (InnoScan30Microarray Scanner, INNOPSYS, Parc d'Activité Activestre, Carbonne, France) using a green channel (excitation frequency 532 nm).

Transfection with siRNA

Knockdown of GSK-3β or β-catenin was performed by transfection with siRNA (GenePharma, Shanghai, China). The targeted sequences GSK-3β were as follows: sense, 5'-GGA CUA UGU UCC GGA AAC ATT-3'; antisense, 5'-UGU UUC CGG AAC AUA GUC CTT-3'. The targeted β-catenin sequences were as follows: sense, 5'-GCA GUU GUA AAC UUG AUU ATT-3'; antisense, 5'-UAA UCA AGU UUA CAA CUG CTT-3'. A total of 3×10^5 cells were plated in six-well plates for 16 h and then transfected with 80 pMol siRNA using Lipofectamine 2000 (Invitrogen) in a serum-free medium for 5 h. Then, the medium was replaced with a serum-supplemented medium for 24 h. Negative controls using nontransfected cells and empty vectors were performed in parallel. Cells were then used for functional assays based on RNA and protein extraction.

Overexpression of β-catenin

β-catenin is a protein that in humans is encoded by the CTNNB1 gene. Nthy-ori 3-1 cells were infected with adenovirus-expressing CTNNB1 (Hanbio Biotechnology, Shanghai, China) at a multiplicity of infection of 100 for

approximately 6 h. The control group included cells with control adenovirus Ad-RFP.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software). Descriptive data are expressed as the mean ± S.D., and numerical data between two groups were compared using the homogeneity of variance test or Wilcoxon Mann–Whitney *U*-test, as appropriate. Differences in various groups' mean values were analyzed using one-way ANOVA with Tukey–Kramer multiple comparison tests. Correlations between variables were determined by Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

Decreased β-catenin and enhanced IFN-γ expression in thyroid tissues from HT patients

To evaluate the expression of β-catenin and IFN-γ in thyroid tissues of HT patients, we used IHC analysis. The results indicated that both HT and goitrous tissues expressed β-catenin and IFN-γ. However, HT tissues ($n = 10$) expressed low levels of β-catenin and high levels of IFN-γ

compared with goiters, while goiter-adjacent normal tissues ($n = 5$) expressed a high level of β-catenin ($P < 0.01$) and a low level of IFN-γ ($P < 0.01$). Our results suggested the occurrence of reduced β-catenin and increased IFN-γ in HT tissues compared with goiter-adjacent normal tissues (Fig. 1A). The results of IHC quantification from all samples are shown in Fig. 1B. Further analysis suggested that the level of β-catenin expression was negatively correlated with the number of infiltrating lymphocytes in the same tissue area of HT patients by IHC analysis ($n = 10$, $P < 0.05$; Fig. 1C).

IFN-γ downregulated β-catenin expression in TFCs

Nthy-ori 3-1 cells, a human immortalized thyroid follicular cell line, were treated with gradient concentrations of exogenous recombinant human IFN-γ, and the expression of β-catenin was detected by Western blot and RT–PCR analysis. The results showed that the protein levels of β-catenin significantly decreased in IFN-γ-treated Nthy-ori 3-1 cells in a concentration-dependent manner ($P < 0.01$; Fig. 2A) but not in the mRNA levels of β-catenin (Fig. 2B). Further analysis showed that the level of nuclear β-catenin (n-β-catenin) was decreased in IFN-γ-treated Nthy-ori 3-1 cells, along with a decrease in the level of cytoplasmic β-catenin (c-β-catenin) by Western blot (Fig. 2C).
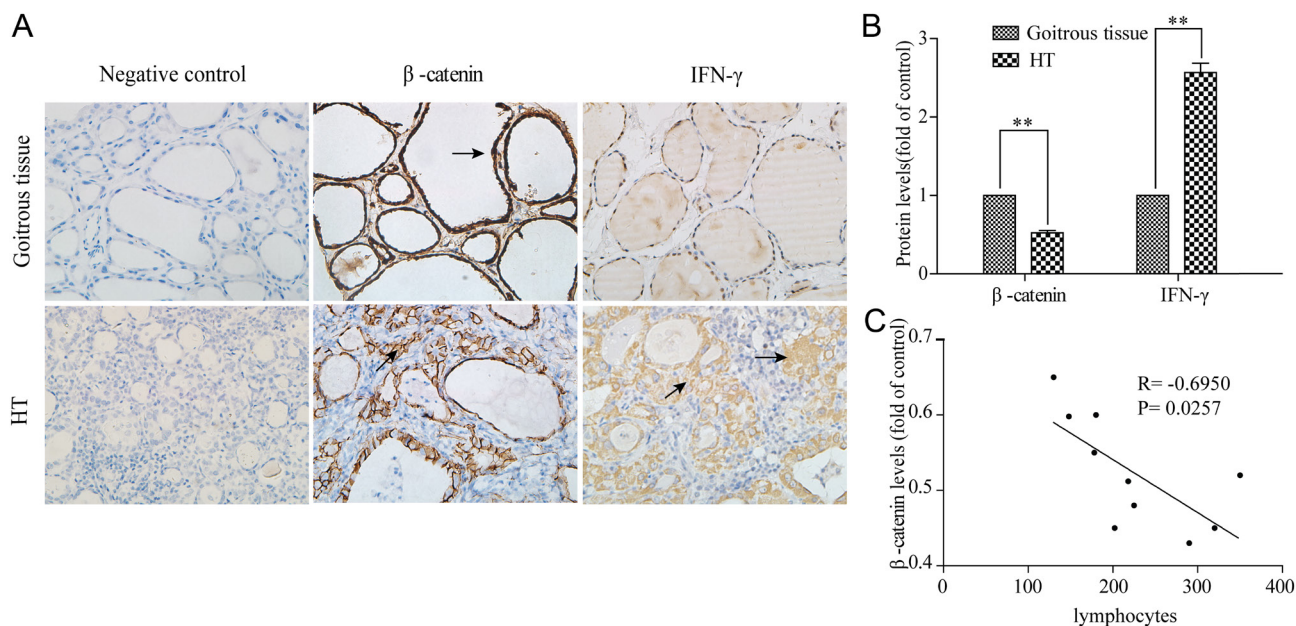

Figure 1

β-Catenin and IFN-γ expression in HT tissues and goiter-adjacent normal tissues. (A) Representative results of immunohistochemical staining for β-catenin and IFN-γ in HT tissues ($n = 10$) and goiter-adjacent normal tissues ($n = 5$) are shown. Goiter-adjacent normal tissues were obtained from patients with simple goiter of the thyroid gland. Brown regions represent positive expression (original magnification, 400×). (B) The results of IHC quantification from all samples are shown. The Mann–Whitney *U*-test was used to calculate significant differences and *P* values. **$P < 0.01$ vs controls. (C) The relationship between β-catenin expression and the number of infiltrating lymphocytes in the same tissue area of HT patients by IHC analysis is shown ($n = 10$).

GSK-3β/β-catenin signaling contributes to IFN-γ-induced chemokine secretion in TFCs

The culture supernatant from Nthy-ori 3-1 cells was analyzed using the RayBio Human Chemokine Antibody Array (AAH-CHE-G1) to investigate the change in the IFN-γ-induced chemokine profile in the TFCs. The results showed that the expression of five chemokines (CCL2, CCL5, CXCL10, CXCL16, and GRO) was increased significantly in the presence of IFN-γ compared with the control ($P < 0.05$; Fig. 3A).

To further determine the mechanism of IFN-γ-induced chemokine secretion, GSK-3β/β-catenin protein, the important molecules of Wnt signaling pathway, which play a key role in many biological aspects, were analyzed after IFN-γ treatment in Nthy-ori 3-1 cells. Western blot analysis was performed to examine the protein expression levels of phosphorylated and total GSK-3β and total β-catenin in IFN-γ-treated Nthy-ori 3-1 cells at different times. The results showed that IFN-γ treatment induced the phosphorylation of GSK-3β in Nthy-ori 3-1 cells, and the increased phosphorylated protein levels were significant at 6 h (Fig. 3B). Similarly, the expression of β-catenin began to decrease at 6 h (Fig. 3B).

These findings suggested that IFN-γ could activate GSK-3β, which is further responsible for β-catenin degradation, which might contribute to chemokine secretion in TFCs. Inhibition of GSK-3β with 10 mM lithium, which was reported to inhibit GSK-3β fully (17), particularly reduced the expression of chemokines CCL5, CXCL16, and GRO (Fig. 3A). Similarly, pretreatment with the GSK3β inhibitor lithium inhibited changes in chemokine (CCL5, CXCL16, GRO-β, and GRO-γ) gene expression in Nthy-ori 3-1 cells in the presence of IFN-γ, as shown by RT–PCR analysis (Fig. 3C). To exclude the indeterminate effects of the GSK-3β inhibitor, GSK-3β expression was silenced in Nthy-ori 3-1 cells and then the cells were treated with IFN-γ. RT–PCR analysis showed that silencing the GSK-3β gene significantly also reduced IFN-γ-induced chemokine (CCL5, CXCL16, GRO-β, and GRO-γ) expression (Fig. 3D). To prove the effect of β-catenin, we found that IFN-γ-induced chemokines were decreased by adenovirus-mediated CTNNB1 overexpression (Fig. 3E). Additionally, silencing β-catenin expression in Nthy-ori 3-1 cells enhanced IFN-γ-induced chemokines (Fig. 3F). These results suggested that the production of IFN-γ-induced chemokines (CCL5, CXCL16, GRO-β, and GRO-γ) was mediated by the GSK-3β/β-catenin pathway.
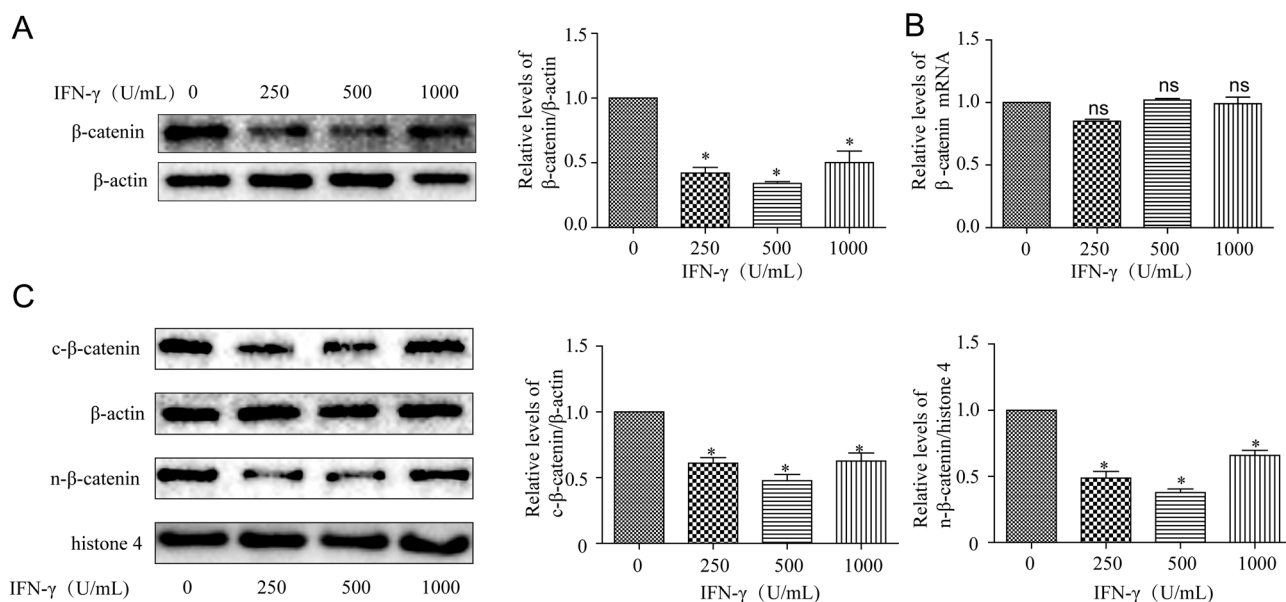

Figure 2

Effects of IFN-γ exposure on the expression of β-catenin in Nthy-ori 3-1 cells. Nthy-ori 3-1 cells were treated with gradient concentrations of IFN-γ. (A) Cell lysates were harvested after 24 h of IFN-γ treatment. The images presented are Western blots (left upper panel) probed for β-catenin (β-actin served as the loading control). The mean values of all three replicates are shown (right upper panel). (B) Relative expression levels of β-catenin mRNA were examined by RT–PCR assay after 5 h of IFN-γ treatment. The analysis of the CT values of Nthy-ori 3-1 cells was normalized to GAPDH for each sample. The normalized values (ΔCT) of all samples were compared with the control in each group (ΔΔCT). The results are expressed as $2^{-\Delta\Delta CT}$, and the resulting data were derived from at least three independent experiments. (C) Western blot results showed the changes in n-β-catenin and c-β-catenin expression levels in Nthy-ori 3-1 cells treated with IFN-γ for 24 h. Histone 4 and β-actin were used as protein loading controls. Significant differences and *P* values were calculated with one-way ANOVA. ns, not significant. *$P < 0.05$ vs controls.
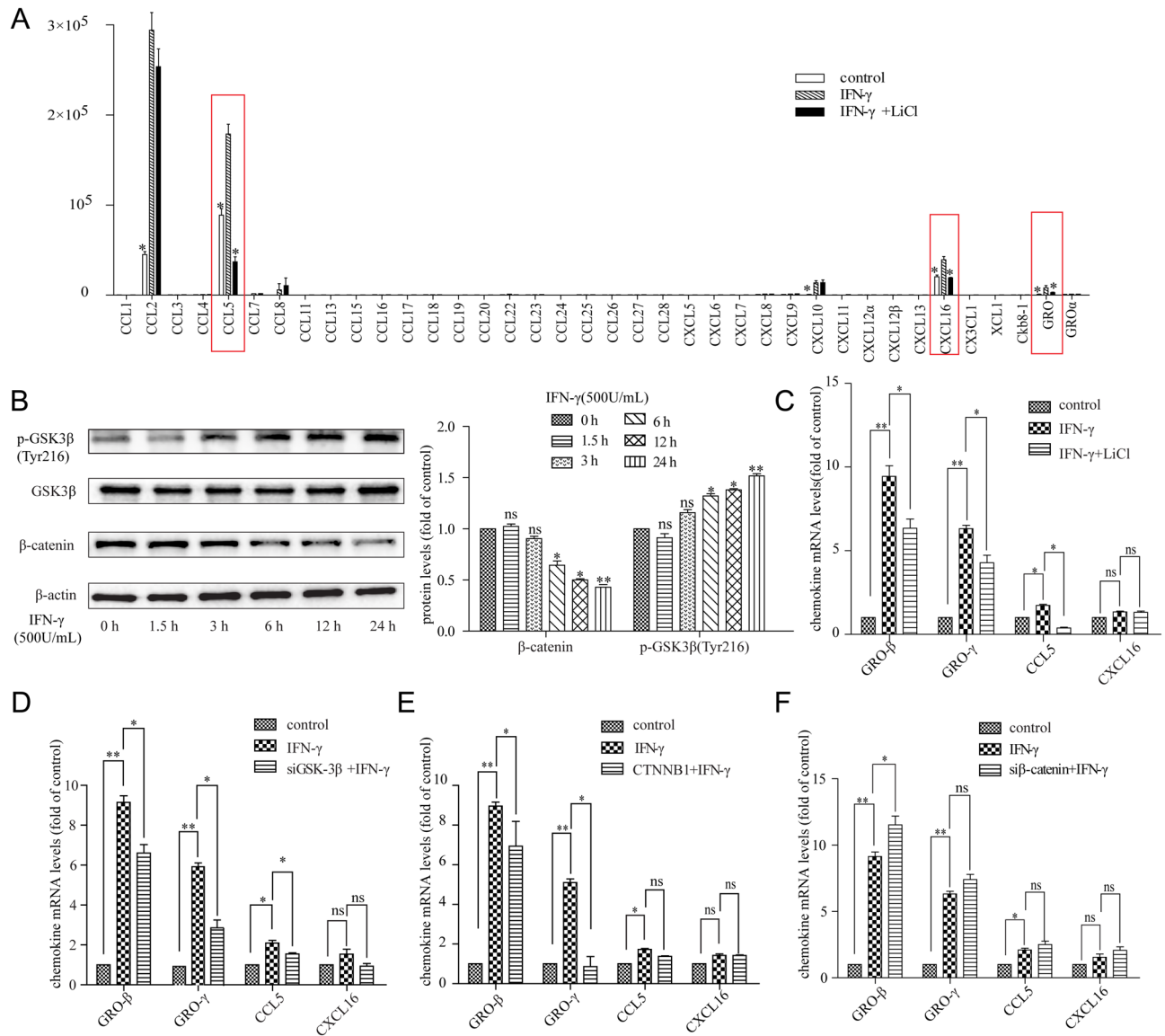
Figure 3

The effects of GSK-3β/β-catenin signaling on the expression of IFN-γ-induced chemokines in Nthy-ori 3-1 cells. (A) Effect of IFN-γ on chemokine expression: Nthy-ori 3-1 cells were treated with IFN-γ (500 U/mL) for 24 h. The supernatants were then collected for chemokine antibody array analysis. The relative signal intensity of each of the indicated chemokines is shown. (B) Western blot results showed the changes in GSK-3β, p-GSK-3β, and β-catenin expression levels in Nthy-ori 3-1 cells treated with IFN-γ (500 U/mL) at different time points. (C) and (D) The changes in GRO-β and GRO-γ, CCL5, and CXCL16 expression in the presence of IFN-γ (500 U/mL) after pretreatment with LiCl (10 mM) or with siRNA targeting GSK-3β by RT–PCR analysis. (E) and (F) Changes in GRO-β and GRO-γ, CCL5, and CXCL16 expression in the presence of IFN-γ (500 U/mL) after pretreatment with overexpression of Ad-CTNNB1 or siRNA targeting β-catenin by RT–PCR analysis. Significant differences and *P* values were calculated with one-way ANOVA. *$P < 0.05$, **$P < 0.01$ vs controls.

Discussion

HT is characterized by the destruction of thyroid cells and the tendency to hypothyroidism, the unique pathologic feature of which is the infiltration of many lymphocytes. Chemokines, inflammatory cytokines, and lymphocytes work together to attract lymphocytes to inflammatory sites. Most studies have found a significantly higher expression of

IFN-γ in HT patients' serum and thyroid tissue, suggesting that the cytokine profile in HT has a Th1 bias (22, 23, 24). Our IHC data revealed a significantly decreased expression of β-catenin and an increased expression of IFN-γ in the thyroid tissue of HT patients compared to goiter-adjacent normal tissues. This suggested that IFN-γ was associated with the decreased expression of β-catenin in the thyroid tissue of HT patients. This finding is consistent with other

autoimmune inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis. A study showed that Treg depletion renders thyroiditis-resistant IL-17 KO, but not IFN-γR KO, NOD.H-2h4 mice susceptible to thyroiditis (25), suggesting that IFN-γ plays an important role in the development of thyroiditis; however, the mechanism of this is not clear. More interestingly, our study found that IFN-γ-induced expression of five chemokines (CCL2, CCL5, CXCL10, CXCL16, and GRO) increased significantly in TFCs. As reported by Caturegli (26), IFN-γ is able to directly affect thyroid function independently of the destructive effect of infiltrating lymphocytes. In this nonclassical model, hypothyroidism results from chronic inhibition of thyroid function induced by exposure of thyrocytes to proinflammatory cytokines such as IFN-γ. On the other hand, IFN-γ, by stimulating the intrathyroidal secretion of chemokines, which in turn recruit Th1 lymphocytes secreting IFN-γ, also plays a role in the classical model of hypothyroidism, which results from the loss of thyroid follicles due to massive lymphocytic infiltration (6). Our data thus cast new light on the mechanism of IFN-γ as a primary regulator of chemokine profiles in the development of HT.

Wnt signaling has been demonstrated to play crucial roles in several biological aspects, including cellular proliferation, embryonic development, tissue homeostasis, development of the immune system, and other systemic effects (27). In addition to its dispensable roles in the development of T cells and the immune system, mounting evidence has recently suggested that this signaling pathway is involved in the pathogenesis of many types of autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and ankylosing spondylitis (AS) (28, 29, 30, 31, 32, 33, 34). However, the role of Wnt signaling in the development of HT remains unclear. Spranger and colleagues recently reported an inverse relationship between melanoma intrinsic β-catenin signaling and intratumoral T cell infiltration, providing an explanation for potential mechanisms of T cell exclusion (15). In contrast, with the infiltration of a large number of lymphocytes in HT thyroid tissue, our results showed the occurrence of reduced β-catenin expression. We also found that β-catenin at the protein level was significantly decreased in IFN-γ-treated TFCs in a concentration-dependent manner but not at the mRNA level. More importantly, this decrease was accompanied by an increase in GSK-3β, suggesting that IFN-γ-induced chemokine secretion was associated with a reduction in β-catenin expression and activation of GSK-3β and that GSK-3β activation was responsible for the degradation of the β-catenin protein. To further determine

the relationship, application of the GSK-3β inhibitor LiCl to mimic the effect of canonical Wnt activation (17) was particularly able to reduce the expression of chemokines (CCL5, CXCL16, and GRO) in IFN-γ-stimulated TFCs. This study provided evidence that the GSK-3β/β-catenin pathway might regulate IFN-γ-induced chemokine secretion of TFCs in the development of HT.

In conclusion, our findings showed that in the pathogenesis of HT, IFN-γ, an important inflammatory cytokine, activates GSK-3β signaling molecules, increases β-catenin degradation, restrains the Wnt signaling pathway. It also promotes the secretion of chemokines, lymphocyte infiltration, and chemotactic thyroid tissue, which contribute to the development of Hashimoto's thyroiditis. The decreased expression of β-catenin might play an important role in determining the nature of the infiltrating lymphocytes in HT.

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