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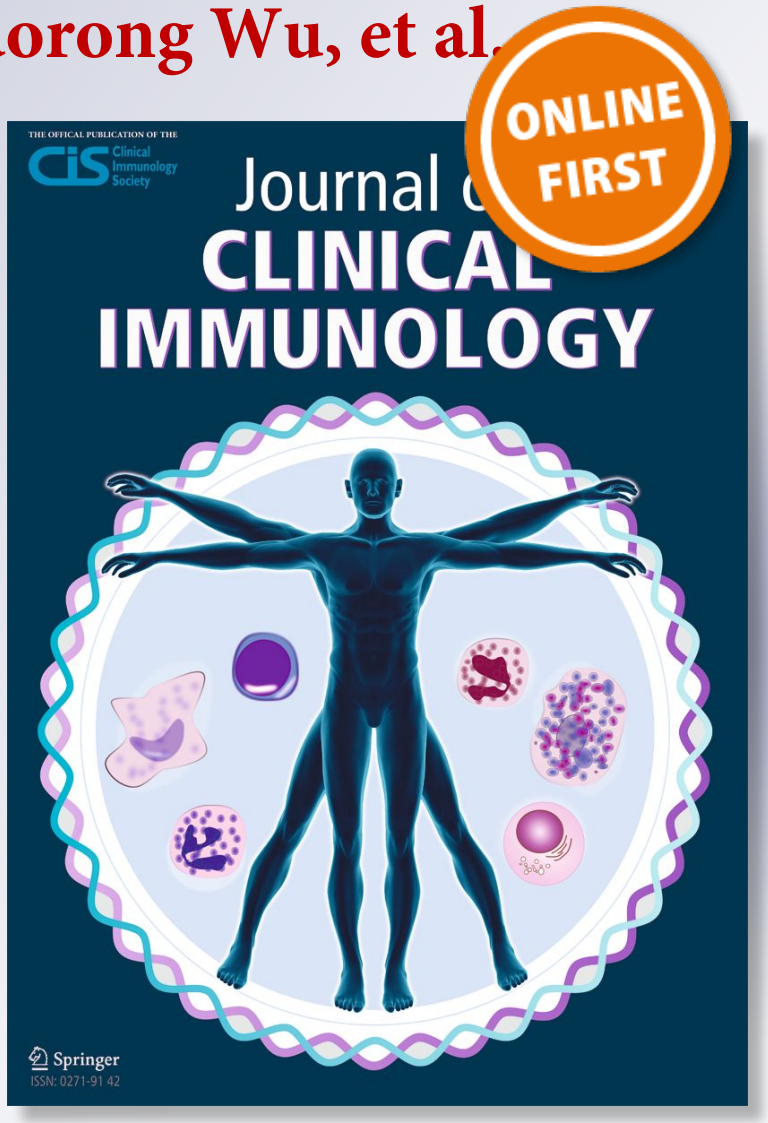

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Involvement of Inducible Costimulator Ligand (ICOSL) Expression in Thyroid Tissue in Hyperthyroidism of Graves' Disease Patients

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Abstract
Background The role of costimulatory molecules expressed on lymphocytes and thyrocytes in hyperthyroidism has attracted increasing attention and research has shown a close correlation between variant expression of these molecules on lymphocytes and thyrocytes and the development of GD.
Meterials and Methods Thyroid tissues were collected from GD patients during surgery and from Hashimoto disease (HT) and non-toxic goiter (NTG) patients as controls. ICOSL expression on infiltrated B cells and TFC was detected by flow cytometry (FCM), reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC). Variation in ICOSL expression on TFC in primary cultures was analyzed in the absence or presence of cytokines using FCM assays. The role of ICOS-ICOSL signaling in proliferation, thyroid hormone production and thyroglobulin (Tg) release was investigated in primary TFC cultures using ICOS gene transfected L929 cells (ICOS-L929 cells) and the blocking ICOSL antibody (11 C4) in MTT assays and radioimmunoassays.
Results and Discussion ICOSL expression on infiltrated B cells and TFC was detected in GD patient tissue. However, ICOSL expression was only detected on infiltrated B cells in control HT and NTG patient tissue. ICOSL expression on TFC was induced in vitro by the proinflammatory cytokines IFN-γ, IL-6 and TNF-α. Compared with mock transfected L929 (mock-L929) control cells, ICOS-L929 cells promoted significant proliferation of primary cultured TFC, with increased thyroid hormone and Tg production (all $P<0.01$). TFC proliferation and production of thyroid hormones and

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Tg were inhibited significantly in the presence of ICOSL blocking antibody (11 C4) (all $P<0.05$). Our observations suggest that ICOS-ICOSL signal plays a direct role in proliferation and differentiation of TFC and may exert important effects in the initiation, maintenance and exaggeration of autoimmune responses in local tissue.

Keywords Graves' disease · ICOSL · thyroid follicular cells

Introduction

Graves' disease (GD), also known as toxic diffuse goiter, is an organ-specific autoimmune disease, caused by the overstimulation of thyroid cells through the ligation of stimulating anti-thyrotropin receptor (TSHR) and the thyrotropin receptor autoantibody (TRAb) [1]. The disease is accompanied by thyromegaly, hyperthyroidism symptoms, infiltrative exophthalmos, pretibial myxedema and the production of TRAb. Abnormal immunologic function is known to exist in GD patients. The interaction of autoreactive T cells and thyroid follicular cells (TFC) via abnormally expressed immune molecules could promote the migration, activation, proliferation and differentiation of antigen-specific T cells, and subsequently lead to the cytokine release that mediates the immune injury [2, 3]. Furthermore, B cell differentiation is promoted, resulting in generation of the thyroid tissue specific autoantibodies responsible for damage to the structure and function of thyroid glands. Recently, the role of costimulatory molecules expressed on lymphocytes and thyrocytes in hyperthyroidism has attracted increasing attention and research has shown a close correlation between variant expression of these molecules on lymphocytes and thyrocytes and the development of GD [4–6].

The inducible costimulatory (ICOS) molecule was identified on activated T cells by Hutloff et al. [7] in 1999 and shown to be structurally and functionally similar to CD28. As the third member of the CD28 family, expression of ICOS was shown to occur on activated T cells and memory T cells. In contrast to CD28, ICOS has no MYPPPY motif and therefore should have a specific ligand. The same year, Yoshinaga [8] and colleagues first discovered the mouse B7RP-1 molecule, which was confirmed as the specific ligand of ICOS. The ligand of human ICOS, ICOSL [9] (also known as hGL50 [10], hB7RP-1[11], B7-H2 [12], hLICOS [13]) is detected predominantly on activated B cells, and on macrophage and dendritic cells to some extent. Previous studies have shown that ICOSL expression on mice fibroblasts and non-lymphoid tissues is induced by TNF-α. IL-1β and ICOSL expression on endothelial cells is obviously increased by TNF-α [14]. Thus, ICOSL expression on cells and tissues is regulated by a variety of cytokines.

ICOS-ICOSL signaling plays an important role in immune regulation. ICOS expression is increased on activated T cells, indicating that the interaction of ICOS and ICOSL promotes T cells activation and proliferation and cooperates with CD28-B7 signaling in immunoregulation of immune responses [15, 16]. ICOS-ICOSL signaling is also involved in humoral immune responses. Although ICOS-ICOSL signaling does not influence B cell development , it exerts effects in the maturation of B cells. T cell-dependent B cells response are severely impaired in the absence of ICOS-ICOSL signaling [17–19]. In humoral immune responses, ICOS-ICOSL signaling participate in the cross-talk of T lymphocyte and B lymphocyte, promoting proliferation of activated B cells and antibody production. ICOS-ICOSL signaling also plays a key role in local immunity and inflammation responses due to ICOSL expression on non-lymphoid tissues [20].

The functions and mechanisms of ICOS-ICOSL signaling in the immunological pathogenesis of hyperthyroidism remain to be elucidated. In the present study, ICOSL expression in GD patient thyroid tissue was chatacterized by RT-PCR, flow cytometry and immunohistochemistry analyses. In addition, in vitro investigations demonstrated increased ICOSL expression on TFC in the presence of proinflammatory cytokines. Further studies showed that ICOS-L929 promoted TFC growth, thyroid hormone production and Tg release. In combination, our study has established an important role for ICOS-ICOSL signaling in the pathogenesis of hyperthyroidism.

Materials and Methods

Study Subjects

The paraffin blocks of thyroid tissues were obtained from the Departments of Pathology at the Second Affiliated Hospital of Soochow University and The Affiliated Suzhou Hospital of Nanjing Medical University, Jiangsu Suzhou, China. All specimens were identified with hematoxylin and eosin (H&E) staining, including 10 experimental subject cases with GD (3 men, 7 women; mean age, 45.8 years), 10 cases with Hashimoto's disease (HT) (3 men, 7 women, mean age, 43.6 years) and 10 cases with non-toxic goiter (NTG) (4 men, 6 women, mean age, 46.4 years) as controls. Thyroid tissues were obtained for primary cell culture from six patients undergoing partial thyroidectomy as a treatment of GD or NTG.

All of HT patients were positive for anti-thyroid peroxidase antibody (TPO-Ab) and had normal hormone levels. Eight of the HT patients had a nodular goiter while one had a micro-carcinoma. Lymphocytic infiltration was detected in all goiters evaluated in the HT patients.

The diagnosis of GD was established according to a typical clinical presentation (e.g., weight loss, hyperkinesis,

tachycardia), physical findings (diffusely enlarged goiter and occasional ophthalmopathy), diffuse and homogeneous uptake in thyroid gammagraphy with technetium-99 m, as well as increased serum concentrations of free triiodothyronine (FT3), free thyroxine (FT4), decreased serum concentrations of thyroid-stimulating hormone (TSH) and mean serum anti-TSH-receptor antibodies (TRAb). Serum concentrations of TSH, FT4 and FT3 were measured using commercial kits (Elecsys and cobas e analyzers, Roche Diagnostics GmbH, Germany), and TRAb using a commercial radioreceptor assay (TRAK-Assay, Henning, Berlin, Germany). All of the GD patients received more than 3 years of methimazole or propylthiouracil therary but were still positive for TRAb. In addition, all of the patients had not received corticosteroids or other immunosuppressive agents for at least 6 months prior to surgery. All patients gave their informed consent to participation in this study, in accordance with the local medical ethics committee.

Cell Lines and Cell Culture

ICOS transfectant L929 (ICOS-L929) cells and mock transfectant (mock-L929) cells were generated in our laboratory[21, 22] and were cultured in RPMI1640 (Gibco, USA), containing 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator supplemented with 5 % CO_2.

Cytokines and Antibodies

Cytokines TNF-α and IFN-γ were purchased from Biosource, USA. Cytokine IL-6 was produced in our institute. PE-labeled mouse anti-human ICOSL (clone No.MIH12) and ICOS (clone No.ISA-3) antibodies were purchased from eBioscience, USA. FITC-labeled mouse anti-human CD4, CD19 antibody, PE-Cy5-labeled mouse anti-human CD3 antibody and isotype antibodies were purchased from Immunotech, France. Mouse anti-human ICOSL monoclonal antibody, Clone No.11 C4, established and characterized in our institute[23], was used to perform the vitro experiment and immunohistochemistry assay.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Thyroid tissues were cut into several small pieces around 0.5 cm^3 in size and transported to the laboratory in ice-cold saline containing RNAlater® (Invitrogen) to prevent RNA degradation. Total RNA was isolated from the tissue lysed with Trizol reagents (Takara, Kyoto, Japan) according to the instructions provided by the manufacturer. Total RNA (2 μg) was reverse transcribed into cDNA using an RT-PCR kit (Takara, Kyoto, Japan) as previously described [24]. Primer sequences were as follows: ICOSL, 5′-ATGCGGCTGGGCAGTCCTG GACTG-3′ and 5′-CTCTGTCTCCGGACTCACAG-3′; for β-actin, 5′- ATCTGGCACCACACCTTCTACA-3′ and 5′-GATAGCAC AGCCTGGATAGCAA-3′.

Isolation of Infiltrating Lymphocytes from Thyroid Tissues

Specimens were stored in 50 ml tubes in phosphate-buffered saline during transportation. All preparations were performed under sterile conditions in a laminar airflow bench. All plastic materials were sterile and disposable. Isolation of cells was initiated immediately upon arrival in the laboratory. After 15 min of washing in a rotating vessel, connective tissue and nodular structures were separated from macroscopically regular tissue components and discarded.

Regular thyroid parenchyma was selected exclusively for tissue culture. The tissue samples were minced into small pieces using fine surgical forceps and scissors. Tissue fragments were collected in a tube containing 1 % collagenase IV (Sigma, USA) and incubated for 45 min in the rotating tube to yield a suspension of isolated cells. The suspension was filtered by a monofilament polypropylene mesh (pore size, 250 μm) to retain connective tissue components. Infiltrating lymphocytes were separated by Ficoll-Hypaque (BIOCHROME KG, Berlin, Germany) density-gradient centrifugation.

Immunofluorescence and Flow Cytometry Analyses

For flow cytometry analysis, infiltrated lymphocytes were washed with PBS and incubated with the PE-Cy5-labeled mouse anti-human CD3, FITC-labeled mouse anti-human CD4 and PE-labeled mouse anti-human ICOS (ICOS expression on T lymphocytes) or FITC-labeled mouse anti-human CD19 and PE-labeled mouse anti-human ICOSL (ICOSL expression on B lymphocytes) monoclonal antibodies for 20 min on ice and washed. Primary thyroid follicular cells were collected after cultivation for 72 h with or without cytokines and incubated with PE-labeled ICOSL mAb for 20 min on ice and washed. The results were analyzed at the Flow Cytometry Facility (Beckman Coulter, Altra, USA) with Beckman Coulter EXPO32 MultiComp software.

Immunohistochemistry Procedures

Immunohistochemistry (IHC) was performed using the Dako EnVision™ method according to the instructions provided by the manufacturer. In brief, 3-μm thick consecutive sections were cut using a microtome, dewaxed in xylene and rehydrated through a graded series of ethanol solutions. Antigens were retrieved by heating the tissue sections at 100°C for 30 min in citrate solution or EDTA solution as required. Sections were cooled down and immersed in 0.3 % H_2O_2 solution for 20 min to block endogenous peroxidase activity and then rinsed in PBS for 5 min, blocked with 3 %

BSA at room temperature for 20 min and incubated with primary antibodies against specific mouse anti-human ICOS-L mAb 11 C4 at a 1/200 dilution for 60 min at 4°C overnight. Negative controls were generated by replacing the specific primary antibody with PBS. After three PBS washes, sections were incubated with secondary antibodies for 30 min at room temperature. Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain. Sections were dehydrated, cleared and mounted.

Preparation of Human Thyroid Primary Cells

Isolation of thyroid primary cells was performed by a combination of mechanical disintegration and enzymatic digestion using the protocol previously described for isolation of infiltrating lymphocytes from thyroid tissues. The resulting cells suspended in Hank's buffer and centrifuged for 10 min at 500×*g*. The cell pellet was resuspended in 10 ml of a culture medium (6 H medium) based on Ham's F12 solution (Gibco, Germany), supplemented with six hormones and growth factors (10 mg/l insulin [Gibco], 5 mg/l transferrin [Gibco], 10 μg/l somatostatin [Sigma, Deisenhofen, Germany], 3.625 μg/l hydrocortisone [Sigma] and 10 μg/l Gly-His-Lys [Sigma]) and 10 IU/l bovine TSH (Sigma) [25]. All media were enriched with 10 % heat-inactivated FBS (Gibco, USA) and with a pre-mixed solution of antibiotics (5 ml/l streptomycin/penicillin; Gibco). Cells were seeded into T25 tissue culture flasks (Greiner, Germany). Media were added to give a total volume of 10 ml. The flasks were deposited with released caps in an incubator with a humidified atmosphere containing 5 % CO_2 at a temperature of 37°C. Whenever possible, multiple (2–4) cultures were prepared to perform duplicate or triplicate measurements. The number of cultures prepared was determined by the size of the tissue samples.

Storage of Cultured Cells

Cells were cultured for at least 4 d to allow adherence. Integrity of cell cultures was confirmed by morphologic criteria under an inverted microscope (Olympus America Inc., Melville, NY). The absence of microbial contamination was confirmed by the observation of clear culture media. Before uptake was measured, culture media were changed several times to allow cells to adapt to culture conditions.

Cytokine Stimulation Analysis

Primary thyroid cells were digested with 0.25 % trypsin and cultured in triplicate wells in 24-well plates at 1×10^5 cells/well in 1 ml/well RPMI1640+10 % FCS and were stimulated for 72 h with the following cytokines: IL-6 (300 ng/ml), IFN-γ (50 ng/ml) and TNF-α (20 ng/ml). Cells were harvested for FACS analysis.

Primary Thyroid Cell Proliferation Assay

Primary thyroid cells were digested with 0.25 % trypsin and washed. The resulting cells (5×10^4/ml) were co-cultured with mitomycin treated ICOS-L929 transfected cells (2.5×10^4/ml) or mock-L929 tranfected controls with or without anti-ICOSL monoclonal antibody 11 C4 in 96-well flat-bottom culture plates at at 37°C, 5 % CO_2. Mouse IgG1 was used as a negative control. Following cell culture for 4 d, MTT (3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyl-tetrazolium bromide) solution was added to each well to a final concentration of 10 mg/l and the cells were continuously incubated for 5 h. After centrifugation for 5 min at 3000×*g*, the supernatant was removed and the wells were washed thee times with PBS before addition of 100 ml isopropyl alcohol to dissolve MTT crystals and absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). All measurements were performed in triplicate.

Quantification of FT3, FT4 and Ig

Primary thyroid cells were cultured for 72 h in 24-well flat-bottom plates and supernatant was collected. Concentrations of FT3, FT4 and Ig in the culture media were measured by radioimmunoassay (Beijing Atom HighTech Co., Ltd., China) according to the instructions provided by the manufacturer.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 4.0 software package (GraphPad Software, Inc., San Diego, USA). Paired or unpaired Student's *t*-tests were used,

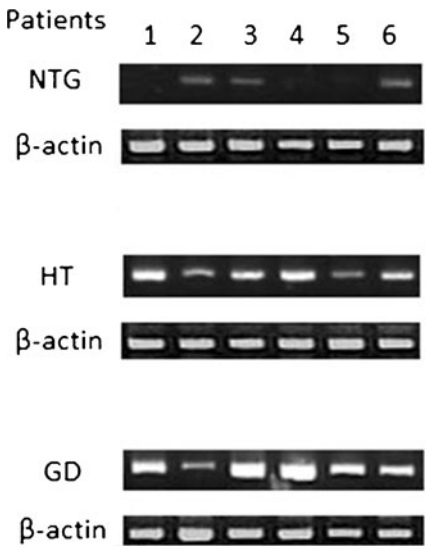

Fig. 1 ICOSL messenger RNA expression in thyroid tissue of GD, HT and NTG patients. The level of ICOSL expression was increased in GD patient thyroid tissue compared with both HT and NTG patients

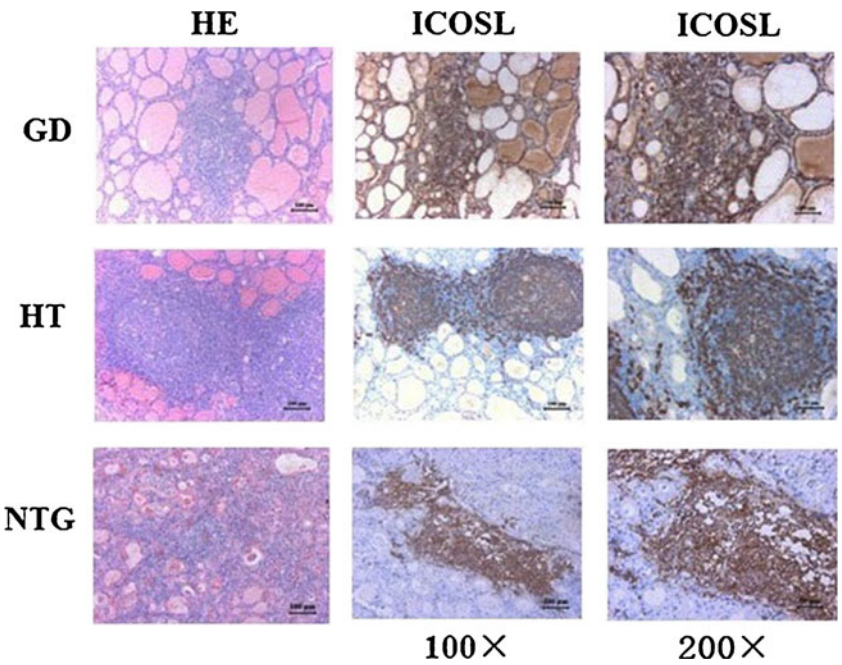

Fig. 2 Immunohistochemistry analysis of ICOSL molecule in thyroid tissue from GD, HT and NTG patients. Noteworthily, expression of ICOSL could be detected with strong staining on the TFC and lymphocytes in thyroid tissue from GD patients, but only on the infiltrating lymphocytes from HT and NTG patients

where appropriate. *P*-values less than 0.05 were considered to be statistically significant.

Results

ICOSL Expression in GD Patient Thyroid Tissue

ICOSL messenger RNA (mRNA) expression in thyroid tissue was analyzed by RT-PCR. As shown in Fig. 1, the level of ICOSL expression was increased in GD patient thyroid tissue compared with both HT and NTG patients. To further determine the ICOSL immunolocalization in tissue, ICOSL expression was characterized in resected specimens from 10 GD patients as well as 10 HT and 10 NTG patients by immunohistochemistry (IHC). Consistent with the RT-PCR analysis results, IHC staining showed that expression of ICOSL in the thyroid tissues of GD, HT and NTG patients. Interestingly, IHC analysis of thyroid sections revealed variable expression of ICOSL, with strong staining on the TFC and lymphocytes in thyroid tissue from GD patients, but only on the infiltrated lymphocytes from HT and NTG patients (Fig. 2).

ICOSL Expression on Infiltrated B Lymphocytes and Primary Cultured TFC

Thyroid tissues resected from six patients during surgery were used for isolation of infiltrating lymphocytes and preparation of primary TFC cultures. Flow cytometry analysis demonstrated moderate expression of ICOS and ICOSL in T and B lymphocytes from GD patients

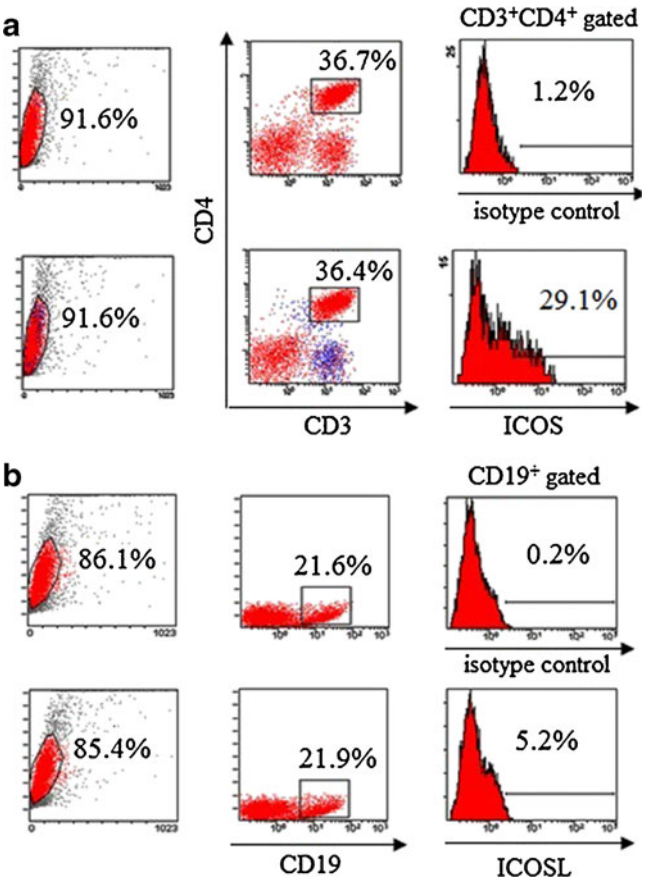

Fig. 3 Detection of ICOS **a** and ICOSL **b** expression on infiltrating lymphocytes from GD patient thyroid tissue. Infiltrating lymphocytes were gated on $CD3^+CD4^+$ T cells for ICOS expression **a** and $CD19^+$ B cells for ICOSL expression **b**

(Fig. 3). Surprisingly, low expression of ICOSL was detected in primary cultured TFC, with no significant difference compared with NTG patients (Fig. 4A). It is known that ICOSL expression in cells and tissues is regulated by various cytokines. Numerous studies have reported detection of high levels of proinflammatory cytokine secretion in peripheral blood and glands of GD patients [26]. Therefore, the roles of proinflammatory cytokines such as TNF-α (20 ng/ml), IFN-γ (50 ng/ml) and IL-6 (300 ng/ml) were individually investigated in the regulation of ICOSL expression in primary cultures of TFC. Flow cytometry analysis showed that these proinflammatory cytokines significantly influenced ICOSL expression in primary cultures of TFC (Fig. 4B).

ICOS-ICOSL Signaling Promoted Proliferation, Hormones Production and Thyroglobulin (Tg) Release in Primary TFC Cultures

To investigate the role of the interaction between ICOS expressed on infiltrated T cells and ICOSL expressed on TFC in GD pathogenesis, ICOS transfected L929 cells (ICOS-L929 cells) were co-cultured for 72 h with primary TFC. MTT assays and radioimmunoassays showed that ICOS-L929 cells significantly promoted proliferation of primary TFC, with increased production of thyroid hormones and Tg (all $P<0.01$), compared with mock transfected L929 (mock-L929) control cells. Proliferation of primary TFCs and thyroid hormone and Tg production were inhibited significantly in the presence of the ICOSL blocking antibody (11 C4) (all $P<0.05$) (Fig. 5).

Discussion

Optimal activation of antigen-specific T cells requires not only the engagement of the T cell receptor (TCR) with the antigen/major histocompatibility (Ag/MHC) complex but also costimulatory signals provided by antigen presenting cells (APCs) [27]. CD28 ligation with its counterpart ligand B7 are required to promote T cell clonal expansion and rescue T cells from anergy or apoptosis in the initial period of an immune response[28, 29]. However, proliferation, differentiation and biological functions of various subsets of T cells and T/B cell cross-talk rely on the participation and regulation of costimulatory molecules [30–33] such as CD40-CD40L and ICOS-ICOSL.

Accumulating evidence has demonstrated that costimulatory molecules are expressed in thyroid tissue and that costimulatory signals play an immportant role in cross-talk between infiltrated lymphocytes, TFC and other immunocytes. It has been reported that CD80 is expressed on TFC

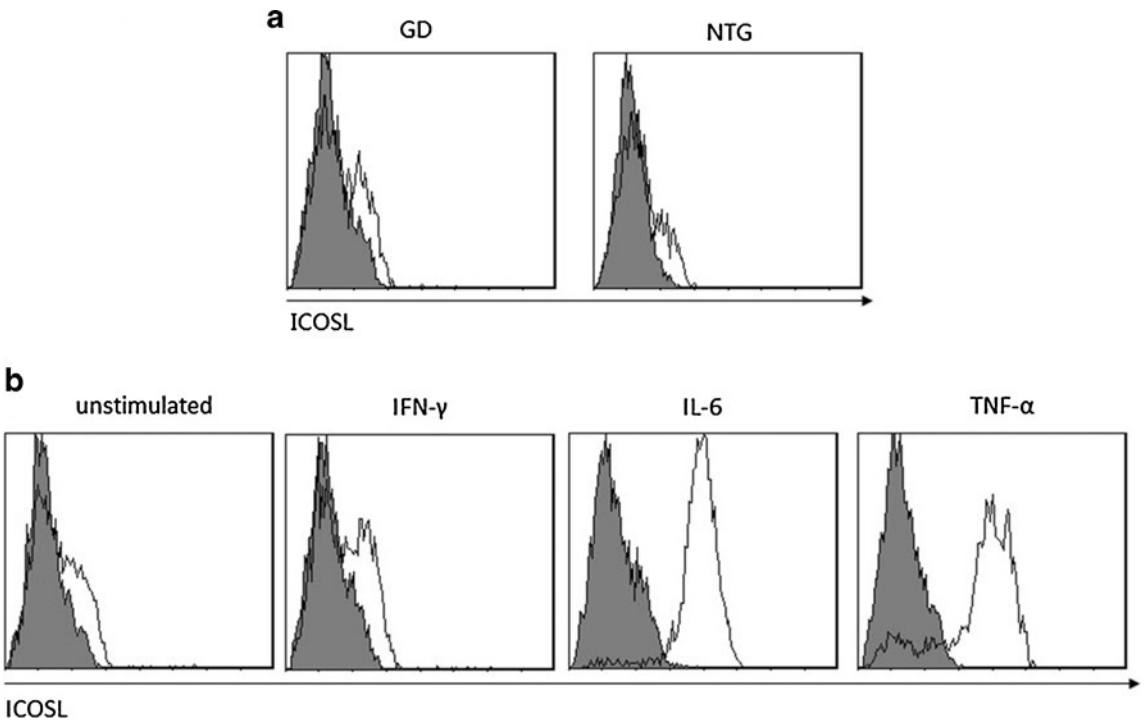

Fig. 4 **a**, ICOSL expression on primary cultured TFCs form GD and NTG patients. **b**, Increased ICOSL expression on primary cultured TFCs from GD patients stimulated by proinflammatory cytokines IL-6 (300 ng/ml), IFN-γ (50 ng/ml) and TNF-α (20 ng/ml), respectively. Increased ICOSL expression was detected when TFCs stimulated by above proinflammatory cytokines for 72 h cultivation in contrast to the negative control

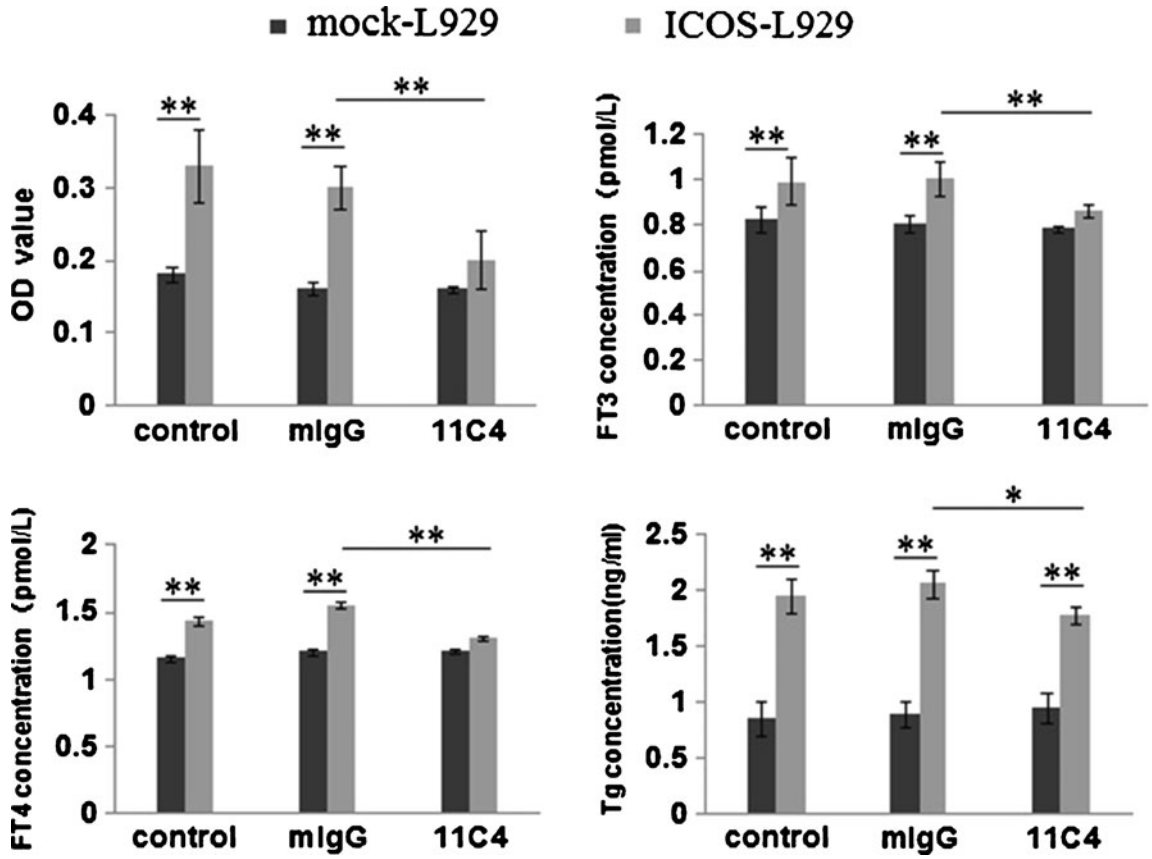

Fig. 5 ICOS-ICOSL signaling promoted proliferation, hormones production and thyroglobulins (Tg) secretion of primary TFC. ICOS transfected L929 cells (ICOS-L929 cells) were co-cultured for 72 h with primary TFC. MTT assays and radioimmunoassays showed that ICOS-L929 cells significantly promoted proliferation of primary TFC, with increased production of thyroid hormones and Tg (all $P < 0.01$), compared with mock transfected L929 (mock-L929) control cells. Proliferation of primary TFCs and thyroid hormone and Tg production were inhibited significantly in the presence of the ICOSL blocking antibody (11 C4)

from HT patients but not from GD patient groups[34]. CD40 is expressed on various epithelial cells including TFC and exerts regulatory effects on TFC proliferation and differentiation. Moreover, CD40-CD40L costimulatory signaling inhibits autoreactive T cell apoptosis, triggers clonal expansion and abnormal activation of lymphocytes, stimulates and amplifies immune responses in local tissues in GD patients, finally resulting in continuing and protracted antoimmunity[35, 36].

The present study revealed ICOSL mRNA expression in thyroid tissue from GD patients. Subsequent immunohistochemical staining showed ICOSL expression on the infiltrating lymphocytes and TFC in thyroid tissue. Primary TFC cultures were established to investigate the hypothesis that ICOS-ICOSL signaling is involved in the biological function of TFCs. ICOSL expression was detected on primary cultured TFC in vitro, although there was no significant difference compared with the NTG group. In previous studies, ICOSL expression on cells and tissues are regulated by various cytokines, and many markedly elevated serum concentration of various proinflammatory cytokines such as TNF-α, IFN-γ and IL-6 were found in patients with GD. Thus, in our further investigation it was observed that proinflammatory cytokines increased ICOSL expression on TFC in GD patients by cell culture and flow cytometry assays in vitro. These observations suggested that the interaction between ICOS on the T cell surface and ICOSL on B cells promoted B cell activation, triggered Th2 responses, increased secretion of many proinflammatory cytokines and stimulated autoantibody production. Those cytokines further enhanced ICOSL expression on TFC, which could ligate ICOS molecules expressed on infiltrating T cells. Therefore, immune responses will be stimulated, maintained and amplified in the thyroid microenvironment composed of infiltrating T and B cells and thyroid follicular cells.

TFCs are a type of epithelial cell possessing endocrine function and produce thyroid hormones and thyroglobulins. Previous studies have reported that the biological function of TFCs is mainly regulated by the iodine titre and thyrotropin. However, recent studies support the role of costimulatory

signaling in the biological function of TFC. It has been reported that a stimulatory CD40 antibody promotes the proliferation of primary TFCs, increases hormone production and Tg release in vitro[37]. Thus, the role of ICOS-ICOSL signaling in TFC biological function was investgated in vitro. It was observed that ICOS-L929 cells induced significant proliferation in primary TFC, with increased production of thyroid hormones and Tg compared with mock transfected L929 (mock-L929) control cells. Furthermore, TFC proliferation and thyroid hormone and Tg production were inhibited significantly in the presence of the ICOSL blocking antibody (11 C4).

Based on previous reports and the findings of the present study, we concluded that costimulatory signals play an important role in the pathogenesis of GD. Loss of CD80 expression on TFC induces Th1 cell anergy or apoptosis, resulting in partial or complete inhibition of immune responses. In contrast, abnormal expression of CD40 and ICOSL induces CD40-CD40L and ICOS-ICOSL signaling, which promote T cell-dependent humoral immune responses, induce B cell activation and increase production of autoantibodies, ultimately resulting in disease development.

In conclusion, this study demonstrated expression of the costimulatory molecule ICOSL on TFC from GD patients and in vitro studies were performed to investigate the role of ICOS-ICOSL signaling in the proliferation and function of TFC. In thyroid the microenvironment composed of TFC and infiltrating autoreactive T and B cells, ICOS-ICOSL costimulatory signal plays an important role in the initiation, maintenance and amplification of immune responses. Therefore, investigation of the role of ICOS-ICOSL signaling in immune regulation is required to further elucidate the mechanisms of immunological pathogenesis in GD and to explore novel means of immune intervene.

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