Caveolin-1 regulates autophagy activity in thyroid follicular cells and is involved in Hashimoto’s thyroiditis disease

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Abstract. Hashimoto’s thyroiditis (HT) is considered a T helper-type 1 (Th1) cytokine-dominant autoimmune thyroid disease. Caveolin-1 (Cav-1), a part of the thyroxisome multiprotein complex, is localized at the apical pole of thyrocytes and is indispensable for synthesis of thyroid hormones and modulation of oxidative stress in order to avoid cell damage and apoptosis. Reduced autophagy induces thyroid follicular cells (TFC) apoptosis by activating reactive oxygen species (ROS) in HT patients. Nevertheless, whether Cav-1 has roles in the regulation of autophagy remains largely unclear. In this study, we examined Th1 cytokines and Cav-1 expression in HT thyroid tissues, determined the effects of interleukin-1beta (IL-1β) and interferon-gamma (IFN-γ) on Cav-1 and autophagy activity in TFC, and investigated the association between Cav-1 and autophagy activity in vitro. Our results indicate that higher levels of IL-1β and IFN-γ and lower levels of Cav-1 were expressed in thyroid tissues of HT patients than in those of normal controls. Cav-1 mRNA and protein levels were significantly decreased in TFC exposed to IL-1β and IFN-γ, accompanied by decreased expression of autophagy-related protein LC3B-II. Interestingly, small interfering RNA (siRNA)-mediated Cav-1 knockdown in TFC reduced LC3B-II protein expression. Taken together, these results suggest that lack of Cav-1 expression inhibited autophagy activity in TFC exposed to Th1 cytokines (IL-1β and IFN-γ), which might be a novel pathogenetic mechanism of HT.

Key words: Th1 cytokines, Caveolin-1, Autophagy, Hashimoto’s thyroiditis

HASHIMOTO’S THYROIDITIS (HT), a chronic thyroid inflammation described over a century ago as a pronounced lymphoid goiter affecting predominantly women [1, 2], is now considered the most prevalent organ-specific autoimmune disease [3-5] and the most common endocrine disorder [6] as well as the most common cause of hypothyroidism [2, 7]. It is pathologically characterized by local infiltration of lymphocytes, with subsequent destruction of thyroid follicles and hypothyroidism [4, 8], accompanied by higher levels of serum auto-antibodies specific for thyroid components, such as thyroglobulin (Tg) and thyroperoxidase (TPO) [9]. It is generally understood that morphological and functional alterations in HT are prominently mediated by Th1 cytokines [10, 11] through apoptotic cell death [12-14].

Caveolae are small flask-shaped plasma membrane invaginations, which can be viewed as a subset of lipid rafts due to a high content of cholesterol and glycosphingolipids [15]. Caveolin-1 (Cav-1), a 22-kDa protein of 178 amino acids, plays a critical role in the structure and function of caveolae [16]. By creating raft-like membrane structures, Cav-1 is associated with the apical membranous localization of the TPO and Dual Oxidase.
(DUOX) protein complex to form thyroxisomes in human primary thyroid cells, directly modulating thyroid hormone synthesis [17, 18]. The absence of Cav-1 leads to the cytoplasmic localization of TPO and DUOX, together with a loss of normal hormonal synthesis, intracellular iodination and excess hydrogen peroxide (H₂O₂) production, which are connected to high oxidative stress, cell destruction, increased apoptosis and finally hypothyroidism [18, 19]. Thus, the aberrant expression of Cav-1 is a key event in HT pathogenesis.

Autophagy and apoptosis are cellular processes that regulate cell survival and death, the former by eliminating intracellular dysfunctional components and extracellular organisms through a lysosomal degradation and the latter by programmed cell death [20]. Autophagy plays significance roles in cellular homeostasis and adaptation to adverse environments and represents a primarily survival mechanism of cells [21], although the regulation of this process remains incompletely understood. Inhibition of autophagy may contribute to a bioenergetic shortage and favour oxidative reactions that trigger cell death characterized by hallmarks of apoptosis [22]. Autophagy activity is decreased and apoptosis level is increased in HT thyroids compared to in control thyroids [23, 24]. Cav-1 has been associated with the apoptosis of thyroid cells [18]. However, until now, no research has been performed to explore the relationship between Cav-1 and autophagy in HT.

In the present study, we investigate the relationship between Cav-1 and autophagy activity in human thyroid follicular cells (TFC) under stimulation with the Th1 cytokines IL-1β and IFN-γ. Our findings about the impact of Cav-1 on autophagy activity could provide a novel pathogenetic mechanism of HT.

Materials and Methods

Reagents

The primary antibodies rabbit anti-human Cav-1, rabbit anti-human LC3B-II, and rabbit anti-β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). HRP-labelled goat anti-rabbit IgG, mouse anti-human IL-1β, and mouse anti-human IFN-γ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of tissue samples

Specimens of thyroid tissues were obtained from five patients with HT who underwent thyroidectomy and five multinodular goiters used as controls based on documented clinical evaluations. The diagnoses of HT were based on clinical criteria and Japanese guidelines as described previously [25, 26]. All samples were confirmed by pathology obtained in accordance with the regulations and approval of the Institutional Review Board of the Affiliated Hospital of Jiangsu University, shown in the Supplemental file. All tissues were obtained after patients gave informed consent.

Immunohistochemistry (IHC) for IL-1β, IFN-γ and Cav-1

The thyroids were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h, embedded in paraffin, serially cut into 4-μm-thick sections and mounted on slides. Following deparaffinization and rehydration, the sections were rinsed using PBS three times and then microwaved for 10 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. After washing the slides with PBS, 3% hydrogen peroxide was added to block endogenous peroxidase for 20 min. Sections were blocked with 2% bovine serum albumin (Shenggong, Shanghai, China) in PBS for 30 min. After the incubation with a primary antibody (rabbit anti-human Cav-1 antibody, mouse anti-human IL-1β antibody, or mouse anti-human IFN-γ) overnight at 4°C, the sections were rinsed with PBS and then were incubated with the corresponding streptavidin peroxidase-conjugated secondary antibody (Maixin Biotechnology Co., Ltd.) for 40 min. Finally, tissue sections were counterstained with 3,3’-diaminobenzidine (DAB) and haematoxylin. Positive cells were observed under an optical microscope (Olympus). The results of all samples were quantitatively analysed by the number of cells with a defined intensity using Image-Pro plus 6.0 software (Version X; Media Cybernetics, USA) and are presented graphically.

Cell culture and IL-1β/IFN-γ treatment

The human thyroid follicular epithelial cell line Nthy-ori 3-1, from the European Collection of Animal Cell Cultures, was cultivated in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum and 2 mM L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel). Nthy-ori 3-1 cells were seeded into 6-well plates and incubated with a cytokine cocktail comprising recombinant human 2 ng/mL IL-1β (PeproTech, Rocky Hill, NJ, USA) and recombinant human 10 ng/mL IFN-γ (PeproTech, Rocky Hill, NJ, USA) in a humidified atmosphere of 5% CO₂. Cells
were harvested at 4 h or 48 h after stimulation, as appropriate.

**Small interfering RNA (siRNA) and transfection**

The target siRNA constructs for human Cav-1 (si-h-Cav-1) and negative control siRNA (siCon) were designed and constructed by Suzhou GenePharma Co., Ltd. (Suzhou, China). To select the siRNA with the best transfection efficiency, we designed three different si-h-Cav-1 sequences and one siCon sequence. The sequences are shown in Table 1. Prior to transfection with siRNA, the medium was replaced with penicillin/streptomycin-free RPMI-1640 complete medium. Nthy-ori 3-1 cells were then transfected with si-h-Cav-1 targeting constructs (20 μM) or siCon using Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) (0.15%v/v) in OPTI-MEM (Gibco-Life Technologies). Six hours after transfection, the medium was replaced with RPMI-1640 complete medium, and cells were cultured for 48 h prior to harvest.

**RNA extraction and reverse transcription**

Total RNA was extracted from Nthy-ori 3-1 cells using RNAiso Plus reagent (Takara Bio Company, Kusatsu, Japan) following the manufacturer’s instructions. The purity and integrity were assessed by measuring the absorbance ratio at 260/280 nm (1.8–2.0 was considered eligible). The PrimeScript™ RT Reagent Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) (0.15%/v/v) in OPTI-MEM (Gibco-Life Technologies). Six hours after transfection, the medium was replaced with RPMI-1640 complete medium, and cells were cultured for 48 h prior to harvest.

**RT-PCR analysis of Cav-1**

Primers were designed and synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The sequences used are follows: Cav-1: forward primer, 5′-TCAACCGCGACCCCTAAACACC-3′; reverse primer, 5′-TGAAATAGCTCAGAAGAGACA-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward primer, 5′-GGGTGGAATCATATTGGAACA-3′, reverse primer, 5′-GGGTTGGAATCATATTGGAACA-3′. SYBR® Premix Ex Taq™ was employed to detect the mRNA expression of Cav-1. For RT-PCR, cDNA samples (1 μL) were mixed with 10 μmol/L of the primers and 2 × SYBR Premix Ex Taq (10 μL) to a final volume of 20 μL. Reactions were performed using the real-time PCR Mx3000PTM System (Genetimes Technology, China). The cycling parameters were 95°C for 2 min followed by 40 cycles of 95°C for 15 s, the appropriate annealing temperature for 20 s, and 72°C for 1 min. Relative Cav-1 mRNA levels are expressed as the relative fold change in threshold cycle (Ct) values and calculated using the following formula: $2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct(Sample) - \Delta Ct(Calibrator))}$, where each $\Delta Ct = \Delta C_{Cav-1} - \Delta C_{GAPDH}$. One sample without any treatment was designated a calibrator. Each sample was analysed in triplicate.

**Immunoblot analysis for Cav-1 and LC3B-II**

Protein lysates were extracted from Nthy-ori 3-1 cells using a whole-cell extraction kit (Merck Millipore, Billerica, MA, USA). Protein concentration was determined using a BCA protein concentration kit (Beyotime, Shanghai, China). The 5 × loading buffer (Solarbio, Beijing, China) was added, and simples were heated at 100°C for 10 min. First, 5 μg of protein was subjected to electrophoresis on a 10%–15% SDS-PAGE gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). After saturation with 5% non-fat powdered milk in PBS-Tween 0.1% (PBST) (blocking buffer) for 1 h at room temperature, the membrane was incubated with primary antibodies overnight at 4°C. Following several washes with PBST, the membranes were incubated with corresponding HRP-conjugated secondary antibodies. The proteins were visualized using the ChemiDoc™ XRS + System (Bio-Rad, CA, USA). The relative expression levels of Cav-1 and LC3B-II were quantified using ImageJ software. The expression level of each protein was normalized to GAPDH.
temperature, the membranes were incubated with primary antibodies (1:1,000 in PBST containing 2% BSA) overnight at 4°C. β-actin was used as a loading control. Following washing three times with PBST for 10 min at room temperature, membranes were probed with peroxidase-conjugated secondary antibody (anti-rabbit IgG whole-molecule diluted 1:5,000 in PBST) for 1 h at room temperature. After three washings, the signal was detected using the Pierce ECL-plus substrate (Thermo Fisher Scientific, Waltham, MA, USA) and scanned by a Fluor Chem FC3 camera system (Protein-Simple, California, USA). Images were analysed by densitometry using Alpha View software (AIC, California, USA). The results of quantitative analyses are presented graphically. Values were normalized to the loading control.

**Data analysis and statistics**

Data are expressed as the mean ± SEM. Statistical analyses of the data were performed using the t-test or, for non-normally distributed data, the Mann-Whitney test (GraphPad Prism 6.01 Software, Inc., San Diego, CA, USA). All experiments were carried out at least three times separately. A p value of less than 0.05 was considered significant.

**Results**

**Increased IL-1β and IFN-γ as well as reduced Cav-1 expression in thyroid tissues from HT patients**

To investigate the relationship between cytokines and Cav-1 in HT patients, the expression of the two cytokines, IL-1β and IFN-γ, and Cav-1 were detected by IHC. The results indicated that in HT tissues, Cav-1 was significantly lower in thyrocytes, especially where lymphocytes heavily infiltrated. Meanwhile, IL-1β and IFN-γ were abundant in both lymphocytes and thyrocytes compared to those of normal control thyroid tissues (n = 5) (Fig. 1a). The statistical analysis of the quantification of the IL-1β, IFN-γ, and Cav-1 levels in five pairs of tissue samples is shown in Fig. 1b. Compared with the control tissues, HT tissues expressed higher levels of IL-1β and IFN-γ and lower levels of Cav-1.

**IL-1β and IFN-γ inhibit the expression of Cav-1 and LC3B-II in TFC**

To further determine whether the decreases in Cav-1 and LC3B-II were due to the increase in IL-1β and IFN-γ in HT tissues, we used Nthy-ori 3-1 cells, an immortalized human thyroid follicular epithelial cell line, for further experiments. Nthy-ori 3-1 cells were treated with 2 ng/mL IL-1β and 10 ng/mL IFN-γ. The results showed that mRNA expression of Cav-1 was significantly decreased compared to that of the control (Fig. 2a). In addition, the Cav-1 and LC3B-II proteins were decreased by IL-1β and IFN-γ treatment (Fig. 2b and c).

**Cav-1 knockdown impairs autophagy activity in TFC**

To evaluate the association between the Cav-1 decrease and autophagy inhibition in the presence of IL-1β and IFN-γ, the expression of LC3B-II was detec-
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Fig. 2  The effect of IL-1β and IFN-γ on Cav-1 and LC3B-II expression in Nthy-ori 3-1 cells. Nthy-ori 3-1 cells were treated with 2 ng/mL IL-1β and 10 ng/mL IFN-γ. (a) After treatment for 4 h, Cav-1 mRNA expression was analysed by real-time reverse transcription PCR. The Cav-1 mRNA expression relative to that of GAPDH is expressed as the mean ± SEM of three independent experiments. (b) Cell lysates were harvested after 48 h treatment, and Cav-1 and LC3B-II were detected by immunoblot analysis. The representative images were probed for Cav-1 and LC3B-II (β-actin served as the loading control). (c) The cumulative data are expressed as the mean ± SEM, representative of four independent experiments. **p < 0.01 vs. control.

Fig. 3  Effect of Cav-1 knockdown in Nthy-ori 3-1 cells on the autophagy activity. (a) Nthy-ori 3-1 cells were transfected with siRNA against human Cav-1 (si-h-Cav-1-439, si-h-Cav-1-548, si-h-Cav-1-710) or negative-control siRNA (siCon) for 48 h, and then, cell lysates were analysed by immunoblot using Cav-1 antibody. β-actin was used as the loading control. (b) Nthy-ori 3-1 cells were transfected with si-h-Cav-1-548 or siCon. Forty-eight hours after transfection, immunoblot analysis was performed to determine the effects of Cav-1 knockdown on LC3B-II expression. The representative images were probed for Cav-1 and LC3B-II (β-actin served as the loading control). (c) The cumulative data are expressed as the mean ± SEM, representative of three independent experiments. **p < 0.01 vs. siCon.

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ned by immunoblot analysis after Cav-1 was knocked down in Nthy-ori 3-1 cells. First, cells were transfected with si-h-Cav-1 or siCon, and the successful knockdown of Cav-1 was confirmed using immunoblot analysis. The protein level of Cav-1 in the si-h-Cav-1 cells was significantly lower than in the siCon group; in particular, si-h-Cav-1-548 had the best efficiency of Cav-1 knockdown (Fig. 3a). Furthermore, Cav-1 knockdown markedly reduced LC3B-II protein expression in Nthy-ori 3-1 cells (Fig. 3b and c), suggesting that Cav-1 may regulate autophagy activity.
Discussion

HT is caused by an interaction of genetic susceptibility and environmental triggers that lead to the breakdown of tolerance to thyroid antigens, which initiates thyroid autoimmunity and recruits lymphocytes into the thyroid [27, 28]. On the one hand, lymphocytes infiltrating the thyroid gland can be directly cytoxic to TFC and disturb TFC integrity [29, 30]. On the other hand, these lymphocytes produce cytokines that result in perpetuation of the autoimmune process [31, 32]. Th1 cytokines are the predominant immunological environment in HT and play important roles in the pathogenesis of HT [27, 28, 30, 33, 34]. For example, cytokines not only modulate TFC growth and function [12, 32, 34] but also can even confer TFC with immunological properties, such as increased expression of major histocompatibility complex class II (MCH-II), adhesion molecules and Fas [28, 34]. In addition, thyroid cells from HT patients are able to produce many proinflammatory cytokines under inflammatory conditions, such as IL-1β, IFN-γ, and IL-23 [31-36].

In the present study, IHC showed that intense staining for IL-1β occurred in the aggregation of lymphocytes in HT thyroid tissues. Thyrocytes close to lymphocytes also had detectable expression. IFN-γ expression was elevated in HT thyroid tissues, which corresponded to the increase in IL-1β. The finding further demonstrated that TFC are able to secrete IL-1β and IFN-γ. It is reported that cytokines can stimulate thyrocytes to secrete some chemokines, especially CXCL10 (the prototype of the IFN-γ-inducible Th1 chemokines), which in turn attract more lymphocytes to infiltrate the thyroid gland, finally creating an amplification feedback loop [27]. Therefore, a vicious cycle probably also exists between thyrocytes and lymphocytes involving the production of IL-1β and IFN-γ, perpetuating the autoimmune process of HT. However, the combined effect of the two Th1 cytokines on TFC is still unclear.

Notably, we found that Cav-1 expression was significantly reduced in thyrocytes from HT patients, especially where lymphocytes heavily infiltrated. Therefore, we postulated that the decreased Cav-1 in thyroids glands of HT might be due to up-regulation of IL-1β and IFN-γ released by the inflammatory infiltrate. As demonstrated by in vitro our experiments, Cav-1 mRNA and protein were strongly decreased in response to IL-1β/IFN-γ in Nthy-ori 3-1 cells. Nthy-ori 3-1 cells, an immortalized thyroid follicular epithelial cell line, are highly suitable for studies of the control of growth and function in the human thyroid [37, 38]. Our results are consistent with previous reports that a combination of IL-1α and IFN-γ induces Cav-1 reduction in primary cultures of human thyrocytes [18, 39]. Collectively, these results confirm that Th1 cytokines are at least in part responsible for Cav-1 down-regulation. Furthermore, the effect of Th1 cytokines on Cav-1 expression is of particular importance, considering that Cav-1 plays important roles in diverse mammalian cells in the regulation of multiple plasma-membrane-initiated signalling molecules and receptors, primarily through the interaction of the Cav-1 scaffolding domain (CSD) with target proteins, such as mitogen-activated protein kinase (MAPK) [40], transforming growth factor-beta (TGF-β) receptor [41] and AKT [42].

Cav-1 is an essential structural constituent of lipid rafts in the plasma membrane that are enriched in glycosphingolipids and cholesterol [43-45]. Induction of autophagy is involved in various intracellular membrane structures, including autophagosomes, lysosomes, and autolysosomes [46, 47]. Light chain 3 (LC3), an autophagy-related protein, exists in two forms, called LC3B-I and LC3B-II. LC3B-I is changed into LC3B-II and then incorporated into autophagosomes in the process of autophagy [48]. Therefore, the LC3B-II level serves as a well-characterized autophagy marker. Cav-1 might participate in regulating autophagy under several stress conditions [49, 50]. Cav-1 negatively regulates autophagy by directly binding LC3B-II localized in caveolae in the plasma membrane [51, 52] or through interactions with ATG12-ATG5 in the cytosol [53]. In addition, in mouse embryonic fibroblasts, Cav-1 knock-out promotes lysosomal function and increases LC3B-II expression via the disruption of lipid rafts to promote cell survival under starvation [49]. In contrast, Cav-1 can also function as a positive regulator of 17β-oestradiol mediated autophagy in BT474 human breast cancer cells [50] or autophagy-mediated claudin-5 degradation in oxygen-glucose deprivation-treated endothelial cells [54] and oxidative stress-activated autophagy [55], which involves the Cav-1/HMGB1 pathway [50], the autophagy-lysosome pathway and an interaction with Beclin-1. Moreover, some components of lipid rafts are reported to promote autophagy [56, 57]. Since Cav-1 is a component of lipid rafts, it remains to be further determined whether the Cav-1-dependent lipid rafts or lipid rafts per se are involved in the regulation of autophagy presently. Therefore, the question of how Cav-1 can regulate autophagy
negatively or positively may depend on the specific cell types, tissue functions, stimuli, lipid raft dependence, disease conditions, and the context that arises from these factors.

Increasing evidence indicates the importance of autophagy in the pathogenesis and development of inflammation [58, 59]. Previous work from our laboratory has found that the LC3B-II protein expression in TFC was sharply decreased in HT tissues compared with that of the healthy controls [23, 24]. In this study, LC3B-II expression in TFC was significantly decreased following treatment with IL-1β and IFN-γ, suggesting that autophagy activity in TFC was suppressed under a Th1 cytokines environment. Based on the findings above, we speculate that the IL-1β- and IFN-γ-mediated decrease in autophagy activity in TFC was associated with the Cav-1 protein level. More interestingly, Cav-1 knockdown markedly inhibited LC3B-II protein expression in TFC, further providing evidence of the pivotal role of Cav-1 in the regulation of autophagy activity in HT.

In conclusion, our study demonstrated the effect of IL-1β and IFN-γ on Cav-1 and LC3B-II expression and the correlation between Cav-1 and autophagy activity in TFC. However, the exact molecular mechanisms underlying the regulatory role of these inflammatory cytokines and Cav-1 in autophagy of TFC remain to be further investigated. The adverse effect of down-regulated Cav-1 on autophagy activity in TFC could yield novel insight into the potential pathogenic mechanisms of HT.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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