BMB
*Reports*

**BMB Rep. 2021; 54(2): 136-141**
www.bmbreports.org

# Ganglioside GT1b increases hyaluronic acid synthase 2 via PI3K activation with TLR2 dependence in orbital fibroblasts from thyroid eye disease patients

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**Thyroid eye disease (TED) is a complex autoimmune disease with a spectrum of signs. we previously reported that trisialoganglioside (GT)1b is significantly overexpressed in the orbital tissue of TED patients, and that exogenous GT1b strongly induced HA synthesis in orbital fibroblasts. However, the signaling pathway in GT1b-induced hyaluronic acid synthase (HAS) expression in orbital fibroblasts from TED patients have rarely been investigated. Here, we demonstrated that GT1b induced phosphorylation of Akt/mTOR in a dose-dependent manner in orbital fibroblasts from TED patients. Both co-treatment with a specific inhibitor for PI3K and siRNA knockdown of TLR2 attenuated GT1b-induced Akt phosphorylation. GT1b significantly induced HAS2 expression at both the transcriptional and translational level, which was suppressed by specific inhibitors of PI3K or Akt/mTOR, and by siRNA knockdown of TLR2. In conclusion, GT1b induced HAS2 in orbital fibroblasts from TED patients via activation of the PI3K-related signaling pathway, dependent on TLR2. [BMB Reports 2021; 54(2): 136-141]**

## INTRODUCTION

Thyroid eye disease (TED) is an autoimmune disorder in which stimulatory antibodies bind to the thyrotropin receptor, resulting in active inflammation of orbital tissues such as orbital fat and extraocular muscle (1). While most TED patients experience only mild ocular surface discomfort, approximately 3-5% of patients suffer from more severe ophthalmopathy, characterized by proptosis, eyelid retraction, chemosis, double vision, limitation of ocular motion, and even vision loss due to corneal ulceration or compressive optic neuropathy (2, 3). Most of these clinical findings result from progressive enlargement of orbital tissues within the limited space of the bony orbit (4). Although the pathogenesis of TED is not completely understood, the volumetric expansion of orbital tissues is known to originate from edematous changes of connective tissue and extraocular muscles, which is caused by extracellular accumulation of hyaluronic acid (HA) (5).

HA, which belongs to the glycosaminoglycan family, is one of the most prominent macromolecules of extracellular matrices. It is synthesized by a class of integral membrane proteins called hyaluronic acid synthase (HAS), of which vertebrates have three types: HAS1, HAS2, and HAS3. These enzymes lengthen hyaluronan by repeatedly adding glucuronic acid and N-acetylglucosamine to the nascent polysaccharide as it is extruded via an ABC-transporter through the cell membrane into the extracellular space (6), which occurs according to the rheological properties of HA including hydrophilia, polyanionic charge, and high osmotic pressure.

The extracellular accumulation of HA attracts water into the tissue, resulting in edema (7). Taken together with the characteristics of orbital fibroblasts and resident cells in orbital fatty connective tissue, which possess the capability to produce a significant amount of HA in response to proinflammatory cytokines such as interleukin (IL)-1β and transforming growth factor (TGF)-β (8-10), the effective suppression of HA production in orbital fibroblasts is thought to be a therapeutic target in TED.

Ganglioside is an acidic glycosphingolipid that has a variable sialic acid-containing oligosaccharide structure attached to an acylated ceramide core (11). More than 60 gangliosides exist, according to the numbers and sites of sialic acid residues. They are classified into a-, b-, and c- series gangliosides according to the numbers and sites of sialic acids bound to the galactose portion of the molecule (12). The a-series includes monosialoganglioside (GM)3, GM2, GM1, disialoganglioside (GD)1a, and trisialoganglioside (GT)1a; the b-series includes GD3, GD2, GD1b, GT1b, and quatrosialoganglioside (GQ)1b; and the c-series includes GT3, GT2, GT1c, GQ1c, and pentasialoganglioside (GP)1c (13). They are widely distributed in the plasma membrane

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https://doi.org/10.5483/BMBRep.2021.54.2.178

**Received 26 August 2020, Revised 10 September 2020, Accepted 17 December 2020**

**Keywords:** Orbital fibroblast, Phosphoinositide 3-kinase, Thyroid eye disease, Toll-like receptor 2, Trisialoganglioside 1b

ISSN: 1976-670X (electronic edition)

of vertebrate tissue and are known to play a key role in cell differentiation, adhesion, proliferation, and signal transduction (14). In our previous study, we reported that the expression of GT1b was increased in the orbital fatty connective tissues of TED patients, and that GT1b induced an increase of both HAS and HA in orbital fibroblasts, suggesting a possible role of gangliosides in the pathogenesis of TED (15). However, the signaling pathway of GT1b, which could be a potential therapeutic target, has not been completely elucidated.

In the present study, we describe a signaling pathway through which GT1b increased HAS2 expression in orbital fibroblasts from TED patients. We showed that GT1b induced phosphorylation of both protein kinase B (Akt) and mammalian target of rapamycin (mTOR) via phosphoinositide 3-kinase (PI3K) activation; moreover, the increased HAS2 expression induced by GT1b was effectively attenuated by co-treatment with specific inhibitors of PI3K or mTOR in orbital fibroblasts from TED patients. Additionally, we showed that loss of Toll-like receptor (TLR)2 attenuated the GT1b-induced HAS2 increase in cells. Taken together, our results indicated that GT1b induced HAS2 expression by activating the PI3K/Akt/mTOR signaling cascade, with dependence on the TLR2. Our results provide insight into the signaling pathways through which GT1b stimulates HA synthesis in orbital fibroblasts from TED patients, suggesting a possible specific therapeutic target for the treatment of this disease.

## RESULTS

### PI3K inhibitor reduced GT1b-induced Akt/mTOR phosphorylation

We initially examined whether the Akt/mTOR signaling pathway was activated in response to GT1b in orbital fibroblasts from TED patients. Cells were treated by commercially available GT1b with graded concentrations up to 40 μg/ml for 5 minutes. GT1b induced a dose-dependent increase in the levels of phosphorylated Akt up to a concentration of 40 μg/ml, with no changes in the amount of Akt or GAPDH proteins (Fig. 1A). A significant increase in Akt phosphorylation was detected at 5 minutes in orbital fibroblasts from TED patients treated with 40 μg/ml GT1b, which showed a gradual decrease with time but persisted at a significant level for up to 30 minutes (Fig. 1B). The effects of GT1b on mTOR phosphorylation were also examined. Immunoblot analyses revealed that mTOR phosphorylation reached significantly increased levels at 30 minutes after GT1b treatment (Fig. 1C). The Akt/mTOR signaling pathways are known to be activated mainly by PI3K (16). To examine whether GT1b-induced phosphorylation of Akt/mTOR was mediated by PI3K in orbital fibroblasts from TED patients, we utilized a PI3K inhibitor (LY294002). GT1b-induced phosphorylation of Akt was significantly blocked in orbital fibroblasts from TED patients by LY294002 in a dose-dependent manner, up to a concentration of 10 μg/ml (Fig. 1D). Both Akt and mTOR were phosphorylated in response to GT1b (40 μg/ml); this GT1b-induced phosphorylation of Akt/mTOR was significantly reduced

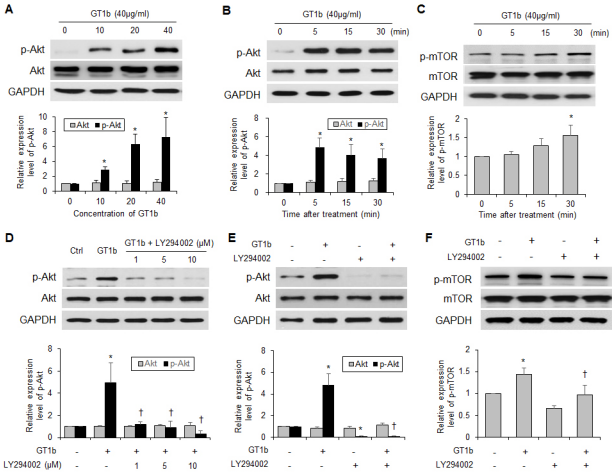


**Fig. 1.** GT1b-induced Akt/mTOR phosphorylation was mediated by phosphoinositide 3-kinase in orbital fibroblasts from thyroid eye disease (TED) patients. Orbital fibroblasts were cultivated from TED patients (n = 5) (A) Cells were treated with gradual increments of GT1b (10, 20, 40 μg/ml) for 5 minutes, followed by immunoblotting for phosphorylated Akt (p-Akt) or Akt. (B, C) Following treatment of cells with 40 μg/ml GT1b for the indicated times, immunoblotting was performed for p-Akt/Akt and p-mTOR/mTOR, respectively. (D) Cells were treated with 40 μg/ml of GT1b with or without addition of gradual increments of LY294002 (1, 5, and 10 μM) for 5 minutes, followed by immunoblotting for p-Akt or Akt. (E) Cells were treated with 40 μg/ml of GT1b in the presence or absence of 10 μM of LY294002 for 5 minutes and immunoblotting was performed for p-Akt or Akt. (F) Following treatment with GT1b and/or LY294003 for 30 minutes as in (E), immunoblotting was performed for phosphorylated mTOR. Representative blots from independent experiments, for cells from each TED patient, are shown. Quantification of each protein (fold increase) was calculated by dividing the densitometric value of each lane by the corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) value (bar graphs, means ± SD; *$P < 0.05$ vs. untreated control; †$P < 0.05$ vs. GT1b-treated cells).

by LY294002 (Fig. 1E, F), indicating that PI3K was directly involved in the GT1b-induced phosphorylation of Akt/mTOR signaling pathways in orbital fibroblasts from TED patients.

### Akt phosphorylation by GT1b was mediated by TLR2

Because there have been reports suggesting that TLR2 is involved in gangliosides-mediated intracellular signaling (17), and that TLR2 stimulation activates PI3K/Akt signaling (18), we next examined whether downregulation of TLR2 expression using specific siRNA affected Akt phosphorylation in orbital fibroblasts from TED patients. Fig. 2A, B shows that transfection of cells with TLR2 siRNA decreased endogenous TLR2 expression. In the presence of TLR2 siRNA, GT1b failed to induce an increase in Akt phosphorylation (Fig. 2C), which supported the hypothesis that GT1b induced Akt phosphorylation and activation via TLR2.

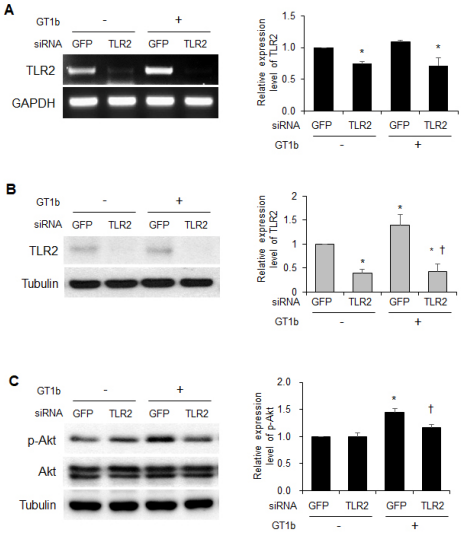


**Fig. 2.** Akt phosphorylation by GT1b was mediated by Toll-like receptor (TLR)2. (A, B) Orbital fibroblasts were transiently transfected with TLR2 siRNA or control siRNA. One day after transfection, cells were treated with or without GT1b (40 μg/ml) for 24 hours and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting. (C) Cells transiently transfected with TLR2 siRNA or control siRNA were treated with GT1b (40 μg/ml) for 0, 0.5, 1.5, 3, and 6 hours and analyzed by immunoblotting with phosphorylation-specific antibodies for Akt. Effective siRNA-mediated suppression of TLR2 mRNA/protein expression was verified for each assay by RT-PCR and immunoblotting. Tubulin was used as the loading control. Data are presented as the fold increase compared with untreated control cells. Data shown are representative of at least three experiments (bar graphs, means ± SD; *P < 0.05 vs. untreated control; †P < 0.05 vs. GT1b-treated cells).

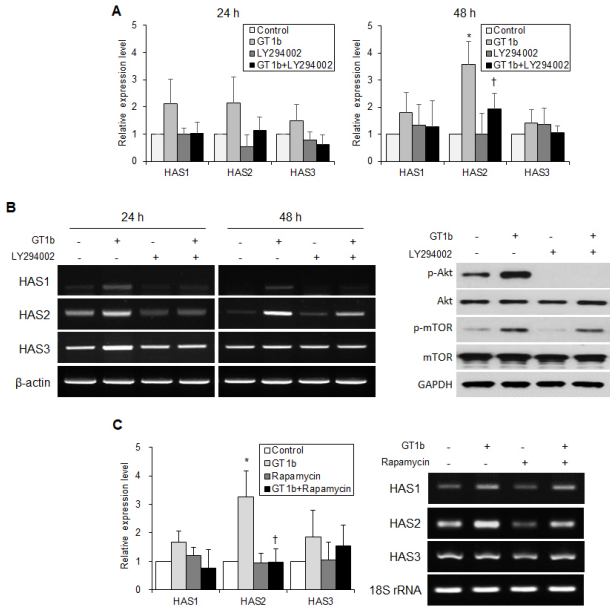


**Fig. 3.** Involvement of the PI3K/Akt/mTOR signaling pathway in GT1b-induced hyaluronic acid synthase (HAS) transduction. (A) Orbital fibroblasts from TED patients were treated with 40 μg/ml of GT1b/10 μM or LY294002, alone or in combination at the same dose, for 24 hours or 48 hours and transcriptional levels of HAS1, HAS2, and HAS3 in cells were measured by the quantitative real-time polymerase chain reaction (qRT-PCR). (B) Following the same treatment protocol as (A), RT-PCR analyses for HAS1, HAS2, or HAS3 were performed. Cells were treated with 40 μg/ml of GT1b in the presence or absence of 10 μM of LY294002 and immunoblotting was performed for p-Akt/Akt and p-mTOR/mTOR. (C) Cells were treated with 40 μg/ml of GT1b/5 ng/ml of rapamycin, alone or in combination at the same dose, for 48 hours and the transcriptional levels of HAS1, HAS2, and HAS3 in cells were measured by qRT-PCR (left) or RT-PCR (right). Representative blots from independent experiments for cells from each TED patient are shown. *P < 0.05 vs. untreated control; †P < 0.05 vs. GT1b-treated cells.

## The PI3K/mTOR inhibitors abrogated GT1b-induced HAS expression

We previously reported that GT1b induced HA production in orbital fibroblasts, which might have resulted from increased expression of HAS by GT1b (15). We therefore examined the contribution of PI3K to GT1b-induced HAS expression by utilizing a PI3K inhibitor. To determine whether LY294002 affected HAS expression at the transcriptional level, orbital fibroblasts from TED patients were treated with GT1b (40 μg/ml) in the presence or absence of LY294002 (10 μg/ml) for 24 hours or 48 hours, and RNA was then extracted and subjected to qRT-PCR. At 24 hours of incubation, the expression levels of HAS1, HAS2, and HAS3 were increased by GT1b treatment, and attenuated by co-treatment with LY294002, but the changes were not significant (Fig. 3A, left). At 48 hours of incubation, the expression level of HAS2 was significantly increased by GT1b treatment, and inhibition of PI3K suppressed GT1b-induced HAS2 expression. HAS1 and HAS3 showed similar results as those at 24 hours (Fig. 3A, right). These results were verified by RT-PCR, which showed that GT1b increased mRNA transcription of HAS1, HAS2 and HAS3, and LY294002 dramatically suppressed the GT1b-induced increase in HAS mRNA levels; this was the most striking for HAS2 (Fig. 3B, left). LY294002 alone did not show suppression of HAS mRNA levels. GT1b-induced phosphorylation of Akt/mTOR was inhibited by LY294002 (Fig. 3B, right).

To explore whether activation of mTOR was involved in the signaling pathway of GT1b-induced PI3K-mediated HAS expression, qRT-PCR was performed using rapamycin, a specific inhibitor of mTOR. Fig. 3C (left) shows that GT1b strikingly increased HAS2 transcription, and co-treatment with rapamycin drastically suppressed it. Furthermore, these results were verified by RT-PCR (Fig. 3C, right). Taken together, these results indicated that GT1b induced expression of HAS2 through the PI3K/Akt/mTOR signaling pathways in orbital fibroblasts from TED patients.

### Loss of TLR2 and PI3K/mTOR inhibitors attenuated GT1b-induced HAS2 transcription and translation, respectively

Orbital fibroblasts transfected with TLR2 siRNA displayed reduced levels of endogenous TLR2 expression, as confirmed by RT-PCR (data not shown), in a manner similar to Fig. 2A. In the presence of TLR2 siRNA, GT1b failed to induce an increase in HAS2 mRNA/protein expression (Fig. 4A, B). Because transcription of HAS2 was substantially elevated in GT1b-treated orbital fibroblasts from TED patients, and this increase was significantly suppressed by LY294002 as well as rapamycin, we measured relevant protein levels of HAS2 in cell lysates of GT1b-treated orbital fibroblasts using immunoblotting. Consistent with the qRT-PCR and RT-PCR results, phosphorylation of Akt/mTOR and HAS2 protein expression was enhanced by GT1b treatment and suppressed by LY294002 and rapamycin treatments, respectively (Fig. 4C).

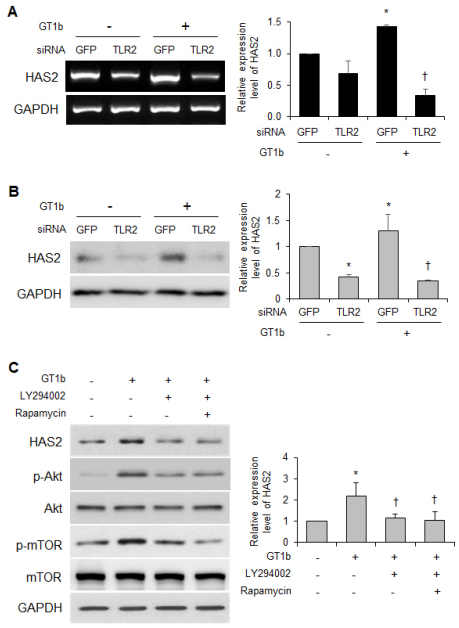


**Fig. 4.** Dependence on TLR2 and PI3K/Akt/mTOR signaling pathway in GT1b-induced HAS2 expression. (A, B) Orbital fibroblasts were transiently transfected with TLR2 siRNA or control siRNA. One day after transfection, cells were treated with or without GT1b (40 μg/ml) for 24 hours and analyzed by the RT-PCR and immunoblotting. Values for HAS2 mRNA/protein expression were normalized to GAPDH levels for each experimental condition. The value of HAS2 in the untreated control transfected with control siRNA was set as 1. (C) Cells were treated with 40 μg/ml of GT1b with or without addition of 10 μM of LY294002 or 5 ng/ml of rapamycin, and immunoblotting was performed for HAS2, p-Akt/Akt and p-mTOR/mTOR. Representative blots from independent experiments, for cells from each TED patient, are shown. The data represent three independent experiments. Quantification of each protein (fold increase) was calculated by dividing the densitometric value of each lane by the corresponding GAPDH value (bar graphs, means ± SD; *$P < 0.05$ vs. untreated control; $^{\dagger}P < 0.05$ vs. GT1b-treated cells).

## DISCUSSION

Ganglioside is an acidic glycosphingolipid that can be classified according to the number of sialic acid residues. Ganglioside is known to play important roles in the nervous system, including neuronal plasticity and regeneration. Experimental data have shown that exogenous gangliosides exhibit properties similar to neurotrophins, which are important in the survival and maintenance of neurons (19). Among them, increasing evidence has shown that the b-series ganglioside, GT1b, has a potential role in modulating the immunological process. For example, GT1b indirectly suppresses immunoglobulin production in human peripheral blood mononuclear cells without affecting the viability of the cells, indicating that GT1b may act as an important inhibitor of the human humoral immune response (20). In another report, the b-series gangliosides, including GT1b, enhanced IL-2 and IFN-γ production in phytohemagglutinin-stimulated human T cells (21). Regarding the pathogenic role of gangliosides in TED, we previously reported that GT1b is significantly overexpressed in the orbital tissue of TED patients, and that exogenous GT1b strongly induced HA synthesis in orbital fibroblasts; this suggests a pathological role of GT1b in the pathogenesis of TED (15). In terms of developing a selective molecular treatment for a particular disease based on its pathogenic mechanism, identifying the related signaling pathway is important.

In the present study, GT1b induced Akt phosphorylation by PI3K activation in orbital fibroblasts from TED patients. The PI3K/Akt signaling pathway is an intracellular signaling pathway that is closely associated with cell proliferation, transformation, metabolism, motility, and the development and progression of tumors (22). Regarding its role in pathogenesis, clinical trials for some cancers have been performed using PI3K inhibitors as therapeutic agents, which have shown promising results (23-25). Akt is also known as an important mediator of insulin-activated insulin-like growth factor (IGF)-1 receptor signaling, which plays a significant role in the regulation of adipocyte differentiation (26). Regarding orbital fibroblasts, several stimulants, such as IGF-1 (27), thyroid stimulating antibody (28), and thyroid stimulating hormone (29), have been reported to induce phosphorylation of Akt, suggesting pathogenic roles of its signaling cascade in TED, as well as its potential as a target for novel TED therapies by suppression of the PI3K/Akt signaling pathway. Our results showing that LY294002, a specific PI3K inhibitor, effectively attenuated GT1b-induced Akt phosphorylation in orbital fibroblasts from TED patients supported this possibility.

TLRs are evolutionarily conserved receptors belonging to a family of pattern recognition receptors (30). They are expressed by a variety of cell types, and their activation triggers an inflammatory response by activating regulation pathways of innate and adaptive immunities. However, aberrant activation of TLR signaling pathways leads to the development of chronic inflammatory diseases (31). Regarding ganglioside-mediated intracellular

signaling, the involvement of TLR2 has been proposed, where brain ganglioside mixtures were shown to enhance the expression of TLR2 in murine microglia (BV2) (17). The relevance of TLR2 in PI3K/Akt signaling pathways was revealed by another study of the signaling cascade through which Pam3Cys (a TLR2 agonist) induces cellular proliferation, including PI3K/Akt-mediated proliferation in human colorectal cancer cells (18). Consistent with these two previous reports, our results showed that GT1b induced activation of the PI3K/Akt signaling cascade, and the induction of HAS expression was dependent on TLR2, suggesting a possible link between gangliosides, TLR2, and PI3K/Akt (17, 18). To our knowledge, there has been no previous report of the participation of TLR2 in the pathogenesis of TED.

HA, a high molecular weight glycosaminoglycan, is known as an important mediator of TED. Because HA-accumulated tissues can expand by hydration due to the remarkable hydrophilic nature of HA, abnormal HA accumulation in orbital tissues is considered the hallmark of the connective tissue remodeling found in TED patients (5). In addition, we suggested an additional role of HA in the pathogenesis of TED in our previous report, suggesting that HA increased COX-2 expression in orbital fibroblasts from TED patients via CD44 through MAPK- and NF-κB-mediated signaling pathways, which in turn suggested that HA had a proinflammatory role in addition to its known role in the swelling of orbital tissue during the pathogenesis of TED (32). HA production is mediated by HAS, a membrane-bound enzyme. There are three known isoforms, HAS1, 2, and 3, in mammalian cells. Although human orbital fibroblasts express low levels of all three HA synthases, HAS2 is the most abundantly expressed isoform (33). In addition, HAS2 protein is strongly increased by db-cAMP in orbital preadipocytes, while HAS1 is undetectable by the same treatment (34). In accordance with these previous reports, GT1b strikingly induced HAS2 mRNA expression, while it did not show a significant effect on HAS1 or HAS3 mRNA expression in our system. These results suggested that HAS2 had a role in the production of HA in orbital tissue of TED patients.

Involvement of Akt signaling in the HAS2 expression of various cells was suggested by previous studies. In dermal fibroblasts (35), fibroblasts from *Smpd3* gene-deleted mouse (36), and breast cancer cell line MCF7 (37), the increased expression of HAS2 was positively regulated by phosphorylation of Akt. Consistent with these previous findings, our results showed that GT1b-induced HAS2 expression was mediated by activation of the PI3K/Akt pathway. This activated pathway mediated downstream responses by phosphorylating a range of intracellular target proteins, including mTOR, which is also a member of the PI3K protein kinase family and an important downstream component of the PI3K pathway (38). Our data showing attenuation of GT1b-induced HAS2 expression by co-treatment with a specific mTOR inhibitor supported this possible involvement of the PI3K/Akt/mTOR signal cascade.

In conclusion, our results provided evidence that GT1b activated the PI3K/Akt/mTOR signaling pathways, with dependence on TLR2 when inducing HAS2, further suggesting that it could be a pathogenesis-based therapeutic target for TED.

## MATERIALS AND METHODS

Detailed information is provided in Supplementary Information.

## ACKNOWLEDGEMENTS

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2016R 1A2B1014704) (K.H.K) and by RP-Grant 2018 of Ewha Womans University (H.P.).

## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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