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An Alpha7 Nicotinic Acetylcholine Receptor Agonist (GTS-21) Promotes C_2C_{12} Myonuclear Accretion in Association with Release of Interleukin-6 (IL-6) and Improves Survival in Burned Mice

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Abstract

The role of interleukin-6 (IL-6) in physiological processes and disease is poorly understood. The hypothesis tested in this study was that selective alpha7 acetylcholine receptor (α7AChR) agonist, GTS-21, releases of IL-6 in association with myonuclear accretion and enhances insulin signaling in muscle cells, and improves survival of burn injured (BI) mice. The *in vitro* effects of GTS-21 were determined in C_2C_{12} myoblasts and 7-day differentiated myotubes (myotubes). The *in vivo* effects of GTS-21 were tested in BI wild type (WT) and IL-6 knockout (IL6KO) mice. GTS-21 dose-dependently (0, 100 and 200 μM) significantly increased IL-6 levels in myoblasts and myotubes at 6 and 9 h. GTS-21-induced IL-6 release in myotubes was attenuated by methyllycaconitine (α7AChR antagonist), and by Stat-3 or Stat-5 inhibitors. GTS-21 increased MyoD and Pax7 protein expression, myonuclear accretion and insulin-induced phosphorylation of Akt, GSK-3β and Glut4 in myotubes. The glucose levels of burned IL6KO mice receiving GTS-21 decreased significantly compared to sham-burn IL6KO mice. Superimposition of BI on IL6KO mice caused 100% mortality; GTS-2 reduced mortality to 75% in the IL6KO mice. The 75% mortality in burned WT mice was reduced to 0 % with GTS-21. These *in vitro* findings suggest that GTS-21-induced IL-6 release from muscle is mediated via α7AChRs upstream of Stat-3 and -5 pathways and is associated with myonuclear accretion, possibly via MyoD and Pax7 expression. GTS-21 *in vivo* improves survival in burned WT mice and IL6KO mice, suggesting a potential therapeutic application of α7AChR agonists in treatment of BI.

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INTRODUCTION

The role of interleukin-6 (IL-6) in normal physiological and pathological processes is broad, multifaceted and controversial.[1–8] IL-6 is a ubiquitous cytokine that participates in inflammation, cell proliferation and glucose homeostasis. Skeletal myocytes, keratinocytes, adipocytes, hepatocytes, neurons, endothelial cells, and immune cells including monocytes and/or macrophages produce IL-6; the released amounts and its physiological effects are stimuli- and tissue-dependent.[1,6–14] Some studies have described IL-6 as a detrimental (pro-inflammatory) cytokine in certain pathological conditions. Local and transient IL-6 release, however, has been shown to have proliferative effects in skeletal muscle cells.[2,6] Plasma IL-6 levels in burned patients greater than 1000 pg/ml were associated with increased mortality, while patients with IL-6 levels lower than 500 pg/ml survived.[15]

The beneficial functions of IL-6 have been demonstrated using IL-6 knockout (IL6KO) or IL-6Rα (IL-6 receptor alpha) knockout mice. IL6Rα deficient mice had glucose dyshomeostasis, insulin resistance, inflammation, and shift of macrophage to M1-phenotype during diet-induced obesity.[7] IL-6 regulates glucose homeostasis, when released by contracting skeletal muscle fibers during exercise or at rest.[1,6,10,16,17] Knockdown of IL-6 mRNA in differentiating myotubes impaired expression of the myogenic markers myogenin and Myosin Heavy Chain IIb and inhibited myotube fusion. Primary muscle cells isolated from skeletal muscle of IL6KO mice showed significant reduction in the number of Myosin Heavy Chain IIb-positive cells, associated with impaired myotube fusion.[18] Strong evidence exists that exercise-induced local and transient release of IL-6 promotes muscle growth by acting as an essential regulator of the satellite cell marker Pax7 in human skeletal muscle.[19]

GTS-21, an alpha7 acetylcholine receptor (α7AChR) selective agonist, is a synthetic analog of anabaseine, a naturally occuring animal alkaloid.[20] It has already undergone several phase II clinical trials for potential treatment of cognitive deficits in Alzheimer's disease and schizophrenia, and endotoxemia-induced inflammation.[20,21,22] The role of α7AChR in inflammation is well demonstrated by the electrical stimulation of vagus nerve. The stimulation of this nerve releases acetylcholine, an endogenous agonist of α7AChR, that activates α7AChR in macrophages, leading to the inhibition of pro-inflammatory cytokine release and inflammatory responses.[23] Similarly, the exogenous agonist, GTS-21 has also been documented to have anti-inflammatory properties including attenuation of sepsis- and burn injury (BI)-induced tumor necrosis factor alpha (TNFα) release.[24, 25] The hypothesis, tested in the *in vitro* experiments was that α7AChR agonist, in a concentration- and time-dependent manner, induces IL-6 release from myoblasts and 7-day differentiated myotubes (myotubes), upregulate satellite cell marker Pax7 and myogenic transcription factor MyoD together with myonuclear accretion. The hypothesis tested in the *in vivo* experiments was that IL-6 is pivotal for survival in the early phase of post-burn phase, and GTS-21 *in vivo* improves survival in both wild type (WT) and IL-6KO mice.

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MATERIALS AND METHODS

Drugs

GTS-21 dihydrochloride (MW 381) was synthesized as previously described[20] and stored in the dark in a tightly sealed container before use. The anesthetics ketamine and xylazine were purchased from Vedco Inc. (St. Joseph, MO) and Lloyd Inc. (Shenandoah, IW), respectively. Buprenorphine was obtained from Reckitt Benckiser Pharmaceuticals Inc. (Richmond, VA). The Stat-3 (5, 15)-DPP) and Stat-5 inhibitors were purchased from Sigma (St. Louis, MO) and Cayman Chemicals (Ann Arbor, MI), respectively.

Cell culture

All *in vitro* experiments were performed in C_2C_{12} cells (ATCC, Rockville, MD) derived from murine skeletal muscle. The cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS), and 1% penicillin and streptomycin (ATCC, Rockville, MD) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C until confluence to 1×10^6 cells/ml. To cause differentiation of myotubes, the 10% FBS was replaced with 2% horse serum and cultured for 7 days with daily changes of medium. To test whether GTS-21 effects IL-6 release, the myoblasts and myotubes were treated with GTS-21 (0, 100, 200 μM) alone for 6 or 9 h. Previous studies have documented that GTS-21 decreases release of inflammatory cytokines (e.g., tumor necrosis factor, TNF-α).[24] Therefore, in separate experiments, we determined whether lipopolysaccharide (LPS)-induced release of IL-6 by myoblasts and myotubes is modulated by GTS-21. The cells were either treated with LPS (1μg/ml) or GTS-21 (0, 10, 100, 200 μM) alone and in combination for 6 h. In order to test the binding of GTS-21, a potent antagonist of α7AChR, methyllycaconitine (100 μM), was used. To elucidate downstream pathways mediating IL-6 release by GTS-21, myotubes were treated with GTS-21 in the absence or presence of 20 μM of Stat-3 (5, 15-DPP) or Stat-5 inhibitors for 6 h. To measure *in vivo* local release of IL-6 in skeletal muscle by GTS-21, the gastrocnemius muscle of mice received intramuscular injection of 100 μl of 10 mM GTS-21 and the local production of IL-6 in the muscle was examined after 1 and 6 h.

GTS-21-induced IL-6 release in the gastrocnemius muscle and GTS-21- or LPS-induced IL-6 and TNF-α release into the culture media of myoblasts and myotubes were measured by Sandwich ELISA Kit (R&D Systems Inc., Minneapolis, MN) by interpolating the sample's optical density at 450 nm into the linear range from a standard curve generated with serial concentrations of standard TNF-α and IL-6. The cultured C_2C_{12} cells were harvested using trypsin-EDTA, centrifuged and the cell pellets were stored at −80 °C until analyzed. Frozen cell pellets were thawed and proteins extracted using cell lysis buffer (Sigma, St. Louis, MO) containing 1 tablet of complete protease inhibitor (Roche, Indianapolis, IN) in 10 ml of cell lysis buffer. The resultant supernatant was used to detect the expression of proteins reflecting markers of muscle cell proliferation (Pax7 and MyoD), insulin signaling (Akt, GSK-3β and GLUT4) and Stat-3 phosphorylation.

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Protein expression and phosphorylation by immunoblot analysis

Immunoblot analysis was performed to detect protein expression of Stat-3, and MyoD and Pax7 (myogenic transcripton factor and satellite cell activation markers, respectively), and phosphorylation of Akt, Glut4, GSK-3β (markers of insulin signaling) and Stat-3 in myotubes. For protein analysis, 40 μg of protein extract per lane was fractionated by Nu-PAGE and blotted onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA). For regular antibodies, the membrane was blocked by 5% nonfat dry milk and 1% nonfat dry milk for phospho antibodies for 30 min. The membranes were stained with anti-phospho Akt, anti-phospho Glut4, anti-phospho Stat-3 (Santa Cruz, Dallas, TX), anti-phospho GSK-3β (Cell Signaling, Danvers, MA), anti-Stat-3, anti-MyoD and anti-Pax7 (Santa Cruz, Dallas, TX), and anti-actin (1:10,000) (Sigma, St. Louis, MO) primary antibodies overnight at 4 °C. The membranes were incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies for 30 min (dilution of 1:5,000) and proteins detected by exposing membranes to Kodak X-Omat films.

Staining of myonuclei and determination of myonuclear number in C_2C_{12} myotubes

To determine the myonuclear accretion in myotubes, the cells were subjected to EZBlue staining. Briefly, the myotubes treated in the absence or presence of GTS-21 (10 μM) for 24 h were washed with PBS, and fixed with 100% methanol for 5 min. The myotubes were then incubated with EZBlue stain (Sigma, St. Louis, MO) for 10 min at room temperature and washed three times with H_2O. To examine the fusion of nuclei-rich myotubes, three different random phase contrast images were taken in each well at a 20× magnification using EVOS microscope (Thermofischer Scientific, Waltham, MA).

In addition to myonuclear accretion studied by EZblue stain, the proliferation of myonuclei in myotubes induced by GTS-21 (10 μM) vs saline treatment for 24 h was studied using a propidium Iodide method[24] to stain nuclei. Briefly, the nuclei from dead muscle cells were counted first by mixing only cell suspension and stabilizing buffer. To determine total number of myonuclei in live myotubes, the cell samples were mixed with an equal volume (100 μl) of cell suspension, lysis buffer and stabilizing buffer. The lysis of cell membrane rendered cellular DNA susceptible to staining with the propidium iodide fluorescence dye giving total cell count of myonuclei in lysed myotubes. Nuclei number in myotubes was evaluated by nucleocounter (Nucleocounter® NC-100, Brunswick, NJ). The cell numbers were determined in triplicate.

Mouse model of burn injury and survival

All animal experiments were performed in accordance with the National Institutes of Health and ARRIVE guidelines and protocol (# 2013N000193) was approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Male WT mice (n=12) and IL6KO mice (n=8) with C57Bl/6J background, age between 8–12 weeks, and weighing 25–30 g (Jackson Laboratories, Bar Harbor, ME) were used after acclimatization for 1 week.

The improvised model of scald BI used for our experiments (Supplemental Fig. S1) has been previously described.[24] In order to measure mortality/survival rates, a larger than usual body burn was produced. The mice were anesthetized using mixture of ketamine (50–100 mg/kg)/

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xylazine (5–10 mg/kg, i.p.). A Scald burn was produced by immersing approximate 40% of total body surface area (TBSA) into the hot water at 80 °C for 8s, 6s and 5s for abdomen, back and each flank, respectively. This exposure produced a third degree BI to the skin only and no injury to deeper tissues.[26] The injured animals were kept warm and 1% silver sulfadiazine cream (Silvadene®, Marion Labs. Inc. Kansas City, MO) was applied to area of BI followed by i.p. administration of 1.0 mL of 0.9% saline/mouse for fluid resuscitation. A single dose of buprenorphine (0.1 mg/kg/bw, i.p.) was administered, immediately after BI, as analgesic. Sham-burned mice (control) were treated similarly after anesthesia except immersing them in lukewarm (37 °C) water. Thereafter, the animals were observed for survival for 7 days post burn. A Kaplan-Meier survival plot was used to assess survival rate.

Both WT and IL6KO mice were also studied. The WT mice received BI and were administered saline or GTS-21. IL6KO mice also received BI or sham burn and received saline or GTS-21 resulting in four experimental groups: IL6KO mice receiving sham-burn and given saline or GTS-21 and IL6KO mice receiving BI and treated with saline or GTS-21. GTS-21 (4 mg/kg/bw, i.p.) or saline was administered once daily.

Blood glucose levels and burn injury in WT and IL6KO mice

Blood glucose levels in response to BI or sham BI were studied. Male WT mice (n=4) and IL6KO mice (n=4) with C57Bl/6J background, age between 8–12 weeks, and weighing 25–30 g (Jackson Laboratories, Bar Harbor, ME) were used after acclimatization for 1 week.

The following four groups of IL6KO mice were studied: IL6KO mice receiving sham-burn and given saline or GTS-21 and IL6KO mice receiving BI and given saline or GTS-21. The WT mice receiving BI were treated with saline. The mice in all groups were anesthetized using a mixture of ketamine (50–100 mg/kg)/xylazine (5–10 mg/kg, i.p.) and then BI was produced as described previously,[26] but for this experiment, body surface area burn was reduced approximately to 30% to minimize mortality induced with larger burns of approximately 40%). This was achieved by avoiding injury to the flanks area. The injured animals were kept warm and 1% silver sulfadiazine cream (Silvadene®, Marion Labs. Inc. Kansas City, MO) was applied to area of BI followed by i.p. administration of 1.0 mL of 0.9% saline/mouse as fluid resuscitation. A single dose of buprenorphine (0.1 mg/kg/bw, i.p.) was used once as analgesic immediately after BI. Naive mice were used as controls.

Basal blood glucose level was measured for 7 days in burned WT and IL6KO mice treated either with saline or GTS-21. The glucose levels after 3 h of fasting were measured by obtaining blood from snipped mouse tail. Precision Xtra Glucometer using calibrated Precision Xtra glucose test strip (Abbott Laboratories, Alemada, CA) was used to measure glucose.

Statistical Analysis

Results are presented as means ± SE. For statistical analysis, one way ANOVA was used and subjected to Tukey's multiple comparison tests. For survival analysis, log-rank test was used. $p < 0.05$ was considered statistically significant. All analyses were performed using Prism 6.0 (GraphPad, San Diego, CA).

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RESULTS

GTS-21-mediated cytokine (IL-6 and TNFα) release in myoblasts and myotubes

In order to assess the effects of GTS-21 on IL-6 and TNFα release in C_2C_{12} cells, myoblasts and myotubes were treated with different concentrations of GTS-21 (0, 100 and 200 μM) for two different time periods (6 or 9 h). GTS-21 exposure to myoblasts showed concentration- and time-dependent increase in IL-6 levels from 15 ± 10 pg/ml at 0 μM to 34.6 ± 19 pg/ml at 100 μM and to 50.5 ± 4 pg/ml at 200 μM at 6h, and from 10 ± 4 pg/ml at 0 μM, to 61 ± 8 pg/ml at 100 μM and to 94 ± 3 pg/ml at 200 μM at 9 h (Fig. 1A). In myotubes, GTS-21-induced IL-6 levels were increased from 42 ± 25 pg/ml at 0 μM to 119 ± 9 pg/ml at 100 μM and to 499 ± 12 pg/ml at 200 μM at 6 h, and from 13 ± 6 pg/ml at 0 μM to 99 ± 6 pg/ml at 100 μM and to 1160 ± 66 pg/ml at 200 μM at 9 h. (Fig. 1B). GTS-21 did not release TNFα in culture medium of both myoblasts and myotubes. Thus, GTS-21 was capable of inducing IL-6 release even in the absence of pro-inflammatory partner (TNFα) in C_2C_{12} muscle cells. In the *in vivo* study, the intramuscular injection of GTS-21 into gastrocnemius muscle significantly increased IL-6 levels to 46 ± 2.9 pg/ml compared to untreated muscle (22 ± 3.1 pg/ml) at 6 h. No significant differences were observed in the gastrocnemius IL-6 levels between GTS-21-treated- (31 ± 2.6 pg/ml) and untreated- (22 ± 3.8 pg/ml) gastrocnemius muscle at 1 h (Fig. 1C).

GTS-21 fails to modulate LPS-induced IL-6 in myoblasts and myotubes

In a previous study, we showed that GTS-21 inhibited LPS-induced TNFα release in macrophages.[24] In the following experiments, the modulation by GTS-21 of LPS-induced IL-6 release in myoblasts and myotubes was assessed. The myoblasts were treated with LPS (1μg/ml) alone and in combination with different concentrations of GTS-21 (0, 1, 10 and 100 μM) for 6 h. Treatment of myoblasts with GTS-21 (100 μM) alone significantly increased IL-6 release (61 ± 17 pg/ml). Myoblasts treated with saline or LPS (1 μg/ml) showed a significant increase in IL-6 release from 9 ± 4 pg/ml to 613 ± 115 pg/ml at 6 h. In myotubes, saline vs. GTS-21 (200 μM) caused significant elevation of IL-6 levels from 42 ± 25 pg/ml to 1,320 ± 131 pg/ml at 6 h. Treatment of myotubes with saline vs. LPS (1 μg/ml) alone significantly increased IL-6 levels from 42 ± 25 pg/ml to 3,081 ± 4 pg/ml at 6 h. In myoblasts, co-treatment of LPS with varying concentrations of GTS-21 (0, 1, 10 and 100 μM) did not affect levels of IL-6 (613 ± 115 pg/ml, 654 ± 109 pg/ml, 601 ± 98 pg/ml and 840 ± 157 pg/ml) respectively, compared to LPS-treated myoblasts cells (613 ± 115 pg/ml) (Fig. 2A). Similarly in myotubes, co-treatment of LPS with maximal (100 μM) concentration of GTS-21 used in myoblasts and even higher concentration (200 μM) did not change the LPS-induced levels of IL-6 (2,767 ± 67 pg/ml and 3,048 ± 98 pg/ml) compared to LPS-treated cells (3,081 ± 4 pg/ml) at 6 h (Fig. 2B). Thus, GTS-21 did not modulate LPS-induced IL-6 release, but GTS-21 *per se* induced modest increases of IL-6 both in myoblasts and myotubes.

GTS-21 upregulates MyoD and Pax7 protein expression, and myonuclear accretion in myotubes

In several reports, IL-6 has been described as a myokine that supports muscle growth and development, especially during exercise.[27, 28] To examine whether GTS-21-induced IL-6

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accelerates myogenesis, the upregulation of muscle cell myogenic differentiation marker MyoD and satellite cell marker Pax7 in association with accretion of myonuclei was determined in myotubes. Immunoblot analysis revealed that GTS-21 treatment increased MyoD protein expression (~ three-fold) in myotubes compared to control (without GTS-21) at 6 h. Myoblasts were used as additional control to show that MyoD is not expressed in myoblasts (Fig. 3A). GTS-21 treatment increased Pax7 protein expression compared to untreated control at 6 h (Fig. 3B). Actin was used as a loading control. Moreover, GTS-21 (10 μM) treatment of myotubes significantly increased the number of myonuclei (2.7 *vs* 3.2 $\times 10^6$, $p < 0.05$) and promoted myonuclear accretion compared to GTS-21 naïve cells after 24 h, as shown in Figs. 3C and D. Thus, GTS-induced release of IL-6 is associated with proliferative signals in the myotubes.

GTS-21-induced IL-6 is mitigated by antagonist of α7AChR, Stat-3 or -Stat-5

In the above experiments (Fig. 1A & B), it was demonstrated that GTS-21 induced the release of IL-6 into the culture medium. In order to confirm that IL-6 production is occurring through α7AChR, myotubes were treated with GTS-21 (100 μM) alone and together with potent α7AChR antagonist, methyllycaconitine (100 μM). The saline treated IL-6 level was 24 ± 6 pg/ml. GTS-21-induced IL-6 release was significantly reduced from 190 ± 8.5 to 141 ± 3.3 pg/ml in the presence of methyllycaconitine (Fig. 4A). Myotubes were then treated with GTS-21 in the absence or presence of Stat-3 or −5 inhibitors (20 μM). GTS-21-induced IL-6 release was significantly attenuated from 190 ± 8.5 to 137 ± 12 pg/ml by Stat-3 inhibitor (Fig. 4B). Stat-5 inhibitor also significantly attenuated GTS-21-induced IL-6 release from 190 ± 8.5 to 142 ± 3.4 pg/ml) at 6 h (Fig. 4C). Treatment of myotubes with GTS-21 in the presence of Stat-3 inhibitor significantly decreased Stat-3 phosphorylation (~60%) compared to untreated or ~30% compared to GTS-21-treated myotubes (Fig. 4D and E). These results suggest that GTS-21 acts upstream through the α7AChR and its effects are mediated downstream through Stat-3 and possibly Stat-5 protein also.

GTS-21 enhances insulin sensitivity *in vitro* and improves glucose dyshomeostasis in burn injured IL6KO mice *in vivo*

Secretion of IL-6 and its importance in glucose homeostasis has been well documented.[1,7] The initial studies examined if GTS-21enhances insulin signaling in myotubes. Myotubes were treated with saline or insulin (100 nM) alone and insulin with GTS-21 (100 and 200 μM). Compared to basal (no insulin) controls, insulin treatment of myotubes increased phosphorylation of Akt, GSK-3β and Glut4, which are the pivotal downstream signaling molecules of insulin receptor. Co-treatment of insulin with GTS-21 of myotubes further enhanced the phosphorylation of Akt, GSK-3β and Glut4 compared to both control and insulin alone (Fig. 5A).

It has been reported that mice lacking IL-6 gene are predisposed to hyperglycemia.[29] Therefore, the following studies determined the effects of BI or sham-burn on blood glucose levels in IL6KO mice with saline or GTS-21 treatment. The glucose levels in IL6KO mice were compared to WT mice with BI and naive mice. On day of BI the glucose levels were elevated in all animals receiving BI or sham-burn, probably related to anesthesia and/or stress of BI or sham-burn (data not shown). These glucose levels were not different from

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each other. Time-related (post-burn) glucose levels for each animal are depicted in Fig. 5B and Table 1. The glucose levels of WT burned mice treated with saline were higher than naive animals. In IL6KO mice with BI treated with saline, the glucose levels were 286 mg/dl and 289 mg/dl on 1, and 2 days respectively, but did not survive beyond 2 days. Hence, the glucose levels cannot be reported beyond two days. However, mean glucose levels of IL6KO mice with BI treated with GTS-21 were decreased significantly at day 3 and 7 compared to glucose levels in sham-burn IL6KO mice receiving saline or GTS-21. At day 3, the glucose levels in IL6KO mice with BI treated with GTS-21 (111 ± 5.5 mg/dl) were not significantly different compared to naive animals (99 ± 6 mg/dl). This suggests that superimposition of BI in the absence of IL-6 could cause severe hyperglycemia compared to IL6KO mice without BI. GTS-21 treatment of burned IL6KO mice corrected some of the glucose dyshomeostasis in burned IL6KO mice with time in the later period.

IL6KO mice are prone to burn injury-induced mortality

In this study, the role of IL-6 in survival after BI was investigated. In spite of the smaller BI (approximately 30% vs 40% in the survival studies), IL6KO mice caused 100% mortality by 3 days. Treatment of IL6KO mice with GTS-21 reduced mortality to 75%. That is, GTS-21 administration to IL6KO burned mice improved survival despite the absence of IL-6. WT mice in the presence of BI also had 75% mortality, but GTS-21 treatment of WT burned mice showed 0% mortality at 7 days. The IL6KO mice given saline or GTS-21 without BI used as controls, had 100% survival rate (Fig. 6). Thus the absence of IL-6 causes 100% lethality in burn injured mice, while the administration of GTS-21 reduced the mortality to 75%.

DISCUSSION

The principal findings of this study are that (i) GTS-21 treatment of C_2C_{12} myoblasts and myotubes induces release of IL-6 in a concentration- and time-dependent manner; (ii) GTS-21 also releases IL-6 at 6 h after intramuscular injection in gastronemius muscle. (iii) Although GTS-21has anti-inflammatory effects, it does not attenuate LPS-induced IL-6 release; (iv) GTS-21 upregulates myogenic transcription factor MyoD and satellite cell marker Pax7 protein expression, and promotes myonuclear accretion in myotubes; (v) GTS-21-induced IL-6 release is inhibited by methyllycaconitine and Stat-3/−5 inhibitors; (vi) GTS-21 enhances post receptor insulin signaling in myotubes evidenced by the increased phosphorylation of Akt, GSK-3β and Glut 4; (vii) mortality of 100% in IL6KO mice with BI injury is reduced to 75% (25% survival) by GTS-21 treatment and (viii) mortality in WT mice with BI is decreased from 75% (25% survival) to 0% (100% survival) with GTS-21 treatment. The aforementioned findings support the notion that GTS-21-induced IL-6 not only has a beneficial proliferative role in muscle cells but also a favorable *in vivo* effect on burn-induced mortality.

In a previous study, we have shown that GTS-21 significantly decreased LPS-induced TNFα release from J774A.1 macrophages as well as in peritoneal macrophages from burned mice.[24] This observation led us to test if GTS-21 modulates LPS-induced IL-6 release in C_2C_{12} muscle cells. GTS-21 *per se* induced IL-6 release in myoblasts and myotubes in a

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concentration- and time-dependent manner. Surprisingly, GTS-21 did not modulate LPS-induced IL-6 levels in myoblasts and myotubes at both concentrations (100 and 200 μM). Although LPS treatment causes TNFα release in macrophages, LPS does not release TNFα in C_2C_{12} muscle cells. The failure of myotubes to release TNFα with LPS indicates that the secretion of IL-6 is independent of TNFα release. The fact that GTS-21 does not attenuate IL-6 release may be considered an advantage: GTS-21 administered to BI subjects, while decreasing inflammatory cytokine response (e.g., TNFα release) will not attenuate the beneficial effects of IL-6 release on muscle.

To determine the relationship between the GTS-21-mediated α7AChR activation and IL-6 release, we used an α7AChR antagonist, methyllycaconitine, to mitigate the upstream α7AChR signaling. Methyllycaconitine attenuated GTS-21-induced IL-6 release, indicating the binding of GTS-21 to the receptor. Our studies also elucidated the downstream signaling pathways leading to release of IL-6 by GTS-21. GTS-21 induced IL-6 release was attenuated by Stat-3 or Stat-5 inhibitor. GTS-21 also decreased phosphorylation of Stat-3 protein. These results are in line with a study by Rayanade et. al. who showed that protective effects of IL-6 were abolished by Stat-3 inhibitor and Stat-3 siRNA.[30] It has also been reported that cholinergic agonists inhibit Jak2/Stat-3 phosphorylation through modulating phosphatase activity.[31] Thus, the decrease in Stat-3 phosphorylation by GTS-21 may be the result of complex interplay between the phosphatases and kinases that dephosphorylate and phosphorylate their target proteins.[31,32] Our *in vitro* results of GTS-21-induced decreased Stat-3 phosphorylation in myotubes is in agreement with the above findings.[31] In another report, Kashiwagi et. al. also demonstrated that the decreased Stat-3 phosphorylation by GTS-21 in tibialis muscle of burn-injured mice.[33] IL-6, however, has also been identified as an essential regulator of satellite cell-mediated hypertrophic muscle growth.[6, 19] Thus, in agreement with those studies,[6,19] our data with myotubes demonstrate that GTS-21-induced IL-6 release is associated with proliferation of muscle cells by upregulating the protein expression of Pax7 and MyoD along with the increase in myonuclear accretion in myotubes. It is tempting to speculate that this increase in myonuclear accretion is related to enhance proliferative signaling via downstream insulin signaling proteins (Akt, GSK-3β and Glut 4). Our future studies will address if GTS-21 *in vivo* can attenuate inflammation-induced muscle wasting *via* its effects on IL-6 and satellite cells.

Distinct from leukocytes, energy providing cells such as adipocytes, hepatocytes and myocytes also release IL-6, and participate in glucose homeostasis. Skeletal muscle accounts for up to 80% of the total insulin-mediated glucose disposal in humans. Insulin resistance in skeletal muscle has been shown to contribute significantly to most, if not all to the hyperglycemia of patients with diabetes and diet-induced obesity.[7,34] Acute IL-6 exposure has been shown to increase glucose metabolism in resting human skeletal muscle.[17] Evaluation of the insulin-sensitizing effect of GTS-21 in myotubes shows that GTS-21potentially enhances the insulin signaling by increasing phosphorylation of Akt, GSK-3β and Glut4 in insulin signaling pathway.

Our data in IL6KO mice show that burn injury causes further aggravation of hyperglycemia evidenced by the higher glucose levels early in the animals that died, but it is unclear whether the hyperglycemia caused the death. What is evident is that GTS-21 treatment of

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ILKO mice resulted in healthier glucose levels than in the animals that died, but the pathway for its beneficial effects is unclear. The statistical validity of this statement cannot be tested in view of paucity of numbers in the one group (IL6KO mice) with a 100% mortality by three days. The additional insult of systemic inflammation of BI makes IL6KO burned mice vulnerable to 100% mortality. However, the treatment of IL6KO burned mice with GTS-21 showed improved glucose response at day 3 and 7. Most importantly, BI-induced mortality of 100% in IL6KO mice was attenuated by GTS-21 treatment that had improved-survival (from 0% to 25%) after 7 days. Similarly, even treatment of burned WT mice (which had 75% mortality) with GTS-21 resulted in 100% survival after 7 days. We speculate that 25% survival of IL6KO mice with GTS-21 in contrast to 0% survival of ILKO mice after BI may be mediated by oncostatin M, an endogenous homologue of IL-6, which possibly functions as of surrogate during the absence of IL-6 *in vivo*. This notion is presented on the basis that oncostatin M mimics IL-6 activity and has also shown to induce up to 500 pg/ml of IL-6 *in vitro* in astrocytes[35] similar to that observed with GTS-21 in skeletal muscle cells *in vitro* and *in vivo*. Additionally, it is also possible that anti-inflammatory effect of GTS-21 through the stimulation of α7AChR in BI mice lacking IL-6 may also be responsible for the improved 7 day survival.

In conclusion, a considerable amount of data has been accumulated over time to demonstrate that skeletal muscle is an endocrine gland and IL-6 is a myokine.[28] The concentration of IL-6 present in the cell/tissue seems critical for determining whether the effects of IL-6 will be beneficial or detrimental factor. In other words, IL-6 can either be a pro-inflammatory or anti-inflammatory cytokine in a given cell or tissue. The experiments reported herein document the beneficial effects of IL-6 both in terms of effects on muscle *in vitro* and upon survival and glucose homeostasis *in vivo*. These effects of IL-6 were studied using GTS-21 as an inducer of IL-6 through α7AChR (Fig. 7). These observations open more questions that require further investigations to characterize the role of GTS-21-induced IL-6 in normotrophic/hypertrophic skeletal muscle growth, insulin action and survival that has been demonstrated to involve α7AChR/IL-6 and/or IL-6/Stat-3 or −5 pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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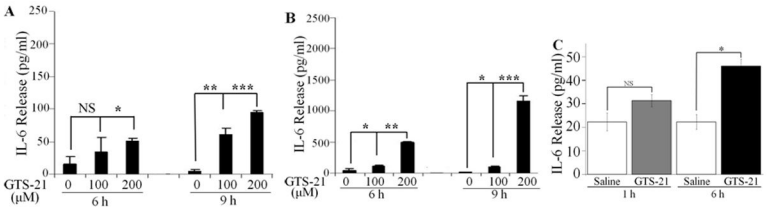

Fig. 1. GTS-21 induces IL-6 release in a concentration- and time-dependent manner in C_2C_{12} myoblasts and differentiated myotubes, and in gastrocnemius muscle

GTS-21 treatment of C_2C_{12} myogenic cells and gastrocnemius muscle. (A) Myoblasts and (B) 7-day differentiated myotubes treated with different concentration of GTS-21 (0, 100 or 200 μM) for 6 and 9 h. Untreated cells were used as controls. (C) Gastrocnemius muscle treated with GTS-21 (100 μl of 10mM solution) for 1 and 6h. The data represent the mean ± standard error (n=3). Statistical analysis using one-way ANOVA with Tukey's multiple comparison tests or unpaired t-test was used for statistical analysis. Asterisks represent *P* values (*$p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$), as compared to control. GTS-21 increases IL-6 release into the culture media of C_2C_{12} myoblasts and myotubes, and into gastrocnemius muscle tissue *in vivo*.

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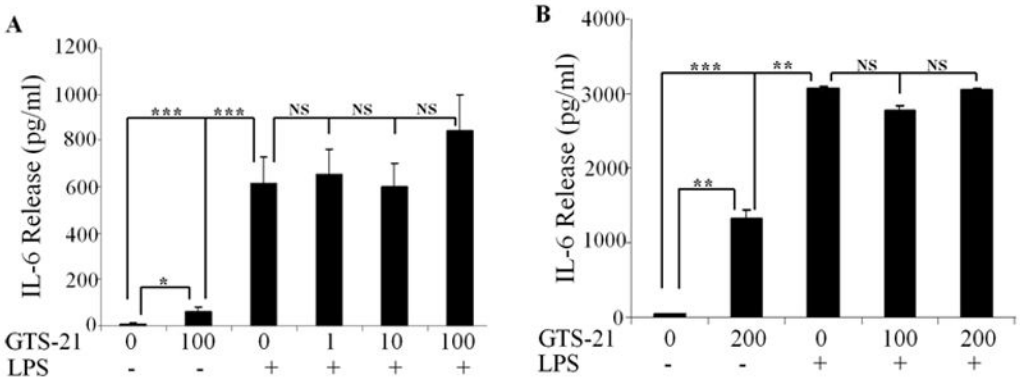

Fig. 2. LPS-induced IL-6 release in C_2C_{12} muscle cells is not modulated by GTS-21

Treatment of LPS-induced C_2C_{12} (A) myoblasts and (B) 7-day differentiated myotubes with different concentration of GTS-21 (0, 1. 10, 100 or 200 μM) for 6 h. Untreated cells were used as controls. The data represent the mean ± standard error (n=3). Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison tests. Asterisks represent *p* values (*$p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$), as compared to control. GTS-21 does not modulate LPS-induced IL-6 levels both in myoblasts and myotubes.

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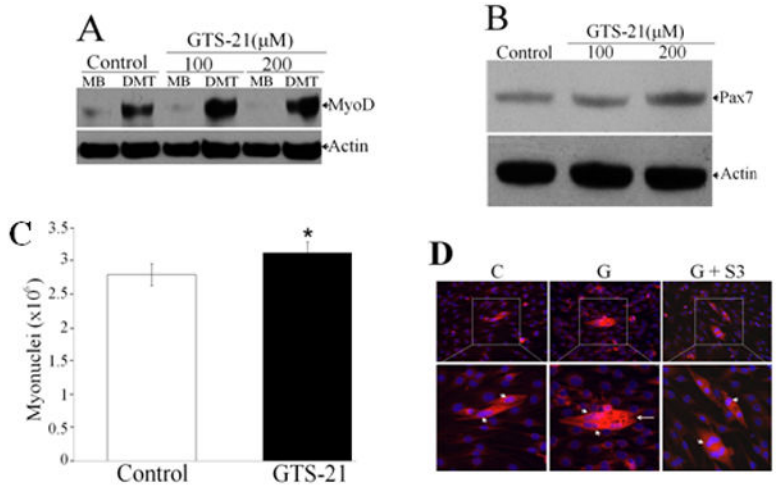

Fig. 3. GTS-21 enhances muscle differentiation and myonuclear accretion

Expression of (A) myogenic differentiation marker MyoD and (B) satellite cell marker Pax7 were studied in 7-day differentiated myotubes treated with 0, 100 or 200 μM of GTS-21 for 6 h. Actin was used as a loading control. MB and DMT represent myoblasts and differentiated myotubes, respectively. (C) Analyses of myonuclei and (D) EZblue staining of untreated (controls), treated myotubes with GTS-21 alone and in the presence of Stat-3 inhibitor. Myonuclei are indicated by arrow heads and myotube fusion is indicated by an arrow. The data represent the mean ± standard error (n=3). Student-t tests were used for statistical analysis. Asterisks represent p values (*$p < 0.05$), as compared to control. The letters C, G, and S3 represent Control, GTS-21, Stat-3 inhibitor, respectively. GTS-21 increases MyoD and Pax7 protein expression, along with myonuclear accretion in myotubes.

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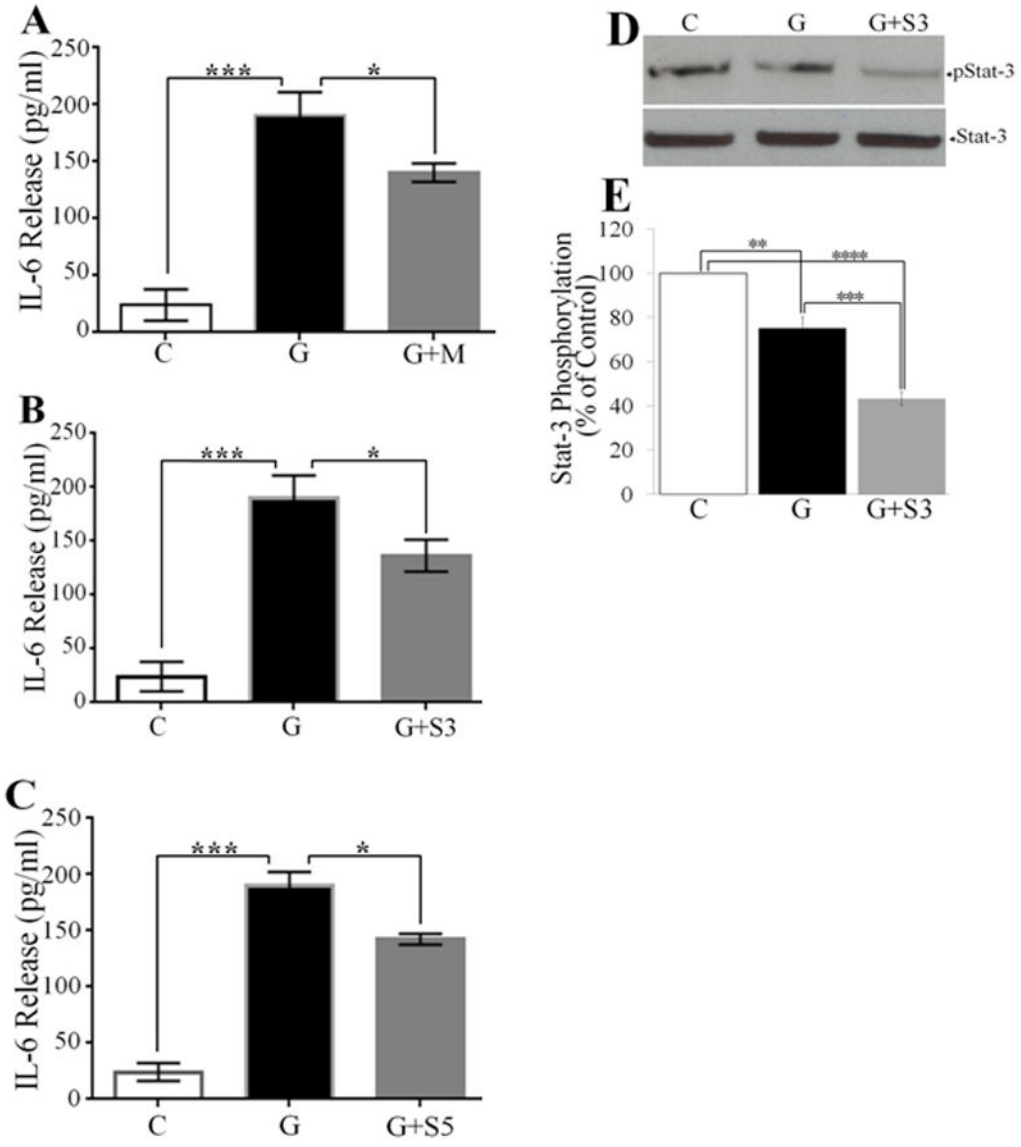

Fig. 4. Selective α7AChR antagonist, methyllycaconitine, and Stat-3 or −5 inhibitors attenuate GTS-21-induced IL-6 release

GTS-21treatment of 7-day differentiated myotubes in the presence of (A) methyllycaconitine, (B) Stat-3 inhibitor or (C) Stat-5 inhibitor for 6 h. IL-6 levels in culture media of myotubes were measured by ELISA. D. Immunoblot analyses of Stat-3 protein expression (*upper panel*) and phosphorylation (*lower panel*). E. Densitometric analysis of Stat-3 phosphorylation. The letters C, G, M, S3 and S5 represent Control, GTS-21, methyllycaconitine, Stat-3 inhibitor or Stat-5 inhibitor, respectively. The data represent the mean ± standard error (n=3). One-way ANOVA with Tukey's multiple comparison tests was used for statistical analysis. Asterisks represent p values (*$p < 0.05$; (**$p < 0.005$; (***$p <$ 0.0005; ****$P < 0.00005$) as compared to control. GTS-21-induced IL-6 is attenuated by α7AChR antagonist, and Stat-3 or Stat-5 inhibitors. Treatment with GTS-21 in the presence of Stat-3 inhibitor attenuated the phosphorylation of Stat-3.

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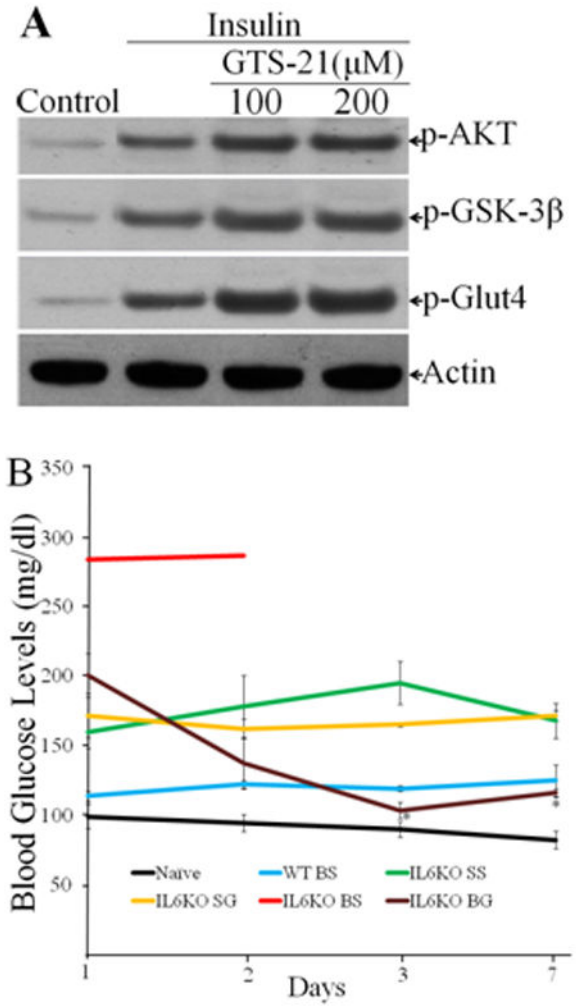

Fig. 5. GTS-21 enhances insulin receptor downstream signaling *in vitro* and partly affects glucose dyshomeostasis *in vivo*

(A) Immunoblot analysis of protein extract from GTS-21-treated 7-day differentiated myotubes showed increased phosphorylation of Akt, GSK3β and Glut4 after insulin (100 nM) treatment. The phosphorylation of these proteins were further enhanced by GTS-21 (100 or 200 μM) treatment compared to untreated controls at 6 h. Actin was used as a loading control. (B) Measurement of blood glucose levels in WT burned mice treated with saline or IL6KO sham-burn or burned mice without and with GTS-21 (4 mg/kg/bw, i.p.). Naive mice were used as controls. The blood glucose levels of IL6KO mice with BI treated with saline could not be determined after days 2 due to early mortality in this group. The measured glucose levels in saline-treated burn injured IL6KO mice was elevated in first 2 days compared to all IL6KO mice in other groups (IL6KO mice receiving sham-burn and given saline or GTS-21 and IL6KO mice receiving BI and given GTS-21) and saline-treated burn injured WT mice. Burn injured IL6KO mice receiving GTS-21showed improved glucose levels at day 3 and 7. The data for all animals except the burned IL6KO mice treated with saline represent the mean ± standard error (n=4). One-way ANOVA with Tukey's multiple comparison tests was used for statistical analysis.

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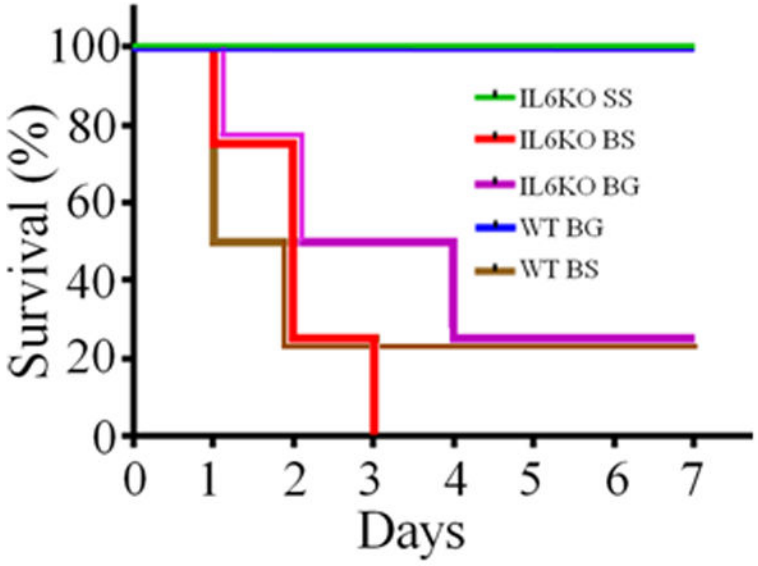

Fig. 6. Kaplan-Meier plot of WT and IL6KO mice survival after burn injury

After sham burn or scald of approximately 40% body surface area burn, to IL6KO and WT mice, they were treated with either saline or GTS-21 (4 mg/kg/bw, i.p.) for 7 days. WT burned mice given saline had 75% mortality (25% survival) rate at 7 days (brown line). IL6KO burned mice given saline showed 100% mortality (0% survival) by day 3 (red line). In IL6KO burned mice mortality improved from 100% to 25 % with GTS-21 treatment (purple line). WT burned mice given GTS-21 showed 100% survival rate (0% mortality) at 7 days (blue line) compared to 25% survival without GTS-21. IL6KO mice with sham-burn were used as sham-burn controls (green line). The 100% mortality in IL-6KO burned mice treated with saline indicated that IL-6 is essential for early survival after burns.

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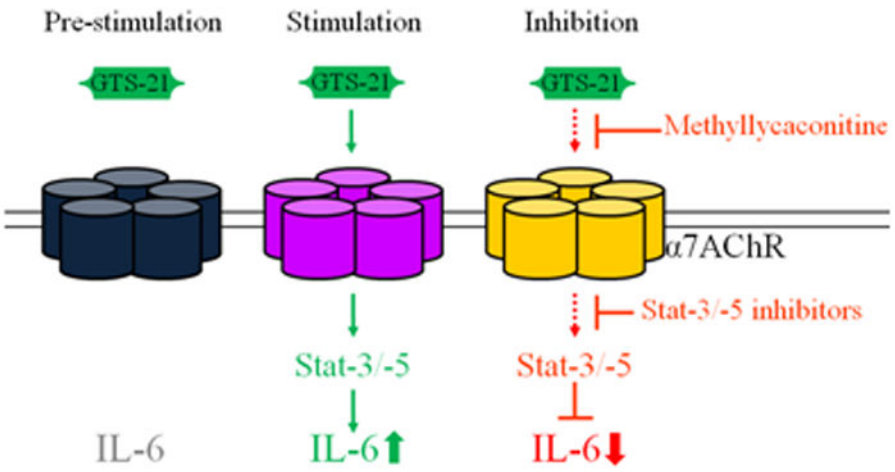

Fig. 7. Schematic diagram shows interactions between agonist and antagonist of α7AChR. or Stat-3/−5 inhibitors on attenuation of GTS-21-induced IL-6 release

GTS-21-induced IL-6 release was blocked upstream by potent α7AChR antagonist, methyllycaconitine. GTS-21-induced IL-6 release was attenuated downstream by Stat-3/−5 inhibitors. Green hexagonal shape represents GTS-21, indigo, purple and gold pentameric cylinders represent α7AChR subunits in three different states of activation: pre-stimulation, stimulation by GTS-21and inhibition during stimulation by GTS-21, respectively. The letters IL-6 in grey color represents basal expression level of IL-6. Green arrows denote stimulation of GTS-21-induced IL-6 release, broken red arrows and red lines denote inhibition of GTS-21-induced IL-6 release, solid upward green arrow indicates upregulation of IL-6 release after GTS-21 induction and solid downward red arrow indicates downregulation of GTS-21-induced IL-6 release after methyllycaconitine and Stat-3/−5 inhibitors treatment.

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Table 1

Post-burn blood glucose levels in IL6KO mice after GTS-21 treatment

Days →	1	2	3	7
Groups ↓	Blood Glucose Levels (mg/dl)			
Naive	107 ± 8	103 ± 6	99 ± 6	91 ± 6
IL6KO SS	166 ± 11	184 ± 22	200 ± 15	174 ± 12
IL6KO SG	178 ± 16	168 ± 7	171 ± 1	177 ± 4
IL6KO BS	286	289	ND	ND
IL6KO BG	205 ± 15	144 ± 17	111 ± 6*	123 ± 3*
WT BS	121 ± 4	130 ± 3	127 ± 2	132 ± 11

*Significant difference from IL6KO mice with BI received saline and IL6KO mice with sham-burm received saline or GTS-21.

ND, Not determined due to mortality.

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