Published in final edited form as:

Shock. 2012 August ; 38(2): 213–219. doi:10.1097/SHK.0b013e31825d628c.

Lipopolysaccharide Up-regulates Alpha7 Acetylcholine Receptors: Stimulation with GTS-21 Mitigates Growth Arrest of Macrophages and Improves Survival in Burned Mice

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Abstract

Nicotinic stimulation of the alpha7 acetylcholine receptors ($\alpha 7$ AChRs) mitigates the lipopolysaccharide (LPS)-induced TNF- α and other cytokines release in macrophages. This effect is blocked by $\alpha 7$ AChR antagonist, α -bungarotoxin (BTX). We tested and confirmed the hypotheses that LPS up-regulates $\alpha 7$ AChRs and the prototypical $\alpha 7$ AChR antagonists, vecuronium, and BTX do not block the effects of GTS-21, a specific $\alpha 7$ AChR agonist, on TNF- α release. With the knockdown of $\alpha 7$ AChR expression by siRNA, GTS-21 effects on inhibition of TNF- α release were not demonstrable. In addition, GTS-21 mitigated the LPS-induced growth arrest of macrophages *in vitro* in J774A.1 cells and *ex vivo* in peritoneal macrophages obtained from mice at three days after burn. Moreover, GTS-21 reduced mortality after burn injury in mice. These results indicate that (i) LPS up-regulates $\alpha 7$ AChRs; (ii) the therapeutic beneficial effects of GTS-21 on cytokine release are specifically mediated via $\alpha 7$ AChRs and are preserved even when co-treated with prototypical antagonist, BTX, or clinically used muscle nicotinic antagonist, vecuronium; (iii) activation of $\alpha 7$ AChRs by GTS-21 partially reverses the LPS-induced proliferation arrest and (iv) GTS-21 reduces mortality in mice with burn injury. The *in vivo* beneficial effects of GTS-21 in burn injury warrant further studies.

Keywords

alpha7 acetylcholine receptor agonist; alpha7 acetylcholine receptor antagonists; α -bungarotoxin; tumor necrosis factor- α ; up-regulation; vecuronium

INTRODUCTION

Macrophage activation is integral to the inflammatory reaction occurring during bacterial infection or burn injury. Although excessive macrophage activation can lead to increased morbidity and mortality, their activation and function, however, are vital to the host defense system (1, 2). Possible causes of the higher morbidity and mortality rate in critically ill patients are alterations in the immune status. Migration of macrophages and infiltration of other inflammatory cells have been seen in skin and muscles of severely burned and immobilized mice (3, 4). Activated macrophages are capable of inducing apoptosis of neutrophils, enhance phagocytosis of debris and release factors that can regulate cell proliferation and myogenesis (4). These activated macrophages secrete tissue specific pro- and anti-inflammatory mediators, and express a number of receptors, which are pivotal for

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innate immune responses (5). The $\alpha 7$ acetylcholine receptors ($\alpha 7$ AChRs), previously described in neuronal cells only, have recently been shown to exist in non-excitable cells including macrophages (6, 7–9) and the $\alpha 7$ AChR-mediated effects produced by nicotine can be blocked by α -bungarotoxin (BTX) (6, 9, 10). The $\alpha 7$ AChRs are ligand-gated ion channels, whose activation results in modulation of the release of pro-inflammatory cytokines in macrophages (1, 6, 7–9).

Many non-specific $\alpha 7$ AChR agonists including nicotine, choline and acetylcholine have been used to modulate cytokine release in various *in vitro* and *in vivo* models of inflammation (11). Recent studies have shown that GTS-21, 3-(2, 4)-dimethoxybenzylidene)-anabaseine (DMXB-A) as a specific agonist of $\alpha 7$ AChR with a potential to exert a variety of anti-inflammatory effects including decreased cytokine release, inhibiting infiltration of neutrophils and improving survival rate in mice following LPS treatment (12–15). Phase I and II clinical trials are ongoing for the use of GTS-21 to attenuate LPS-induced systemic cytokine release, which can lead to systemic inflammatory response syndrome (16). GTS-21 can also bind to $\alpha 4\beta 2$ AChRs and displace BTX binding from the $\alpha 7$ AChRs (17, 18). Furthermore, stimulation of $\alpha 7$ AChRs enhances signaling via phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (AKT) in neuronal cells resulting pro-survival and anti-apoptotic effects (19). The previous observations of pro-survival effect of GTS-21 in LPS- and cecal ligation and puncture-induced severe sepsis in mice (14) led us to hypothesize that LPS up-regulates $\alpha 7$ AChRs, and their stimulation with GTS-21 will mitigate the LPS-induced arrest of proliferation of macrophages *in vitro* and *ex vivo* and also improve survival rate of mice with burn injury. The potency of GTS-21 was tested using prototypical $\alpha 7$ AChR antagonist BTX and muscle relaxant, vecuronium. The specificity of the GTS-21 was also tested by knock-down of $\alpha 7$ AChRs using short interference ribonucleic acid (siRNA).

MATERIALS AND METHODS

Cell culture

Most of the *in vitro* experiments were performed in J774A.1 murine macrophages (ATCC, Rockville, MD), cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin and streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C, and routinely passaged to maintain an optimal density of $0.5\text{--}1.3 \times 10^6$ cells per mL. Cells were split every 3–5 days. Immediately before the various treatments described herein, the cells were centrifuged and re-suspended in fresh medium and the cell density was adjusted to 1×10^6 cells/mL.

Isolation of mouse peritoneal macrophages

Peritoneal fluid from anesthetized 10-week old male C57Bl/6J mice at three days after burn injury was collected by washing the peritoneal cavity with 5 ml ice-cold sterile PBS containing 10% FBS and 1% penicillin and streptomycin. It is noteworthy that obtained peritoneal macrophages were not elicited by thioglycollate because this procedure may change some of their physiological properties. Therefore, the harvest of these macrophages without thioglycollate yielded 10 times less macrophages than the thioglycollate elicitation. However, the collected cell suspension was centrifuged at 1000 RPM for 10 min and resuspended in DMEM growth medium followed by seeding the equal number of cells in culture plates. The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. After the overnight incubation, the floating cells were removed by decantation of the medium, and the adherent macrophage cells were incubated for 7 to 10 days and used for the *ex vivo* experiments.

Immunocytochemical detection of $\alpha 7$ AChRs

After treatment with and without 1 μ g/mL LPS for 9 h, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and then blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)-Tween for 30 min followed by incubation with $\alpha 7$ AChR antibody (dilution of 1:200) overnight at 4 °C. After washing with PBS, the $\alpha 7$ AChR-antibody-bound cells were incubated with FITC-conjugated mouse secondary antibody in 1% BSA at room temperature in dark for 1 h. Then, they were washed and mounted for viewing by fluorescent microscope. In another set of experiments to detect $\alpha 7$ AChRs, the J774A.1 cells or peritoneal macrophages exposed to LPS or saline (controls) for 9 h were incubated with 1.5 μ g/mL FITC-labeled BTX or Alexa Flour 488-labeled BTX (Molecular Probes, Carlsbad, CA) in the cell culture medium for 15 min at 4 °C. After washing with DMEM medium, the cells were fixed for 15 min at room temperature in 4% paraformaldehyde and mounted for viewing. Images were acquired by Axiovision Release 4.6.3 software on a Zeiss Axiovert 200M inverted microscope under 10X and 40X objective (Carl Zeiss, Thornwood, NY) or by spot advanced software on a Nikon Eclipse E800 microscope.

Immunoblot analysis

For immunoblot analysis, 40 μ g of protein per lane were fractionated by Nu-PAGE and blotted onto nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membrane was blocked by 5% nonfat dry milk. Anti- $\alpha 7$ AChR (1:1,000), anti-GAPDH (Abcam, Cambridge, MA) and anti-actin (1:10,000) (Sigma, St. Louis, MO) were used as primary antibodies. The membranes were incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies for 30 min (dilution of 1:5,000). Specific proteins were detected by exposing membranes to Kodak X-Omat films.

Expression of $\alpha 7$ AChRs by immunoprecipitation

For immunoprecipitation, 1 mL aliquot of immunoprecipitation buffer-solubilized cell extracts were incubated with 15 μ l of $\alpha 7$ AChR specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C on a rotator. Twenty microliters of magnetic Dynabeads M380 coated with sheep secondary antibody (Invitrogen, Carlsbad, CA) were then added to antigen-antibody complex, and the incubation continued for another 1 h. The immunoprecipitates were collected using magnet, washed with immunoprecipitation buffer, pelleted, resuspended in 30 μ l of NuPage LDS buffer and then heated at 100 °C for 5 min. Immunoprecipitated $\alpha 7$ AChR protein was separated on 4–20% NuPage gel and immunoblot was performed using $\alpha 7$ AChR specific antibody.

TNF- α levels during GTS-21 treatment with and without $\alpha 7$ AChR antagonists

After treatment of the J774A.1 cells with 1 μ g/mL LPS or saline (controls) for 9 h in the presence or absence of 10 or 100 μ M GTS-21 with/without vecuronium (1 μ g/mL) or BTX (1.5, 4.5 and 45 μ g/mL), the cell culture media were subjected to Enzyme-linked immunosorbent assay (ELISA) for TNF- α (Sandwich ELISA Kit, R&D Systems Inc., Minneapolis, MN). Additionally, the murine peritoneal macrophages were also treated with the same of concentration of LPS and in combination with GTS-21 as described above. The TNF- α concentrations in the media were determined by interpolating the sample's optical density at 450 nm into the linear range from a standard curve generated with serial concentrations of TNF- α .

Test of specificity of GTS-21 by knock-down of $\alpha 7$ AChR

For RNA interference experiment, the cells were seeded in a 6-well plate and incubated overnight in normal growth medium to reach 30% confluency. The cells were then

transfected with 100 nM of either scrambled siRNA or siRNA of SMART pool containing 5 nmol of a mixture of four different oligonucleotide sequences to target multiple domains and functional regions of $\alpha 7$ AChR mRNA, in the presence of 4 μ l Dharmafect transfection reagent (Dharmacon RNA Technologies, Lafayette, CO) in 2 mL of complete growth medium containing 10% fetal bovine serum, according to the protocol provided by the company. Following 96 h of transfection, the cells were treated for 9 h with LPS (1 μ g/mL) in presence or absence of GTS-21 (100 μ M) in complete growth medium. The conditioned medium was stored at -20°C for later analysis of the TNF- α levels using ELISA. The cells were harvested by the addition of lysis reagent for immunoblots.

Determination of proliferation and morphology of macrophages during LPS treatment

The cells ($\sim 1.27 \times 10^6$) were exposed to saline or LPS (1 μ g/mL) with and without GTS-21 (10 μ M) for 0, 3, 6 and 9 h. Propidium Iodide (PI) exclusion method was employed to monitor the total and dead cell population by nucleocounter (Nucleocounter® NC-100, Brunswick, NJ). When cell samples are mixed with equal volume (100 μ l) of cell suspension, lysis buffer (100 μ l) and stabilizing buffer (100 μ l), lysis of cell membrane takes place rendering cellular DNA susceptible to staining with the PI fluorescence dye giving total cell count; cells stained directly with PI without any treatment, reflects dead cell count. The cell numbers were determined in triplicate. For morphological analysis, three different phase contrast images were taken in each assay at a 40 $\times$ magnification using microscope (Carl Zeiss, Thornwood, NY) at 0, 3, 6 and 9 h.

Mouse model of burn injury

Male (C57BL/6J) mice, 25–30 g, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed to acclimatize for 1 week. Mice were divided into 4 groups (n=12), sham-burn with or without GTS-21 and burn with or without GTS-21. All the animal experiments were performed as per the protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital in accordance with the National Institutes of Health guidelines. This model was previously described for studies related to oxidative stress and insulin signaling (20). Briefly, mice were anesthetized with pentobarbital (50 mg/kg). For the burn on the back, the mice were immersed into the hot water (maintained at 80°C) for 8 sec followed by flanks for 5 sec each and then abdomen for 8 sec. A 40% total body surface area was burn inflicted. This exposure produced a third degree burn to the skin but caused no injury to deeper tissues. The injured animals were kept warm and 1% silver sulfadiazine cream (Silvadene®, Marion Labs. Inc. Kansas City, MO) was applied to burned area, followed by i.p. administration of 1.0 mL 0.9% saline/mouse as fluid resuscitation and a single dose of buprenorphine (0.1 mg/kg i.p.). After burn injury, the mice were subjected to a daily injection of two doses of GTS-21 at 4 mg body weight dose or saline. Sham-burn mice (controls) were treated after anesthesia by immersing in lukewarm (37°C) water. Thereafter, the animals were observed for the survival for 7 days post burn. Survival percentage of mice was determined using Kaplan-Meier survival curve.

Statistical Analysis

Results are presented as means $\pm$ SE. For statistical analysis, one way ANOVA was used and subjected to Tukey's multiple comparison tests. For survival analysis, Logrank test was used. $P < 0.05$ was considered statistically significant. All analyses were performed using Prism 4.0 (GraphPad, San Diego, CA).

RESULTS

LPS promotes expression and recruitment of $\alpha 7$ AChRs to cell surface

Cells exposed to LPS were larger in size with strong binding of the cells to FITC-labeled BTX (Fig. 1A, *right upper panel*) or to FITC-conjugated $\alpha 7$ AChR antibody (Fig. 1A, *right middle and lower panel*). The FITC fluorescence was distributed along the periphery of the cell surface in the form of a ring. Cells without LPS showed a lower intensity of the fluorescence, which was seen at a niche on one edge of the cell surface (Fig. 1A, *left upper, middle and lower panel*). These experiments strongly indicate that LPS up-regulates $\alpha 7$ AChRs on the plasma membrane. The up-regulated $\alpha 7$ AChRs expression was confirmed by immunoblot that showed a band corresponding to 54 kDa molecular weight; brain extract was used as a positive control (Fig. 1B). GAPDH was used as a protein loading control. Increased expression of $\alpha 7$ AChRs was corroborated by immunoprecipitation with $\alpha 7$ AChR-specific antibody followed by immunoblotting with anti- $\alpha 7$ AChR antibody (Fig. 1C). Densitometric analysis indicated three-fold greater protein expression of $\alpha 7$ AChRs in LPS-treated compared control cells (Fig. 1D). Macrophages isolated from murine peritoneum were also used to determine the expression of $\alpha 7$ AChRs with LPS treatment in *ex vivo*. *Ex vivo* stimulation of peritoneal macrophages with LPS up-regulated the expression of $\alpha 7$ AChRs when compared with control peritoneal macrophages (Fig. 1E).

Potency and Specificity of GTS-21

In the following experiments, we examined the specificity of GTS-21 and whether its effects can be blocked by its prototypical $\alpha 7$ AChR antagonists, vecuronium and BTX (21, 22). The concentrations of vecuronium and BTX used were therapeutic and pharmacologic, respectively. Vecuronium is a drug used in humans to cause muscle paralysis both in the operating room and intensive care unit. GTS-21 strongly inhibited the LPS-induced release of TNF- α into the medium in J774A.1 cells in a concentration-dependent manner (Fig. 2A). To determine whether GTS-21 has the similar effects on inhibition of TNF- α release in LPS-induced peritoneal macrophages, we performed *ex vivo* stimulation of the harvested peritoneal macrophages with LPS in combination with or without GTS-21 and the medium was examined for TNF- α release. As expected, GTS-21 significantly inhibited TNF- α release (Fig. 2B). Co-administration of vecuronium with GTS-21 (10 and 100 μ M) failed to attenuate the beneficial GTS-21 effects on TNF- α release (Fig. 2C). Similarly, BTX (1.5, 4.5 or 45 μ g/mL) failed to block the effects of GTS-21 on TNF- α release in J774A.1 cells (Fig. 2D).

Since GTS-21 is known to bind to $\alpha 7$ and $\alpha 4\beta 2$ AChRs (12, 13), we next examined the whether GTS-21 effects are dependent of $\alpha 7$ AChRs. This was achieved by knock-down of $\alpha 7$ AChRs using siRNA. The $\alpha 7$ AChR siRNA almost completely abrogated $\alpha 7$ AChRs expression in macrophages (Fig. 3A). In the cells that were treated with siRNA for $\alpha 7$ AChRs GTS-21 failed to decrease LPS-induced increased TNF- α release in the cells (Fig. 3B). In contrast, GTS-21 treatment of cells transfected with scrambled siRNA for $\alpha 7$ AChRs significantly decreased TNF- α levels. These results confirm that the effects of GTS-21 are dependent and mediated by $\alpha 7$ AChRs and not by other receptors. Of note, knock-down of $\alpha 7$ AChRs enhanced LPS-induced TNF- α release in the absence of GTS-21 treatment. These findings indicate that in the LPS-treated cells with no knock-down, the $\alpha 7$ AChRs can inhibit TNF- α release even in the absence of exogenous agonist.

GTS-21 partially reverses LPS-induced decreased cell proliferation

LPS has cytotoxic effects in cells causing cell death by apoptotic and necrotic pathways (23). In the following experiments, the effects of LPS with and without GTS-21 on cell proliferation and morphology were tested. In the absence of LPS, compared to baseline and

at 3 h, there was a significant increase in cell numbers at 6 and 9 h. In contrast, the LPS-treated cells had arrest in their proliferative activity throughout the observation period of 9 h. Treatment of LPS exposed cells with 10 μ M GTS-21 partially reversed the inhibitory effects of LPS on proliferation of J774A.1 cells (Fig. 4A). We also observed morphological changes; cells treated with LPS showed cell swelling at 3, 6 and 9 h and had a severely deformed morphology of the cell membrane (Fig. 4B). Co-treatment of LPS with 10 μ M GTS-21 reversed some of the deformity, although some cell swelling was still present. Similarly, the treatment of peritoneal macrophages with GTS-21 in the presence of LPS showed increase in proliferative activity at 9h (Fig. 4C).

GTS-21 improves survival rate in burn injury-induced mice

In the following studies, we tested the *in vivo* effects of GTS-21 on survival following burn injury of 40% body surface area. The survival rate in burned mice treated with saline was 25%, whereas the survival rate in burned mice treated with GTS-21 was 100%, similar to that of sham-burn treated with saline or GTS-21 (Fig. 5).

DISCUSSION

The salient new, previously unreported findings of our study are that (i) LPS up-regulates α 7AChRs in macrophages; (ii) the beneficial effects of α 7AChRs cannot be antagonized by concomitant therapy with α 7AChR antagonist, vecuronium. (iii) LPS induced arrest of proliferation of cells can be mitigated by GTS-21; and (iv) GTS-21 reduces mortality in severely burned mice.

AChRs directly or indirectly regulate cellular and biochemical processes. Certain pathologic states up-regulate nicotinic AChRs in the central or peripheral nervous system (21). Although the expression of α 7AChRs has been documented in non-neuronal cells including macrophages, whether these AChRs are already present or are expressed *de novo* after LPS exposure is unknown. Our studies document for the first time that LPS triggers increased expression of α 7AChRs on the cell surface of macrophages. Immunostaining of LPS-induced J774. A1 cells and mouse peritoneal macrophages using FITC- and Alexa Fluor 488-labeled BTX, respectively, revealed increased cell surface expression of α 7AChRs at 9 h. BTX also binds to other subunits α 1, α 4 and α 9 of AChRs in mammalian cells. This broad spectrum binding capacity of BTX to other subunits may raise questions about specificity of BTX in immunocytochemical detection of α 7AChRs on the surface of macrophages. Previous studies have shown that α 4 and α 9 receptor subunits are not found in macrophages or other immune cells (6, 24, 25). Although, the α 1 receptor subunits are expressed in macrophages, they are down-regulated during differentiation of peripheral blood mononuclear cells to macrophages (6) diminishing the possibility that our BTX experiments bound to AChRs expressing α 1 subunit. However, the immunostaining with specific FITC-conjugated antibody to α 7AChR and the immunoblotting experiments, which indicated increased expression of ~54 kDa protein, confirm that the up-regulation of α 7AChRs does indeed occur following exposure to LPS. Densitometric analysis demonstrated a three-fold increase when compared to control. We used brain extract as a positive control, which also migrated as a ~54 kDa band protein, confirms the expression of α 7AChR. The molecular mechanism of up-regulation of α 7AChRs was not within the scope of this study.

GTS-21 potently inhibits production of pro-inflammatory cytokines and mediates innate immune responses (13, 14, 16). In agreement with previous studies (13, 14, 16), our *in vitro* and *ex vivo* experiments were also clearly demonstrated the significant inhibition of LPS-induced TNF- α release followed by GTS-21. Unexpectedly, the α 7AChRs antagonists, vecuronium and BTX did not antagonize the effects of GTS-21 in J774A.1 cells. BTX and

vecuronium are prototypical antagonists blocking nicotinic- and acetylcholine-mediated effects on $\alpha 7$ AChRs (17, 18, 22). Our study documents that GTS-21 preserves its therapeutic beneficial effects even when co-treated with BTX and vecuronium in cultured macrophages. In conjunction with the recent study of GTS-21, which attenuated the mechanical ventilation-induced TNF- α release- associated lung injury (26), our data raise the possibility that GTS-21 may continue to have anti TNF- α effects even when prototypical $\alpha 7$ AChR antagonist, muscle relaxant, vecuronium, is concomitantly used during mechanical ventilation of critically ill patients.

One might therefore pose the question if GTS-21 has effects independent of the $\alpha 7$ AChRs because the effects of TNF- α release were not blocked by $\alpha 7$ AChR antagonists. The siRNA experiments with knock-down of the $\alpha 7$ AChRs confirmed that the effects of GTS-21 are specifically mediated by $\alpha 7$ AChRs (discussed in detail later). Consistent with our findings with vecuronium, previous studies have also shown that GTS-21 is a potent agonist even in the presence of nicotinic antagonists other than vecuronium such as BTX (13). It is notable that we used pharmacological concentrations of BTX (45 μ g/mL) and yet could not block the beneficial effects of GTS-21 on TNF- α release. The inability of vecuronium and BTX to block the effects of GTS-21 may be due to the higher affinity GTS-21 exhibits for the $\alpha 7$ AChRs compared to BTX and vecuronium.

Tracey et al. have shown that TNF- α level in serum, liver and spleen was significantly higher in $\alpha 7$ AChR deficient mice as compared with wild type mice (5). GTS-21 binds to $\alpha 4\beta 2$ AChRs in addition to $\alpha 7$ AChRs, although it is more specific to latter. To establish the specificity of GTS-21 on $\alpha 7$ AChRs-dependent versus -independent effects on LPS-induced inflammation, we knocked-down the $\alpha 7$ AChR expression using specific siRNA. The protein expression of $\alpha 7$ AChRs was down-regulated in cells treated with $\alpha 7$ AChR siRNA, whereas the transfection of scrambled siRNA did not inhibit the expression of $\alpha 7$ AChRs. Our data demonstrate that the down-regulation of $\alpha 7$ AChR expression by siRNA, resulted in ineffectiveness of GTS-21 to reverse TNF- α release when compared to LPS-treated macrophages treated with scrambled siRNA. The $\alpha 7$ AChR siRNA alone without LPS treatment had no effect on TNF- α release (Fig. 3B). It is of interest to note that TNF- α levels were increased more than 200 percent in $\alpha 7$ AChR knock-down cells when compared to cells that had been treated with scrambled siRNA without GTS-21. That is, the TNF- α levels are lower in cells expressing $\alpha 7$ AChRs as compared to cells not expressing $\alpha 7$ AChRs, even in the absence of GTS-21. We postulate a reason for this difference. The complete absence of any functional $\alpha 7$ AChRs on the surface of siRNA treated macrophages results in the inability of endogenous macrophage-released acetylcholine to act on $\alpha 7$ AChRs. In the presence of $\alpha 7$ AChRs, the endogenously released acetylcholine can act on the receptors reducing TNF- α levels even in the absence of GTS-21. Rosas-Ballina et al. have recently demonstrated that lymphocytes (T cells) are capable of synthesizing acetylcholine (27), which raises the possibility that other type of circulating cells such as macrophages could also produce acetylcholine. Thus, the acetylcholine released from the macrophages itself may account for the difference in TNF- α levels in LPS-treated macrophages expressing or lacking $\alpha 7$ AChRs.

Administration of GTS-21 (4mg/kg) to endotoxemic mice improved survival rate upto 90% as compared to 20% in the control (without GTS-21) (14). A similar survival benefit was observed following cecal ligation and puncture-induced severe sepsis (14). Similarly, majority of patients with burn injury also suffer from systemic inflammatory response syndrome, which causes immunosuppressive effects (28) and its early prevention using anti-inflammatory drug could significantly decrease mortality and improve the survival rate. Therefore, we chose a mouse model of burn injury-induced inflammation to demonstrate the pro-survival effect of GTS-21.

In our survival study, since most of the deaths in our study occurred in first 3 days of burn injury and macrophages are also reported to accumulate on third day of inflammation (29), we isolated macrophages from peritoneal cavity of burn-injured mice at three days. The induction of peritoneal macrophages with LPS *ex vivo* showed a significant up-regulation of $\alpha 7$ AChRs. In addition, these peritoneal macrophages when co-treated with LPS and GTS-21 demonstrated a significant increase in the proliferative activity with a strong inhibitory effect on TNF- α release.

Our studies provide evidence for pro-survival effects of GTS-21 *in vitro*, *ex vivo* and *in vivo*. The experiments with macrophages indicate that activation of $\alpha 7$ AChRs with its agonist GTS-21 mitigates proliferation arrest of macrophages induced by LPS. In our *in vivo* study of 7 days duration, we found that all of the burn injury-related deaths were completely prevented by GTS-21 compared to vehicle all of whom had the same fluid resuscitation volume. Thus, excessive depletion of fluids from the body leading to early death cannot account for the mortality differences. Future studies are required to clarify the precise mechanisms by which GTS-21 promotes survival after burn injury in mice.

Acknowledgments

This work was supported grants from the NIH RO1-055082 and P50-2500 project IV and from Shriners Hospitals for Children®.

Abbreviations

BTX	α -bungarotoxin
GTS-21	(3-[(2,4-Dimethoxy) benzylidene]-anabaseine dihydrochloride)
LPS	lipopolysaccharide
nAChRs	nicotinic acetylcholine receptors
$\alpha 7$AChR	alpha7 acetylcholine receptor
siRNA	short interference Ribonucleic Acid
TNF-α	tumor necrosis factor-alpha

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FIG. 1.

Immunocytochemistry, Immunoblot and immunoprecipitation of LPS-induced expression of $\alpha 7$ AChRs. (A) FITC-conjugated BTX binding to $\alpha 7$ AChRs (*upper panel*) and FITC-labeled $\alpha 7$ AChR antibody staining (*middle and lower panel*) in macrophages. Images were acquired by Axiovision Release 4.6.3 software on a Zeiss Axiovert 200M inverted microscope under 10X (*upper and middle panel*) and 40X (*lower panel*) objective (Carl Zeiss, Thornwood, NY). LPS increased expression of $\alpha 7$ AChR on cell surface at 9 h. (B) Immunoblot, (C) Immunoprecipitation and (D) Densitometric analysis of $\alpha 7$ AChRs expression in J774A.1 cells. (E) Expression of Alexa Fluor 488-labeled $\alpha 7$ AChRs (*upper panel*) and phase contrast (*lower panel*) in mouse peritoneal macrophages. Cells were treated with 1 μ g/mL LPS for 9h. A band corresponding to 54 kDa protein, which is the molecular weight of $\alpha 7$ AChR, together with brain extract which expresses $\alpha 7$ AChRs is indicated by arrow. GAPDH was used as a loading control. The representative immunoblot is shown from three independent experiments. The data represent the mean $\pm$ standard error (n=3). Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison tests. Asterisks represent *P* value (**P* 0.05), as compared to control and LPS treated cells. The letters C, L and BE stand for Control, LPS-treated samples and Brain Extract (positive control), respectively. LPS up-regulated $\alpha 7$ AChRs at 9h.

FIG. 2.

Receptor specificity of GTS-21 on TNF- α release. (A) J774A.1 cells were co-treated with different concentration (0, 0.1, 1.0, 10, 100 μ M) of specific α 7AChR agonist, GTS-21 and 1 μ g/mL LPS for 9 h. (B) Peritoneal macrophages obtained from mice were co-treated with 100 μ M of GTS-21 and 1 μ g/mL LPS for 9 h. Cells were concomitantly treated with LPS (1 μ g/mL) for 9 h with and without α 7AChR agonist, GTS-21 (10 or 100 μ M) and in the presence and absence of (C) α 7AChR blocker, vecuronium and (D) α 7AChR antagonist, BTX. The culture media were analysed by ELISA for TNF- α release. # indicates a higher concentration (100 μ M) of GTS-21. The data represent the mean $\pm$ 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 and LPS treated cells or are shown to compare LPS plus GTS-21 and/or together with vecuronium treatment against LPS treatment only. GTS-21 significantly decreased TNF- α levels. This effect was not antagonized by vecuronium or BTX.

FIG. 3.

GTS-21 is ineffective with α7AChR siRNA knock-down. The macrophages cultured in 6 well plates were transfected with 100 nmol scrambled or α7AChR siRNAs and after 96 h post-transfection the cells were replaced with fresh culture medium and co-treated with and without 10 μM of GTS-21 and 1 μg/mL LPS for 9 h. (A) Immunoblotting with anti-α7AChR antibody. Actin was used as a protein loading control. The letters S and α7 stand for scrambled and α7AChR siRNA, respectively. (B) Densitometric analysis of α7AChR expression in scrambled or α7AChR knock-down in macrophages. (C) TNF-α levels by ELISA. The data represent the mean ± standard error (n=4). Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison tests. Asterisks represent *P* values (**P* < 0.05), as compared to LPS treated cells against LPS plus GTS-21 treatment. NS = not significant. Immunoblot and densitometric analyses reveals that knock-down of α7AChR siRNA decreases expression of the α7AChRs. Knock-down of α7AChR by siRNA did not decrease TNF-α levels when treated with GTS-21, indicating that the specific effect of GTS-21 is via α7AChRs.

FIG. 4.

GTS-21 mitigates LPS-induced proliferation arrest. Macrophages seeded in culture plates at 1×10^6 cells/mL were treated with saline or $1 \mu\text{g/mL}$ LPS alone (white box) in the presence or absence of $10 \mu\text{M}$ of $\alpha 7\text{AChR}$ agonist GTS-21 (hatched box) for 0, 3, 6 and 9 h. Untreated (dark box) cells served as controls. (A) Cell proliferation, (B) cell morphology in J774A.1 cells and (C) cell proliferation in peritoneal macrophages from mice. The data represent the mean $\pm$ standard error ($n=4$). Statistical analysis was done using one-way ANOVA with Tukey's multiple comparison tests. Asterisks represent P values ($*P < 0.05$), as compared to LPS treated cells against control and LPS plus GTS-21 treatment. Cell numbers were determined using Propidium Iodide uptake. LPS-treated macrophages showed proliferation arrest and changes in morphology at 3, 6 and 9 h. The changes in morphology included cell swelling and distortion of the cell membrane. Treatment with GTS-21 partially reversed the proliferation arrest and the morphological changes induced by LPS.

FIG. 5.

Kaplan-Meier survival curves of burn injury-induced mice. Kaplan-Meier plot of sham-burned or burned mice treated either with saline or GTS-21 (4mg/kg) for 7 days. Burned mice treated with saline were compared with sham-burned saline, sham-burned GTS-21 and burned GTS-21 groups. 'n' is the number of animals in each group. The *P* value from the Logrank test is shown in each panel. GTS-21 improves the survival in burned mice.