

## RESEARCH ARTICLE

# GTS-21 has cell-specific anti-inflammatory effects independent of $\alpha 7$ nicotinic acetylcholine receptors

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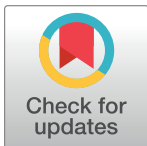
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## Abstract

$\alpha 7$  Nicotinic acetylcholine receptors (nAChRs) reportedly reduce inflammation by blocking effects of the important pro-inflammatory transcription factor, nuclear factor kappa-light chain-enhancer of B cells (NF $\kappa$ B). The  $\alpha 7$  nAChR partial agonist GTS-21 reduces secretion of pro-inflammatory cytokines including interleukin-6 (IL6) and tumor-necrosis factor (TNF) in models of endotoxemia and sepsis, and its anti-inflammatory effects are widely ascribed to  $\alpha 7$  nAChR activation. However, mechanistic details of  $\alpha 7$  nAChR involvement in GTS-21 effects on inflammatory pathways remain unclear. Here, we investigate how GTS-21 acts in two cell systems including the non-immune rat pituitary cell line GH4C1 expressing an NF $\kappa$ B-driven reporter gene and cytokine secretion by *ex vivo* cultures of primary mouse macrophages activated by lipopolysaccharide (LPS). GTS-21 does not change TNF-stimulated NF $\kappa$ B signaling in GH4C1 cells expressing rat  $\alpha 7$  nAChRs, suggesting that GTS-21 requires additional unidentified factors besides  $\alpha 7$  nAChR expression to allow anti-inflammatory effects in these cells. In contrast, GTS-21 dose-dependently suppresses LPS-induced IL6 and TNF secretion in primary mouse macrophages endogenously expressing  $\alpha 7$  nAChRs. GTS-21 also blocks TNF-induced phosphorylation of NF $\kappa$ B inhibitor alpha (I $\kappa$ B $\alpha$ ), an important intermediary in NF $\kappa$ B signaling. However,  $\alpha 7$  antagonists methyllycconitine and  $\alpha$ -bungarotoxin only partially reverse GTS-21 blockade of IL6 and TNF secretion. Further, GTS-21 significantly inhibited LPS-induced IL6 and TNF secretion in macrophages isolated from knockout mice lacking  $\alpha 7$  nAChRs. These data indicate that even though a discrete component of the anti-inflammatory effects of GTS-21 requires expression of  $\alpha 7$  nAChRs in macrophages, GTS-21 also has anti-inflammatory effects independent of these receptors depending on the cellular context.

## Introduction

Chronic inflammation may lead to several inflammatory disorders including sepsis, rheumatoid arthritis, asthma, diabetes and Crohn's disease [1, 2] and involves production and secretion of various pro-inflammatory cytokines [3] including IL6, TNF and high mobility group



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box-1 (HMGB1). Koopman et al. [4] recently proposed vagus nerve stimulation as therapy against the inflammation found in rheumatoid arthritis, based in part on previous work [5] that vagus nerve stimulation requires cholinergic activation to prevent TNF secretion in response to endotoxins, such as lipopolysaccharide (LPS). LPS is a gram-negative bacteria cell wall component that activates NF $\kappa$ B-mediated inflammatory signaling in cells expressing Toll-like 4 receptors (TLR4) [6]. Later, Wang et al. [7] reported that activated  $\alpha$ 7 nicotinic acetylcholine receptors (nAChRs) inhibit LPS-induced pro-inflammatory cytokine secretions without affecting anti-inflammatory mediators such as IL-10. Wang et al. [8] found that  $\alpha$ 7 nAChRs mediate anti-inflammatory signaling in part by blocking NF $\kappa$ B activation in a mouse sepsis model. Follow-up studies have since established that during macrophage activation, the afferent vagus nerve senses pro-inflammatory mediators in the periphery to relay the message to the brain which releases acetylcholine via efferent vagus activity to inhibit inflammation locally through  $\alpha$ 7 nAChRs activation [9]. This pathway is known as the ‘cholinergic anti-inflammatory pathway’ [9, 10] and has increased interest in  $\alpha$ 7 nAChR agonists, such as GTS-21, as potential anti-inflammatory drugs.

$\alpha$ 7 nAChRs are pentameric ion-channel receptors involved in memory and cognition and are implicated in various disorders including Alzheimer’s, Parkinson’s, schizophrenia, cystic fibrosis and inflammation [7, 11–15]. These receptors are found throughout human brain and the peripheral nervous system. Also, these receptors have been observed recently on certain immune cells such as macrophages and lymphocytes [16], and  $\alpha$ -bungarotoxin ( $\alpha$ BGT), a snake neurotoxin relatively specific for  $\alpha$ 7 nAChRs, shows the possible presence of these receptors on macrophage cells [7]. *In vivo* animal models of inflammation suggest that nicotine, like ACh, can reduce pro-inflammatory cytokines and even improve survival in mouse models of endotoxemia and sepsis [8, 17–21]. However, nicotine lacks receptor specificity and possesses major toxicity challenges [22, 23]. Hence, recent studies have focused on  $\alpha$ 7 nAChR-selective ligands to find an anti-inflammatory drug devoid of nicotine-like toxic side-effects, including GTS-21, 3-(2,4-dimethoxybenzylidene) anabaseine, a partial  $\alpha$ 7 nAChR agonist [24].

GTS-21 (also known as DMBX-anabaseine) inhibits pro-inflammatory cytokines like TNF, IL-1 $\beta$ , IL6 and HMGB1 and improves survival in murine endotoxemia and sepsis models [8, 24–27]. Further, its anti-inflammatory effects are widely ascribed to actions as a partial  $\alpha$ 7 nAChR agonist. However, Nullens et al. [28] found that GTS-21 decreased systemic and colonic levels of IL-6, colonic permeability and levels of infection in both wild-type (WT) and  $\alpha$ 7 knockout septic mice. These *in vivo* results suggest that GTS-21 has anti-inflammatory effects that are independent of its effects as a partial  $\alpha$ 7 agonist. We sought to investigate GTS-21 effects at a molecular level in macrophages derived from both WT and alpha7 knockout animals. We also investigated whether GTS-21 causes anti-inflammatory signaling in rat GH4C1 cells, a pituitary-derived cell line transfected with  $\alpha$ 7 nAChR. GH4C1 cells do not express mRNA for known nicotinic receptor  $\alpha$  subunits [29] but readily express electrophysiologically-functional receptors when transfected with the  $\alpha$ 7 nAChR gene [30]. Our results suggest that  $\alpha$ 7 nAChR expression in the heterologous cell line does not suppress inflammatory signaling and that GTS-21 has both  $\alpha$ 7 nAChR-dependent and  $\alpha$ 7 nAChR-independent anti-inflammatory effects in mouse macrophages.

## Materials and methods

### Cell cultures and animals

All studies involving mice were approved by Northeastern University’s Institutional Animal Care and Use Committee (NU-IACUC). Northeastern’s animal care and use program holds

an assurance with the Office of Laboratory Animal Welfare (OLAW) and is accredited by the Association for the Assessment and Accreditation for Laboratory Animal Care (AAALAC). In our NU-IACUC approved protocol, 15-0522R-“Nicotinic anti-inflammatory effects in mouse macrophages”, mice were injected intraperitoneally with sterile thioglycolate and euthanized four days later by CO<sub>2</sub> inhalation followed by cervical dislocation in accordance with the American Veterinary Medical Association Guidelines for Euthanasia of Animals. All efforts were made to minimize suffering. GH4C1 rat pituitary cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown at 37°C in 5% CO<sub>2</sub>. GH4C1 cells were grown in F-10 complete growth medium: Ham’s F-10 basic medium containing 1% penicillin-streptomycin (both from Thermo-Fisher) supplemented with 10% fetal bovine serum (Premium Select from Atlanta Biologicals, Lawrenceville, GA). Male C57BL/6 wild type and  $\alpha 7$  nAChR knockout mice (8–12 weeks old; purchased from Jackson Laboratory, Bar Harbor, Maine) were housed under standard conditions in an Institutional Animal Care and Use Committee approved animal facility. Animals were provided with food and water *ad libitum*. Mouse primary macrophages were isolated from peritoneal cavities as described in Zhang et al. [31]. Briefly, 1ml sterile thioglycollate medium (Fisher Scientific catalog #L21199) was injected into mouse peritoneal cavities, the liquid contents of the cavities were harvested four days later, and the resulting cells were grown in DMEM complete growth medium, consisting of DMEM basic medium containing 1% penicillin-streptomycin (both from Thermo-Fisher) supplemented with 10% fetal bovine serum (Hyclone from GE Healthcare Life Sciences, Pittsburgh, PA).

### Plasmids and RNA

Total GH4C1 and primary macrophage RNA was extracted using a TRIzol Plus RNA purification kit (Invitrogen, Carlsbad, CA), and quantified with a NanoDrop ND-1000 UV-Vis spectrophotometer. Complementary DNA (cDNA) synthesis from 1  $\mu$ g of total RNA for each reaction was carried out using the AffinityScript<sup>TM</sup> QPCR cDNA synthesis kit, (Agilent Technologies, Santa Clara CA).  $\alpha 7$  nAChR primers (5′ : ACATGTCTGAGTACCCCGGA and 3′ : AGGACCACCCTCCATAGGAC) were found using Pubmed primer-BLAST (NCBI, Bethesda MD) and obtained from Integrated DNA Technologies (Coralville, IA). The primers were designed to amplify both mouse and rat  $\alpha 7$  nAChR cDNA using 32 cycles of 95 °C denaturation (30 sec, 5 min before first cycle), 57.5°C annealing (30 sec) and 68°C extension steps (1 min) to get an expected 264 bp amplicon. Approximately 100 ng of cDNA (equivalent to 1/10<sup>th</sup> of the starting RNA amount) was used to perform polymerase chain reaction (PCR) analysis using Platinum Taq polymerase (Invitrogen, Carlsbad, CA). PCR product was analyzed by gel electrophoresis on a 1.5% agarose gel and sequenced by Genewiz (Cambridge, MA). The full-length rat  $\alpha 7$  nAChR sequence cloned into Invitrogen pRep4 plasmids have been previously described (Lee et al., 2009). pEGFP-N1 was obtained from Clontech, Mountain View, CA. NF $\kappa$ B reporter assays were performed using the PNiFty-SEAP plasmid (Invivogen, San Diego, CA., SEAP being a SEcreted Alkaline Phosphatase reporter gene). The NF $\kappa$ B promoter element together with the SEAP open reading frame was excised from this plasmid and inserted into an episomal pRep9 plasmid (Invitrogen) modified to have blasticidin antibiotic resistance (P9KB, the final sequence is available upon request).

### Reagents and antibodies

$\alpha$ -Bungarotoxin ( $\alpha$ BGT) was obtained from Biotoxins Inc., St Cloud, FL, and radioiodinated using iodogen (Pierce Chemical, Rockford, IL) as previously described [32]. Rat TNF (catalog# CYT-393) was purchased from ProSpecbio, East Brunswick, NJ. GTS-21 (# SML0326),

Pyrrolidine dithiocarbamate (PDTC, # P8765), methyllycaconitine citrate (MLA, # M168), and LPS (# L6529) were purchased from Sigma Aldrich, St. Louis, MO. Rabbit polyclonal anti-GAPDH (# PA1-988) and monoclonal anti-phospho-I $\kappa$ B $\alpha$  pSer32 (#PIMA515087) were obtained from Thermo-Fisher Scientific, Waltham, MA. Alexa Fluor 488 anti-mouse/human CD11b #101219 and Alexa Fluor 647 anti-mouse F4/80 Antibody #123121 were purchased from BioLegend, San Diego, CA. Secondary HRP-conjugated anti-rabbit (# 7074) antibody was purchased from Cell Signaling Technology and used at 1:1000 dilution.

## Transfections

GH4C1 cells were transfected as previously described [33]. Briefly, cells were plated at 500,000 cells/well in a BD Falcon 6-well plate in F-10 complete growth medium 24 h prior to transfection with lipofectamine LTX (Invitrogen, Carlsbad, CA). Cells were serum starved (1 ml F-10 medium only) for 1 h before adding transfection reagents consisting of 3  $\mu$ g plasmid DNA, 9  $\mu$ l lipofectamine LTX and 3  $\mu$ l Plus reagent per well in 300  $\mu$ l reduced serum Opti-MEM (Invitrogen) using green fluorescent protein (pEGFP-N1) as a control transfection. Four hr post-transfection, 1 ml complete growth medium was added to make the final volume of 2 ml/well. Next day, supernatant medium was replaced by complete growth medium to maintain good cell viability. Transfection efficiency was monitored by fluorescence microscopy 48–72 hr post-transfection. Three days later,  $\alpha$ 7 nAChR transfected cells were selected with hygromycin (100  $\mu$ g/ml, Invivogen, San Diego CA), and NF $\kappa$ B-SEAP transfected cells were selected with blasticidin (10  $\mu$ g/ml, Invivogen). Cells transfected with both  $\alpha$ 7 nAChR and NF $\kappa$ B-SEAP were selected with both 100  $\mu$ g/ml hygromycin and 10  $\mu$ g/ml blasticidin. Transfected cells were cultured and used for PCR analysis,  $^{125}$ I- $\alpha$ BGT binding studies, and SEAP experiments.

## Western blots

For pI $\kappa$ B western blots, cells were grown in a 6-well BD Falcon plate and were pretreated for 30 min with different concentrations of GTS-21 (followed by 1 h treatment with TNF or LPS) and washed with ice-cold phosphate buffered saline on the experimental day. After washing, cells were immediately scraped off the surface and centrifuged at 12,000 g at 4°C for 10 min. Supernatant was discarded, and the cells were resuspended in radio-immunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 20 mM Tris, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM EDTA, pH 8.0) containing Halt protease inhibitor cocktail (Thermo Fischer Scientific, Waltham, MA). Cell suspensions were sonicated for 10–15 seconds to ensure cell lysis. Cell suspensions were kept on ice for 30–40 min and vortexed every 5–10 min before centrifugation at 9600 g at 4°C for 10 min. Supernatants were collected in a fresh 1.5 ml centrifuge tube, and total protein was quantified using a Pierce BCA protein assay kit. Cell lysates were reduced using Bolt 1X lithium dodecyl sulfate loading buffer containing 0.1 M dithiothreitol from Thermo Fisher Scientific, followed by heating the samples at 70°C for 10 min. Samples were kept on ice for 5 min before 40  $\mu$ g total protein of each sample was loaded in each well on a Bolt™ 4–12% Bis-Tris protein gel for protein separation. Manufacturer's recommended running and transfer buffers were used with the iBlot 2 dry blotting system to transfer the protein onto nitrocellulose. Blocking was done with 5% bovine serum albumin (BSA) containing tris-buffered saline with 0.1% Tween-20 (TBS-T); the membrane was incubated in primary antibody solution (made in 5% BSA at recommended dilution) overnight at 4°C on a rocker. On the following day, the blot was washed with TBS-T 3–5 times and incubated with secondary antibody conjugated to horseradish peroxidase (HRP) at 1:1000 dilution for 1 h. Protein-antibody complex was visualized on the blot using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) and the image was captured by a ChemiDoc XRS Imager from

Biorad. GAPDH immunostaining was performed on each blot to confirm equivalent protein loading across the wells.

### Enzyme-linked immunosorbent assay (ELISA)

Primary mouse macrophages were plated at 50,000 cells/well in a 96-well plate 24 h prior to drug treatment. The following day, cells were pretreated with various concentrations of GTS-21 for 30 min before bringing the final volume to 200  $\mu$ l/well with or without 10 ng/ml LPS. Sixteen h after LPS treatment, TNF and IL6 ELISAs were performed on 20  $\mu$ l samples as per manufacturer's instructions (eBioscience, San Diego, CA catalog#s 88-7324-22 (TNF), 88-7064-88 (IL6)). When used, pretreatments with  $\alpha$ BGT or MLA were 1 h. Absorbances were read at 630 nm on a BioTek Synergy plate reader (Winooski, VT).

### [<sup>125</sup>I]-labeled- $\alpha$ BGT binding assay

Radioactive binding assays were performed to detect surface  $\alpha$ 7 nAChR expression as previously described [34]. Cells were plated at 200,000 density per well in a 24-well plate on day 1. <sup>125</sup>I- $\alpha$ BGT binding assay was performed when cells were 80% confluent. Cells were washed three times with sodium bicarbonate and 0.1% BSA containing Hank's balanced salt solution (HBSS). BSA washing was used to reduce non-specific binding. Cells were incubated with 10 nM <sup>125</sup>I- $\alpha$ BGT (unless stated otherwise) for 3 h at 4°C to measure total surface binding. Non-specific binding was determined by the addition of 1  $\mu$ M  $\alpha$ BGT. After washing the cells three times in HBSS + BSA, cells were lysed for 10–15 minutes on ice by the addition of 100  $\mu$ l extraction buffer (0.5 M NaOH + 1% Triton X-100). Lysates were transferred into polypropylene tubes and counted for 1 min with a Packard gamma counter. Specific binding was determined as the mean of quadruplicate samples of total binding minus the mean of quadruplicate nonspecific binding. The associated errors represent the square root of the sum of the standard deviations for total and nonspecific binding squared.

### Secreted alkaline phosphatase (SEAP) assay

GH4C1 cells transfected with  $\alpha$ 7 and NF $\kappa$ B SEAP were plated at 100,000 cells/well in a 24-well plate in 0.5 ml complete medium (without antibiotics). On day 2, a 30-min pre-treatment was performed with either 100  $\mu$ M PDTC or varying concentrations of GTS-21, followed by treatment with 20 ng/ml rat TNF. Cells without any treatment were used as negative controls. Twenty-four h later, 20  $\mu$ l supernatant was collected from each well and mixed with 200  $\mu$ l Invivogen SEAP substrate per manufacturer's instructions to quantify relative SEAP secretion. Absorbance readings were taken at 630 nm.

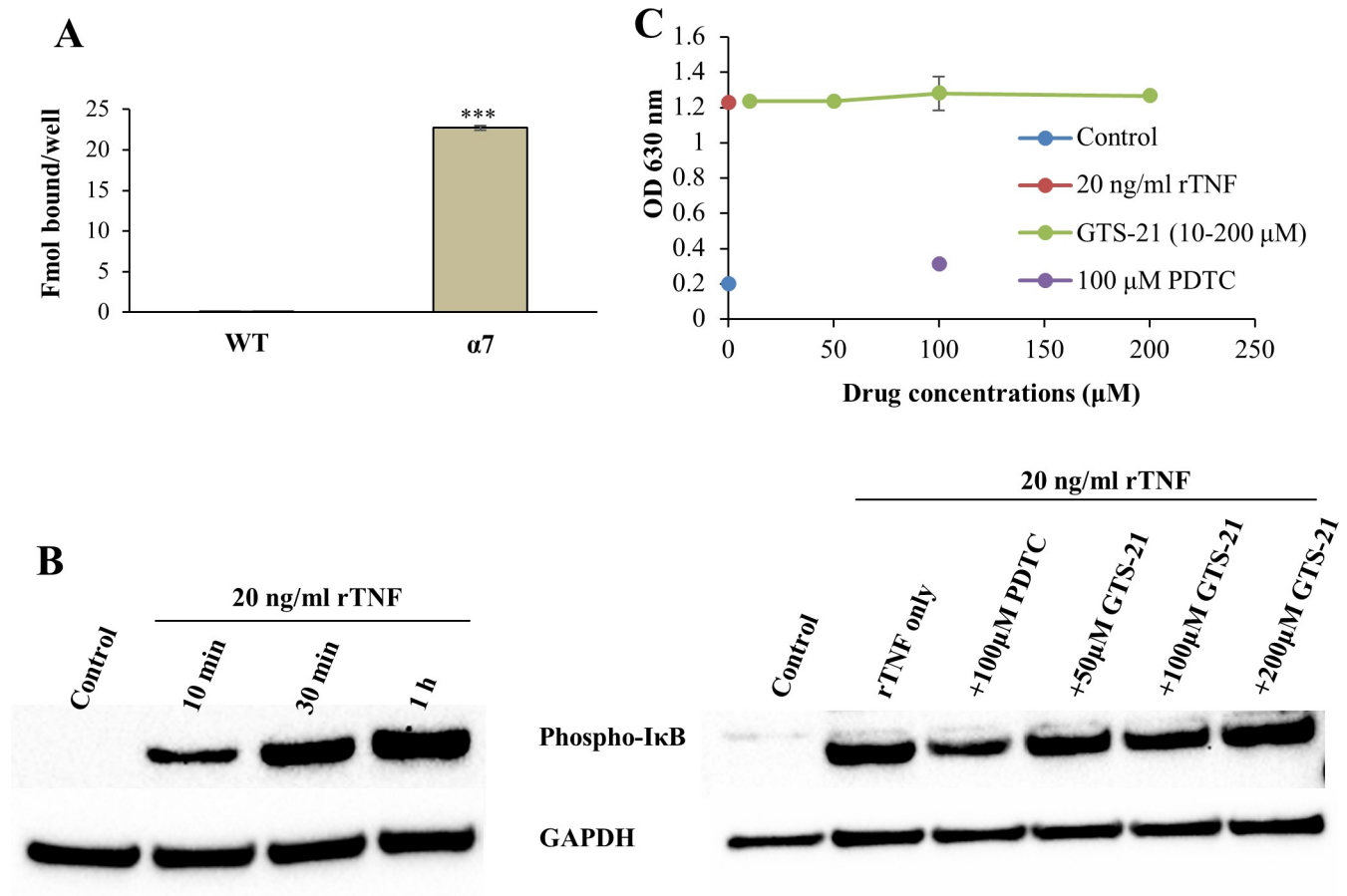
### Statistical analysis

Quantitative data were expressed as mean  $\pm$  standard error of the mean. Statistical analysis on ELISA data was conducted by one-way ANOVA followed by post-hoc Tukey HSD test to compare differences between multiple treatment groups. Student t-test was performed to analyze <sup>125</sup>I- $\alpha$ BGT binding data.

## Results

### GTS-21 does not block NF $\kappa$ B-driven SEAP secretion in GH4C1 cells expressing $\alpha$ 7 nAChR

GH4C1 cells transfected with  $\alpha$ 7 nAChR, but not WT cells, exhibit surface <sup>125</sup>I- $\alpha$ BGT binding (Fig 1A). A 1 h treatment of cells transfected with both  $\alpha$ 7 nAChR and an NF $\kappa$ B-driven SEAP



**Fig 1.  $\alpha 7$  nAChR expression and GTS-21 stimulation do not block TNF-induced NF $\kappa$ B-mediated signaling in GH4C1 cells.** (A)  $^{125}\text{I}$ - $\alpha\text{BGT}$  binding to WT GH4C1 cells compared to  $\alpha 7$  nAChR transfected cells ( $\alpha 7$ ). Concentrations and error bars are as described in methods. \*\*\*  $P < 0.0001$  compared to WT. (B) Left: Western blot data showing a time-dependent TNF-induced I $\kappa$ B phosphorylation levels. Right: Different concentrations of GTS-21 added 30 min prior to a 1 h TNF exposure (20 ng/ml), had no effect on pI $\kappa$ B levels in  $\alpha 7$  nAChR transfected GH4C1 cells. In contrast, the NF $\kappa$ B blocker PDTC decreased the levels of pI $\kappa$ B. GAPDH is the loading control. (C) Effects of different concentrations of GTS-21 on TNF-induced SEAP. PDTC, an NF $\kappa$ B inhibitor, blocked TNF-induced SEAP, confirming that inflammatory signaling is intact in GH4C1 cells and that these cells can be used to study NF $\kappa$ B-mediated signaling. GTS-21 was unable to block TNF-induced SEAP in GH4C1 cells transfected with  $\alpha 7$  nAChR and NF $\kappa$ B-SEAP plasmids.

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reporter gene with 20 ng/ml rat TNF (Fig 1B, left) causes robust phosphorylation of inhibitor of NF $\kappa$ B (I $\kappa$ B), an important inflammatory marker [6]. Briefly, TNF acting through its receptor activates I $\kappa$ k to eventually phosphorylate I $\kappa$ B, releasing NF $\kappa$ B. However, 30 min pre-treatment with various concentrations of GTS-21 did not change TNF-activated phospho-I $\kappa$ B levels, whereas 100  $\mu\text{M}$  PDTC, an NF $\kappa$ B inhibitor that can block I $\kappa$ B degradation and phosphorylation [35, 36], partially blocked I $\kappa$ B phosphorylation (Fig 1B, right). TNF-mediated SEAP secretion instigates when TNF binds to TNF receptors to activate I $\kappa$ B through a cascade of downstream pathways. Phosphorylated I $\kappa$ B degrades, releasing NF $\kappa$ B which, when phosphorylated, dimerizes and translocates to the nucleus, where it acts as a transcription factor to bind a NF $\kappa$ B promoter sequence. Twenty ng/ml rat TNF at 24 h gave the optimal response for NF $\kappa$ B-driven SEAP secretion in GH4C1 cells transfected with the reporter gene (not shown). TNF-treated GH4C1 cells transfected with  $\alpha 7$  nAChR and NF $\kappa$ B-SEAP showed a robust SEAP secretion into the cell medium, that acts as an indicator of NF $\kappa$ B activation, which was substantially inhibited by PDTC (Fig 1C). However, GTS-21 (10–200  $\mu\text{M}$ ) had no effect on TNF-

driven SEAP secretion. Hence, GTS-21 inhibited neither TNF-dependent I $\kappa$ B phosphorylation nor TNF-dependent NF $\kappa$ B-driven SEAP secretion in GH4C1 cells expressing  $\alpha$ 7 nAChRs.

### Mouse macrophages express $\alpha$ 7 nAChRs and GTS-21 blocks LPS-induced I $\kappa$ B phosphorylation

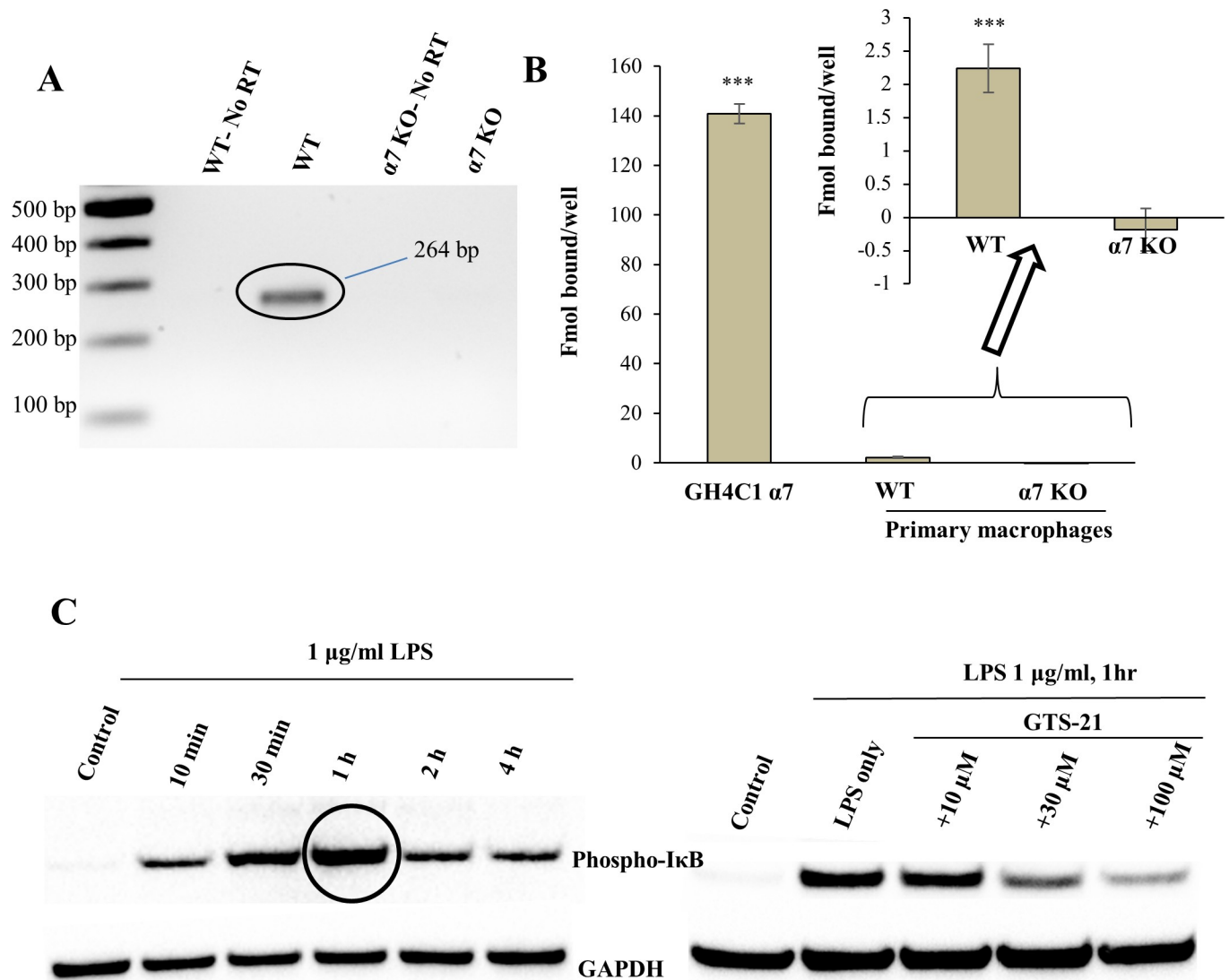
Primary macrophage cultures were stained with F4/80 and CD11b (Figure A in [S1 File](#)), two antibodies commonly used as macrophage markers, and over 90% of the cells stained with both markers. PCR analysis confirmed that  $\alpha$ 7 nAChR mRNA is expressed in macrophages from WT, but not *Chrna7*<sup>-/-</sup> mice (Fig 2A). Sequencing confirmed the 264 bp PCR product identity. Binding studies corroborated these results, since WT macrophages showed specific  $\alpha$ BGT binding, whereas no specific  $\alpha$ BGT binding was observed in  $\alpha$ 7 nAChR knockout murine macrophages (Fig 2B). LPS induced significant levels of pI $\kappa$ B after 1 h treatment, and GTS-21 pre-treatment reduced LPS-induced pI $\kappa$ B in macrophages isolated from WT mice (Fig 2C).

### GTS-21 exhibits both $\alpha$ 7 nAChR-mediated and $\alpha$ 7 nAChR-independent anti-inflammatory effects in macrophages

Primary mouse macrophages from both WT and  $\alpha$ 7 nAChR knockout mice showed robust TNF and IL6 secretion in response to LPS treatment. Ten ng/ml LPS applied to WT mouse macrophages gave submaximal but significant IL6 and TNF secretion, and this LPS concentration was used in subsequent experiments. GTS-21 dose-dependently attenuated TNF and IL6 secretion in macrophages isolated from both WT and  $\alpha$ 7 nAChR knockout mice, confirming that GTS-21 possess  $\alpha$ 7 nAChR-independent anti-inflammatory effects (Fig 3). This effect was not due to gross changes in cell viability (Figure B in [S1 File](#)). Fifty  $\mu$ M GTS-21 pre-treatment reduced >50% cytokine response in mouse macrophages and was used to perform further studies using  $\alpha$ 7 nAChR antagonists ( $\alpha$ BGT and MLA) [37]. The  $\alpha$ 7 nAChR antagonist  $\alpha$ BGT showed about 10% reversal of GTS-21 induced block of TNF release by LPS in macrophages isolated from WT mice, but MLA elicited no such reversal (Fig 4A). Additionally,  $\alpha$ BGT reversed about 40%, and MLA about 30% of the GTS-21 block of IL6 release by LPS in WT macrophages (Fig 4B). The partial  $\alpha$ BGT and MLA reversals of GTS-21 blockade suggest some  $\alpha$ 7 nAChR-dependent anti-inflammatory effects of GTS-21. However, GTS-21 blocked TNF and IL6 secretion in  $\alpha$ 7 nAChR knockout macrophages, and neither  $\alpha$ BGT nor MLA has any effect on either TNF or IL6 secretion (Fig 4C & 4D). These data strongly suggest that GTS-21 mediates anti-inflammatory effects in mouse macrophages at least in part via an  $\alpha$ 7 nAChR-independent pathway.

## Discussion

Chronic inflammation often occurs due to either unresolved injuries or pathogenic infections that cause over-production of multiple pro-inflammatory cytokines such as TNF, IL1, IL6, IL17, HMGB1 and other mediators such as nitric oxide. Monoclonal antibodies are used clinically to prevent inflammation associated with various disease states by binding to specific pro-inflammatory cytokines. An example is etanercept, a TNF antibody used to treat rheumatoid arthritis [38]. However, a major challenge has been to find orally bioavailable small molecules that simultaneously suppress multiple pro-inflammatory cytokines. Wang et al. [7] reported that vagus nerve stimulation activates cholinergic signaling via  $\alpha$ 7 nAChRs to inhibit chronic inflammation. Since then, many reports (based on *in vivo* and *in vitro* experiments) have shown that like vagus nerve stimulation,  $\alpha$ 7 nAChR agonists inhibit multiple pro-



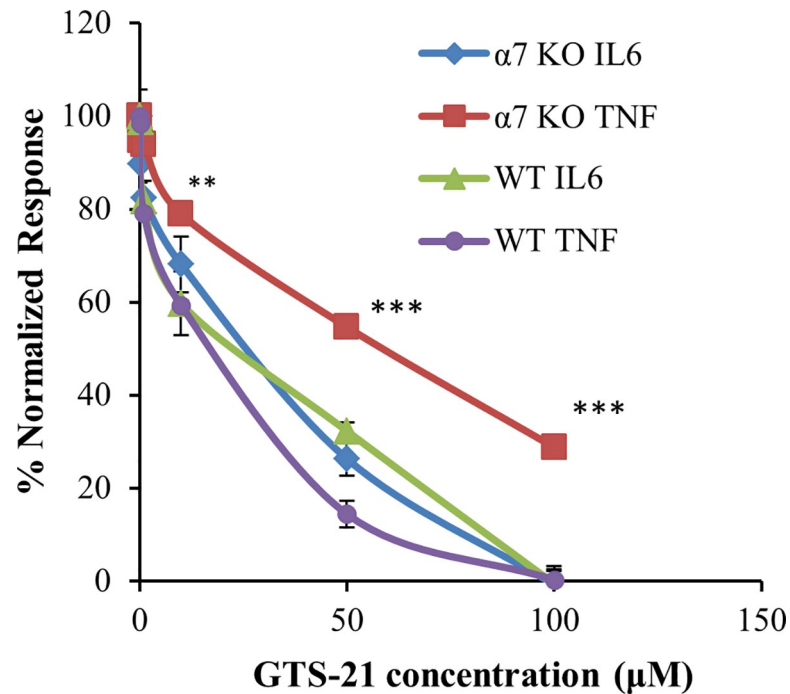
**Fig 2. Confirmation of  $\alpha 7$  nAChR surface expression on mouse macrophages.** (A) PCR analysis showing a band of 264 bp size confirming the presence of  $\alpha 7$  nAChR mRNA in macrophages isolated from WT but not  $\alpha 7$  nAChR knockout mice. The 100 base pair ladder was from New England Biolabs. No RT: No reverse transcriptase control. (B)  $^{125}$ I- $\alpha$ BGT binding data comparing  $\alpha 7$  nAChR expression on macrophages isolated from WT and  $\alpha 7$  nAChR knockout murine model.  $\alpha 7$  nAChR transfected GH4C1 cells were used as a positive control. Macrophages from WT mouse showed a small but significant  $\alpha$ BGT specific binding site (detected with 100 nM  $^{125}$ I- $\alpha$ BGT). Bars in the figure represent specific binding. \*\*\*  $P < 0.0001$  compared to  $\alpha 7$  nAChR knockout mouse macrophages. (C) Left: Western blot data suggested LPS induced maximal pI $\kappa$ B levels in WT mouse macrophages at 1 h (circled). Right: GTS-21 attenuated LPS-stimulated pI $\kappa$ B levels in mouse macrophages in a dose-dependent manner. GAPDH was the loading control.

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inflammatory cytokines [10, 17]. GTS-21 is an  $\alpha 7$  nAChR partial agonist commonly used as an anti-inflammatory agent [24–27]. In this report, we investigated whether  $\alpha 7$  nAChRs are required for GTS-21's proposed anti-inflammatory effects, both in immune-derived cells and in a heterologous expression model in a non-immune cell system.

GH4C1 cells, derived from rat pituitary do not express any known nicotinic receptor subunits, while  $\alpha 7$  nAChRs heterologously expressed in GH4C1 cells [29] show classic properties of  $\alpha 7$  ligand-gated ion channels. Activation with agonist causes cell depolarizations that desensitize rapidly [30] show high calcium permeability [39, 40] and even mutant  $\alpha 7$  receptors can show single channel currents as well as inward rectification [41]. Receptor activation in

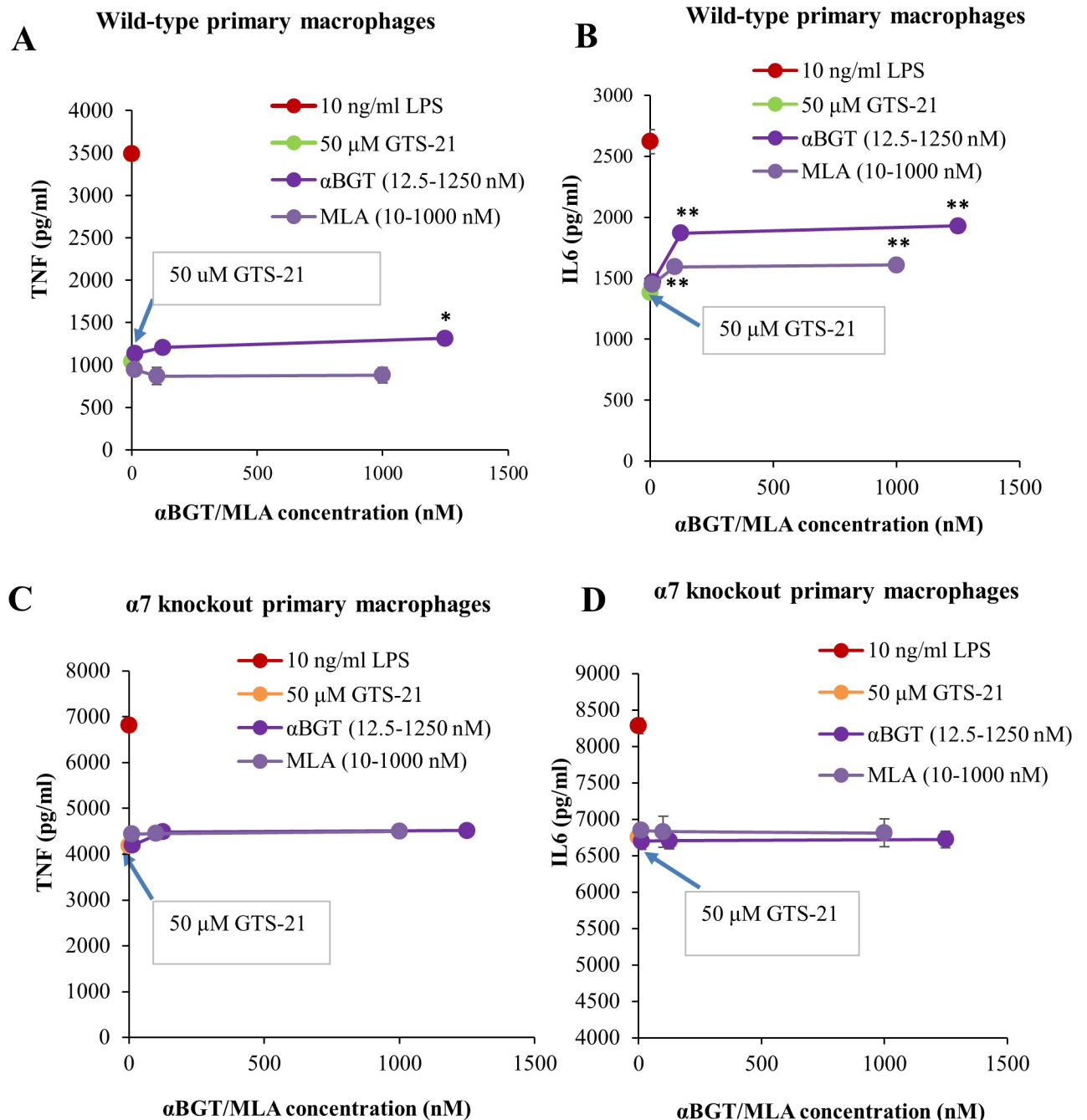




**Fig 3. GTS-21 attenuates IL6 and TNF secretion in macrophages from WT and  $\alpha 7$  nAChR knockout mice.** Macrophages from WT and  $\alpha 7$  nAChR knockout mice were isolated and activated by LPS before assaying the amount of IL6 and TNF release measured by ELISA as indicated in the methods section. Quadruplicate responses are normalized to cytokine release by LPS in the absence of GTS-21. A repetition of this experiment yielded similar results. One-way ANOVA (analysis of variance) with post-hoc Tukey HSD (honestly significant difference) test used to compare differences between multiple treatment groups. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  for the  $\alpha 7$  KO TNF curve, other curves had similar P values (not shown).

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GH4C1 cells is blocked by  $\alpha 7$  antagonists such as MLA [30], and agonist-induced ion channel activity can be dramatically increased by  $\alpha 7$  positive allosteric modulators such as PNU-120596 [40]. The concentration of MLA necessary to block  $\alpha 7$  nAChRs is 1 nM in GH4C1 cells [30], while that of  $\alpha$ BGT in other heterologous expression systems is around 10 nM [42, 43]. Therefore, it is a reasonable assumption that appearance of  $\alpha$ BGT binding sites after transfection of  $\alpha 7$  nAChR DNA in GH4C1 cells correlates with expression of electrophysiologically-functional  $\alpha 7$  nAChRs ion channels. In contrast, nAChRs expressed on myeloid immune-derived cell systems have to date shown no evidence of electrophysiological function. Nicotine blocked TNF release in rat microglia via intracellular calcium release in the absence of cell surface ion channel function, but the effect was blocked by 10 nM  $\alpha$ BGT or MLA, suggesting involvement of  $\alpha 7$  nAChRs [44]. Similarly, nicotine, acetylcholine, choline, and several choline derivatives effectively blocked ATP-induced release of the pro-inflammatory cytokine IL-1 $\beta$  from rat and human monocytes expressing  $\alpha 7$ ,  $\alpha 9$  and/or  $\alpha 10$  subunits, but nicotine induced no current in these cells, unlike ATP [45]. In those monocyte cultures, 1  $\mu$ M  $\alpha$ BGT blocked the effects. Also, nicotine blocked the ability of ATP to increase intracellular calcium in rat alveolar macrophages expressing  $\alpha 9$  and  $\alpha 10$  subunits, and 100 nM  $\alpha$ BGT blocked the effects, but no nicotinic channel function was observed, and extracellular calcium was not required for these effects [46]. Thomsen and Mikkelsen [47] demonstrated that the weak partial  $\alpha 7$  agonists GTS-21 (50–100  $\mu$ M) and NS6740 (50  $\mu$ M) and the  $\alpha 7$  antagonist MLA (10 nM) significantly attenuated LPS-induced TNF release from cultured rat microglia, while adding positive allosteric modulators such as PNU120596 had no further effect. Together, these



**Fig 4.  $\alpha 7$  nAChR-independent anti-inflammatory effects of GTS-21 in WT and *Chrna7*<sup>-/-</sup> mouse macrophages.** (A) 50  $\mu$ M GTS-21 pre-treatment significantly blocked endotoxin-stimulated TNF and IL6 secretions and this concentration was selected to further study the effects of  $\alpha 7$  nAChR antagonists,  $\alpha$ BGT and MLA. ELISA data showing approx. 10% reversal of GTS-21 block on TNF secretion by 1.25  $\mu$ M  $\alpha$ BGT. No reversal was observed with MLA. ELISA data in panel (B) demonstrates 40% reversal of GTS-21 block on IL6 secretion by 1.25  $\mu$ M  $\alpha$ BGT and 30% reversal by 1  $\mu$ M MLA. Neither  $\alpha$ BGT nor MLA produced significant changes in the GTS-21 block of LPS-induced (C) TNF or (D) IL6 secretion in  $\alpha 7$  nAChR absence. One-way ANOVA (analysis of variance) with post-hoc Tukey HSD (honestly significant difference) test used to compare differences between multiple treatment groups. \*  $P < 0.01$ , \*\*  $P < 0.001$ , \*\*\*  $P < 0.0001$ . A duplicate of this experiment showed similar effects.

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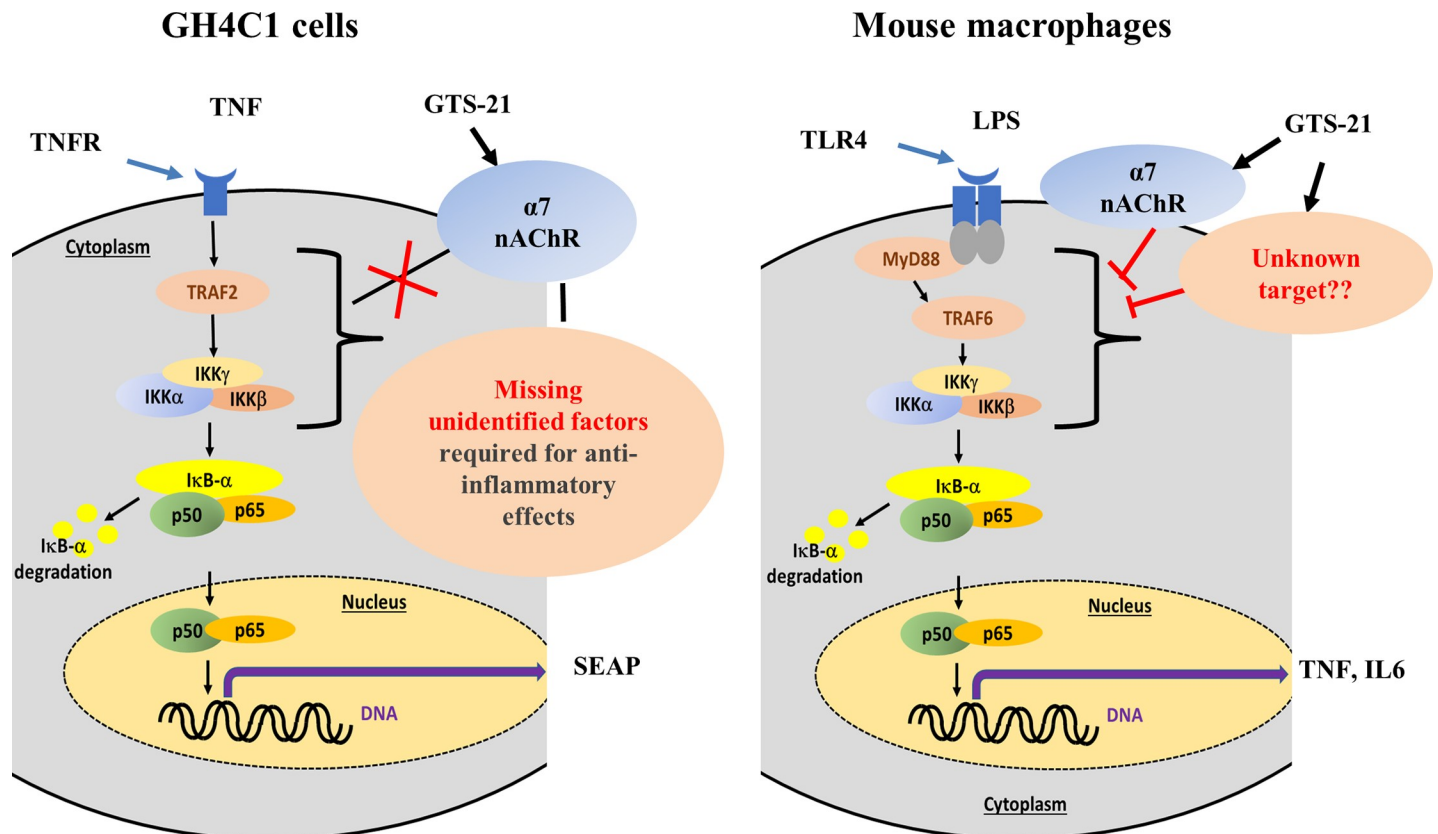
results have led to the hypothesis that ionotropic channel action is not required for nicotinic receptor anti-inflammatory actions in myeloid tissues, and that “silent agonists” can bind to

$\alpha 7$  nAChRs to produce metabotropic-like activity that is responsible for anti-inflammatory actions [48]. The basis for functional differences between nicotinic receptors expressed in myeloid tissues and neuronal tissues is not known.

We used GH4C1 cells expressing both  $\alpha 7$  nAChR and the gene reporter NF $\kappa$ B-SEAP (Fig 1) to check whether  $\alpha 7$  nAChR expression alters GTS-21 effects on NF $\kappa$ B signaling.  $^{125}$ I- $\alpha$ BGT binding studies validated that  $\alpha 7$  nAChR are being expressed, while SEAP secretion assays and western blots of I $\kappa$ B $\alpha$  phosphorylation measured rat TNF-activated NF $\kappa$ B signaling. However, GTS-21 (100 nM–200  $\mu$ M) had no blocking effect on TNF effects. These data suggest that GTS-21 is unable to alter TNF signaling by activating  $\alpha 7$  nAChR ion channels in GH4C1 cells, unlike the GTS-21 blockade we observed for LPS-induced signaling in macrophages. This could be due to differences in downstream inflammatory signaling between TNF receptors and LPS-activated TLR4 receptors. TNF receptors signal through TNF receptor associated factor 2 (TRAF2) and other components upstream of NF $\kappa$ B, while LPS signals through myeloid differentiation primary response gene 88 (MyD88) and TRAF6, among others (Fig 5, [6]). GTS-21 may not affect TNF-induced inflammatory signaling in GH4C1 cells via  $\alpha 7$  nAChR, as it may need adaptor molecules like MyD88 and TRAF6. We hypothesize that GTS-21 effects on NF $\kappa$ B signaling may require such adaptor molecules and additional unidentified factors which are not present in non-immune GH4C1 cells. It is possible that the same unidentified factors required to link  $\alpha 7$  receptors to dampen inflammatory signaling also silence the ion channel properties of the receptors in myeloid tissue.

Wang et al. [7] showed that human macrophages possess  $\alpha 7$  nAChR RNA signal by PCR analysis and FITC-labeled  $\alpha$ BGT staining. Similarly, we found subtle but significant  $\alpha 7$  nAChR RNA expression and  $^{125}$ I- $\alpha$ BGT specific binding in macrophages isolated from WT mice, but both were absent in  $\alpha 7$  nAChR knockout mouse macrophages. Wang et al. [7] used the  $\alpha 7$  nAChR agonist nicotine, to demonstrate  $\alpha 7$  nAChR-mediated anti-inflammatory effects on LPS-induced cytokines secretion by comparing the effects in WT and  $\alpha 7$  nAChR knockout mouse macrophages. However, nicotine toxicity, i.e., nausea, headache, and addiction, has raised concerns over the specificity of anti-inflammatory effects of nicotine [22, 49], and has raised the question of whether more selective  $\alpha 7$  nAChR agonists, such as GTS-21, should be substituted to achieve anti-inflammatory effects.

GTS-21 blocks LPS-induced cytokine RNA levels [27, 50, 51] and LPS-driven IL1, IL6, and TNF release [27] measured by ELISA in leukocyte or macrophage cultures or in whole animals. Likewise, in our experiments, 50  $\mu$ M GTS-21 blocked >50% LPS-induced pI $\kappa$ B levels and TNF and IL6 secretion. Nullens et al. [28] studied a murine cecal ligation and puncture-induced sepsis model and found that GTS-21 reduced colon and serum IL6 levels in both WT and  $\alpha 7$  nAChR knockout animals. In contrast, Rosas-Ballina et al. [50] reported that GTS-21 alleviates LPS-induced inflammatory signaling in macrophages via  $\alpha 7$  nAChRs. Our results establish that GTS-21 retains its anti-inflammatory effects at the cellular level, inhibiting TNF and IL6 secretion in macrophages from the *Chrna7*<sup>-/-</sup> mouse. Moreover,  $\alpha 7$  nAChR antagonists, MLA and  $\alpha$ BGT, partially reversed GTS-21 blockade of LPS-induced TNF and IL6 secretion, but only in macrophages isolated from WT mice and had no effect on  $\alpha 7$  nAChR knockout macrophages. Further, the concentrations of these antagonists we used were well in excess of concentrations necessary to block function at ionotropic  $\alpha 7$  nAChRs, and the maximum effects were very modest. These *ex vivo* data confirm that GTS-21 has both  $\alpha 7$  nAChR-mediated and  $\alpha 7$  nAChR-independent anti-inflammatory effects. GTS-21 might produce  $\alpha 7$  nAChR-independent anti-inflammatory effects via other nicotinic subtypes like  $\alpha 4\beta 2$  [52], as some reports claim that  $\alpha 4\beta 2$  nAChRs also play a crucial role in suppressing inflammation [53, 54]. Another report indicates that the nAChR agonist anatabine exhibits  $\alpha 7$  nAChR-independent anti-inflammatory effects in HEK-293 cells that lack nAChRs [55].



**Fig 5. Schematic comparing cell-dependent effects of GTS-21 in GH4C1 cells and primary mouse macrophages.** Top panel: GTS-21 was unable to suppress TNF-induced secreted alkaline phosphatase in a  $\alpha 7$  nAChR and NF $\kappa$ B-SEAP expressing GH4C1 cell system. Bottom panel: GTS-21 reduced LPS-induced TNF and IL6 levels through both an  $\alpha 7$  nAChR-dependent and an  $\alpha 7$  nAChR-independent mechanism in primary mouse macrophages. In both cases, activation of the NF $\kappa$ B pathway leads to phosphorylation of I $\kappa$ B, and dimerization and translocation of two subunits of cytoplasmic NF $\kappa$ B (usually p50 and p65) to the nucleus to act as a transcription factor (Lawrence et al., 2009). In spite of the common NF $\kappa$ B pathway, the immediate effects of TNF and LPS differ in terms of the receptors and linkage to NF $\kappa$ B signaling, where GTS-21 non-nicotinic receptor actions may be taking place.

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In summary, GTS-21 effects are highly cell-type dependent, and the presence of  $\alpha 7$  nAChR is insufficient to allow GTS-21 to block NF $\kappa$ B signaling in all cell types. Further, GTS-21 can block NF $\kappa$ B signaling in cells lacking  $\alpha 7$  nAChRs. Thus, anti-inflammatory effects of GTS-21 should not necessarily be ascribed to its effects as a partial  $\alpha 7$  nAChR agonist.

## Supporting information

**S1 File. Supplementary methods and figures.**  
(PDF)

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**Writing – original draft:** Brijesh K. Garg.

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