Biochemical Pharmacology 78 (2009) 863–872

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

GTS-21 inhibits pro-inflammatory cytokine release independent of the Toll-like receptor stimulated via a transcriptional mechanism involving JAK2 activation

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ARTICLE INFO

Article history:
Received 22 April 2009
Accepted 23 June 2009

Keywords:
Inflammation
Cholinergic anti-inflammatory pathway
Nicotine
Monocytes
Peripheral blood mononuclear cells
α7 nicotinic acetylcholine receptor

ABSTRACT

The vagus nerve can limit inflammation via the α7 nicotinic acetylcholine receptor (α7nAChR). Selective pharmacological stimulation of the α7nAChR may have therapeutic potential for the treatment of inflammatory conditions. We determined the anti-inflammatory potential of GTS-21, an α7nAChR-selective partial agonist, on primary human leukocytes and compared it with nicotine, the nAChR agonist widely used for research into the anti-inflammatory effects of α7nAChR stimulation. Furthermore, we investigated whether the effects of both nicotinic agonists were restricted to specific Toll-like receptors (TLRs) stimulated and explored the mechanism behind the anti-inflammatory effect of GTS-21.

GTS-21 and nicotine inhibited the release of pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs), monocytes and whole blood independent of the TLR stimulated, with higher potency/efficacy for GTS-21 compared to nicotine. The anti-inflammatory cytokine IL-10 was relatively unaffected by both nicotinic agonists. The effects of GTS-21 and nicotine could not be reversed by nAChR antagonists, while the JAK2 inhibitor AG490 abolished the anti-inflammatory effects. GTS-21 downregulated monocyte cell-surface expression of TLR2, TLR4 and CD14. qPCR analysis demonstrated that the anti-inflammatory effect of GTS-21 is mediated at the transcriptional level and involves JAK2-STAT3 activation.

In conclusion, GTS-21 has a profound anti-inflammatory effect in human leukocytes and that GTS-21 is more potent/efficacious than nicotine. The absence of a blocking effect of nAChR antagonists in human leukocytes might indicate different pharmacological properties of the α7nAChR in human leukocytes compared to other cell types. GTS-21 may be promising from a therapeutic perspective because of its suitability for human use.

1. Introduction

In the past few years, a novel link between the vagus nerve and the inflammatory responses has been established. In addition to "sensing" focal inflammation in the periphery and relaying it to the brain via afferent fibers [1–3], recent work has demonstrated that the efferent vagus nerve can modulate the inflammatory response in a reflex-like fashion, termed "the cholinergic anti-inflammatory pathway" [4]. It has become clear that the α7 nicotinic acetylcholine receptor (α7nAChR), expressed in various cell types including human leukocytes [5], is an essential regulator of this anti-inflammatory effect of the vagus nerve [6]. Consequently, more specific agonists of this receptor were identified or developed and used in various studies [7–9]. To date, one of the most effective α7-selective partial agonists for modulating inflammatory responses is 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) which has proven to be effective in attenuating the immune response and improving outcome in animal models of pancreatitis [9], endotoxemia [8,7], sepsis [7], acute lung injury [10,11] and ischemia-reperfusion injury [12]. However, its anti-inflammatory potential in human inflammatory cells has never been evaluated. This is of particular interest because GTS-21, which has been primarily developed for the treatment of Alzheimer's disease, has been administered to human volunteers and patients and is well tolerated without clinically significant safety findings [13]. Therefore, GTS-21 may have therapeutic potential for the treatment of inflammatory conditions and is preferred above nicotine which lacks pharmacologic specificity and has toxic side effects and the potential to produce physical dependence (addiction). Although comparisons between GTS-21 and non-selective nAChR agonists have been reported at α7nAChR receptor activation level in

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doi:10.1016/j.bcp.2009.06.096

electrophysiological studies using heterologous expression in *Xenopus laevis* oocytes [14,15], the immunomodulating effects of selective and non-selective agonists of the α7nAChR have not been compared. Furthermore, in vitro studies investigating the cholinergic anti-inflammatory pathway almost exclusively used the principal Toll-like receptor 4 (TLR4) agonist LPS as a trigger for inflammation. It is unknown whether the cholinergic anti-inflammatory pathway is restricted to certain TLRs stimulated or is a general mechanism not constrained to a specific stimulus.

Finally, the mechanism by which α7nAChR stimulation attenuates pro-inflammatory cytokine production has not been fully elucidated and sparcely studied in human cells. A role for the JAK2-STAT3 pathway [16–19] as well as suppression of NFκB transcriptional activity [20,21] is implied. However, the cholinergic anti-inflammatory pathway is believed to be regulated at a post-transcriptional level [4,16]. Mechanistic studies regarding the anti-inflammatory effect of GTS-21 are limited to one study reporting decreased NFκB activity in a murine cell line [7].

In this study, we investigated for the first time the anti-inflammatory potential of GTS-21 on primary human leukocytes and compared it with nicotine. Furthermore, we investigated whether the effects of both nicotinic agonists were restricted to specific TLRs stimulated and whether they affected cell-surface expression of receptors involved in the innate immune response. Finally, we studied whether the anti-inflammatory effects of GTS-21 and nicotine are regulated at the transcriptional level and determined the involvement of the JAK-STAT signal transduction pathway.

2. Methods

2.1. General reagents

RPMI culture medium (RPMI 1640 Dutch modification, ICN Biomedicals; Costa Mesa, CA, USA) was supplemented with gentamicin 10 μg/mL, L-glutamine 10 mM and pyruvate 10 mM. GTS-21 was obtained from the University of Florida (a kind gift of Prof. Dr. Roger L. Papke) and from Comentis Inc. (South San Francisco, CA, USA). No differences in potency/efficacy between GTS-21 from the University of Florida and from Comentis Inc. were observed (data not shown). Nicotine (liquid, naturally occurring isomer), mecamylamine, α-bungarotoxin, methyllycaconitine (MLA), tyrphostin AG490, D-tubocurarine and *Escherichia coli* lipopolysaccharide (LPS, serotype O55:B5) were obtained from Sigma–Aldrich (St Louis, MO, USA). LPS was further purified as described previously [22]. LPS concentration was 1 ng/mL in all experiments. Pam3Cys was purchased from EMC Micro-collections (Tübingen, Germany). Flagellin and polyI:C were obtained from InvivoGen (San Diego, CA, USA). All stimuli except AG490, which was dissolved in ethanol, were dissolved in RPMI.

2.2. Peripheral blood mononuclear cells, monocyte and whole blood stimulation

After obtaining informed consent venous blood was drawn from the cubital vein of healthy non-smoking male volunteers into EDTA or lithium heparin tubes (Vacutainer System, BD Biosciences, Plymouth, UK). All volunteers refrained from caffeine-containing beverages/food for at least 12 h before blood collection. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA anticoagulated blood by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden), washed three times in ice-cold sterile phosphate-buffered saline (B Braun Melsungen AG, Melsungen, Germany), and resuspended in RPMI 1640 culture medium supplemented with 10% autologous serum obtained by centrifugation (6000 rpm, 10 min) of lithium-heparin anticoagulated blood. Cells were counted in a Bürker hemocytometer and viability was assessed using trypan blue (viability was >95%). The number of cells was adjusted to 2.5×10^6/mL and 5×10^5 cells per well (200 μL) were seeded in duplicate in 96-well flat bottom plates (Greiner, Alphen a/d Rijn, The Netherlands) and stimulated for 22 h (37 °C, 95% O_2, 5% CO_2) with various compounds. Incubation with RPMI alone served as a negative control. Nicotine or GTS-21 were added 30 min before TLR agonists. Nicotinic antagonists or the JAK2 inhibitor AG-490 were added 30 min before nicotine or GTS-21.

Primary monocytes were obtained from PBMCs using magnetic bead negative depletion (Monocyte Isolation kit II and LS columns, Miltenyi Biotec, Utrecht, The Netherlands). This procedure yields untouched monocytes suitable for short-term stimulation experiments where magnetic beads attached to cell surface epitopes are unwanted. Monocyte purity was evaluated using flow cytometry and was 85–90%. 1×10^5 cells per well (200 μL) in duplicate were stimulated in the presence of 10% autologous serum for 22 h as described above.

After stimulation, PBMC/monocyte well plates were centrifuged (1700 rpm, 8 min) and supernatants were stored at −80 °C until assayed.

For whole blood stimulation experiments, venous blood was drawn into 2 mL lithium-heparin containing vacutainers (Vacutainer System, BD Biosciences). Whole blood was diluted 1:5 in RPMI and stimulated for 24 h as described above. After stimulation, whole blood cultures were centrifuged (14,000 rpm, 5 min) after which supernatants were stored at −80 °C until assayed.

2.3. Cytokine measurements

Cytokines in supernatants of whole blood cultures were determined using a simultaneous Luminex Assay according to the manufacturer's instructions (Bio-plex cytokine assay, BioRad, Hercules, CA, USA). Cytokines in supernatants of PBMC and monocytes cultures were determined using enzyme-linked immunosorbent assays. TNF-α was determined by a specific ELISA using four antibodies as described previously [23]. IL-1β, IL-6 and IL-10 were measured by commercial ELISA kits (IL-1β: R&D systems, Minneapolis, MN, USA; IL-6 and IL-10: Pelikine Compact, Sanquin, Amsterdam, The Netherlands, according to the manufacturer's instructions).

2.4. Viability assays

Monocyte viability was assessed using the *in vitro* toxicology assay kit, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based (Sigma–Aldrich). Monocytes were stimulated as described in section 2.2 (22 h). After stimulation, plates were centrifuged (1700 rpm, 8 min), 100 μL of supernatant was stored at −80 °C for cytokine analysis and 10 μL of MTT stock solution (5 mg/mL) was added to the wells. Monocytes were incubated at 37 °C for an additional 4 h after which formazan crystals were solubilized and absorbance at 570 nm was measured in a plate reader. Additionally, lactate dehydrogenase (LDH) was determined in supernatants of PBMCs stimulated for 22 h as described in Section 2.2.

2.5. Flow cytometry

Flow cytometry was performed on PBMCs stimulated as described in section 2.2 (22 h), After stimulation, plates were centrifuged (1700 rpm, 8 min), supernatants were stored at −80 °C for cytokine analysis and adherent cells were detached by adding 200 μL of ice-cold FACS buffer (PBS with 0.5% BSA) and incubating the plate for 15 min on ice. Subsequently, cells were collected by

vigorous resuspending and scraping of the bottom of the wells and washed twice in ice-cold FACS buffer. TLR2, TLR4 and CD14 expression was determined with the following directly conjugated antibodies: CD282 PE (mouse IgG2a, TLR 2.1 clone, eBioscience, San Diego, CA, USA), CD284 PE-Cy7 (TLR4, mouse IgG2a, HTA125 clone eBioscience), CD14 ECD (mouse IgG2a, RMO52 clone Immunotech, Beckman Coulter, Mijdrecht, The Netherlands). Expression of α7nAChR on monocytes was determined with a primary antibody against α7nAChR (rat IgG1 monoclonal, 319 clone, Abcam, Cambridge, UK) and CD14 ECD followed by a FITC labeled secondary antibody (donkey anti-rat FITC, Beckman Coulter). After antibody incubation, cells were washed with FACS buffer, resuspended and analyzed on a Beckman Coulter FC500 flow cytometer. TLR2, TLR4, CD14 and α7nAChR expression were analyzed within CD14 positive monocytes.

2.6. Quantitative PCR and JAK-STAT signaling arrays

Untouched primary monocytes were isolated as described in Section 2.2 and seeded in duplicate at a density of 1×10^6/well in 24-well plates in the presence and absence of LPS and nicotinic agonists. After 4 h, plates were centrifuged (1700 rpm, 8 min) and supernatants were aspirated. Subsequently, RNA was isolated using RNeasy plus mini kits (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Per experiment, equal amounts of RNA (150–400 ng) were used for cDNA synthesis using iScript cDNA synthesis kits (Bio-Rad). Negative control reactions were performed in the absence of reverse transcriptase.

The sequences of the primer pairs used were:

TLR2	Forward:	5′-GAATCCTCCAATCAGGCTTCTCT-3′
	Reverse:	5′-GCCCTGAGGGAATGGAGTTTA-3
TLR4	Forward:	5′-GGCATGCCTGTGCTGAGTT-3′
	Reverse:	5′-CTGCTACAACAGATACTACAAGCACACT-3′
TNF-α	Forward:	5′-TGGCCCAGGCAGTCAGA-3′
	Reverse:	5′-GGTTTGCTACAACATGGGCTACA-3′
IL-1β	Forward:	5′-CAGCTACGAATCTCCGACCAC-3′
	Reverse:	5′-GGCAGGGAACCAGCATCTTC-3′
IL-6	Forward:	5′-AATTCGGTACATCCTCGACGG-3′
	Reverse:	5′-GGTTGTTTTCTGCCAGTGCCT-3′
IL-10	Forward:	5′-CAACCTGCCTAACATGCTTCG-3′
	Reverse:	5′-TCATCTCAGACAAGGCTTGGC-3′
CD14	Forward:	5′-ACGCCAGAACCTTGTGAGC-3′
	Reverse:	5′-GCATGGATCTCCACCTCTACTG-3′
α7nAChR	Forward:	5′-AAACTCACAGATGGGCAAGG-3′
	Reverse:	5′- AGGGAACACTGGAGTTGTGG-3′
B2M	Forward:	5′-ATGAGTATGCCTGCCGTGTG-3′
	Reverse:	5′-CCAAATGCGGCATCTTCAAAC-3′

Primers were obtained from Biolegio (Nijmegen, The Netherlands). The quantitative PCR (qPCR) reaction was performed using Power SybrGreen master mix (Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands) on an ABI Prism 7300 Real Time PCR system (Applied Biosystems). Negative control reactions were cycled alongside test samples to ensure the absence of contaminating genomic DNA. The amplification of a single product was ensured by melt-curve analysis for each primer pair. A standard curve constructed from 1:5 serial dilutions of a mixture of cDNA of the samples in the same run was included for each primer pair in every run to perform relative quantification of mRNA expression.

To investigate the involvement of the JAK-STAT signaling pathway we used RT^2 Profiler JAK-STAT qPCR arrays (PAHS-039, SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. For these experiments, isolated monocytes of three different donors were stimulated and mRNA was isolated as described above. Four housekeeping genes, hypoxanthine phosphoribosyltransferase (HPRT1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) and β2 microglobulin (B2M) present on the PCR array were used for normalization.

2.7. Calculations

Data are expressed as mean ± SEM except for whole blood stimulation data which is expressed as median ± interquartile range as it was not normally distributed. Statistical significance in the dose-response curves of nicotine and GTS-21 was evaluated using repeated measures one-way analysis of variance with Bonferroni post-hoc test. Elsewhere, the paired Student's *t*-test was used to test for statistical significance except for the whole blood data which was analyzed using the Wilcoxon matched pairs test and the qPCR JAK-STAT array data for which the analysis procedure is described below. A *p*-value below 0.05 (except for qPCR JAK-STAT array data, $p < 0.01$) was considered statistically significant.

For whole blood experiments, % inhibition/stimulation was calculated as follows: (TLR-agonist-induced cytokine release in the presence of GTS-21 or nicotine/TLR-agonist-induced cytokine release in the absence of GTS-21 or nicotine) − 100. If more than 50% of the subjects had a cytokine response to a TLR-agonist in the absence of a nicotinic agonist that was lower than four times the detection limit, no inhibition/stimulation calculations for this TLR-agonist-cytokine combination were performed.

In the experiments where nAChR antagonists were used, the % blocking effect of the nAChR antagonists was calculated by subtracting the % inhibition of the LPS-response by GTS-21 or nicotine in absence of the nAChR antagonist from the % inhibition of the LPS-response by GTS-21 or nicotine in the presence of the nAChR antagonist.

qPCR data was analyzed using ABI Prism software and calculations were performed as follows: per sample the relative quantity of mRNA of the gene of interest (e.g. TNF-α) was divided by the relative quantity of the housekeeping gene B2M. To calculate fold induction compared to the control sample, we divided the TNF-α/B2M ratio by the TNF-α/B2M ratio of the unstimulated control (RPMI) sample. If fold induction was <1, it was represented as −(1/fold induction).

For the JAK-STAT qPCR array experiments each replicate cycle threshold (CT) was normalized to the average CT of four housekeeping genes on a per plate basis. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantification of gene expression in stimulated samples compared to RPMI samples. A gene was considered not detectable when CT >35. CT was defined as 35 for the $2^{-\Delta\Delta CT}$ calculation when the signal was below detectable limits. If for a specific gene both the stimulated and control sample expression was below detectable limits the sample was excluded from analysis. Genes were considered differentially expressed if mean up- or downregulation was equal or greater than 2-fold and $p < 0.01$ (permutation test, 100 permutations).

Statistical calculations were performed using Graphpad Prism V4.03 (Graphpad software) except for the permutation tests in the JAK-STAT qPCR array experiments which were analyzed with Multi-experiment Viewer V4.3 (TM4 software suite).

3. Results

3.1. Nicotine and GTS-21 dose-dependently inhibit LPS-induced cytokine production in PBMCs

To determine the effect of nAChR agonists on LPS-induced cytokine release we incubated human PBMCs with 1 ng/mL LPS

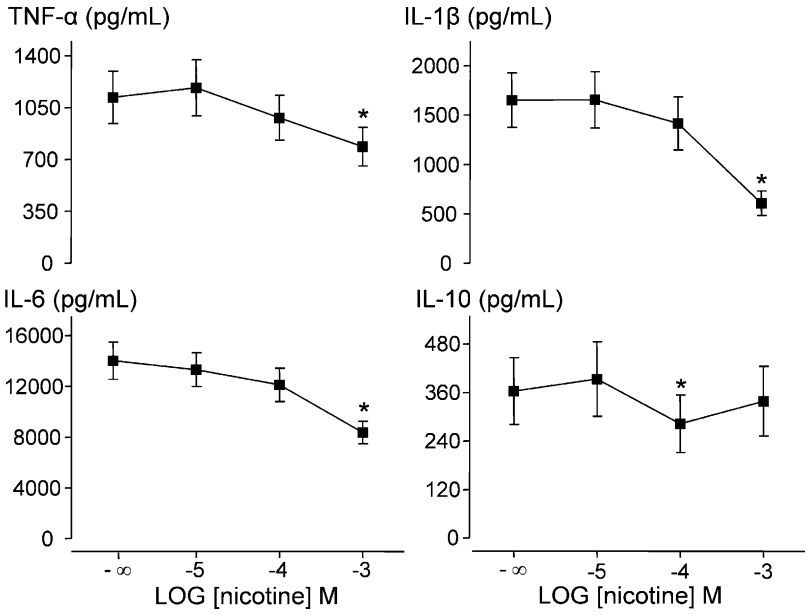

Fig. 1. Dose–response curves of the effect of nicotine on cytokine production in human PBMC stimulated with 1 ng/mL LPS for 22 h. Data are represented as mean ± SEM (eight different donors, two independent experiments). *$p < 0.05$ compared to the LPS response in the absence of nicotine (repeated measures ANOVA with Bonferroni post-hoc test).

in combination with nicotine or GTS-21 for 22 h. The classic nAChR agonist nicotine dose-dependently inhibited production of the pro-inflammatory cytokines TNF- α, IL-1β and IL-6 (Fig. 1) with a maximum inhibition of 30 ± 4%, 65 ± 2% and 36 ± 5%, respectively at the highest dose of nicotine used (1 mM). The anti-inflammatory cytokine IL-10 was relatively unaffected, with a small but significant inhibition at a submaximal concentration of nicotine (21 ± 3% at 100 μM). The selective α7nAChR agonist GTS-21 also dose-dependently inhibited TNF-α and IL-1β (Fig. 2), but was more potent and efficacious than nicotine with a maximum inhibition of 87 ± 2% (IC_{50}: 8.9 μM) and 89 ± 3% (IC_{50}: 17.9 μM) respectively at the highest dose of GTS-21 used (100 μM). Production of IL-6 was not affected by GTS-21. IL-10 was only inhibited by the highest dose of GTS-21 used (42 ± 20% at 100 μM) but not at 10 μM GTS-21, a dose which strongly inhibited release of TNF-α and IL-1β.

3.2. Inhibition of pro-inflammatory cytokine production by nicotine and GTS-21 is mediated by monocytes and does not involve cell death

To investigate which cell type is involved in the inhibition of pro-inflammatory cytokine production by nAChR agonists we performed stimulation experiments on isolated primary human monocytes using the same experimental conditions as in PBMCs. GTS-21 and nicotine both significantly inhibited TNF-α production to a similar extent as in PBMCs with GTS-21 showing a distinctively more pronounced effect than nicotine (84 ± 3% and 39 ± 8%, respectively, Fig. 3A). To exclude the possibility that loss of viability

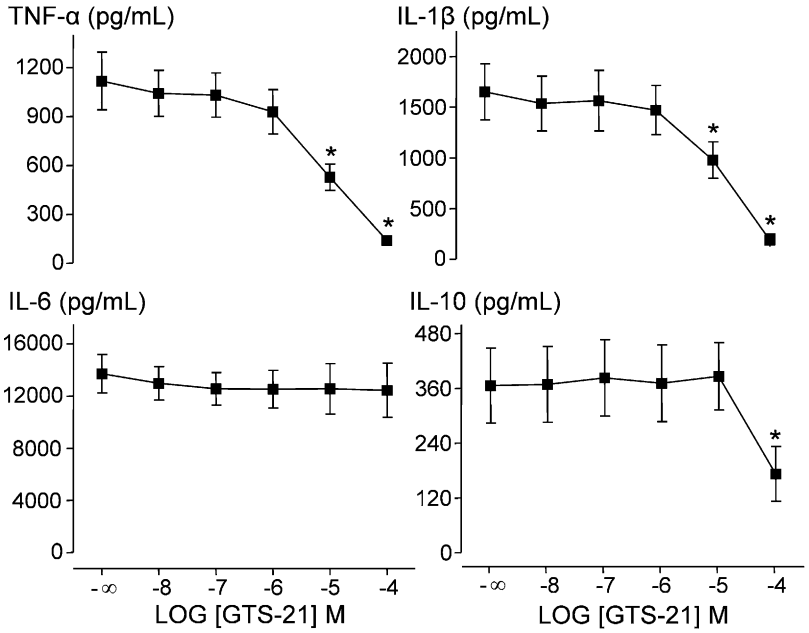

Fig. 2. Dose–response curves of the effect of GTS-21 on cytokine production in human PBMC stimulated with 1 ng/mL LPS for 22 h. Data are represented as mean ± SEM (eight different donors, two independent experiments). *$p < 0.05$ compared to the LPS response in the absence of GTS-21 (repeated measures ANOVA with Bonferroni post-hoc test).

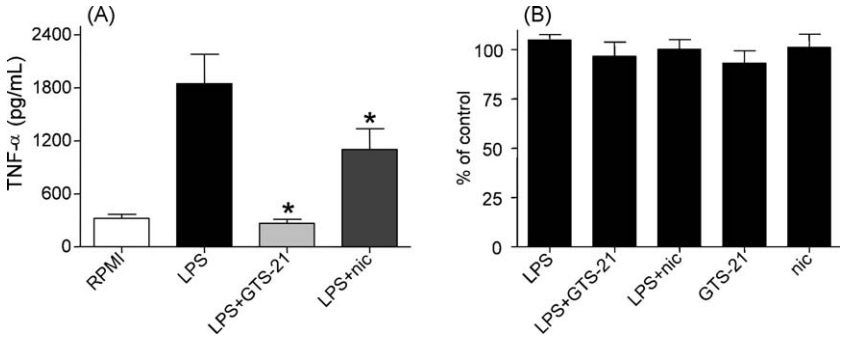

Fig. 3. (A) Effect of nicotine and GTS-21 on TNF-α production in isolated primary human monocytes stimulated with LPS (1 ng/mL) for 22 h. Both GTS-21 (100 μM) and nicotine (1 mM) attenuate TNF-α production in isolated monocytes stimulated with LPS. Data are represented as mean ± SEM (six different donors, two independent experiments). *$p < 0.05$ compared to the LPS alone (paired Student's *t*-test). (B) MTT viability assay on isolated primary human monocytes stimulated with LPS, nicotine and GTS-21. Percentage viability was calculated relative to viability in the control (RPMI) samples. Data are represented as mean ± SEM (six different donors, two independent experiments).

or cell death was responsible for the observed inhibition of cytokine production we performed MTT viability assays on the stimulated monocytes which revealed no loss of cell viability with any of the stimuli used (Fig. 3B). Moreover, lactate dehydogenase levels in supernatants of stimulated PBMCs were not elevated compared to control (RPMI) in any of the samples ($n = 4$; RPMI 75.3 ± 6.2 U/L, LPS 72.0 ± 5.8 U/L, GTS-21 100 μM 57.5 ± 4.1 U/L, nicotine 1 mM 53.5 ± 4.9 U/L, LPS + GTS-21 100 μM 76.5 ± 6.1 U/L, LPS + nicotine 1 mM 66.0 ± 3.4 U/L).

3.3. Inhibition of pro-inflammatory cytokine production by nicotine and GTS-21 is not restricted to a specific TLR stimulated

Pattern recognition of the diverse classes of microbial products causing infection involves various TLRs that modulate the subsequent immune response and cytokine profiles [24]. To explore whether the anti-inflammatory effects of nicotine and GTS-21 are dependent on a specific TLR stimulated we incubated human whole blood cultures with TLR2 (Pam3Cys, 1 μg/mL), TLR3 (PolyI:C, 50 μg/mL), TLR4 (LPS, 1 ng/mL) and TLR5 (flagellin, 1 μg/mL) agonists in combination with nicotine and GTS-21. Incubation with TLR2, TLR4 and TLR5 agonists resulted in production of pro- (TNF-α, IL-1β, IL-6, and IFN-γ) as well as anti-inflammatory (IL-10) cytokines (described in legend of Fig. 4). The TLR3 agonist PolyI:C only evoked a robust IL-6 release while TNF-α and IFN-γ were secreted in very small quantities in whole blood cultures of some but not all of the subjects (and therefore were excluded from inhibition/stimulation calculations). PolyI:C did not result in release of IL-1β and IL-10 in whole blood of any of the subjects.

Co-incubation with 100 μM of nicotine inhibited TLR-agonist induced pro-inflammatory cytokine release in a mild fashion (data not shown) but 1 mM of nicotine inhibited cytokine release up to 100%, independent on the TLR stimulated (Fig. 4, upper panel). The anti-inflammatory cytokine IL-10 was not significantly inhibited by either 100 μM or 1 mM of nicotine.

The selective α7nAChR agonist GTS-21 (100 μM) also significantly inhibited nearly all TLR-agonist induced pro-inflammatory cytokines (up to 95% inhibition, Fig. 4, lower panel). As with nicotine, GTS-21 did not attenuate the anti-inflammatory cytokine IL-10 release to a great extent, only TLR5-induced IL-10 production was significantly inhibited.

3.4. Nicotinic antagonists do not restore cytokine production

As the α7nAChR has been proposed as the receptor responsible for the anti-inflammatory effect of nicotinic agonists [6], we attempted to reverse the effects of nicotine and GTS-21 with nAChR antagonists. However, co-incubation with both α7nAChR-specific antagonists α-bungarotoxin (10 nM–1 μM) and methyllycaconitine (10 μM) as well as non-specific nAChR antagonists mecamylamine (10 nM–100 μM) and D-tubocurarine (10–100 μM) could not reverse the anti-inflammatory effects of any of the concentrations of nicotine and GTS-21. Inhibition of the GTS-21 (100 μM) induced attenuation of the cytokine response by the highest concentration of the nAChR blockers was: α-bungarotoxin 1.9 ± 1.3% ($n = 6$), methyllycaconitine −2.9 ± 1.0% ($n = 4$), mecamylamine −1.8 ± 3.2% ($n = 6$), D-tubocurarine 2.8 ± 2.8% ($n = 4$). % Inhibition of the nicotine (1 mM) induced cytokine attenuation by the highest concentration of the various nAChR blockers was: α-bungarotoxin 5.7 ± 3.4% ($n = 6$), methyllycaconitine 0.6 ± 6.9% ($n = 4$), mecamylamine 2.3 ± 4.1% ($n = 6$), D-tubocurarine −10.3 ± 8.1% ($n = 4$). Co-incubation of other concentrations of GTS-21/nicotine with the various concentrations of the nAChR blockers mentioned above was also ineffective in blocking anti-inflammatory effects (data not shown).

3.5. GTS-21 downregulates cell surface receptors on monocytes

To obtain more insight into the mechanisms by which nicotine and GTS-21 exhibit their anti-inflammatory effects, we stimulated

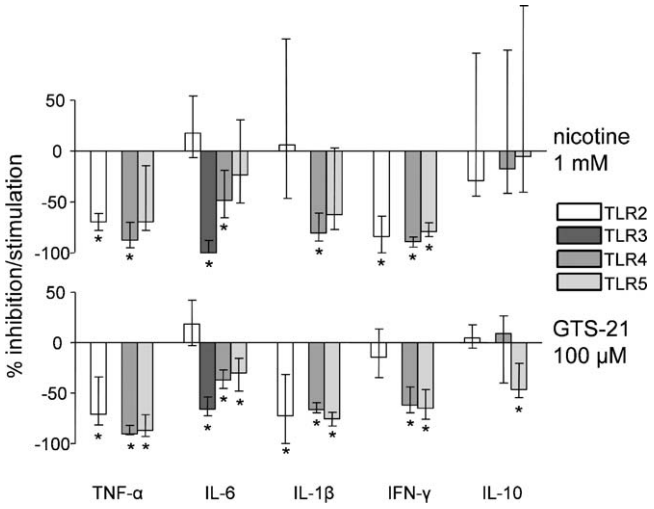

Fig. 4. Percentage inhibition/stimulation of TLR-agonist induced cytokine production in human 1:5 diluted whole blood by 1 mM nicotine and 100 μM GTS-21 (24-h stimulation). TLR agonists used were TLR1 + 2: Pam3Cys (1 μg/mL); TLR3: PolyI:C (50 μg/ml); TLR4: LPS (1 ng/mL); TLR5: flagellin (1 μg/mL). Data are represented as median and interquartile range (six different donors). *$p < 0.05$, Wilcoxon Matched Pairs test. Concentrations of cytokines in absence of nicotine or GTS-21 after stimulation with TLR2, TLR3, TLR4 and TLR5 agonists in pg/mL (interquartile range) were: TNF-α: 154 (41–592), 6 (2–15), 3150 (2020–3927), 2054 (692–4309). IL-6: 1374 (482–4406), 89 (61–134), 6979 (4678–9309), 8341 (4864–11842). IL-1β: 19 (3–80), 0 (0–2), 1637 (922–1963), 1502 (740–2126). IFN-γ 106 (33–349), 9 (0–27), 734 (521–954), 802 (593–1072). IL-10: 43 (6–120), 0 (0-0), 76 (56–116), 221 (99–447).

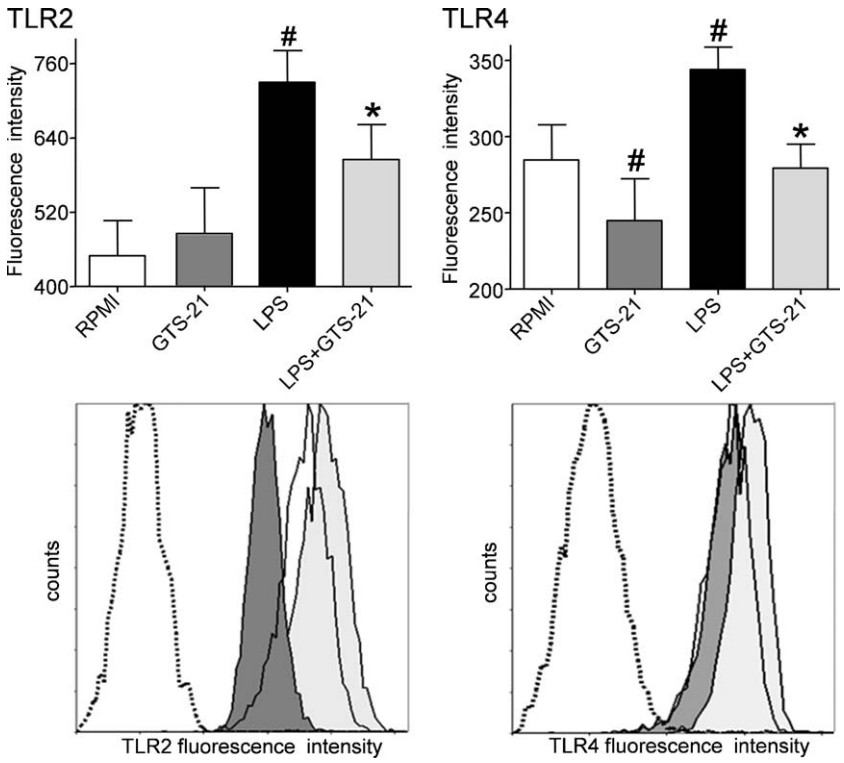

Fig. 5. Flow cytometric analysis of the effects of LPS (1 ng/mL) and GTS-21 (100 μM) on cell-surface expression of TLR2 and TLR4 on primary human monocytes incubated for 22 h. In the lower panels overlays of histograms depicting TLR2 and TLR4 expression on incubated monocytes of a typical subject are displayed. The dashed line represent isotype control, dark grey represents RPMI, light grey represents LPS and white represents LPS + GTS-21. Data are represented as mean ± SEM (eight different donors, two independent experiments). $^{\#}p < 0.05$ compared to RPMI, $^{*}p < 0.05$ compared to LPS (paired Student's *t*-test).

human PBMCs with LPS in combination with nicotine and GTS-21 and determined cell surface expression of TLR2, TLR4, α7nAChR and CD14 on the monocyte fraction by flow cytometry. We chose the 22-h incubation based on similar experiments where LPS induced significant upregulation of TLR2 and TLR4 after 24 h of incubation [25]. As depicted in Fig. 5, LPS incubation upregulated monocyte cell-surface expression of TLR2 and TLR4 compared to control (RPMI). CD14 expression was also upregulated by LPS (2990 ± 237.8 vs. 2173 ± 227.3, $p < 0.05$). GTS-21 inhibited LPS-induced upregulation of TLR2 and CD14 (2467 ± 218.9, $p < 0.05$ compared to LPS) and abolished LPS-induced TLR4 upregulation. Moreover, in the absence of LPS, GTS-21 lowered TLR4 expression below control levels. LPS had no effect on α7nAChR expression, while GTS-21 in the absence of LPS inhibited α7nAChR expression below control level (387 ± 50.6 vs. 713.3 ± 116.3, $p < 0.05$). Nicotine had no effects on expression of any of the measured receptors (data not shown).

3.6. GTS-21, but not nicotine, regulates cytokine production at the transcriptional level

To investigate whether the anti-inflammatory effects of GTS-21 and nicotine are transcriptionally regulated we assessed mRNA levels of pro- and anti-inflammatory cytokines and receptors using quantitative PCR on isolated monocytes stimulated for 4 h. As expected, incubation with LPS significantly upregulated TNF-α, IL-1β, IL-6 and IL-10 mRNA expression (respectively 5.4-, 14.7-, 1715- and 9.7-fold compared to RPMI, Fig. 6). GTS-21 significantly attenuated LPS-induced upregulation of TNF-α and IL-1β (−2.1- and 4.1-fold compared to RPMI, respectively). Moreover, GTS-21 in the absence of LPS significantly decreased TNF-α mRNA levels (−9.6-fold compared to RPMI) while there was a trend towards IL-1β downregulation (−11.9-fold compared to RPMI). There was also a trend towards inhibition of LPS-induced upregulation of IL-6 and IL-10 expression (637.8- and 5-fold compared to RPMI, respectively) by GTS-21 as well as downregulation of IL-10 in the absence of LPS (−4.5 compared to RPMI). TLR2 and TLR4 mRNA expression was not altered by LPS. GTS-21 significantly downregulated TLR2 in the absence of LPS (−3.2-fold compared to RPMI) but had no effect on TLR4 mRNA levels. LPS significantly downregulated CD14 (data not shown, −5.6-fold compared to RPMI), this was inhibited by GTS-21 (−3.5-fold compared to RPMI). Nicotine did not alter expression of any of the cytokine genes assessed (data not shown). α7nAChR gene expression was not altered by any stimulus (data not shown).

3.7. The anti-inflammatory effect of GTS-21 and nicotine are dependent on JAK2 phosphorylation

To evaluate the involvement of the JAK-STAT pathway in the anti-inflammatory effects of nicotine and GTS-21 we incubated PBMCs with LPS, nicotine and GTS-21 in combination with AG490, a selective inhibitor of JAK2 phosphorylation [26,16,17]. 100 μM AG490 inhibited the anti-inflammatory effect of 100 μM GTS-21 while completely restoring the attenuated TNF-α production by 10 μM GTS-21 and 1 mM nicotine (Fig. 7). AG490 alone or vehicle (1% etOH) had no significant effects on LPS-induced TNF-α release (LPS + AG490 2719 ± 379 pg/mL; LPS + 1% etOH 1666 ± 333 pg/mL).

3.8. GTS-21 effects are likely mediated by JAK2/STAT3 signaling

To further explore the role of the JAK-STAT signaling pathway in the anti-inflammatory effects of GTS-21 we used RT^2 profiler qPCR arrays which contain a panel of 84 genes related to JAK-STAT-mediated signaling on isolated monocytes. Genes that were significantly and more than twofold up- or downregulated by 4-h incubations with GTS-21, LPS and LPS + GTS-21 are shown in Table 1. GTS-21 clearly inhibited LPS-induced expression of IFN-γ

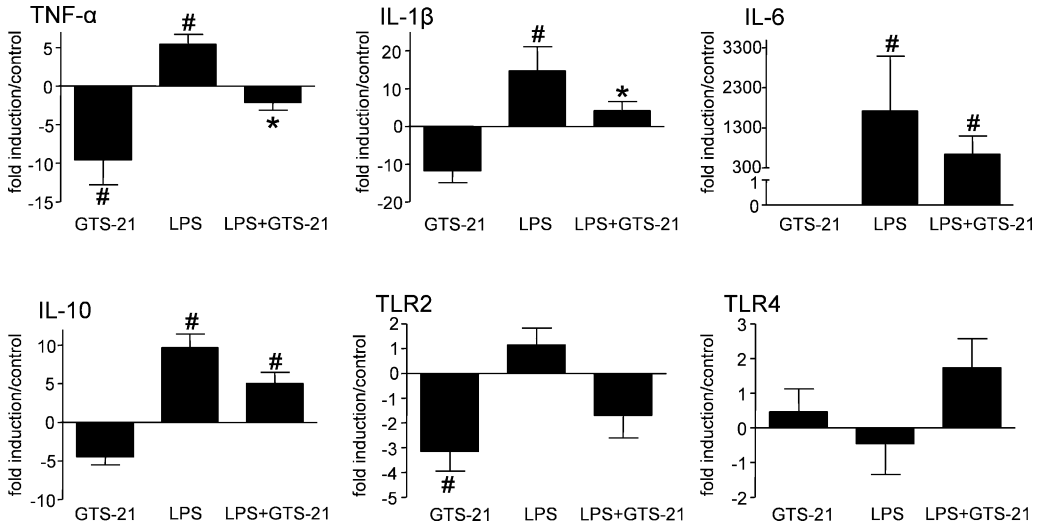

Fig. 6. mRNA expression of TNF-α, IL-1β, IL-6, IL-10, TLR2 and TLR4 in isolated primary human monocytes stimulated with LPS (1 ng/mL) and GTS-21 (100 μM) for 4 h. Data are represented as mean ± SEM fold expression of the gene of interest relative to control (expression in monocytes incubated with medium alone). Data from five different donors, three independent experiments are shown. $^{\#}p < 0.05$ compared to medium, $^{*}p < 0.05$ compared to LPS (paired Student's *t*-test).

and IFN-γ-inducible genes such as CXCL9, GBP1 and OAS1 [27–29]. In concordance with the implicated pivotal role for the JAK2-STAT3 pathway in α7nAChR signaling, GTS-21 upregulated STAT3 expression in the presence of LPS. Furthermore, LPS-induced downregulation of F2 (thrombin), an activator of JAK2/STAT3 pathway and SH2B1, a JAK2 activator, were abolished by GTS-21 whereas GTS-21 upregulated OSM which is involved in STAT3 phosphorylation and PRLR, a receptor which upon ligand binding activates JAK2 [30–33]. In the presence of LPS, GTS-21 induced upregulation of PTPN1, a protein tyrosine phosphatase which dephosphorylates JAK2, which may represent a negative feedback mechanism preventing excessive JAK2 activation [34]. STAT5A was upregulated by GTS-21 in the presence of LPS while LPS-induced downregulation of STAT5B was blocked by GTS-21 indicating activation of the JAK2-STAT5 pathway. The transcription of NFκB and SOCS3 was upregulated by LPS but not affected by co-incubation with GTS-21.

4. Discussion

The cholinergic anti-inflammatory pathway may represent new treatment options for inflammatory conditions such as sepsis, acute lung injury and autoimmune diseases. Vagus nerve stimulation in humans is a very invasive procedure and is not feasible in acute situations. Therefore, pharmacological stimulation of the cholinergic anti-inflammatory pathway via the α7nAChR is a more practical approach. The non-specific nAChR agonist nicotine has little therapeutic potential because of its toxicity and other unwanted side effects. We are the first to show that GTS-21, a compound acting specifically at the α7nAChR, is a strong inhibitor of pro-inflammatory cytokine release in primary human leukocytes while leaving anti-inflammatory cytokine production relatively unaffected. Nicotine has similar effects, but much less pronounced. Therefore, GTS-21 causes a profound

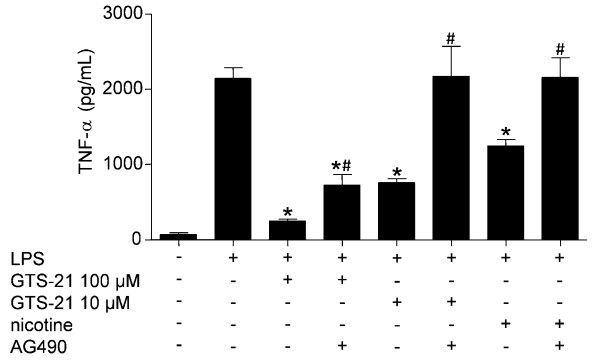

Fig. 7. Effect of the JAK2 inhibitor AG490 (100 μM) on TNF-α production in human PBMCs stimulated with LPS (1 ng/mL), GTS-21 (10 and 100 μM) and nicotine (1 mM) for 22 h. Data are represented as mean ± SEM (5–7 different donors, two independent experiments). $^{*}p < 0.05$ compared to LPS, $^{\#}p < 0.05$ compared to TNF-α response with the same (concentration of) nicotinic agonist in the absence of AG490 (paired Student's *t*-test).

Table 1
Up/down regulation of JAK-STAT related genes compared to RPMI.

GTS-21		LPS		LPS+GTS-21	
SOCS2	8,30	IL2RA	176,11	IL2RA	376,84
INSR	3,11	SOCS1	48,56	STAT4	34,75
EPOR	2,55	ISG15	35,88	SOCS1	24,44
OSM	2,36	CXCL9	24,82	ISG15	16,12
PRLR	2,28	GBP1	18,96	SOCS3	12,92
SMAD1	2,24	SOCS2	18,69	GBP1	9,01
IRF9	−2,32	SOCS3	17,31	NFKB1	3,22
IRF1	−2,50	STAT4	17,29	STAT5A	3,03
GBP1	−2,68	IL20	8,52	SMAD1	2,75
A2M	−2,90	IFNG	6,53	STAT2	2,58
ISG15	−2,97	OAS1	5,34	STAT1	2,45
GATA3	−3,30	STAT1	4,70	IL4R	2,30
CCND1	−3,38	NFKB1	3,09	PTPN1	2,11
IFNAR1	−4,05	STAT2	3,04	STAT3	2,09
OAS1	−4,99	NR3C1	2,35	FCGR1A	−2,25
MYC	−10,96	CDKN1A	2,29	SMAD2	−2,26
		IL6ST	2,14	TYK2	−2,33
		TYK2	−2,38	USF1	−2,66
		USF1	−2,38	IFNGR1	−3,08
		STAT5B	−2,45	EGFR	−3.67
		SMAD3	−2,52	SMAD3	−3,92
		IFNGR1	−2,94	HMGA1	−4,39
		SH2B1	−2,95	FCER1A	−4,75
		SOCS5	−2,97	NOS2A	−4,92
		NOS2A	−4,15	MYC	−5,43
		F2	−5,22	SIT1	−7,36
		FCER1A	−5,43	A2M	−8,83
		EPOR	−5,48	CSF1R	−13,08
		CSF1R	−8,46		
		INSR	−9,77		
		SMAD5	−30,56		

Only genes which are significantly and more than 2-fold up/downregulated are shown.

shift in the pro-/anti-inflammatory balance towards an anti-inflammatory phenotype. While earlier reports state that the anti-inflammatory effect of α7nAChR stimulation relies on a post-transcriptional mechanism [4,16] we present evidence that GTS-21 attenuates the inflammatory response at the transcriptional level.

In our study, GTS-21 and nicotine exerted their anti-inflammatory effects equally on PBMCs and isolated monocytes suggesting that these effects are mainly monocyte-mediated. However, because the monocyte isolation method we employed does not yield completely pure monocytes, effects of the nicotinic agonists on lymphocytes or other cell types present in PBMCs cannot be ruled out. In this respect it is of note that in whole blood experiments the T-cell/NK-cell cytokine IFN-γ was significantly inhibited by both nicotinic agonists. Non-activated lymphocytes generally are not noted for TLR-responses but NK cells have been reported to produce IFN-γ after TLR-stimulation [35]. Whether the observed inhibition of IFN-γ production represents a direct inhibitory effect on NK-cells or an indirect effect via attenuation of cytokines that stimulate TLR-induced IFN-γ production by these cells such as IL-12 and TNF-α, has to be determined. Nonetheless, while we have focused on the effects of GTS-21 and nicotine on innate immune responses, an effect on the adaptive immune system by these compounds is anticipated because of alterations in co-stimulatory factors, cell interactions and interplay between cytokines. Moreover, since primary human lymphocytes express the α7nAChR, a direct effect of α7nAChR agonists on these cells and the adaptive immune response should not be neglected [36,37]. The lack of an inhibitory effect on IL-6 release by GTS-21 is in line with a study in mice which demonstrates a rather selective effect of GTS-21 on TNF-α release [8,37]. There was a trend towards lower IL-6 mRNA levels in GTS-21-treated monocytes but it did not reach statistical significance.

We further demonstrate that the anti-inflammatory effects of both nicotine and GTS-21 are not specific for the TLR stimulated using a whole blood stimulation assay. This is important because pattern recognition of the diverse classes of microbial products involves various TLRs that modulate the subsequent immune response [24,38]. As a consequence, cytokine release profiles evoked by Gram-positive bacteria, Gram-negative bacteria, viruses and various endogenous agonists differ. Previous work in our group has shown that the effect of immunomodulating compounds can differ depending on the TLR stimulated [39]. The data in this paper illustrate that both nicotinic compounds exhibit a generalized anti-inflammatory effect not dependent on the inflammatory stimulus and therefore not confined to a specific sort of infection or endogenous stimulus. Additionally, we affirm the anti-inflammatory potential of GTS-21 and nicotine in whole blood containing all cell types and humoral factors present, which is more reflective of in vivo conditions than an isolated cell model.

While the α7nAChR has been identified as the pivotal receptor in the cholinergic anti-inflammatory pathway [6], we could not reverse the actions of nicotine or GTS-21 with antagonists of this receptor (α-bungarotoxin and MLA) or with non-specific nAChR antagonists (mecamylamine and D-tubocurarine) in human immune cells. In accordance, for nicotine, blockade of the anti-inflammatory effect by α-bungarotoxin has been described by some [16,40] but not all [41]. To date, no attempts have been made to reverse the anti-inflammatory effect of GTS-21 by nAChR antagonists. There are a number of possible reasons to explain the failure to block GTS-21 and nicotine effects by α7 antagonists. First of all the possibility remains that some of the nicotine or GTS-21 effects in human leukocytes are mediated by a non-α7nAChR-related mechanism which remains to be elucidated. Another explanation might rely on differences in the α7nAChR between cell types. In excitable neuronal cells, α7nAChRs are ligand-gated ion channels composed of 5 α7 subunits which upon activation cause depolarization of the cell membrane and influx of Ca^{2+} via voltage-operated Ca^{2+} channels. However, leukocytes do not possess these Ca^{2+} channels and it was demonstrated that in PBMCs α7nAChRs stimulation by nicotine or acetylcholine does not result in detectable membrane currents while it does in neuronal cells [42]. Others have demonstrated that, despite the fact that T-cells express an essentially identical transcript for the α7nAChR subunit as neuronal cells, they do not form functional ligand-gated ion channels [37]. Furthermore, it was shown that leukocytes do not express the "normal" α7 subunit, but an α7 duplicate nicotinic acetylcholine receptor-related protein (dupα7) which lacks the α-bungarotoxin binding site and has most likely different pharmacological properties [42]. This is supported by the aforementioned study in T-cells, where the α7nAChR specific antagonist MLA and α-bungarotoxin did not inhibit nicotine-induced effects [37]. Different pharmacological properties may also explain why the partial α7nAChR agonist GTS-21 has a much more potent effect on cytokine release in primary human leukocytes than the full agonist nicotine, while in classic α7nAChRs, the opposite is true [15]. Interestingly, bone marrow and brain cells were positive for both the normal α7 and the dupα7 subunit [42]. This indicates that the presence of the normal α7nAChR or the dupα7 might vary between different cell types, which could explicate why nicotinic blockers antagonize α7nAChR in some cell types but not in others.

LPS-induced increases in monocyte cell-surface expression of TLR2 and TLR4 have been described previously [25]. We confirm these findings and demonstrate that LPS also increases cell surface expression of CD14 which is essential in LPS-induced cytokine production [43]. GTS-21, but not nicotine, inhibits TLR and CD14 upregulation and this could play a role in the diminished pro-inflammatory cytokine production in GTS-21 treated leukocytes since modulation of cell-surface expression of TLR4 has been linked to the degree of cytokine production [44]. The observed LPS-induced upregulation of TLRs could also be mediated by cytokine production, so-called "priming", which would explain why the non-TLR2 agonist LPS increases TLR2 expression [45]. In this respect inhibition of cell-surface expression of the TLRs by GTS-21 might be a result of inhibition of cytokines rather than a cause. mRNA levels of TLR4 and TLR2 were not affected by LPS and only TLR2 mRNA expression was modestly inhibited by GTS-21. The discrepancies between TLR protein and mRNA expression might result from the different incubation period used in the protein expression experiments compared to the mRNA experiments (22 h vs. 4 h) but can also indicate that the modulation of TLR cell-surface expression relies mainly on post-transcriptional mechanisms. An earlier report demonstrates that nicotine downregulates cell-surface expression of TLR4 and CD14 on human monocytes [46]. This discrepancy with our study might be explained by the modest anti-inflammatory effect of nicotine in our experiments.

De Jonge et al. have demonstrated that the anti-inflammatory effect of nicotine in murine macrophages acts through the recruitment of JAK2 to the α7nAChR and subsequent phosphorylation of JAK2, thereby initiating the anti-inflammatory STAT3 cascade [16]. We show that in primary human leukocytes both GTS-21 and nicotine effects could be inhibited by AG490, a JAK2 phosphorylation inhibitor, confirming a pivotal role for JAK2 activation. We further explored activation of the JAK-STAT pathway using quantitative PCR arrays. Although these arrays do not provide data on phosphorylation of JAK-STAT family members, it evaluates expression of known activators/inhibitors of this signaling cascade at the transcriptional level. Our results indicate that GTS-21 strongly downregulates IFN-γ-inducible genes related to STAT1 activation in monocytes [47]. Therefore, as stated before, GTS-21 may have a pronounced inhibitory effect on the adaptive immune response which warrants future research. JAK2/STAT3 signaling appeared to be activated by GTS-21 reflected

by increased expression of STAT3, receptors activating JAK2 (PRLR) and activators of STAT3 (OSM). Furthermore, GTS-21 blocked LPS-induced downregulation of JAK2/STAT3 activators (F2, SH2B1). The upregulation of STAT5A and the blockade of LPS-induced downregulation of STAT5B by GTS-21 further indicate JAK2 activity as STAT5 is also activated by JAK2 in monocytes [48].

There is much debate regarding the pro- and anti-inflammatory roles of STAT3 which appear to be highly cell- and stimulation-specific. A number of studies implicate pro-inflammatory actions of STAT3, such as a recent paper describing the critical role of STAT3 activation in IL-1β and IL-6 production in a RAW 264.7 macrophage cell line [49]. However, there is also a large body of evidence associating STAT3 activity with anti-inflammatory effects. For instance, STAT3 is essential for responsiveness to IL-10 which is known to deactivate macrophages [50,51] and STAT3 deficient macrophages are constitutively activated and secrete large amounts of pro-inflammatory mediators [51]. Blocking STAT3 in tumor cells increases expression of pro-inflammatory cytokines and mice lacking STAT3 in macrophages and neutrophils are highly susceptible to endotoxic shock [51,52]. Moreover, mice bearing a STAT3 deletion in bone marrow cells display higher levels of circulating TNF-α and IFN-γ a compared to control mice in the absence of an inflammatory process [53]. The mechanism by which GTS-21 (and probably nicotine) inhibits cytokine release may rely on enhanced IL-10 signaling or actions mimicking IL-10 signaling resulting in STAT3 activation. Because IL-10 down-regulates its own production, excessive IL-10 signaling could account for the inhibited production of IL-10 observed with the highest dose of GTS-21 used (100 μM) [50].

Similar to what has been reported for nicotine [16], SOCS3 appears not to play a role in the anti-inflammatory effects of GTS-21 while the LPS-induced upregulation of SOCS3 was not further enhanced by GTS-21. Our quantitative PCR experiments illustrate that GTS-21 inhibits LPS-induced upregulation of pro-inflammatory cytokines at the transcriptional level. While others argue that the anti-inflammatory effects of α7nAChR stimulation predominantly rely on post-transcriptional modulation [4,16], our data are in accordance with IL-10 (-like) signaling which inhibits TNF-α production at both the transcriptional and the translational level [54]. Moreover, GTS-21 and nicotine both have been reported to inhibit LPS-induced NFκB activation which implies transcriptional modulation [7,20,21]. While our JAK-STAT array results demonstrate that the transcription of NFκB is not upregulated by GTS-21 compared to LPS, this does not exclude the possibility that GTS-21 modulates NFκB activity/nuclear translocation as this is not dependent on transcription or translation [55]. Nicotine did not affect mRNA levels of any of the genes assessed. Whether this is due to the relatively low anti-inflammatory potential nicotine displayed in our experiments or to other factors remains to be elucidated.

In conclusion, GTS-21 represents novel opportunities for human research into the cholinergic anti-inflammatory pathway and possibly for future therapeutic applications. It is more effective than nicotine in modulating the immune response in human leukocytes and its suitability for human use makes it a candidate for human *in vivo* trials to further explore the cholinergic anti-inflammatory pathway.

Acknowledgements

The authors would kindly like to thank Prof. Dr. Roger L. Papke of the University of Florida and Comentis Inc. for supplying us with GTS-21 and Trees Jansen for help with the Luminex cytokine measurements. M. Kox and J.C. Pompe are recipients of a grant of the Radboud University Nijmegen Medical Centre Trauma Section/Intensive Care Medicine.

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