



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

Matthijs Kox, Jeroen F. van Velzen, Jan C. Pompe, Cornelia W. Hoedemaekers, Johannes G. van Der Hoeven, Peter Pickkers

► To cite this version:

Matthijs Kox, Jeroen F. van Velzen, Jan C. Pompe, Cornelia W. Hoedemaekers, Johannes G. van Der Hoeven, et al.. GTS-21 inhibits pro-inflammatory cytokine release independent of the Toll-like receptor stimulated via a transcriptional mechanism involving JAK2 activation. *Biochemical Pharmacology*, 2009, 78 (7), pp.863. 10.1016/j.bcp.2009.06.096 . hal-00509509

HAL Id: hal-00509509

<https://hal.science/hal-00509509>

Submitted on 13 Aug 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

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

Authors: Matthijs Kox, Jeroen F. van Velzen, Jan C. Pompe, Cornelia W. Hoedemaekers, Johannes G. van der Hoeven, Peter Pickkers



PII: S0006-2952(09)00567-X
DOI: doi:10.1016/j.bcp.2009.06.096
Reference: BCP 10244

To appear in: *BCP*

Received date: 22-4-2009
Revised date: 12-6-2009
Accepted date: 23-6-2009

Please cite this article as: Kox M, van Velzen JF, Pompe JC, Hoedemaekers CW, van der Hoeven JG, Pickkers P, GTS-21 inhibits pro-inflammatory cytokine release independent of the Toll-like receptor stimulated via a transcriptional mechanism involving JAK2 activation, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2009.06.096

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Matthijs Kox^a, Jeroen F. van Velzen^b, Jan C. Pompe^a, Cornelia W. Hoedemaekers^a,
Johannes G. van der Hoeven^a, Peter Pickkers^a

^aDepartment of Intensive Care Medicine, Radboud University Nijmegen Medical Centre,
Nijmegen, The Netherlands

^bDepartment of Hematology, Radboud University Nijmegen Medical Centre, Nijmegen,
The Netherlands

Corresponding author: P. Pickkers, MD, PhD

Department of Intensive Care Medicine, internal mail 632

Radboud University Nijmegen Medical Centre

PO box 9101, 6500 HB, Nijmegen, The Netherlands

Tel.: +31-24-3617273, Fax: +31-24-3541612

E-Mail: p.pickkers@ic.umcn.nl

Abstract

The vagus nerve can limit inflammation via the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR). Selective pharmacological stimulation of the $\alpha 7$ nAChR may have therapeutic potential for the treatment of inflammatory conditions. We determined the anti-inflammatory potential of GTS-21, an $\alpha 7$ nAChR-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 $\alpha 7$ nAChR 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 $\alpha 7$ nAChR in human leukocytes compared to other cell types. GTS-21
may be promising from a therapeutic perspective because of its suitability for human use.

Key words: inflammation, cholinergic anti-inflammatory pathway, nicotine, monocytes,
peripheral blood mononuclear cells, $\alpha 7$ nicotinic acetylcholine receptor

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 [1,2,3], 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 $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR), 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 [7,8,9]. To date, one of the most effective $\alpha 7$ -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

1
2
3 have been reported at $\alpha 7$ nAChR receptor activation level in electrophysiological studies
4
5 using heterologous expression in *Xenopus laevis* oocytes [14,15], the immunomodulating
6
7 effects of selective and non-selective agonists of the $\alpha 7$ nAChR have not been compared.
8
9 Furthermore, in vitro studies investigating the cholinergic anti-inflammatory pathway
10
11 almost exclusively used the principal Toll-like receptor 4 (TLR4) agonist LPS as a trigger
12
13 for inflammation. It is unknown whether the cholinergic anti-inflammatory pathway is
14
15 restricted to certain TLRs stimulated or is a general mechanism not constrained to a
16
17 specific stimulus.
18
19

20
21 Finally, the mechanism by which $\alpha 7$ nAChR stimulation attenuates pro-inflammatory
22
23 cytokine production has not been fully elucidated and sparsely studied in human cells. A
24
25 role for the JAK2-STAT3 pathway [16,17,18,19] as well as suppression of NF κ B
26
27 transcriptional activity [20,21] is implied. However, the cholinergic anti-inflammatory
28
29 pathway is believed to be regulated at a post-transcriptional level [4,16]. Mechanistic
30
31 studies regarding the anti-inflammatory effect of GTS-21 are limited to one study
32
33 reporting decreased NF κ B activity in a murine cell line [7].
34
35

36
37 In this study, we investigated for the first time the anti-inflammatory potential of GTS-21
38
39 on primary human leukocytes and compared it with nicotine. Furthermore, we
40
41 investigated whether the effects of both nicotinic agonists were restricted to specific
42
43 TLRs stimulated and whether they affected cell-surface expression of receptors involved
44
45 in the innate immune response. Finally, we studied whether the anti-inflammatory effects
46
47 of GTS-21 and nicotine are regulated at the transcriptional level and determined the
48
49 involvement of the JAK-STAT signal transduction pathway.
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 hours 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 μ L) were seeded in duplicate in 96-well flat bottom plates (Greiner, Alphen a/d Rijn, The Netherlands) and stimulated for 22 hours (37 °C, 95% O₂, 5% CO₂) with various compounds. Incubation with RPMI alone served as a negative control. Nicotine or GTS-21 were added 30 minutes before TLR agonists. Nicotinic antagonists or the JAK2 inhibitor AG-490 were added 30 minutes 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 μ L) in duplicate were stimulated in the presence of 10% autologous serum for 22 hours 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 hours as described above. After stimulation, whole blood cultures were centrifuged (14000 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-1 β , IL-6 and IL-10 were measured by commercial ELISA kits (IL-1 β : 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 hours). After stimulation, plates were centrifuged (1700 rpm, 8 min), 100 μ L of supernatant was stored at -80 °C

for cytokine analysis and 10 μ L of MTT stock solution (5 mg/mL) was added to the wells. Monocytes were incubated at 37 °C for an additional 4 hours 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 hours as described in section 2.2.

2.5 Flow cytometry

Flow cytometry was performed on PBMCs stimulated as described in section 2.2 (22 hours). 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 μ L 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 α 7nAChR on monocytes was determined with a primary antibody against α 7nAChR (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 $\alpha 7$ nAChR 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 hours, 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-1 β	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'
$\alpha 7$ nAChR	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 RT² 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 β 2 microglobulin (B2M) present on the PCR array were used for normalization.

2.7 Calculations

Data are expressed as mean \pm SEM except for whole blood stimulation data which is expressed as median \pm 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 in response to a TLR-agonist in the absence of a nicotinic agonist that was lower than 4 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 $-(1/\text{fold induction})$.

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 in combination with nicotine or GTS-21 for 22 hours. The classic nAChR agonist nicotine dose-dependently inhibited production of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 (figure 1) with a maximum inhibition of 30 \pm 4%, 65 \pm 2% and 36 \pm 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 \pm 3% at 100 μ M). The selective α 7nAChR agonist GTS-21 also dose-dependently inhibited TNF- α and IL-1 β (figure 2), but was more potent and efficacious than nicotine with a maximum inhibition of 87 \pm 2% (IC₅₀: 8.9 μ M) and 89 \pm 3% (IC₅₀: 17.9 μ M) respectively at the highest dose of GTS-21 used (100 μ M). Production of IL-6 was not affected by GTS-21. IL-10 was only inhibited by the highest dose of GTS-21 used (42 \pm 20% at 100 μ M) but not at 10 μ M GTS-21, a dose which strongly inhibited release of TNF- α and IL-1 β .

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 \pm 3% and 39 \pm 8% respectively, figure 3A). To exclude the possibility that loss of viability 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 (figure 3B). Moreover, lactate dehydrogenase levels in supernatants of stimulated PBMCs were not elevated compared to control (RPMI) in any of the samples (n=4; RPMI 75.3 \pm 6.2 U/L, LPS 72.0 \pm 5.8 U/L, GTS-21 100 μ M 57.5 \pm 4.1 U/L, nicotine 1 mM 53.5 \pm 4.9 U/L, LPS+GTS-21 100 μ M 76.5 \pm 6.1 U/L, LPS+nicotine 1 mM 66.0 \pm 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 μ g/mL), TLR3 (PolyI:C, 50 μ g/mL), TLR4 (LPS, 1 ng/mL) and TLR5 (flagellin, 1 μ g/mL) agonists in combination with nicotine and GTS-21. Incubation with TLR2, TLR4 and TLR5 agonists resulted in production of pro- (TNF- α , IL-1 β , IL-6, and IFN- γ) as well as anti-inflammatory (IL-10) cytokines (data not shown). 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-1 β and IL-10 in whole blood of any of the subjects.

Co-incubation with 100 μ M 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 (figure 4, upper panel). The anti-inflammatory cytokine IL-10 was not significantly inhibited by either 100 μ M or 1 mM of nicotine.

The selective α 7nAChR agonist GTS-21 (100 μ M) also significantly inhibited nearly all TLR-agonist induced pro-inflammatory cytokines (up to 95% inhibition, figure 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 α 7nAChR 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 α 7nAChR-specific antagonists α -bungarotoxin (10 nM – 1 μ M) and methyllycaconitine (10 μ M) as well as non-specific nAChR antagonists mecamylamine (10 nM-100 μ M) and d-tubocurarine (10-100 μ M) could not reverse the anti-inflammatory effects of any of the concentrations

of nicotine & GTS-21. Inhibition of the GTS-21 (100 μ M) induced attenuation of the cytokine response by the highest concentration of the nAChR blockers was: α -bungarotoxin $1.9 \pm 1.3\%$ (n=6), methyllycaconitine $-2.9 \pm 1.0\%$ (n=4), mecamylamine $-1.8 \pm 3.2\%$ (n=6), d-tubocurarine $2.8 \pm 2.8\%$ (n=4). % inhibition of the nicotine (1 mM) induced cytokine attenuation by the highest concentration of the various nAChR blockers was: α -bungarotoxin $5.7 \pm 3.4\%$ (n=6), methyllycaconitine $0.6 \pm 6.9\%$ (n=4), mecamylamine $2.3 \pm 4.1\%$ (n=6), d-tubocurarine $-10.3 \pm 8.1\%$ (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 human PBMCs with LPS in combination with nicotine and GTS-21 and determined cell surface expression of TLR2, TLR4, α 7nAChR and CD14 on the monocyte fraction by flow cytometry. We chose the 22-hour incubation based on similar experiments where LPS induced significant upregulation of TLR2 and TLR4 after 24 hours of incubation [25]. As depicted in figure 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 $\alpha 7$ nAChR expression, while GTS-21 in the absence of LPS inhibited $\alpha 7$ nAChR 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 hours. As expected, incubation with LPS significantly upregulated TNF- α , IL-1 β , IL-6 and IL-10 mRNA expression (respectively 5.4, 14.7, 1715 and 9.7-fold compared to RPMI, figure 6). GTS-21 significantly attenuated LPS-induced upregulation of TNF- α and IL-1 β (-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-1 β 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). $\alpha 7$ nAChR 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 μ M AG490 inhibited the anti-inflammatory effect of 100 μ M GTS-21 while completely restoring the attenuated TNF- α production by 10 μ M GTS-21 and 1 mM nicotine (figure 7). AG490 alone or vehicle (1% etOH) had no significant effects on LPS-induced TNF- α release (LPS+AG490 2719 \pm 379 pg/mL; LPS+1% etOH 1666 \pm 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 RT² 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-hour incubations with GTS-21, LPS and LPS+GTS-21 are shown in table 1. GTS-21 clearly inhibited LPS-induced expression of IFN- γ and IFN- γ -inducible genes such as CXCL9, GBP1 and OAS1 [27,28,29]. In concordance with the implicated pivotal role for the JAK2-STAT3

1
2
3 pathway in $\alpha 7$ nAChR signaling, GTS-21 upregulated STAT3 expression in the presence
4
5 of LPS. Furthermore, LPS-induced downregulation of F2 (thrombin), an activator of
6
7 JAK2/STAT3 pathway and SH2B1, a JAK2 activator, were abolished by GTS-21
8
9 whereas GTS-21 upregulated OSM which is involved in STAT3 phosphorylation and
10
11 PRLR, a receptor which upon ligand binding activates JAK2 [30,31,32,33]. In the
12
13 presence of LPS, GTS-21 induced upregulation of PTPN1, a protein tyrosine phosphatase
14
15 which dephosphorylates JAK2, which may represent a negative feedback mechanism
16
17 preventing excessive JAK2 activation [34]. STAT5A was upregulated by GTS-21 in the
18
19 presence of LPS while LPS-induced downregulation of STAT5B was blocked by GTS-21
20
21 indicating activation of the JAK2-STAT5 pathway. The transcription of NF κ B and
22
23 SOCS3 was upregulated by LPS but not affected by co-incubation with GTS-21.
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 $\alpha 7$ nAChR 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 $\alpha 7$ nAChR, 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 shift in the pro-/anti-inflammatory balance towards an anti-inflammatory phenotype. While earlier reports state that the anti-inflammatory effect of $\alpha 7$ nAChR 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

1
2
3 nicotinic agonists. Non-activated lymphocytes generally are not noted for TLR-responses
4
5 but NK cells have been reported to produce IFN- γ after TLR-stimulation [35]. Whether
6
7 the observed inhibition of IFN- γ production represents a direct inhibitory effect on NK-
8
9 cells or an indirect effect via attenuation of cytokines that stimulate TLR-induced IFN- γ
10
11 production by these cells such as IL-12 and TNF- α , has to be determined. Nonetheless,
12
13 while we have focused on the effects of GTS-21 and nicotine on innate immune
14
15 responses, an effect on the adaptive immune system by these compounds is anticipated
16
17 because of alterations in co-stimulatory factors, cell interactions and interplay between
18
19 cytokines. Moreover, since primary human lymphocytes express the $\alpha 7$ nAChR, a direct
20
21 effect of $\alpha 7$ nAChR agonists on these cells and the adaptive immune response should not
22
23 be neglected [36,37]. The lack of an inhibitory effect on IL-6 release by GTS-21 is in line
24
25 with a study in mice which demonstrates a rather selective effect of GTS-21 on TNF- α
26
27 release [8,37]. There was a trend towards lower IL-6 mRNA levels in GTS-21-treated
28
29 monocytes but it did not reach statistical significance.
30
31

32
33 We further demonstrate that the anti-inflammatory effects of both nicotine and GTS-21
34
35 are not specific for the TLR stimulated using a whole blood stimulation assay. This is
36
37 important because pattern recognition of the diverse classes of microbial products
38
39 involves various TLRs that modulate the subsequent immune response [24,38]. As a
40
41 consequence, cytokine release profiles evoked by Gram-positive bacteria, Gram-negative
42
43 bacteria, viruses and various endogenous agonists differ. Previous work in our group has
44
45 shown that the effect of immunomodulating compounds can differ depending on the TLR
46
47 stimulated [39]. The data in this paper illustrate that both nicotinic compounds exhibit a
48
49 generalized anti-inflammatory effect not dependent on the inflammatory stimulus and
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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 $\alpha 7$ nAChR 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 $\alpha 7$ antagonists. First of all the possibility remains that some of the nicotine or GTS-21 effects in human leukocytes are mediated by a non- $\alpha 7$ nAChR-related mechanism which remains to be elucidated. Another explanation might rely on differences in the $\alpha 7$ nAChR between cell types. In excitable neuronal cells, $\alpha 7$ nAChRs are ligand-gated ion channels composed of 5 $\alpha 7$ 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 $\alpha 7$ nAChRs 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 $\alpha 7$ nAChR 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” $\alpha 7$ subunit, but an $\alpha 7$ duplicate nicotinic acetylcholine receptor-related protein (dup $\alpha 7$) 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 $\alpha 7$ nAChR specific antagonist MLA and α -bungarotoxin did not inhibit nicotine-induced effects [37].

Different pharmacological properties may also explain why the partial $\alpha 7$ nAChR agonist GTS-21 has a much more potent effect on cytokine release in primary human leukocytes than the full agonist nicotine, while in classic $\alpha 7$ nAChRs, the opposite is true [43].

Interestingly, bone marrow and brain cells were positive for both the normal $\alpha 7$ and the dup $\alpha 7$ subunit [42]. This indicates that the presence of the normal $\alpha 7$ nAChR or the dup $\alpha 7$ might vary between different cell types, which could explicate why nicotinic blockers antagonize $\alpha 7$ nAChR 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 [44]. 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 [45]. 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 [46]. In this respect

1
2
3 inhibition of cell-surface expression of the TLRs by GTS-21 might be a result of
4
5 inhibition of cytokines rather than a cause. mRNA levels of TLR4 and TLR2 were not
6
7 affected by LPS and only TLR2 mRNA expression was modestly inhibited by GTS-21.
8
9
10 The discrepancies between TLR protein and mRNA expression might result from the
11
12 different incubation period used in the protein expression experiments compared to the
13
14 mRNA experiments (22 hours vs. 4 hours) but can also indicate that the modulation of
15
16 TLR cell-surface expression relies mainly on post-transcriptional mechanisms. An earlier
17
18 report demonstrates that nicotine downregulates cell-surface expression of TLR4 and
19
20 CD14 on human monocytes [47]. This discrepancy with our study might be explained by
21
22 the modest anti-inflammatory effect of nicotine in our experiments.
23
24
25
26
27
28

29 De Jonge et al. have demonstrated that the anti-inflammatory effect of nicotine in murine
30
31 macrophages acts through the recruitment of JAK2 to the $\alpha 7$ nAChR and subsequent
32
33 phosphorylation of JAK2, thereby initiating the anti-inflammatory STAT3 cascade [16].
34
35
36 We show that in primary human leukocytes both GTS-21 and nicotine effects could be
37
38 inhibited by AG490, a JAK2 phosphorylation inhibitor, confirming a pivotal role for
39
40 JAK2 activation. We further explored activation of the JAK-STAT pathway using
41
42 quantitative PCR arrays. Although these arrays do not provide data on phosphorylation of
43
44 JAK-STAT family members, it evaluates expression of known activators/inhibitors of
45
46 this signaling cascade at the transcriptional level. Our results indicate that GTS-21
47
48 strongly downregulates IFN- γ -inducible genes related to STAT1 activation in monocytes
49
50 [48]. Therefore, as stated before, GTS-21 may have a pronounced inhibitory effect on the
51
52
53
54
55 adaptive immune response which warrants future research. JAK2/STAT3 signaling
56
57
58
59
60
61
62
63
64
65

1
2
3 appeared to be activated by GTS-21 reflected by increased expression of STAT3,
4
5 receptors activating JAK2 (PRLR) and activators of STAT3 (OSM). Furthermore, GTS-
6
7 21 blocked LPS-induced downregulation of JAK2/STAT3 activators (F2, SH2B1). The
8
9 upregulation of STAT5A and the blockade of LPS-induced downregulation of STAT5B
10
11 by GTS-21 further indicate JAK2 activity as STAT5 is also activated by JAK2 in
12
13 monocytes [49].
14
15

16
17 There is much debate regarding the pro- and anti-inflammatory roles of STAT3 which
18
19 appear to be highly cell- and stimulation-specific. A number of studies implicate pro-
20
21 inflammatory actions of STAT3, such as a recent paper describing the critical role of
22
23 STAT3 activation in IL-1 β and IL-6 production in a RAW 264.7 macrophage cell line
24
25 [50]. However, there is also a large body of evidence associating STAT3 activity with
26
27 anti-inflammatory effects. For instance, STAT3 is essential for responsiveness to IL-10
28
29 which is known to deactivate macrophages [51,52] and STAT3 deficient macrophages
30
31 are constitutively activated and secrete large amounts of pro-inflammatory mediators
32
33 [52]. Blocking STAT3 in tumor cells increases expression of pro-inflammatory cytokines
34
35 and mice lacking STAT3 in macrophages and neutrophils are highly susceptible to
36
37 endotoxic shock [52,53]. Moreover, mice bearing a STAT3 deletion in bone marrow
38
39 cells display higher levels of circulating TNF- α and IFN- γ compared to control mice in
40
41 the absence of an inflammatory process [54]. The mechanism by which GTS-21 (and
42
43 probably nicotine) inhibits cytokine release may rely on enhanced IL-10 signaling or
44
45 actions mimicking IL-10 signaling resulting in STAT3 activation. Because IL-10
46
47 downregulates its own production, excessive IL-10 signaling could account for the
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3 inhibited production of IL-10 observed with the highest dose of GTS-21 used (100 μ M)
4
5 [51].
6

7
8 Similar to what has been reported for nicotine [16], SOCS3 appears not to play a role in
9
10 the anti-inflammatory effects of GTS-21 while the LPS-induced upregulation of SOCS3
11
12 was not further enhanced by GTS-21. Our quantitative PCR experiments illustrate that
13
14 GTS-21 inhibits LPS-induced upregulation of pro-inflammatory cytokines at the
15
16 transcriptional level. While others argue that the anti-inflammatory effects of α 7nAChR
17
18 stimulation predominantly rely on post-transcriptional modulation [4,16], our data are in
19
20 accordance with IL-10 (-like) signaling which inhibits TNF- α production at both the
21
22 transcriptional and the translational level [55]. Moreover, GTS-21 and nicotine both have
23
24 been reported to inhibit LPS-induced NF κ B activation which implies transcriptional
25
26 modulation [7,20,21]. While our JAK-STAT array results demonstrate that the
27
28 transcription of NF κ B is not upregulated by GTS-21 compared to LPS, this does not
29
30 exclude the possibility that GTS-21 modulates NF κ B activity/nuclear translocation as this
31
32 is not dependent on transcription or translation [56]. Nicotine did not affect mRNA levels
33
34 of any of the genes assessed. Whether this is due to the relatively low anti-inflammatory
35
36 potential nicotine displayed in our experiments or to other factors remains to be
37
38 elucidated.
39
40
41
42
43
44
45
46
47

48 In conclusion, GTS-21 represents novel opportunities for human research into the
49
50 cholinergic anti-inflammatory pathway and possibly for future therapeutic applications. It
51
52 is more effective than nicotine in modulating the immune response in human leukocytes
53
54
55
56
57
58
59
60
61
62
63
64
65

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.

Reference List

- [1] Fleshner M, Goehler LE, Schwartz BA, McGorry M, Martin D, Maier SF, Watkins LR. Thermogenic and corticosterone responses to intravenous cytokines (IL-1beta and TNF-alpha) are attenuated by subdiaphragmatic vagotomy. *J.Neuroimmunol.* 1998; 86:134-141.
- [2] Goehler LE, Busch CR, Tartaglia N, Relton J, Sisk D, Maier SF, Watkins LR. Blockade of cytokine induced conditioned taste aversion by subdiaphragmatic vagotomy: further evidence for vagal mediation of immune-brain communication. *Neurosci.Lett.* 1995; 185:163-166.
- [3] Watkins LR, Maier SF, Goehler LE. Immune activation: the role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states. *Pain* 1995; 63:289-302.
- [4] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000; 405:458-462.
- [5] Sato KZ, Fujii T, Watanabe Y, Yamada S, Ando T, Kazuko F, Kawashima K. Diversity of mRNA expression for muscarinic acetylcholine receptor subtypes and neuronal nicotinic acetylcholine receptor subunits in human mononuclear leukocytes and leukemic cell lines. *Neurosci.Lett.* 1999; 266:17-20.
- [6] Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature* 2003; 421:384-388.
- [7] Pavlov VA, Ochani M, Yang LH, Gallowitsch-Puerta M, Ochani K, Lin X, Levi J, Parrish WR, Rosas-Ballina M, Czura CJ, Larosa GJ, Miller EJ, Tracey KJ, Al-Abed Y. Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. *Crit Care Med.* 2007; 35:1139-1144.
- [8] Giebelen IA, van Westerloo DJ, Larosa GJ, de Vos AF, van der PT. Stimulation of alpha7 cholinergic receptors inhibits lipopolysaccharide-induced neutrophil recruitment by a tumor necrosis factor alpha-independent mechanism. *Shock* 2007; 27:443-447.
- [9] van Westerloo DJ, Giebelen IA, Florquin S, Bruno MJ, Larosa GJ, Ulloa L, Tracey KJ, van der PT. The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. *Gastroenterology* 2006; 130:1822-1830.

- [10] Giebelen IA, van Westerloo DJ, Larosa GJ, de Vos AF, van der PT. Local stimulation of alpha7 cholinergic receptors inhibits LPS-induced TNF-alpha release in the mouse lung. *Shock* 2007; 28:700-703.
- [11] Su X, Lee JW, Matthay ZA, Mednick G, Uchida T, Fang X, Gupta N, Matthay MA. Activation of the alpha7 nAChR reduces acid-induced acute lung injury in mice and rats. *Am.J.Respir.Cell Mol.Biol.* 2007; 37:186-192.
- [12] Yeboah MM, Xue X, Duan B, Ochani M, Tracey KJ, Susin M, Metz CN. Cholinergic agonists attenuate renal ischemia-reperfusion injury in rats. *Kidney Int.* 2008; 74:62-69.
- [13] Kitagawa H, Takenouchi T, Azuma R, Wesnes KA, Kramer WG, Clody DE, Burnett AL. Safety, pharmacokinetics, and effects on cognitive function of multiple doses of GTS-21 in healthy, male volunteers. *Neuropsychopharmacology* 2003; 28:542-551.
- [14] Meyer EM, Kuryatov A, Gerzanich V, Lindstrom J, Papke RL. Analysis of 3-(4-hydroxy, 2-Methoxybenzylidene)anabaseine selectivity and activity at human and rat alpha-7 nicotinic receptors. *J.Pharmacol.Exp.Ther.* 1998; 287:918-925.
- [15] Stokes C, Papke JK, Horenstein NA, Kem WR, McCormack TJ, Papke RL. The structural basis for GTS-21 selectivity between human and rat nicotinic alpha7 receptors. *Mol.Pharmacol.* 2004; 66:14-24.
- [16] de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, Berthoud HR, Uematsu S, Akira S, van den Wijngaard RM, Boeckxstaens GE. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat.Immunol.* 2005; 6:844-851.
- [17] Shaw S, Bencherif M, Marrero MB. Janus kinase 2, an early target of alpha 7 nicotinic acetylcholine receptor-mediated neuroprotection against Abeta-(1-42) amyloid. *J.Biol.Chem.* 2002; 277:44920-44924.
- [18] Arredondo J, Chernyavsky AI, Jolkovsky DL, Pinkerton KE, Grando SA. Receptor-mediated tobacco toxicity: cooperation of the Ras/Raf-1/MEK1/ERK and JAK-2/STAT-3 pathways downstream of alpha7 nicotinic receptor in oral keratinocytes. *FASEB J.* 2006; 20:2093-2101.
- [19] Osborne-Hereford AV, Rogers SW, Gahring LC. Neuronal nicotinic alpha7 receptors modulate inflammatory cytokine production in the skin following ultraviolet radiation. *J.Neuroimmunol.* 2008; 193:130-139.
- [20] Sugano N, Shimada K, Ito K, Murai S. Nicotine inhibits the production of inflammatory mediators in U937 cells through modulation of nuclear factor-kappaB activation. *Biochem.Biophys.Res.Comm.* 1998; 252:25-28.

- [21] Yoshikawa H, Kurokawa M, Ozaki N, Nara K, Atou K, Takada E, Kamochi H, Suzuki N. Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7. *Clin.Exp.Immunol.* 2006; 146:116-123.
- [22] Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J.Immunol.* 2000; 165:618-622.
- [23] Grebenchtchikov N, van d, V, Pesman GJ, Geurts-Moespot A, van der Meer JW, Sweep FC. Development of a sensitive ELISA for the quantification of human tumour necrosis factor-alpha using 4 polyclonal antibodies. *Eur.Cytokine Netw.* 2005; 16:215-222.
- [24] Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol.Lett.* 2003; 85:85-95.
- [25] Wittebole X, Coyle SM, Kumar A, Goshima M, Lowry SF, Calvano SE. Expression of tumour necrosis factor receptor and Toll-like receptor 2 and 4 on peripheral blood leucocytes of human volunteers after endotoxin challenge: a comparison of flow cytometric light scatter and immunofluorescence gating. *Clin.Exp.Immunol.* 2005; 141:99-106.
- [26] Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z, Leeder JS, Freedman M, Cohen A, Gazit A, Levitzki A, Roifman CM. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature* 1996; 379:645-648.
- [27] Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, Strieter RM. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J.Immunol.* 2001; 167:623-627.
- [28] Nantais DE, Schwemmle M, Stickney JT, Vestal DJ, Buss JE. Prenylation of an interferon-gamma-induced GTP-binding protein: the human guanylate binding protein, huGBP1. *J.Leukoc.Biol.* 1996; 60:423-431.
- [29] Rebouillat D, Hovanessian AG. The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. *J.Interferon Cytokine Res.* 1999; 19:295-308.
- [30] Huang C, Ma R, Sun S, Wei G, Fang Y, Liu R, Li G. JAK2-STAT3 signaling pathway mediates thrombin-induced proinflammatory actions of microglia in vitro. *J.Neuroimmunol.* 2008; 204:118-125.
- [31] Sakamoto K, Creamer BA, Triplett AA, Wagner KU. The Janus kinase 2 is required for expression and nuclear accumulation of cyclin D1 in proliferating mammary epithelial cells. *Mol.Endocrinol.* 2007; 21:1877-1892.

- [32] O'Brien KB, O'Shea JJ, Carter-Su C. SH2-B family members differentially regulate JAK family tyrosine kinases. *J.Biol.Chem.* 2002; 277:8673-8681.
- [33] Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem.J.* 2003; 374:1-20.
- [34] Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, Parisien JP, Salmeen A, Barford D, Tonks NK. TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. *J.Biol.Chem.* 2001; 276:47771-47774.
- [35] Lauzon NM, Mian F, MacKenzie R, Ashkar AA. The direct effects of Toll-like receptor ligands on human NK cell cytokine production and cytotoxicity. *Cell Immunol.* 2006; 241:102-112.
- [36] De Rosa MJ, Esandi MC, Garelli A, Rayes D, Bouzat C. Relationship between alpha 7 nAChR and apoptosis in human lymphocytes. *J.Neuroimmunol.* 2005; 160:154-161.
- [37] Razani-Boroujerdi S, Boyd RT, vila-Garcia MI, Nandi JS, Mishra NC, Singh SP, Pena-Philippides JC, Langley R, Sopori ML. T cells express alpha7-nicotinic acetylcholine receptor subunits that require a functional TCR and leukocyte-specific protein tyrosine kinase for nicotine-induced Ca²⁺ response. *J.Immunol.* 2007; 179:2889-2898.
- [38] Armant MA, Fenton MJ. Toll-like receptors: a family of pattern-recognition receptors in mammals. *Genome Biol.* 2002; 3:3011.1-3011.6.
- [39] Ramakers BP, Riksen NP, Rongen GA, van der Hoeven JG, Smits P, Pickkers P. The effect of adenosine receptor agonists on cytokine release by human mononuclear cells depends on the specific Toll-like receptor subtype used for stimulation. *Cytokine* 2006; 35:95-99.
- [40] De SR, jnone-Cat MA, Carnevale D, Minghetti L. Activation of alpha7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E2 in rat microglial cultures. *J.Neuroinflammation.* 2005; 2:4.
- [41] Matsunaga K, Klein TW, Friedman H, Yamamoto Y. Involvement of nicotinic acetylcholine receptors in suppression of antimicrobial activity and cytokine responses of alveolar macrophages to *Legionella pneumophila* infection by nicotine. *J.Immunol.* 2001; 167:6518-6524.
- [42] Villiger Y, Szanto I, Jaconi S, Blanchet C, Buisson B, Krause KH, Bertrand D, Romand JA. Expression of an alpha7 duplicate nicotinic acetylcholine receptor-related protein in human leukocytes. *J.Neuroimmunol.* 2002; 126:86-98.

- [43] Stokes C, Papke JK, Horenstein NA, Kem WR, McCormack TJ, Papke RL. The structural basis for GTS-21 selectivity between human and rat nicotinic alpha7 receptors. *Mol.Pharmacol.* 2004; 66:14-24.
- [44] Muta T, Takeshige K. Essential roles of CD14 and lipopolysaccharide-binding protein for activation of toll-like receptor (TLR)2 as well as TLR4 Reconstitution of. *Eur.J.Biochem.* 2001; 268:4580-4589.
- [45] Pearl-Yafe M, Fabian I, Halperin D, Flatau E, Werber S, Shalit I. Interferon-gamma and bacterial lipopolysaccharide act synergistically on human neutrophils enhancing interleukin-8, interleukin-1beta, tumor necrosis factor-alpha, and interleukin-12 p70 secretion and phagocytosis via upregulation of toll-like receptor 4. *Shock* 2007; 27:226-231.
- [46] Bosisio D, Polentarutti N, Sironi M, Bernasconi S, Miyake K, Webb GR, Martin MU, Mantovani A, Muzio M. Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. *Blood* 2002; 99:3427-3431.
- [47] Hamano R, Takahashi HK, Iwagaki H, Yoshino T, Nishibori M, Tanaka N. Stimulation of alpha7 nicotinic acetylcholine receptor inhibits CD14 and the toll-like receptor 4 expression in human monocytes. *Shock* 2006; 26:358-364.
- [48] Liuzzo G, Vallejo AN, Kopecky SL, Frye RL, Holmes DR, Goronzy JJ, Weyand CM. Molecular fingerprint of interferon-gamma signaling in unstable angina. *Circulation* 2001; 103:1509-1514.
- [49] Rosen RL, Winestock KD, Chen G, Liu X, Hennighausen L, Finbloom DS. Granulocyte-macrophage colony-stimulating factor preferentially activates the 94-kD STAT5A and an 80-kD STAT5A isoform in human peripheral blood monocytes. *Blood* 1996; 88:1206-1214.
- [50] Samavati L, Rastogi R, Du W, Huttemann M, Fite A, Franchi L. STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. *Mol.Immunol.* 2009; 46:1867-1877.
- [51] de Waal MR, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J.Exp.Med.* 1991; 174:1209-1220.
- [52] Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, Akira S. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity.* 1999; 10:39-49.

- 1
2
3 [53] Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, Bhattacharya R,
4 Gabrilovich D, Heller R, Coppola D, Dalton W, Jove R, Pardoll D, Yu H.
5 Regulation of the innate and adaptive immune responses by Stat-3 signaling
6 in tumor cells. *Nat.Med.* 2004; 10:48-54.
7
8
9 [54] Welte T, Zhang SS, Wang T, Zhang Z, Hesslein DG, Yin Z, Kano A, Iwamoto Y,
10 Li E, Craft JE, Bothwell AL, Fikrig E, Koni PA, Flavell RA, Fu XY.
11 STAT3 deletion during hematopoiesis causes Crohn's disease-like
12 pathogenesis and lethality: a critical role of STAT3 in innate immunity.
13 *Proc.Natl.Acad.Sci.U.S.A* 2003; 100:1879-1884.
14
15
16 [55] Kontoyiannis D, Kotlyarov A, Carballo E, Alexopoulou L, Blakeshear PJ, Gaestel
17 M, Davis R, Flavell R, Kollias G. Interleukin-10 targets p38 MAPK to
18 modulate ARE-dependent TNF mRNA translation and limit intestinal
19 pathology. *EMBO J.* 2001; 20:3760-3770.
20
21
22 [56] Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-
23 [kappa]B activity. *Annu.Rev.Immunol.* 2000; 18:621-663.
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure Legends

Graphical abstract figure

Model of GTS-21 signaling in primary human monocytes.

Figure 1

Dose-response curves of the effect of nicotine on cytokine production in human PBMC stimulated with 1 ng/mL LPS for 22 hours. Data are represented as mean \pm SEM (8 different donors, two independent experiments). * indicates $p < 0.05$ compared to the LPS response in the absence of nicotine (repeated measures ANOVA with Bonferroni post-hoc test).

Figure 2

Dose-response curves of the effect of GTS-21 on cytokine production in human PBMC stimulated with 1 ng/mL LPS for 22 hours. Data are represented as mean \pm SEM (8 different donors, two independent experiments). * indicates $p < 0.05$ compared to the LPS response in the absence of GTS-21 (repeated measures ANOVA with Bonferroni post-hoc test).

Figure 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 hours. Both GTS-21 (100 μ M) and nicotine (1 mM) attenuate TNF- α production in isolated monocytes stimulated with LPS.

Data are represented as mean \pm SEM (6 different donors, two independent experiments).

* indicates $p < 0.05$ 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 \pm SEM (6 different donors, two independent experiments).

Figure 4

Percentage inhibition/stimulation of TLR-agonist induced cytokine production in human 1:5 diluted whole blood by 1 mM nicotine and 100 μ M GTS-21 (24-hour stimulation).

TLR agonists used were TLR1+2: Pam3Cys (1 μ g/mL); TLR3: PolyI:C (50 μ g/mL);

TLR4: LPS (1 ng/mL); TLR5: flagellin (1 μ g/mL). Data are represented as median and interquartile range (6 different donors). * indicates $p < 0.05$, 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-1 β : 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).

Figure 5

Flow cytometric analysis of the effects of LPS (1 ng/mL) and GTS-21 (100 μ M) on cell-surface expression of TLR2 and TLR4 on primary human monocytes incubated for 22

hours. 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 \pm SEM (8 different donors, two independent experiments). # indicates $p < 0.05$ compared to RPMI, * indicates $p < 0.05$ compared to LPS (paired Student's t-test).

Figure 6

mRNA expression of TNF- α , IL-1 β , IL-6, IL-10, TLR2 and TLR4 in isolated primary human monocytes stimulated with LPS (1 ng/mL) and GTS-21 (100 μ M) for 4 hours. Data are represented as mean \pm SEM fold expression of the gene of interest relative to control (expression in monocytes incubated with medium alone). Data from 5 different donors, three independent experiments are shown. # indicates $p < 0.05$ compared to medium, * indicates $p < 0.05$ compared to LPS (paired Student's t-test).

Figure 7

Effect of the JAK2 inhibitor AG490 (100 μ M) on TNF- α production in human PBMCs stimulated with LPS (1 ng/mL), GTS-21 (10 and 100 μ M) and nicotine (1 mM) for 22 hours. Data are represented as mean \pm SEM (5-7 different donors, two independent experiments). * indicates $p < 0.05$ compared to LPS, # indicates $p < 0.05$ 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. Only genes which are significantly and more than twofold up/downregulated are shown

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		

Figure 1

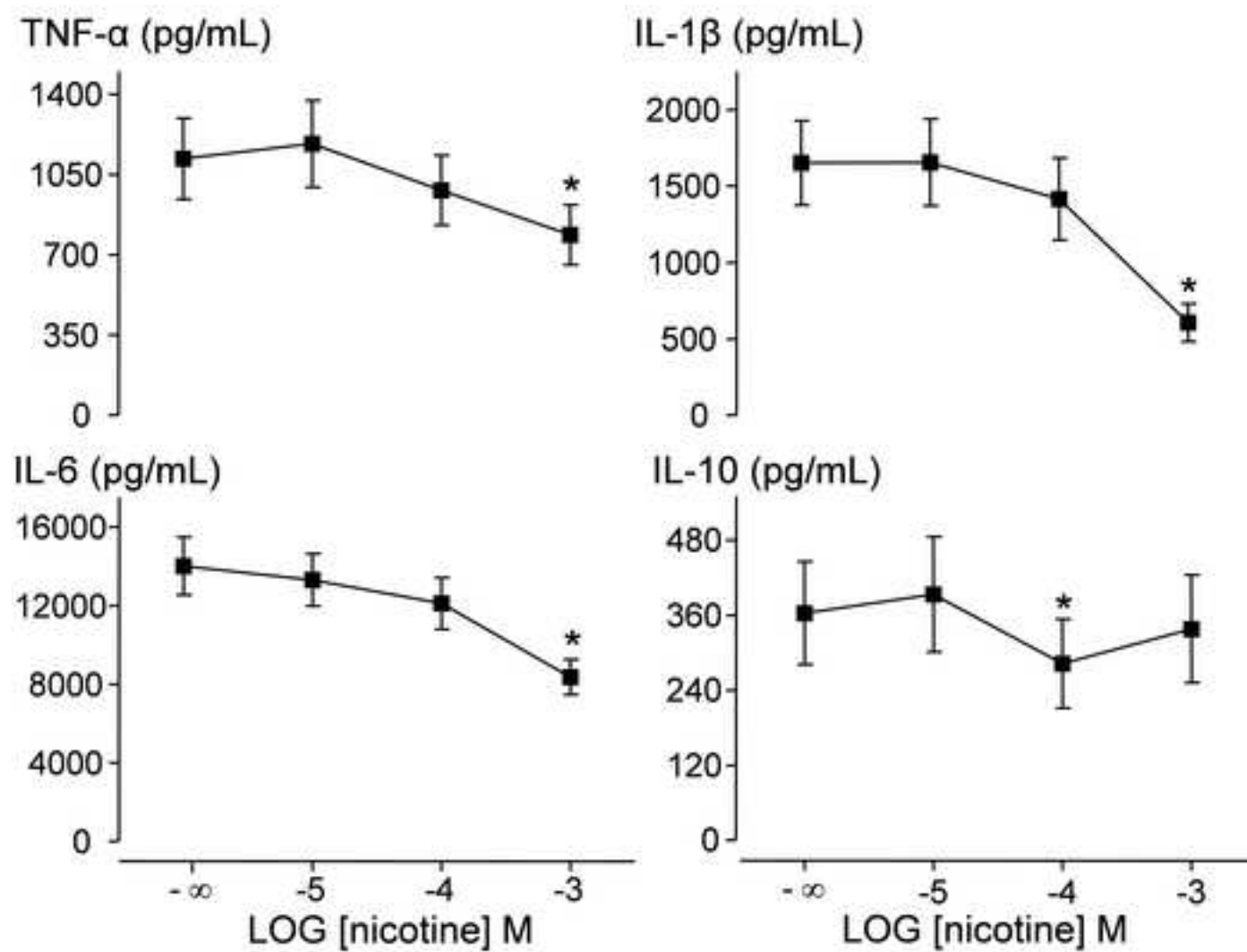


Figure 2

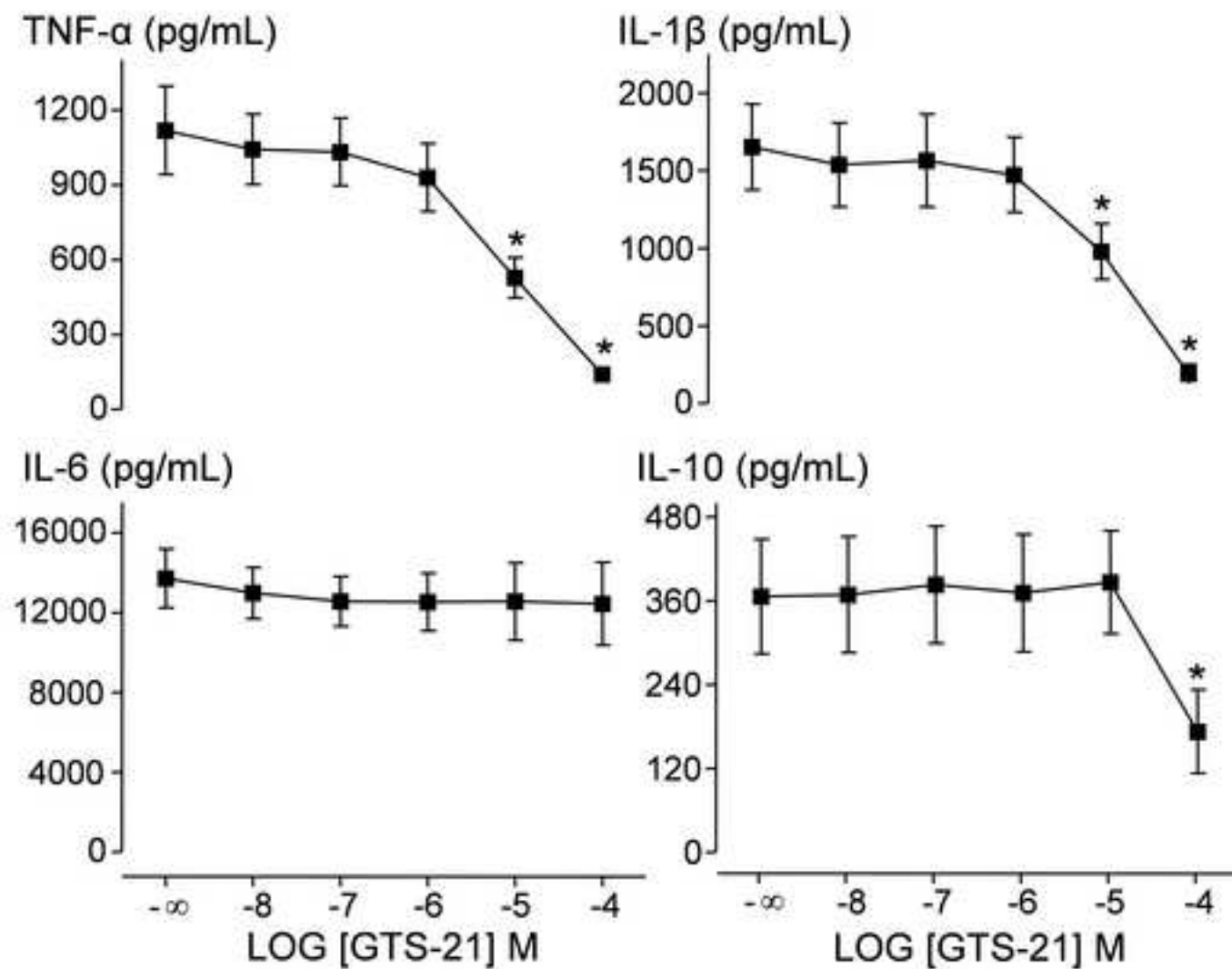


Figure 3

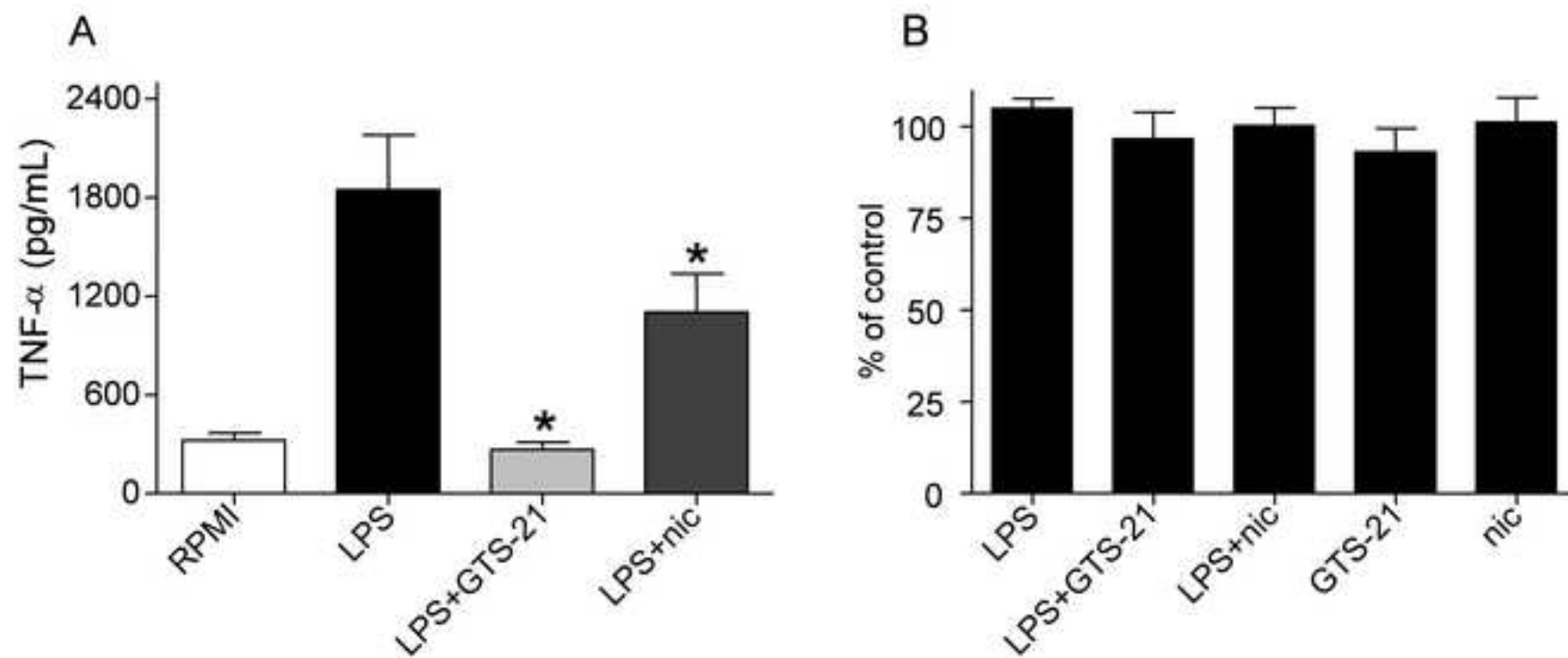


Figure 4

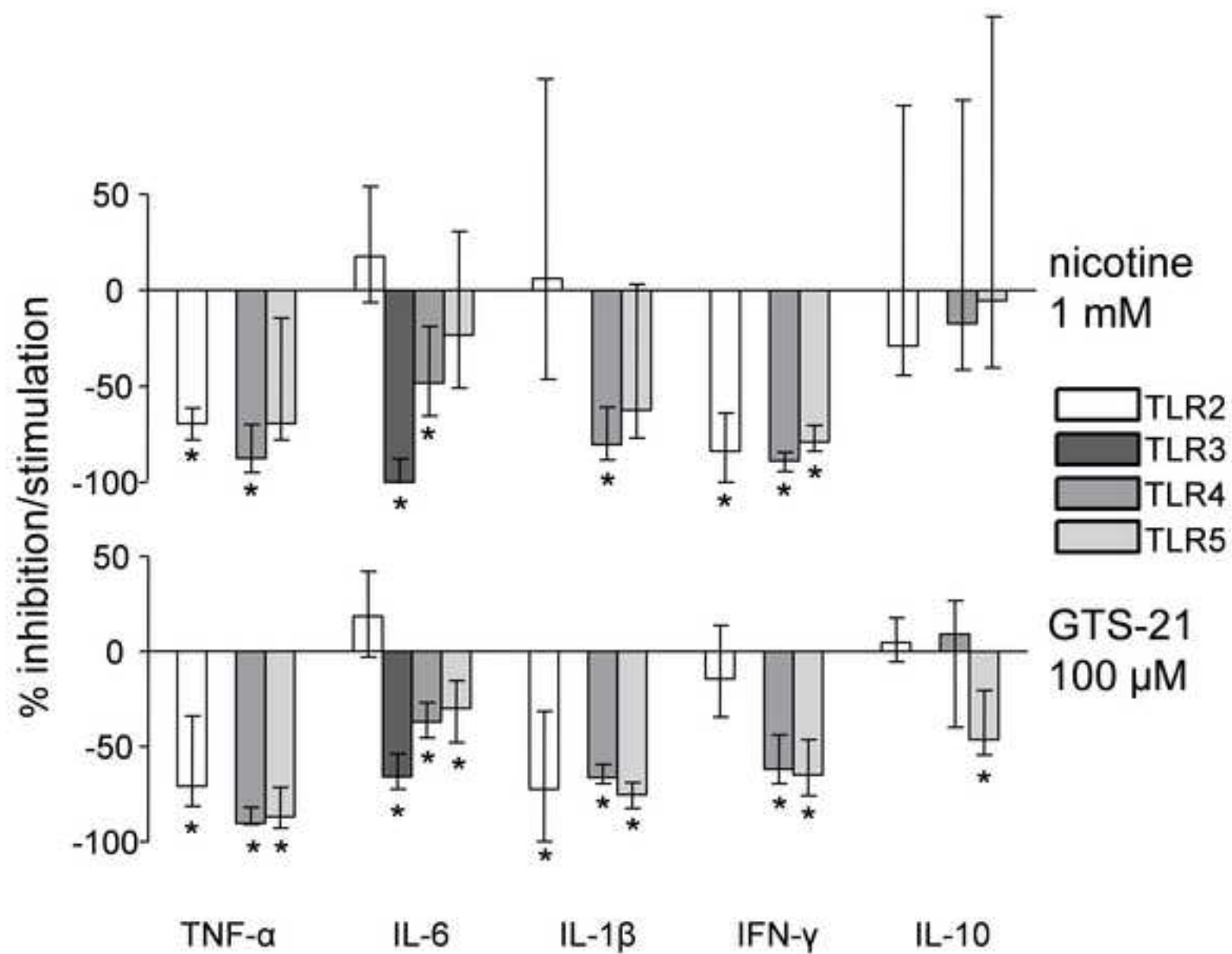


Figure 5

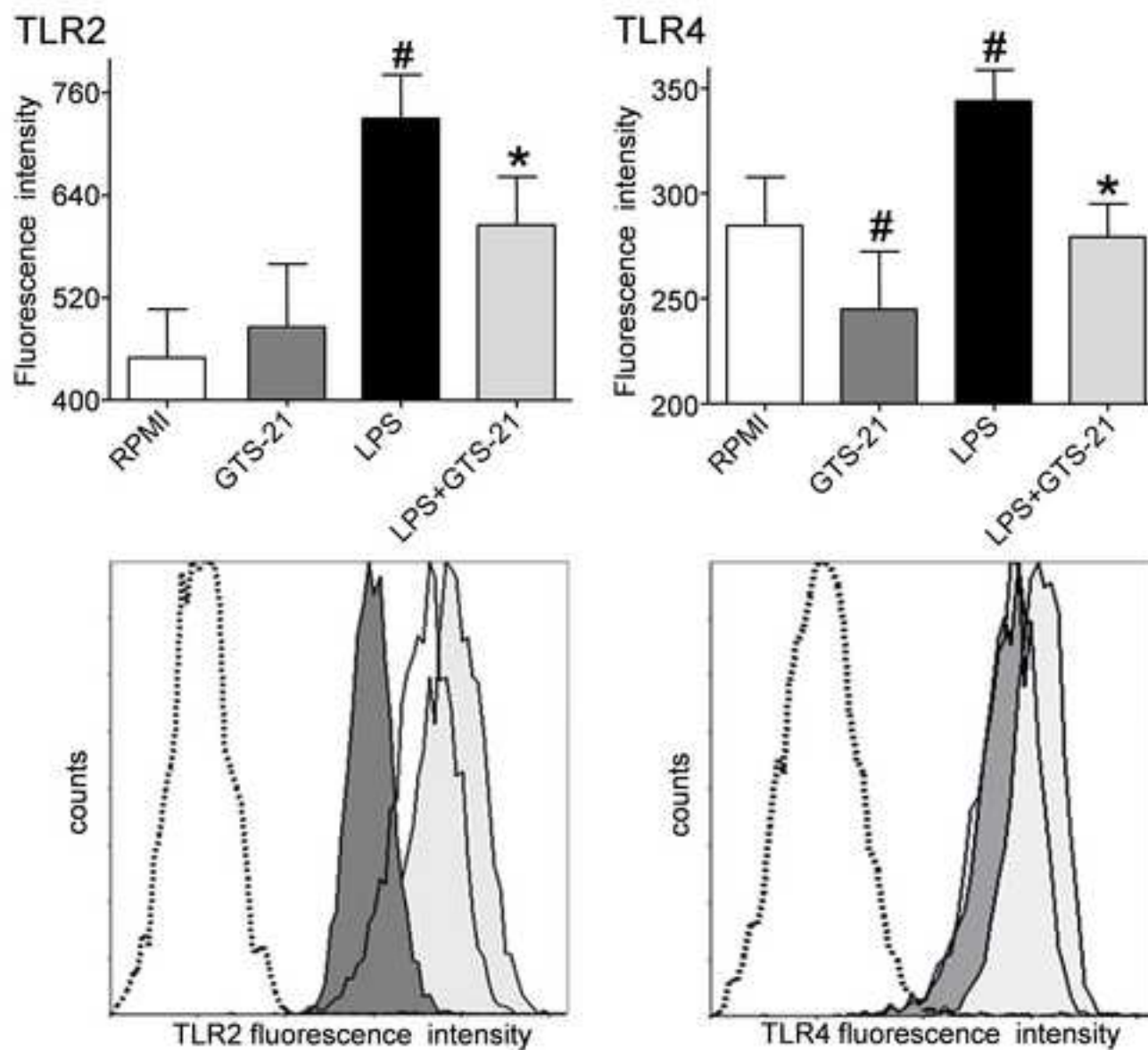
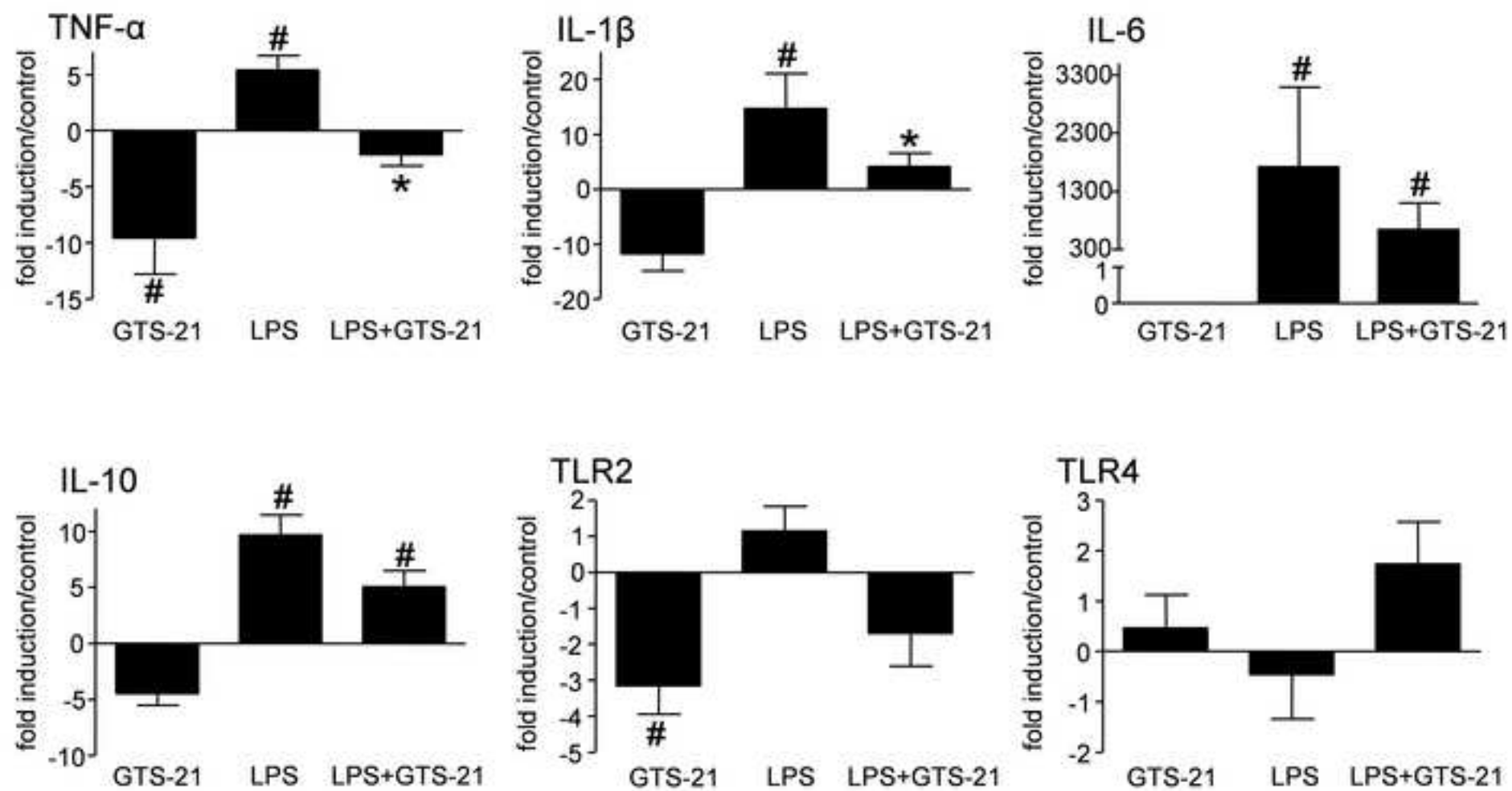


Figure 6



trip

Figure 7

