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3 **GTS-21 inhibits pro-inflammatory cytokine release independent of the Toll-like**
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5 **receptor stimulated via a transcriptional mechanism involving JAK2 activation.**
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8 Matthijs Kox^a, Jeroen F. van Velzen^b, Jan C. Pompe^a, Cornelia W. Hoedemaekers^a,
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10 Johannes G. van der Hoeven^a, Peter Pickkers^a
11
12
13
14
15

16 ^aDepartment of Intensive Care Medicine, Radboud University Nijmegen Medical Centre,
17
18 Nijmegen, The Netherlands
19

20 ^bDepartment of Hematology, Radboud University Nijmegen Medical Centre, Nijmegen,
21
22 The Netherlands
23
24
25
26

27 **Corresponding author:** P. Pickkers, MD, PhD

28 Department of Intensive Care Medicine, internal mail 632

29 Radboud University Nijmegen Medical Centre

30 PO box 9101, 6500 HB, Nijmegen, The Netherlands

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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

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3 properties of the $\alpha 7$ nAChR in human leukocytes compared to other cell types. GTS-21
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5 may be promising from a therapeutic perspective because of its suitability for human use.
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10 **Key words:** inflammation, cholinergic anti-inflammatory pathway, nicotine, monocytes,
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12 peripheral blood mononuclear cells, $\alpha 7$ nicotinic acetylcholine receptor
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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

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3 have been reported at $\alpha 7$ nAChR receptor activation level in electrophysiological studies
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5 using heterologous expression in *Xenopus laevis* oocytes [14,15], the immunomodulating
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7 effects of selective and non-selective agonists of the $\alpha 7$ nAChR have not been compared.
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9 Furthermore, in vitro studies investigating the cholinergic anti-inflammatory pathway
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11 almost exclusively used the principal Toll-like receptor 4 (TLR4) agonist LPS as a trigger
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13 for inflammation. It is unknown whether the cholinergic anti-inflammatory pathway is
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15 restricted to certain TLRs stimulated or is a general mechanism not constrained to a
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17 specific stimulus.
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21 Finally, the mechanism by which $\alpha 7$ nAChR stimulation attenuates pro-inflammatory
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23 cytokine production has not been fully elucidated and sparsely studied in human cells. A
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25 role for the JAK2-STAT3 pathway [16,17,18,19] as well as suppression of NF κ B
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27 transcriptional activity [20,21] is implied. However, the cholinergic anti-inflammatory
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29 pathway is believed to be regulated at a post-transcriptional level [4,16]. Mechanistic
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31 studies regarding the anti-inflammatory effect of GTS-21 are limited to one study
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33 reporting decreased NF κ B activity in a murine cell line [7].
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37 In this study, we investigated for the first time the anti-inflammatory potential of GTS-21
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39 on primary human leukocytes and compared it with nicotine. Furthermore, we
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41 investigated whether the effects of both nicotinic agonists were restricted to specific
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43 TLRs stimulated and whether they affected cell-surface expression of receptors involved
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45 in the innate immune response. Finally, we studied whether the anti-inflammatory effects
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47 of GTS-21 and nicotine are regulated at the transcriptional level and determined the
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49 involvement of the JAK-STAT signal transduction pathway.
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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), mecamlamine, 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-

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3 containing beverages/food for at least 12 hours before blood collection. Peripheral blood
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5 mononuclear cells (PBMCs) were isolated from EDTA anticoagulated blood by density
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7 gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden),
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9 washed three times in ice-cold sterile phosphate-buffered saline (B Braun Melsungen AG,
10
11 Melsungen, Germany), and resuspended in RPMI 1640 culture medium supplemented with
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13 10% autologous serum obtained by centrifugation (6000 rpm, 10 min) of lithium-heparin
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15 anticoagulated blood. Cells were counted in a Bürker hemocytometer and viability was
16
17 assessed using trypan blue (viability was >95%). The number of cells was adjusted to 2.5
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19 $\times 10^6$ /mL and 5 $\times 10^5$ cells per well (200 μ L) were seeded in duplicate in 96-well flat
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21 bottom plates (Greiner, Alphen a/d Rijn, The Netherlands) and stimulated for 22 hours
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23 (37 °C, 95% O₂, 5% CO₂) with various compounds. Incubation with RPMI alone served
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25 as a negative control. Nicotine or GTS-21 were added 30 minutes before TLR agonists.
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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 $\times 10^5$ 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.

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3 For whole blood stimulation experiments, venous blood was drawn into 2 mL lithium-
4 heparin containing vacutainers (Vacutainer System, BD Biosciences). Whole blood was
5 diluted 1:5 in RPMI and stimulated for 24 hours as described above. After stimulation,
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7 whole blood cultures were centrifuged (14000 rpm, 5 min) after which supernatants were
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9 stored at -80 °C until assayed.
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17 *2.3 Cytokine measurements*

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21 Cytokines in supernatants of whole blood cultures were determined using a simultaneous
22 Luminex Assay according to the manufacturer's instructions (Bio-plex cytokine assay,
23 BioRad, Hercules, CA, USA). Cytokines in supernatants of PBMC and monocytes
24
25 cultures were determined using enzyme-linked immunosorbent assays. TNF- α was
26
27 determined by a specific ELISA using four antibodies as described previously [23]. IL-
28
29 1 β , IL-6 and IL-10 were measured by commercial ELISA kits (IL-1 β : R&D systems,
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31 Minneapolis, MN, USA; IL-6 and IL-10: Pelikine Compact, Sanquin, Amsterdam, The
32
33 Netherlands, according to the manufacturer's instructions).
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43 *2.4 Viability assays*

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47 Monocyte viability was assessed using the *in vitro* toxicology assay kit, [3-(4,5-
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49 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based (Sigma-Aldrich).
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52 Monocytes were stimulated as described in section 2.2 (22 hours). After stimulation,
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54 plates were centrifuged (1700 rpm, 8 min), 100 μ L of supernatant was stored at -80 °C
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3 for cytokine analysis and 10 μ L of MTT stock solution (5 mg/mL) was added to the
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5 wells. Monocytes were incubated at 37 °C for an additional 4 hours after which formazan
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7 crystals were solubilized and absorbance at 570 nm was measured in a plate reader.
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10 Additionally, lactate dehydrogenase (LDH) was determined in supernatants of PBMCs
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12 stimulated for 22 hours as described in section 2.2.
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17 *2.5 Flow cytometry*

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21 Flow cytometry was performed on PBMCs stimulated as described in section 2.2 (22
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23 hours), After stimulation, plates were centrifuged (1700 rpm, 8 min), supernatants were
24
25 stored at -80 °C for cytokine analysis and adherent cells were detached by adding 200 μ L
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27 of ice-cold FACS buffer (PBS with 0.5 % BSA) and incubating the plate for 15 min on
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29 ice. Subsequently, cells were collected by vigorous resuspending and scraping of the
30
31 bottom of the wells and washed twice in ice-cold FACS buffer. TLR2, TLR4 and CD14
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33 expression was determined with the following directly conjugated antibodies: CD282 PE
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35 (mouse IgG2a, TLR 2.1 clone, eBioscience, San Diego, CA, USA), CD284 PE-Cy7
36
37 (TLR4, mouse IgG2a, HTA125 clone eBioscience), CD14 ECD (mouse IgG2a, RMO52
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39 clone Immunotech, Beckman Coulter, Mijdrecht, The Netherlands). Expression of
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41 α 7nAChR on monocytes was determined with a primary antibody against α 7nAChR (rat
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43 IgG1 monoclonal, 319 clone, Abcam, Cambridge, UK) and CD14 ECD followed by a
44
45 FITC labeled secondary antibody (donkey anti-rat FITC, Beckman Coulter). After
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47 antibody incubation, cells were washed with FACS buffer, resuspended and analyzed on
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3 a Beckman Coulter FC500 flow cytometer. TLR2, TLR4, CD14 and $\alpha 7nAChR$
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5 expression were analyzed within CD14 positive monocytes.
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8 9 10 *2.6 Quantitative PCR and JAK-STAT signaling arrays*

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14 Untouched primary monocytes were isolated as described in section 2.2 and seeded in
15
16 duplicate at a density of 1×10^6 /well in 24-well plates in the presence and absence of LPS
17
18 and nicotinic agonists. After 4 hours, plates were centrifuged (1700 rpm, 8 min) and
19
20 supernatants were aspirated. Subsequently, RNA was isolated using RNeasy plus mini
21
22 kits (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA
23
24 concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo
25
26 Scientific). Per experiment, equal amounts of RNA (150-400 ng) were used for cDNA
27
28 synthesis using iScript cDNA synthesis kits (Bio-Rad). Negative control reactions were
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30 performed in the absence of reverse transcriptase.
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36 The sequences of the primer pairs used were:

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|----|-----------------|----------|------------------------------------|
| 37 | | | |
| 38 | | | |
| 39 | TLR2 | forward: | 5'-GAATCCTCCAATCAGGCTTCTCT-3' |
| 40 | | reverse: | 5'-GCCCTGAGGGAATGGAGTTTA-3' |
| 41 | TLR4 | forward: | 5'-GGCATGCCTGTGCTGAGTT-3' |
| 42 | | reverse: | 5'-CTGCTACAACAGATACTACAAGCACACT-3' |
| 43 | TNF- α | forward: | 5'-TGGCCCAGGCAGTCAGA-3' |
| 44 | | reverse: | 5'-GGTTTGCTACAACATGGGCTACA-3' |
| 45 | IL-1 β | forward: | 5'-CAGCTACGAATCTCCGACCAC-3' |
| 46 | | reverse: | 5'-GGCAGGGAACCAGCATCTTC-3' |
| 47 | IL-6 | forward: | 5'-AATTCGGTACATCCTCGACGG-3' |
| 48 | | reverse: | 5'-GGTTGTTTTCTGCCAGTGCCT-3' |
| 49 | IL-10 | forward: | 5'-CAACCTGCCTAACATGCTTCG-3' |
| 50 | | reverse: | 5'-TCATCTCAGACAAGGCTTGGC-3' |
| 51 | CD14 | forward: | 5'-ACGCCAGAACCTTGTGAGC-3' |
| 52 | | reverse: | 5'-GCATGGATCTCCACCTCTACTG-3' |
| 53 | $\alpha 7nAChR$ | forward: | 5'-AAACTCACAGATGGGCAAGG-3' |
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3 reverse: 5'-AGGGAACACTGGAGTTGTGG-3'
4 B2M forward: 5'-ATGAGTATGCCTGCCGTGTG-3'
5 reverse: 5'-CCAAATGCGGCATCTTCAAAC-3'
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8 Primers were obtained from Biogio (Nijmegen, The Netherlands). The quantitative
9 PCR (qPCR) reaction was performed using Power SybrGreen master mix (Applied
10 Biosystems, Nieuwekerk a/d IJssel, The Netherlands) on an ABI Prism 7300 Real Time
11 PCR system (Applied Biosystems). Negative control reactions were cycled alongside test
12 samples to ensure the absence of contaminating genomic DNA. The amplification of a
13 single product was ensured by melt-curve analysis for each primer pair. A standard curve
14 constructed from 1:5 serial dilutions of a mixture of cDNA of the samples in the same run
15 was included for each primer pair in every run to perform relative quantification of
16 mRNA expression.
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18

19 To investigate the involvement of the JAK-STAT signaling pathway we used RT²
20 Profiler JAK-STAT QPCR arrays (PAHS-039, SABiosciences, Frederick, MD, USA)
21 according to the manufacturer's instructions. For these experiments, isolated monocytes
22 of three different donors were stimulated and mRNA was isolated as described above.
23 Four housekeeping genes, hypoxanthine phosphoribosyltransferase (HPRT1),
24 glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin (ACTB) and β 2
25 microglobulin (B2M) present on the PCR array were used for normalization.
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28 *2.7 Calculations*

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48 Data are expressed as mean \pm SEM except for whole blood stimulation data which is
49 expressed as median \pm interquartile range as it was not normally distributed. Statistical
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3 significance in the dose-response curves of nicotine and GTS-21 was evaluated using
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5 repeated measures one-way analysis of variance with Bonferroni post-hoc test.
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8 Elsewhere, the paired student's t-test was used to test for statistical significance except
9
10 for the whole blood data which was analyzed using the Wilcoxon matched pairs test and
11
12 the qPCR JAK-STAT array data for which the analysis procedure is described below. A
13
14 p-value below 0.05 (except for qPCR JAK-STAT array data, $p < 0.01$) was considered
15
16 statistically significant.
17

18
19 For whole blood experiments, % inhibition/stimulation was calculated as follows: (TLR-
20
21 agonist-induced cytokine release in the presence of GTS-21 or nicotine / TLR-agonist-
22
23 induced cytokine release in the absence of GTS-21 or nicotine) – 100. If more than 50%
24
25 of the subjects had a cytokine response in response to a TLR-agonist in the absence of a
26
27 nicotinic agonist that was lower than 4 times the detection limit, no inhibition/stimulation
28
29 calculations for this TLR-agonist-cytokine combination were performed.
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33 In the experiments where nAChR antagonists were used, the % blocking effect of the
34
35 nAChR antagonists was calculated by subtracting the % inhibition of the LPS-response
36
37 by GTS-21 or nicotine in absence of the nAChR antagonist from the % inhibition of the
38
39 LPS-response by GTS-21 or nicotine in the presence of the nAChR antagonist.
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43 qPCR data was analyzed using ABI Prism software and calculations were performed as
44
45 follows: per sample the relative quantity of mRNA of the gene of interest (e.g. TNF- α)
46
47 was divided by the relative quantity of the housekeeping gene B2M. To calculate fold
48
49 induction compared to the control sample, we divided the TNF- α /B2M ratio by the TNF-
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51 α /B2M ratio of the unstimulated control (RPMI) sample. If fold induction was < 1 , it was
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53 represented as $-(1/\text{fold induction})$.
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3 For the JAK-STAT qPCR array experiments each replicate cycle threshold (CT) was
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5 normalized to the average CT of four housekeeping genes on a per plate basis. The $2^{-\Delta\Delta CT}$
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7 method was used to calculate the relative quantification of gene expression in stimulated
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9 samples compared to RPMI samples. A gene was considered not detectable when CT
10
11 >35. CT was defined as 35 for the $2^{-\Delta\Delta CT}$ calculation when the signal was below
12
13 detectable limits. If for a specific gene both the stimulated and control sample expression
14
15 was below detectable limits the sample was excluded from analysis. Genes were
16
17 considered differentially expressed if mean up- or downregulation was equal or greater
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19 than 2-fold and $p < 0.01$ (permutation test, 100 permutations).
20
21
22 Statistical calculations were performed using Graphpad Prism V4.03 (Graphpad
23
24 software) except for the permutation tests in the JAK-STAT qPCR array experiments
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26 which were analyzed with Multi-experiment Viewer V4.3 (TM4 software suite).
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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

1
2
3 primary human monocytes using the same experimental conditions as in PBMCs. GTS-
4
5 21 and nicotine both significantly inhibited TNF- α production to a similar extent as in
6
7 PBMCs with GTS-21 showing a distinctively more pronounced effect than nicotine
8
9 (84 \pm 3% and 39 \pm 8% respectively, figure 3A). To exclude the possibility that loss of
10
11 viability or cell death was responsible for the observed inhibition of cytokine production
12
13 we performed MTT viability assays on the stimulated monocytes which revealed no loss
14
15 of cell viability with any of the stimuli used (figure 3B). Moreover, lactate dehydrogenase
16
17 levels in supernatants of stimulated PBMCs were not elevated compared to control
18
19 (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
20
21 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,
22
23 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*

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38 Pattern recognition of the diverse classes of microbial products causing infection involves
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40 various TLRs that modulate the subsequent immune response and cytokine profiles [24].
41
42 To explore whether the anti-inflammatory effects of nicotine and GTS-21 are dependent
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44 on a specific TLR stimulated we incubated human whole blood cultures with TLR2
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46 (Pam3Cys, 1 μ g/mL), TLR3 (PolyI:C, 50 μ g/mL), TLR4 (LPS, 1 ng/mL) and TLR5
47
48 (flagellin, 1 μ g/mL) agonists in combination with nicotine and GTS-21. Incubation with
49
50 TLR2, TLR4 and TLR5 agonists resulted in production of pro- (TNF- α , IL-1 β , IL-6, and
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52 IFN- γ) as well as anti-inflammatory (IL-10) cytokines (data not shown). The TLR3
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3 agonist PolyI:C only evoked a robust IL-6 release while TNF- α and IFN- γ were secreted
4
5 in very small quantities in whole blood cultures of some but not all of the subjects (and
6
7 therefore were excluded from inhibition/stimulation calculations). PolyI:C did not result
8
9 in release of IL-1 β and IL-10 in whole blood of any of the subjects.

10
11
12 Co-incubation with 100 μ M of nicotine inhibited TLR-agonist induced pro-inflammatory
13
14 cytokine release in a mild fashion (data not shown) but 1 mM of nicotine inhibited
15
16 cytokine release up to 100%, independent on the TLR stimulated (figure 4, upper panel).
17
18 The anti-inflammatory cytokine IL-10 was not significantly inhibited by either 100 μ M
19
20 or 1 mM of nicotine.
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24 The selective α 7nAChR agonist GTS-21 (100 μ M) also significantly inhibited nearly all
25
26 TLR-agonist induced pro-inflammatory cytokines (up to 95% inhibition, figure 4, lower
27
28 panel). As with nicotine, GTS-21 did not attenuate the anti-inflammatory cytokine IL-10
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30 release to a great extent, only TLR5-induced IL-10 production was significantly
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32 inhibited.
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34 35 36 37 38 39 *3.4 Nicotinic antagonists do not restore cytokine production*

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43 As the α 7nAChR has been proposed as the receptor responsible for the anti-inflammatory
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45 effect of nicotinic agonists [6], we attempted to reverse the effects of nicotine and GTS-
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47 21 with nAChR antagonists. However, co-incubation with both α 7nAChR-specific
48
49 antagonists α -bungarotoxin (10 nM – 1 μ M) and methyllycaconitine (10 μ M) as well as
50
51 non-specific nAChR antagonists mecamylamine (10 nM-100 μ M) and d-tubocurarine
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53 (10-100 μ M) could not reverse the anti-inflammatory effects of any of the concentrations
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3 of nicotine & GTS-21. Inhibition of the GTS-21 (100 μ M) induced attenuation of the
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5 cytokine response by the highest concentration of the nAChR blockers was: α -
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7 bungarotoxin $1.9 \pm 1.3\%$ (n=6), methyllycaconitine $-2.9 \pm 1.0\%$ (n=4), mecamlamine -
8
9 $1.8 \pm 3.2\%$ (n=6), d-tubocurarine $2.8 \pm 2.8\%$ (n=4). % inhibition of the nicotine (1 mM)
10
11 induced cytokine attenuation by the highest concentration of the various nAChR blockers
12
13 was: α -bungarotoxin $5.7 \pm 3.4\%$ (n=6), methyllycaconitine $0.6 \pm 6.9\%$ (n=4),
14
15 mecamlamine $2.3 \pm 4.1\%$ (n=6), d-tubocurarine $-10.3 \pm 8.1\%$ (n=4). Co-incubation of
16
17 other concentrations of GTS-21/nicotine with the various concentrations of the nAChR
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19 blockers mentioned above was also ineffective in blocking anti-inflammatory effects
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22 (data not shown).
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28 29 *3.5 GTS-21 downregulates cell surface receptors on monocytes*

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34 To obtain more insight into the mechanisms by which nicotine and GTS-21 exhibit their
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36 anti-inflammatory effects, we stimulated human PBMCs with LPS in combination with
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38 nicotine and GTS-21 and determined cell surface expression of TLR2, TLR4, α 7nAChR
39
40 and CD14 on the monocyte fraction by flow cytometry. We chose the 22-hour
41
42 incubation based on similar experiments where LPS induced significant upregulation of
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44 TLR2 and TLR4 after 24 hours of incubation [25]. As depicted in figure 5, LPS
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46 incubation upregulated monocyte cell-surface expression of TLR2 and TLR4 compared
47
48 to control (RPMI). CD14 expression was also upregulated by LPS (2990 ± 237.8 vs.
49
50 2173 ± 227.3 , $p < 0.05$). GTS-21 inhibited LPS-induced upregulation of TLR2 and CD14
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52 (2467 ± 218.9 , $p < 0.05$ compared to LPS) and abolished LPS-induced TLR4 upregulation.
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3 Moreover, in the absence of LPS, GTS-21 lowered TLR4 expression below control
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5 levels. LPS had no effect on $\alpha 7$ nAChR expression, while GTS-21 in the absence of LPS
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7 inhibited $\alpha 7$ nAChR expression below control level (387 ± 50.6 vs. 713.3 ± 116.3 , $p < 0.05$).
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9 Nicotine had no effects on expression of any of the measured receptors (data not shown).
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14 *3.6 GTS-21, but not nicotine, regulates cytokine production at the transcriptional level*

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19 To investigate whether the anti-inflammatory effects of GTS-21 and nicotine are
20
21 transcriptionally regulated we assessed mRNA levels of pro- and anti-inflammatory
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23 cytokines and receptors using quantitative PCR on isolated monocytes stimulated for 4
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25 hours. As expected, incubation with LPS significantly upregulated TNF- α , IL-1 β , IL-6
26
27 and IL-10 mRNA expression (respectively 5.4, 14.7, 1715 and 9.7-fold compared to
28
29 RPMI, figure 6). GTS-21 significantly attenuated LPS-induced upregulation of TNF- α
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31 and IL-1 β (-2.1 and 4.1-fold compared to RPMI respectively). Moreover, GTS-21 in the
32
33 absence of LPS significantly decreased TNF- α mRNA levels (-9.6-fold compared to
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35 RPMI) while there was a trend towards IL-1 β downregulation (-11.9-fold compared to
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37 RPMI). There was also a trend towards inhibition of LPS-induced upregulation of IL-6
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39 and IL-10 expression (637.8 and 5-fold compared to RPMI respectively) by GTS-21 as
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41 well as downregulation of IL-10 in the absence of LPS (-4.5 compared to RPMI). TLR2
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43 and TLR4 mRNA expression was not altered by LPS. GTS-21 significantly
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45 downregulated TLR2 in the absence of LPS (-3.2-fold compared to RPMI) but had no
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47 effect on TLR4 mRNA levels. LPS significantly downregulated CD14 (data not shown,
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49 -5.6 fold compared to RPMI), this was inhibited by GTS-21 (-3.5 fold compared to
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3 RPMI). Nicotine did not alter expression of any of the cytokine genes assessed (data not
4
5 shown). $\alpha 7$ nAChR gene expression was not altered by any stimulus (data not shown).
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10 *3.7 The anti-inflammatory effect of GTS-21 and nicotine are dependent on JAK2*
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12 *phosphorylation*
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16 To evaluate the involvement of the JAK-STAT pathway in the anti-inflammatory effects
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18 of nicotine and GTS-21 we incubated PBMCs with LPS, nicotine and GTS-21 in
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20 combination with AG490, a selective inhibitor of JAK2 phosphorylation [26,16,17]. 100
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22 μ M AG490 inhibited the anti-inflammatory effect of 100 μ M GTS-21 while completely
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24 restoring the attenuated TNF- α production by 10 μ M GTS-21 and 1 mM nicotine (figure
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26 7). AG490 alone or vehicle (1% etOH) had no significant effects on LPS-induced TNF- α
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28 release (LPS+AG490 2719 \pm 379 pg/mL; LPS+1% etOH 1666 \pm 333 pg/mL).
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36 *3.8 GTS-21 effects are likely mediated by JAK2/STAT3 signaling*
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41 To further explore the role of the JAK-STAT signaling pathway in the anti-inflammatory
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43 effects of GTS-21 we used RT² profiler qPCR arrays which contain a panel of 84 genes
44
45 related to JAK-STAT-mediated signaling on isolated monocytes. Genes that were
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47 significantly and more than twofold up- or downregulated by 4-hour incubations with
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49 GTS-21, LPS and LPS+GTS-21 are shown in table 1. GTS-21 clearly inhibited LPS-
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51 induced expression of IFN- γ and IFN- γ -inducible genes such as CXCL9, GBP1 and
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53 OAS1 [27,28,29]. In concordance with the implicated pivotal role for the JAK2-STAT3
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3 pathway in $\alpha 7$ nAChR signaling, GTS-21 upregulated STAT3 expression in the presence
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5 of LPS. Furthermore, LPS-induced downregulation of F2 (thrombin), an activator of
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7 JAK2/STAT3 pathway and SH2B1, a JAK2 activator, were abolished by GTS-21
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9 whereas GTS-21 upregulated OSM which is involved in STAT3 phosphorylation and
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11 PRLR, a receptor which upon ligand binding activates JAK2 [30,31,32,33]. In the
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13 presence of LPS, GTS-21 induced upregulation of PTPN1, a protein tyrosine phosphatase
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15 which dephosphorylates JAK2, which may represent a negative feedback mechanism
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17 preventing excessive JAK2 activation [34]. STAT5A was upregulated by GTS-21 in the
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19 presence of LPS while LPS-induced downregulation of STAT5B was blocked by GTS-21
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21 indicating activation of the JAK2-STAT5 pathway. The transcription of NF κ B and
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23 SOCS3 was upregulated by LPS but not affected by co-incubation with GTS-21.
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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

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3 nicotinic agonists. Non-activated lymphocytes generally are not noted for TLR-responses
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5 but NK cells have been reported to produce IFN- γ after TLR-stimulation [35]. Whether
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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
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11 production by these cells such as IL-12 and TNF- α , has to be determined. Nonetheless,
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13 while we have focused on the effects of GTS-21 and nicotine on innate immune
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15 responses, an effect on the adaptive immune system by these compounds is anticipated
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17 because of alterations in co-stimulatory factors, cell interactions and interplay between
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19 cytokines. Moreover, since primary human lymphocytes express the $\alpha 7$ nAChR, a direct
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21 effect of $\alpha 7$ nAChR agonists on these cells and the adaptive immune response should not
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23 be neglected [36,37]. The lack of an inhibitory effect on IL-6 release by GTS-21 is in line
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25 with a study in mice which demonstrates a rather selective effect of GTS-21 on TNF- α
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27 release [8,37]. There was a trend towards lower IL-6 mRNA levels in GTS-21-treated
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29 monocytes but it did not reach statistical significance.
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36 We further demonstrate that the anti-inflammatory effects of both nicotine and GTS-21
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38 are not specific for the TLR stimulated using a whole blood stimulation assay. This is
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40 important because pattern recognition of the diverse classes of microbial products
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42 involves various TLRs that modulate the subsequent immune response [24,38]. As a
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44 consequence, cytokine release profiles evoked by Gram-positive bacteria, Gram-negative
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46 bacteria, viruses and various endogenous agonists differ. Previous work in our group has
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48 shown that the effect of immunomodulating compounds can differ depending on the TLR
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50 stimulated [39]. The data in this paper illustrate that both nicotinic compounds exhibit a
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52 generalized anti-inflammatory effect not dependent on the inflammatory stimulus and
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3 therefore not confined to a specific sort of infection or endogenous stimulus.
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5 Additionally, we affirm the anti-inflammatory potential of GTS-21 and nicotine in whole
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7 blood containing all cell types and humoral factors present, which is more reflective of in
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9 vivo conditions than an isolated cell model.
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13 While the $\alpha 7$ nAChR has been identified as the pivotal receptor in the cholinergic anti-
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15 inflammatory pathway [6], we could not reverse the actions of nicotine or GTS-21 with
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17 antagonists of this receptor (α -bungarotoxin and MLA) or with non-specific nAChR
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19 antagonists (mecamylamine and d-tubocurarine) in human immune cells. In accordance,
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21 for nicotine, blockade of the anti-inflammatory effect by α -bungarotoxin has been
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23 described by some [16,40] but not all [41]. To date, no attempts have been made to
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25 reverse the anti-inflammatory effect of GTS-21 by nAChR antagonists. There are a
26
27 number of possible reasons to explain the failure to block GTS-21 and nicotine effects by
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29 $\alpha 7$ antagonists. First of all the possibility remains that some of the nicotine or GTS-21
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31 effects in human leukocytes are mediated by a non- $\alpha 7$ nAChR-related mechanism which
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33 remains to be elucidated. Another explanation might rely on differences in the $\alpha 7$ nAChR
34
35 between cell types. In excitable neuronal cells, $\alpha 7$ nAChRs are ligand-gated ion channels
36
37 composed of 5 $\alpha 7$ subunits which upon activation cause depolarization of the cell
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39 membrane and influx of Ca^{2+} via voltage-operated Ca^{2+} channels. However, leukocytes
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41 do not possess these Ca^{2+} channels and it was demonstrated that in PBMCs $\alpha 7$ nAChRs
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43 stimulation by nicotine or acetylcholine does not result in detectable membrane currents
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45 while it does in neuronal cells [42]. Others have demonstrated that, despite the fact that
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47 T-cells express an essentially identical transcript for the $\alpha 7$ nAChR subunit as neuronal
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3 cells, they do not form functional ligand-gated ion channels [37]. Furthermore, it was
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5 shown that leukocytes do not express the “normal” $\alpha 7$ subunit, but an $\alpha 7$ duplicate
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7 nicotinic acetylcholine receptor-related protein (dup $\alpha 7$) which lacks the α -bungarotoxin
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9 binding site and has most likely different pharmacological properties [42]. This is
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11 supported by the aforementioned study in T-cells, where the $\alpha 7$ nAChR specific
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13 antagonist MLA and α -bungarotoxin did not inhibit nicotine-induced effects [37].
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15 Different pharmacological properties may also explain why the partial $\alpha 7$ nAChR agonist
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17 GTS-21 has a much more potent effect on cytokine release in primary human leukocytes
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19 than the full agonist nicotine, while in classic $\alpha 7$ nAChRs, the opposite is true [43].
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21 Interestingly, bone marrow and brain cells were positive for both the normal $\alpha 7$ and the
22
23 dup $\alpha 7$ subunit [42]. This indicates that the presence of the normal $\alpha 7$ nAChR or the dup $\alpha 7$
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25 might vary between different cell types, which could explicate why nicotinic blockers
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27 antagonize $\alpha 7$ nAChR in some cell types but not in others.
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36 LPS-induced increases in monocyte cell-surface expression of TLR2 and TLR4 have
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38 been described previously [25]. We confirm these findings and demonstrate that LPS also
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40 increases cell surface expression of CD14 which is essential in LPS-induced cytokine
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42 production [44]. GTS-21, but not nicotine, inhibits TLR and CD14 upregulation and this
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44 could play a role in the diminished pro-inflammatory cytokine production in GTS-21
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46 treated leukocytes since modulation of cell-surface expression of TLR4 has been linked
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48 to the degree of cytokine production [45]. The observed LPS-induced upregulation of
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50 TLRs could also be mediated by cytokine production, so-called “priming”, which would
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52 explain why the non-TLR2 agonist LPS increases TLR2 expression [46]. In this respect
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3 inhibition of cell-surface expression of the TLRs by GTS-21 might be a result of
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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.
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10 The discrepancies between TLR protein and mRNA expression might result from the
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12 different incubation period used in the protein expression experiments compared to the
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14 mRNA experiments (22 hours vs. 4 hours) but can also indicate that the modulation of
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16 TLR cell-surface expression relies mainly on post-transcriptional mechanisms. An earlier
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18 report demonstrates that nicotine downregulates cell-surface expression of TLR4 and
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20 CD14 on human monocytes [47]. This discrepancy with our study might be explained by
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22 the modest anti-inflammatory effect of nicotine in our experiments.
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29 De Jonge et al. have demonstrated that the anti-inflammatory effect of nicotine in murine
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31 macrophages acts through the recruitment of JAK2 to the $\alpha 7$ nAChR and subsequent
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33 phosphorylation of JAK2, thereby initiating the anti-inflammatory STAT3 cascade [16].
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36 We show that in primary human leukocytes both GTS-21 and nicotine effects could be
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38 inhibited by AG490, a JAK2 phosphorylation inhibitor, confirming a pivotal role for
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40 JAK2 activation. We further explored activation of the JAK-STAT pathway using
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42 quantitative PCR arrays. Although these arrays do not provide data on phosphorylation of
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44 JAK-STAT family members, it evaluates expression of known activators/inhibitors of
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46 this signaling cascade at the transcriptional level. Our results indicate that GTS-21
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48 strongly downregulates IFN- γ -inducible genes related to STAT1 activation in monocytes
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50 [48]. Therefore, as stated before, GTS-21 may have a pronounced inhibitory effect on the
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52 adaptive immune response which warrants future research. JAK2/STAT3 signaling
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3 appeared to be activated by GTS-21 reflected by increased expression of STAT3,
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5 receptors activating JAK2 (PRLR) and activators of STAT3 (OSM). Furthermore, GTS-
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7 21 blocked LPS-induced downregulation of JAK2/STAT3 activators (F2, SH2B1). The
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9 upregulation of STAT5A and the blockade of LPS-induced downregulation of STAT5B
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11 by GTS-21 further indicate JAK2 activity as STAT5 is also activated by JAK2 in
12
13 monocytes [49].
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17 There is much debate regarding the pro- and anti-inflammatory roles of STAT3 which
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19 appear to be highly cell- and stimulation-specific. A number of studies implicate pro-
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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
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27 anti-inflammatory effects. For instance, STAT3 is essential for responsiveness to IL-10
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29 which is known to deactivate macrophages [51,52] and STAT3 deficient macrophages
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31 are constitutively activated and secrete large amounts of pro-inflammatory mediators
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33 [52]. Blocking STAT3 in tumor cells increases expression of pro-inflammatory cytokines
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35 and mice lacking STAT3 in macrophages and neutrophils are highly susceptible to
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37 endotoxic shock [52,53]. Moreover, mice bearing a STAT3 deletion in bone marrow
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39 cells display higher levels of circulating TNF- α and IFN- γ a compared to control mice in
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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
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45 actions mimicking IL-10 signaling resulting in STAT3 activation. Because IL-10
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47 downregulates its own production, excessive IL-10 signaling could account for the
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3 inhibited production of IL-10 observed with the highest dose of GTS-21 used (100 μ M)
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5 [51].
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8 Similar to what has been reported for nicotine [16], SOCS3 appears not to play a role in
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10 the anti-inflammatory effects of GTS-21 while the LPS-induced upregulation of SOCS3
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12 was not further enhanced by GTS-21. Our quantitative PCR experiments illustrate that
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14 GTS-21 inhibits LPS-induced upregulation of pro-inflammatory cytokines at the
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16 transcriptional level. While others argue that the anti-inflammatory effects of α 7nAChR
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18 stimulation predominantly rely on post-transcriptional modulation [4,16], our data are in
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20 accordance with IL-10 (-like) signaling which inhibits TNF- α production at both the
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22 transcriptional and the translational level [55]. Moreover, GTS-21 and nicotine both have
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24 been reported to inhibit LPS-induced NF κ B activation which implies transcriptional
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26 modulation [7,20,21]. While our JAK-STAT array results demonstrate that the
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28 transcription of NF κ B is not upregulated by GTS-21 compared to LPS, this does not
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30 exclude the possibility that GTS-21 modulates NF κ B activity/nuclear translocation as this
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32 is not dependent on transcription or translation [56]. Nicotine did not affect mRNA levels
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34 of any of the genes assessed. Whether this is due to the relatively low anti-inflammatory
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36 potential nicotine displayed in our experiments or to other factors remains to be
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38 elucidated.
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48 In conclusion, GTS-21 represents novel opportunities for human research into the
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50 cholinergic anti-inflammatory pathway and possibly for future therapeutic applications. It
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52 is more effective than nicotine in modulating the immune response in human leukocytes
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3 and its suitability for human use makes it a candidate for human *in vivo* trials to further
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5 explore the cholinergic anti-inflammatory pathway.
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3 **Figure Legends**
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7 **Graphical abstract figure**
8

9 Model of GTS-21 signaling in primary human monocytes.
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14 **Figure 1**
15

16 Dose-response curves of the effect of nicotine on cytokine production in human PBMC
17 stimulated with 1 ng/mL LPS for 22 hours. Data are represented as mean \pm SEM (8
18 different donors, two independent experiments). * indicates $p < 0.05$ compared to the LPS
19 response in the absence of nicotine (repeated measures ANOVA with Bonferroni post-
20 hoc test).
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30 **Figure 2**
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32 Dose-response curves of the effect of GTS-21 on cytokine production in human PBMC
33 stimulated with 1 ng/mL LPS for 22 hours. Data are represented as mean \pm SEM (8
34 different donors, two independent experiments). * indicates $p < 0.05$ compared to the LPS
35 response in the absence of GTS-21 (repeated measures ANOVA with Bonferroni post-
36 hoc test).
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47 **Figure 3**
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49 A) Effect of nicotine and GTS-21 on TNF- α production in isolated primary human
50 monocytes stimulated with LPS (1 ng/mL) for 22 hours. Both GTS-21(100 μ M) and
51 nicotine (1 mM) attenuate TNF- α production in isolated monocytes stimulated with LPS.
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3 Data are represented as mean \pm SEM (6 different donors, two independent experiments).

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5 * indicates $p < 0.05$ compared to the LPS alone (paired Student's t-test).

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7 B) MTT viability assay on isolated primary human monocytes stimulated with LPS,
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9 nicotine and GTS-21. Percentage viability was calculated relative to viability in the
10
11 control (RPMI) samples. Data are represented as mean \pm SEM (6 different donors, two
12
13 independent experiments).
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19 **Figure 4**

20
21 Percentage inhibition/stimulation of TLR-agonist induced cytokine production in human
22
23 1:5 diluted whole blood by 1 mM nicotine and 100 μ M GTS-21 (24-hour stimulation).

24
25 TLR agonists used were TLR1+2: Pam3Cys (1 μ g/mL); TLR3: PolyI:C (50 μ g/ml);

26
27 TLR4: LPS (1 ng/mL); TLR5: flagellin (1 μ g/mL). Data are represented as median and
28
29 interquartile range (6 different donors). * indicates $p < 0.05$, Wilcoxon Matched Pairs test.

30
31 Concentrations of cytokines in absence of nicotine or GTS-21 after stimulation with
32
33 TLR2, TLR3, TLR4 and TLR5 agonists in pg/mL (interquartile range) were; TNF- α : 154
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35 (41-592), 6 (2-15), 3150 (2020-3927), 2054 (692-4309). IL-6: 1374 (482-4406), 89 (61-
36
37 134), 6979 (4678-9309), 8341 (4864-11842). IL-1 β : 19 (3-80), 0 (0-2), 1637 (922-1963),
38
39 1502 (740-2126). IFN- γ 106 (33- 349), 9 (0-27), 734 (521- 954), 802 (593-1072). IL-10:
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41 43 (6-120), 0 (0-0), 76 (56-116), 221 (99-447).
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50 **Figure 5**

51
52 Flow cytometric analysis of the effects of LPS (1 ng/mL) and GTS-21 (100 μ M) on cell-
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54 surface expression of TLR2 and TLR4 on primary human monocytes incubated for 22
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3 hours. In the lower panels overlays of histograms depicting TLR2 and TLR4 expression
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5 on incubated monocytes of a typical subject are displayed. The dashed line represent
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7 isotype control, dark grey represents RPMI, light grey represents LPS and white
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9 represents LPS+GTS-21. Data are represented as mean \pm SEM (8 different donors, two
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11 independent experiments). # indicates $p < 0.05$ compared to RPMI, * indicates $p < 0.05$
12
13 compared to LPS (paired Student's t-test).
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19 **Figure 6**

20
21 mRNA expression of TNF- α , IL-1 β , IL-6, IL-10, TLR2 and TLR4 in isolated primary
22
23 human monocytes stimulated with LPS (1 ng/mL) and GTS-21 (100 μ M) for 4 hours.
24
25 Data are represented as mean \pm SEM fold expression of the gene of interest relative to
26
27 control (expression in monocytes incubated with medium alone). Data from 5 different
28
29 donors, three independent experiments are shown. # indicates $p < 0.05$ compared to
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31 medium, * indicates $p < 0.05$ compared to LPS (paired Student's t-test).
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38 **Figure 7**

39
40 Effect of the JAK2 inhibitor AG490 (100 μ M) on TNF- α production in human PBMCs
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42 stimulated with LPS (1 ng/mL), GTS-21 (10 and 100 μ M) and nicotine (1 mM) for 22
43
44 hours. Data are represented as mean \pm SEM (5-7 different donors, two independent
45
46 experiments). * indicates $p < 0.05$ compared to LPS, # indicates $p < 0.05$ compared to TNF-
47
48 α response with the same (concentration of) nicotinic agonist in the absence of AG490
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50 (paired Student's t-test).
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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

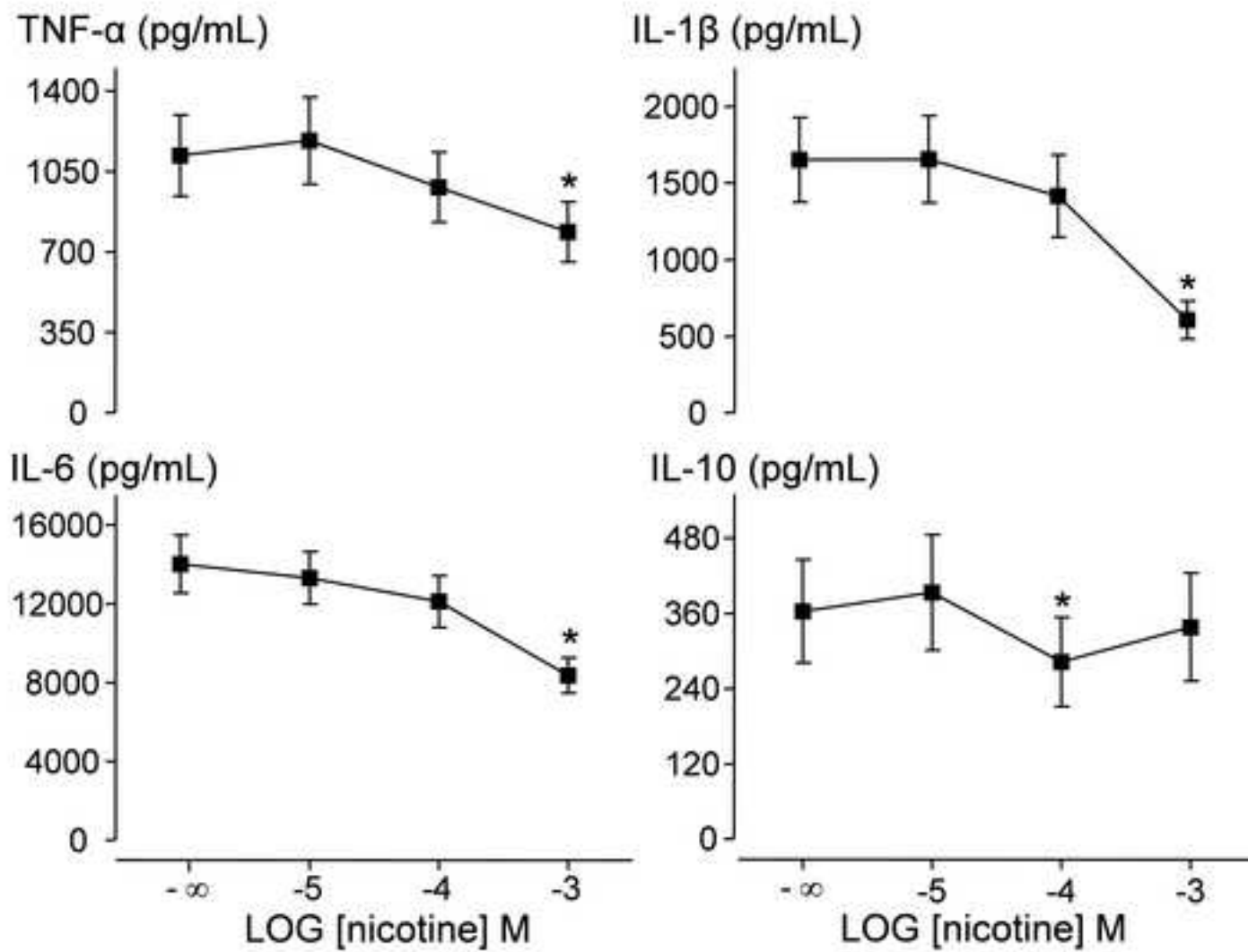




Figure 2

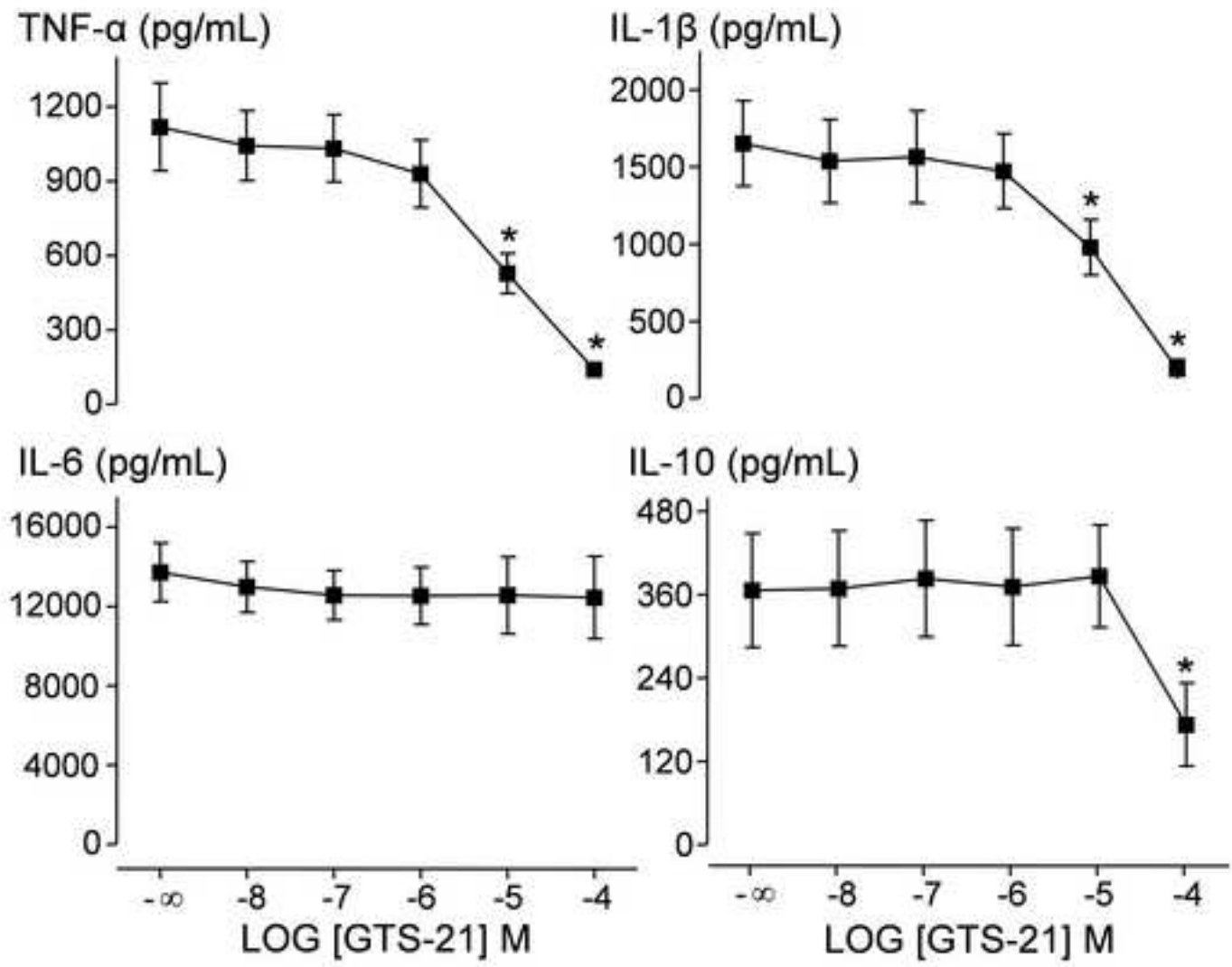


Figure 3

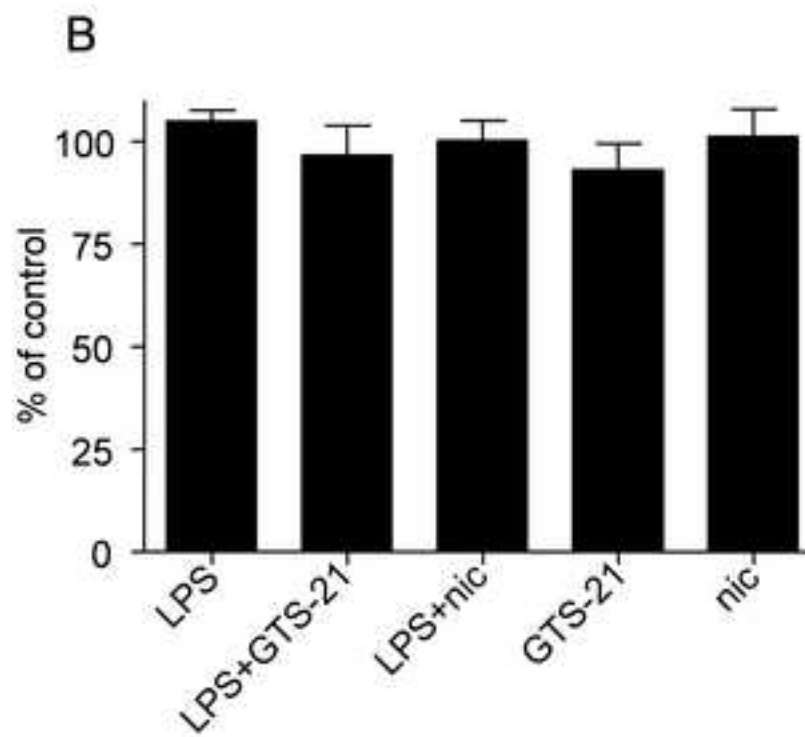
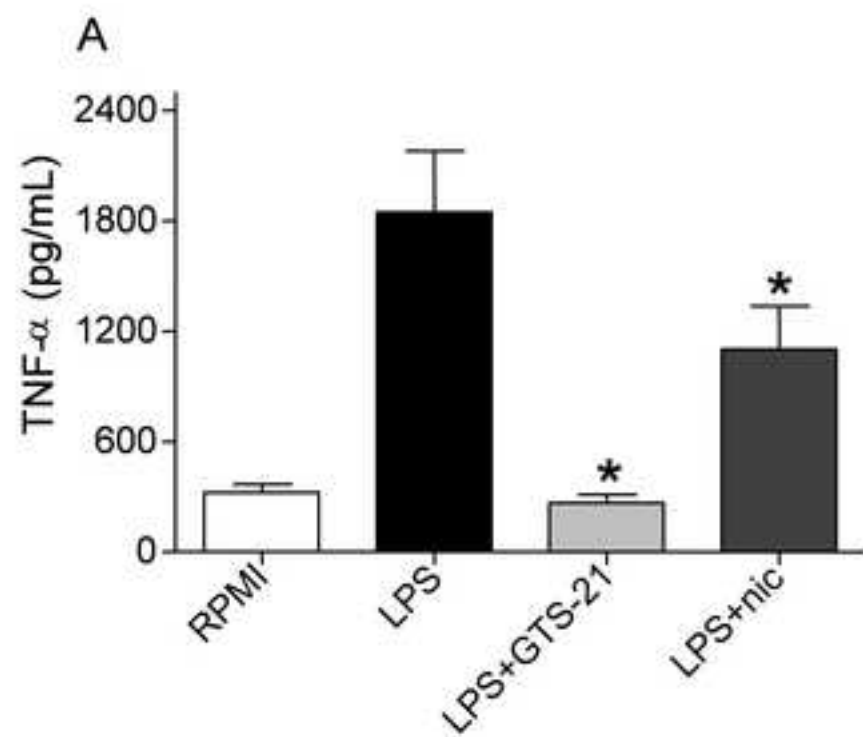


Figure 4

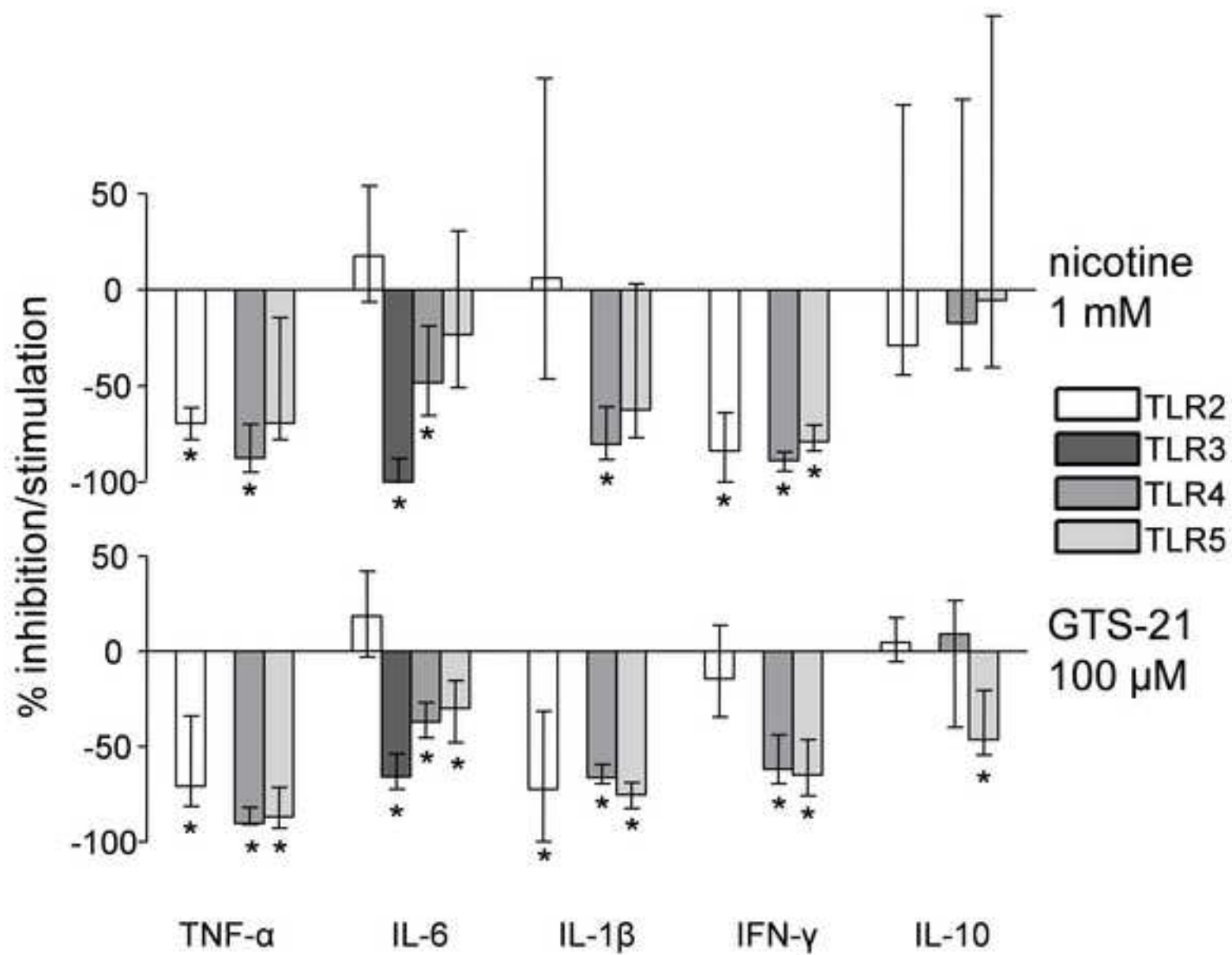




Figure 5

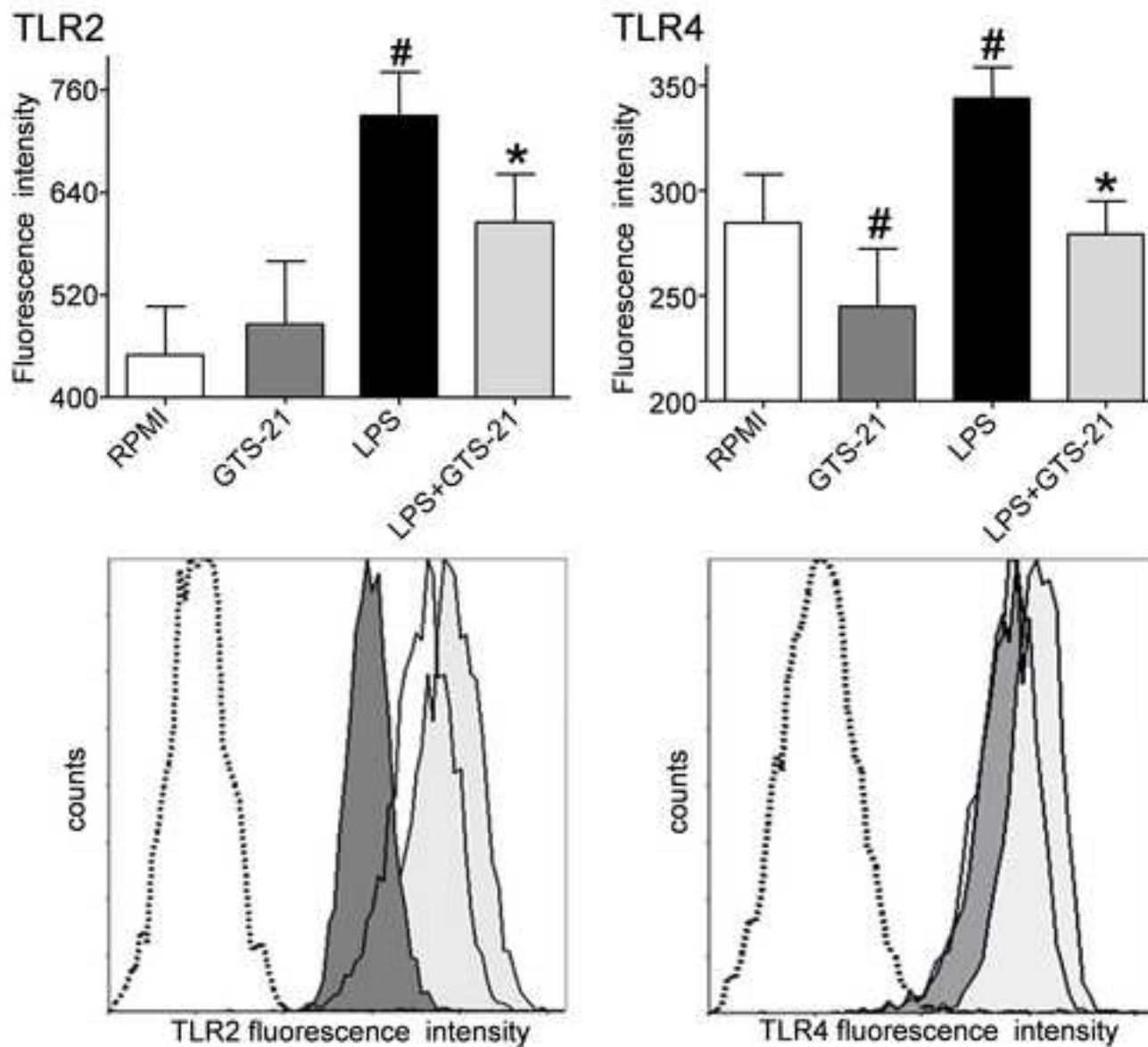




Figure 6

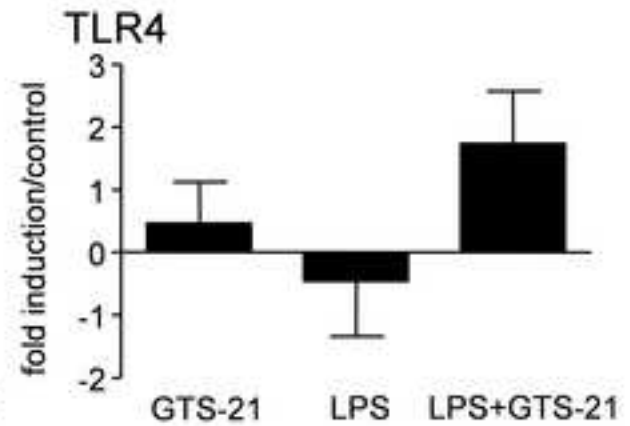
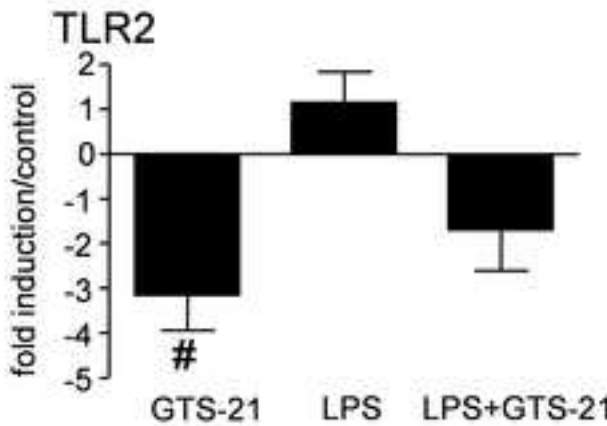
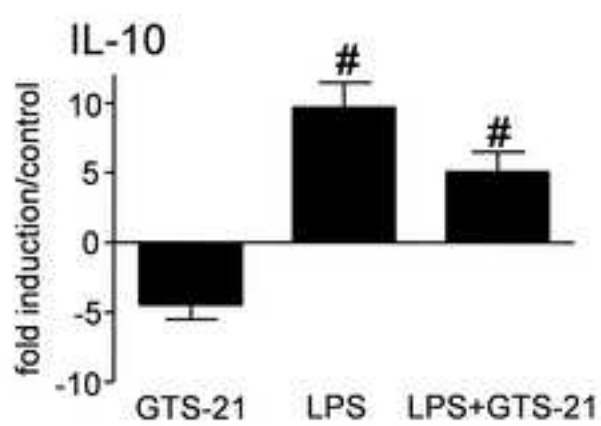
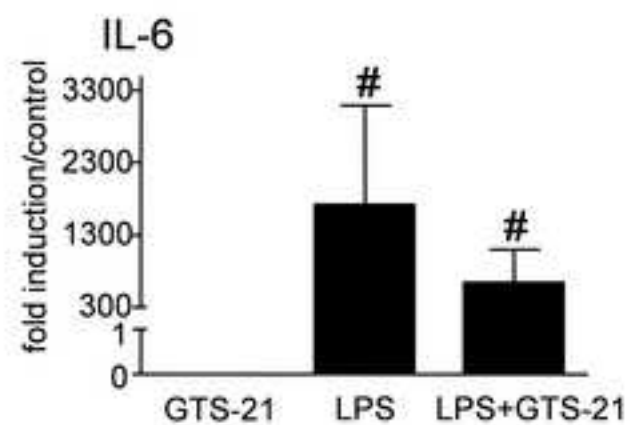
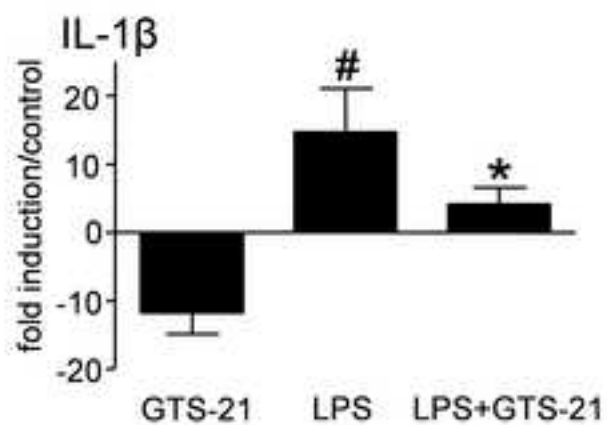
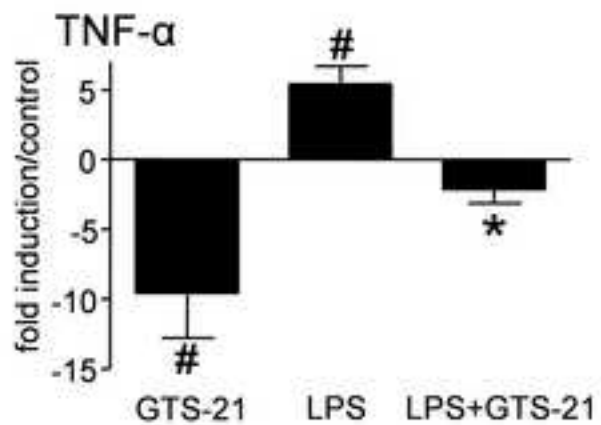




Figure 7

