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Induction of tetraspanin 13 contributes to the synergistic anti-inflammatory effects of parasympathetic and sympathetic stimulation in macrophages

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Abstract

The autonomic nervous system plays an important role in the regulation of peripheral inflammation. Sympathetic nervous activation stimulates inflammatory gene expression and cytokines, whereas parasympathetic nervous activation suppresses the production of inflammatory cytokines by immune cells. However, most studies on the relationship between the autonomic nervous system and immune processes have analyzed a single branch of the autonomic nerves in isolation. Therefore, this study aimed to examine the effects of sympathetic and parasympathetic stimulation on macrophages, which are controlled by autonomic regulation.

Macrophages were stimulated with lipopolysaccharide (LPS) to induce TNF-α. Then, the effects of β2 adrenergic receptor and α7 nicotinic acetylcholine receptor activation on TNF-α production were assessed using concentration-dependent assays. RNA-seq data were also used to identify genes whose expression was enhanced by parasympathetic and sympathetic stimulation.

The simultaneous activation of β2 adrenergic receptors and α7 nicotinic acetylcholine receptors suppressed LPS-induced TNF-α production in a concentration-dependent manner. Moreover, simultaneous activation of these

receptors had synergistic anti-inflammatory effects and induced *Tspan13* expression, thereby contributing to anti-inflammatory mechanisms in macrophages.

Our study revealed the synergistic anti-inflammatory effects of the parasympathetic and sympathetic stimulation of macrophages. Our results suggest that targeting both sympathetic and parasympathetic signaling is a promising therapeutic approach for inflammatory diseases.

Keywords: Neuro-immune system, Synergistic anti-inflammatory effect, Cholinergic anti-inflammatory pathway, α7 nicotinic acetylcholine receptors, β2 adrenergic receptor, Tspan13

Abbreviations: CAP, Cholinergic anti-inflammatory pathway; CREB, cAMP response element-binding protein; ELISA, Enzyme-linked immunosorbent assay; FPKM, Fragments per kilobase of exon per million reads mapped; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; PKA, Protein kinase A; TNF-α, Tumor necrosis factor-alpha.

Introduction

The immune system is essential for the maintenance of homeostasis. An infection triggers an immune response to inhibit foreign invasion, clean debris, and promote repair of the infected tissue. However, inflammation interferes with this process. It contributes to the development of various health conditions, such as sepsis, cancer, inflammatory bowel disease, multiple sclerosis, chronic obstructive pulmonary disease, asthma, and rheumatoid arthritis [1] [2].

The autonomic nervous system also plays an important role in maintaining homeostasis of the organism. The parasympathetic and sympathetic nervous systems cooperate to control most organs. In addition, they appropriately manage blood pressure, pulse, body temperature, fluid and electrolyte balance, metabolism, digestion, and excretion.

The immune and autonomic nervous systems are closely interrelated and exert anti-inflammatory effects. The mechanism underlying nervous system-immune system regulation has attracted significant attention, owing to its potential benefits for patients with inflammatory diseases [3] [4].

Anti-inflammatory and renal protective effects can be achieved by stimulating the vagus [5] [6] and sympathetic nerves [7] respectively. The cholinergic anti-inflammatory pathway (CAP) is triggered by activation of the centrifugal vagus nerve. Furthermore, parasympathetic signals activate noradrenergic neurons in the splenic

ganglion via synapses in the celiac, adrenal, and superior mesenteric ganglia [8]. Norepinephrine released from sympathetic splenic nerve endings activates β2 adrenergic receptors expressed on choline acetyltransferase-positive CD4+ T cells. Similarly, acetylcholine released from these cells activates α7 nicotinic acetylcholine receptors expressed on macrophages and reduces their production of pro-inflammatory cytokines [9]. In addition, β2 adrenergic receptors are expressed on macrophages, and direct stimulation of macrophages with norepinephrine suppresses the production of inflammatory cytokines [7]. The activation of immune cell-mediated anti-inflammatory pathways through autonomic nerve stimulation is considered an effective therapeutic strategy for controlling inflammatory diseases [10] including endotoxemia, inflammatory bowel disease [11] [12], rheumatoid arthritis [13], and renal disease [14].

The autonomic nervous system maintains biological homeostasis by balancing the parasympathetic and sympathetic functions. Immune cells are similarly controlled by the autonomic balance, affecting the anti-inflammatory system via the nervous and immune systems. Although the effects of vagal [5] [6] and sympathetic [7] stimulation alone have been studied, the effects of simultaneous stimulation of the vagal and sympathetic nervous systems remain unknown. Similarly, the balance of autonomic

stimulation that most effectively exerts anti-inflammatory and renoprotective effects is yet to be clarified.

In this study, we explored the effects of parasympathetic and sympathetic stimulation on macrophages under different conditions and the key factors involved in their cooperative anti-inflammatory mechanisms.

Materials and Methods

Cell culture and Reagents

RAW 264 cells (mouse macrophage cell line) were purchased from the European Collection of Authenticated Cell Cultures (ECACC 85062803). The cells were incubated in DMEM-high-glucose medium (D6429; Sigma-Aldrich, Saint Louis, MO, USA) containing 10% fetal bovine serum (F7524; Sigma-Aldrich), 10% L-glutamine (25030081; Thermo Fisher Scientific, Waltham, MA, USA), and 100 units/mL penicillin G sodium and 100 µg/mL streptomycin sulfate (15140122; Thermo Fisher Scientific). The cells were maintained at 37 °C in a humidified incubator containing 5% CO2. Passages 15–30 of the cell culture were used in this study. In all experiments, 1.5 x 10^5 cells were seeded in 24-well plates for 24 h, followed by any drug. TNF-α was induced using 100 ng/mL LPS (4391; Sigma-Aldrich). Moreover,

salbutamol (S8260; Sigma-Aldrich) and GTS-21 (SML0326; Sigma-Aldrich) were used as autonomic nerve receptor agonists.

Enzyme-linked Immunosorbent Assay (ELISA)

Mouse and human TNF-α concentrations were measured according to the manufacturer's instructions using the TNF-α Mouse Uncoated ELISA Kit with Plates (88–7324–86; Invitrogen, Waltham, MA, USA). A Synergy HTX (Agilent, Santa Clara, CA, USA) was used as an ELISA plate reader.

RNA extraction and quantitative Real-Time PCR (qPCR)

The FastGene RNA Basic Kit (FG-80006; NIPPON Genetics, Tokyo, Japan) was used for RNA isolation. Then, RNA concentration was measured based on spectrophotometric determination of the 260/280 ratio (Synergy HTX). Next, cDNA was generated from the resulting RNA using PrimeScript RT Master Mix (RR036A; Takara Bio, Shiga, Japan), as described by the manufacturer. The resultant cDNA was used to measure the relative mRNA expression level of various genes with the iTAC Universal SYBR Green Supermix (1725121; Bio-Rad, Tokyo, Japan). The gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Finally, real-time PCR tests were performed using the CFX

Connect Real-Time PCR Detection System (Bio-Rad) and data were analyzed using the ΔΔCt method. Primer sequences are shown in the Supplemental Table.

Gene Overexpression

DNA fragments encoding *Tspan13* were inserted into the pDsRed-Monomer-N In-Fusion Ready Vector (632498; Takara Bio) using an In-Fusion HD Cloning Kit (639648; Takara Bio). A vector without insertion was used as the control plasmid. Then, the plasmid was confirmed to have no mutations through sequencing. This plasmid was subsequently introduced into E. coli DH5α competent cells (9057; Takara Bio) and amplified. The *Tspan13* plasmid or control plasmid was introduced according to the manufacturer's instructions using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA, USA). RAW 264 cells were washed once with PBS (-) and resuspended in Resuspension Buffer R (Invitrogen) at a concentration of 1×10^6 cells/mL. Next, 9 µL cell suspension with 1 µL of 1 µg/µL amplified plasmid was subjected to electroporation using a 10 µL Neon tip with double pulse at a voltage of 1720 V and pulse width of 10 ms. Following electroporation, 10 µL of cell suspension was added to 500 µL of culture medium and incubated at 37 °C for 24 h. Then, the culture medium was replaced and used for experiments 24 h after transfection. The overexpression of *Tspan13* was

validated by the percentage of cells expressing the fluorescent dye DsRed and by qPCR. The efficiency of plasmid transfection by electroporation was determined using the percentage of cells expressing DsRed through fluorescence microscopy. The average percentage of cells expressing DsRed per four fields of view was used to determine transfection efficiency.

RNA sequencing

RNA sequencing and analysis were performed as previously described, with some modifications [7]. Specifically, RAW 264.7 cells were seeded for 24 h and treated with the vehicle, LPS with 100 mM GTS-21, or LPS with 100nM salbutamol for 4 h. Total RNA was isolated using the RNeasy Mini Kit (74106; Qiagen, Hilden, Germany). Poly (A)-containing mRNA molecules were isolated from the total RNA and converted to cDNA with poly (A) primers with the TruSeq RNA Sample Preparation kit v2 (Illumina). Then, high-throughput mRNA sequencing was performed with the Hiseq2500 (Illumina) system. Sequenced paired-end reads were mapped onto the mouse build mm10 reference genome using hisat2 with the parameter “-q–dta-cufflinks.” The SAM file was converted into BAM format. Furthermore, the fragments per kilobase of exon per million reads mapped (FPKM) were calculated as indicators of gene expression using cuffdiff and cummerBund.

Subsequently, the FPKM values of the genes commonly induced by GTS-21 and salbutamol administration were extracted from the FPKM. Finally, a heat map was created using log (FPKM + 1) through GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA).

Statistical analysis

Data were analyzed using one-way ANOVA and two-tailed Student's t-test, with a significant difference defined as $P < 0.05$. For multiple comparisons, one-way ANOVA was applied, followed by a post-hoc multiple-comparison test (Tukey's test). However, two-tailed Student's t-tests were used for comparisons between two groups. The experiments were repeated using a randomized complete block design. All values are presented as mean ± SEM and as individual values in dot plots. All analyses were performed using GraphPad Prism version 9 (GraphPad Software).

Results

Stimulation of α7 nicotinic acetylcholine receptor or β2 adrenergic receptor on macrophages attenuates TNF-α production induced by LPS in a dose-dependent manner.

The effects of parasympathetic or sympathetic signaling on the inflammatory response were evaluated in macrophages treated with various drug concentrations. Under all conditions, culture supernatants were collected 4 or 24 h after dosing, and TNF-α values were evaluated. GTS-21, an α7 nicotinic acetylcholine receptor agonist, inhibited TNF-α induction in a concentration-dependent manner at different time points in RAW 264 cells (Figure 1A-B). Salbutamol, a β2 adrenergic receptor agonist, also inhibited TNF-α induction in a concentration-dependent manner in the different time points (Figure 1C-D).

Spontaneous stimulation of α7 nicotinic acetylcholine and β2 adrenergic receptors on macrophages synergistically attenuates TNF-α production induced by LPS.

The effects of simultaneous parasympathetic and sympathetic stimulation on macrophages were evaluated using TNF-α 4 or 24 h after administration. When 100 μM GTS-21 and 100 nM salbutamol were simultaneously administered to LPS-stimulated macrophages, TNF-α levels were significantly lower than observed during single administration of each agonist. Furthermore, the combined low concentrations of 10 μM GTS-21 and 10 nM salbutamol substantially reduced TNF -α levels compared to 100 μM GTS-21 or 100 nM salbutamol alone (Figure 2A-B). We also

evaluated whether this synergistic effect could be achieved using cytokines, chemokines, and growth factors other than TNF-α. The expression of *Ccl2* and *Tgfb1*, which are inflammation-inducible factors, was considerably decreased by the simultaneous administration of GTS-21 and salbutamol (Figure 3A-B). In contrast, the expression of *I L10*, an anti-inflammatory factor, was significantly upregulated by the simultaneous administration of GTS-21 and salbutamol (Figure 3C).

Tspan13 expression is enhanced by either the α7 nicotinic acetylcholine or β2 adrenergic receptor agonists in LPS-stimulated macrophages.

A comprehensive genetic analysis identified genes commonly induced by sympathetic and parasympathetic stimulation in inflammatory macrophages. The enhanced genes were selected from RNA-seq data obtained from RAW 264.7 cells treated with LPS and GTS-21 or LPS and salbutamol, or commonly induced by GTS-21 and salbutamol (Supplementary Data). Genes were extracted based on the following criteria: i) FPKM value of the LPS + agonist group > 0 and ii) FPKM value of the LPS group = 0. A total of 581 and 466 genes were screened in the GTS-21 and salbutamol groups, respectively. Moreover, 72 genes were identified as being commonly induced (Figure 4A). The 72 genes are listed on a heat map based on log values (Figure 4B).

***Tspan13* expression is commonly enhanced by either the α7 nicotinic acetylcholine receptor or the β2 adrenergic receptor agonist, exerting an anti-inflammatory effect.**

RT-qPCR was performed for the top five genes commonly included in the top 10 highest FPKM values for LPS + GTS-21 and LPS + Salbutamol. Here, *Tspan13* expression was most enhanced when simultaneously treated with GTS-21 and salbutamol (Figure 5A-B). No synergistic enhancement of gene expression was observed for the other four genes (Supplementary Figure). Gene cloning and overexpression experiments were performed to evaluate the function of *Tspan13*. Subsequently, TNF-α production was induced in RAW 264 cells overexpressing *Tspan13* or the control plasmid by administering LPS. Overexpression of *Tspan13* suppressed TNF-α, an inflammatory cytokine induced by LPS (Figure 5C).

Discussion

This study aimed to investigate the relationship between autonomic regulation and acute inflammation in macrophages and to determine an appropriate sympathetic-parasympathetic balance. Our findings demonstrated the synergistic anti-inflammatory effects of parasympathetic and sympathetic stimulation in

macrophages. To the best of our knowledge, these anti-inflammatory effects have not been previously reported. We demonstrated that simultaneous β2 adrenergic and α7 nicotinic acetylcholine receptor signaling suppressed LPS-induced TNF-α production in a concentration-dependent manner in macrophages (Figure 1). Additionally, their activation had synergistic effects (Figures 2 and 3). Furthermore, the simultaneous stimulation of β2 adrenergic and α7 nicotinic acetylcholine receptors induced *Tspan13* expression, which contributed to the anti-inflammatory mechanism in macrophages (Figures 4 and 5).

The neuroimmune communication theory suggests that autonomic nervous system connections with immune cells are important drivers of inflammation. Animal and human studies have elucidated that sympathetic nervous activation stimulates pro-inflammatory gene expression and cytokines [15] [16], whereas parasympathetic nervous activation reduces inflammatory markers in immune cells [1]. Animal studies have shown the CAP where electrical or drug stimulation of the vagus nerve inhibits systemic inflammation induced by endotoxins. This reduces the production of cytokines, such as TNFα and IL6 [17]. Moreover, this neuro-immunomodulatory circuit regulates splenic macrophage cytokine release via acetylcholine-synthesizing lymphocytes close to catecholaminergic nerve terminals [8] [9] [18]. The CAP

pathway is also closely involved in the regulation of renal disorders, inflammatory bowel disease [12], and rheumatoid arthritis [13]. Stimulation of the vagus nerve and brainstem C1 neurons can protect the kidney from ischemia-reperfusion injury or reduce inflammation by activating CAP via the splenic nerve. In addition, the selective nicotinic acetylcholine receptor agonist suppresses nuclear factor kappa B activation, and reduces kidney fibrosis and inflammation by CAP activation [19].

Sympathetic and parasympathetic interactions are mediated through several pathway including both central and peripheral nerves [20] [21]. Most organs, tissues, and cells in the body function properly under the dual control of autonomic nerves. Therefore, imbalance of sympathetic and parasympathetic interactions may lead to the development of various diseases, including inflammatory, cardiovascular, metabolic, neurological, nonalcoholic fatty liver, and kidney diseases, as well as psychiatric disorders. Indeed, sympathetic and parasympathetic nerves can be simultaneously activated in specific physiological situations. For instance, sexual activity can activate both branches of the autonomic nervous system to varying degrees in response to the phases of sexual response, such as erection, gonadal secretion, ejaculation, and rhythmic vaginal contractions [22] [23]. Another example is urination, in which dual autonomic innervation of the bladder controls urinary

storage and voiding through the simultaneous stimulation of multiple autonomic receptors [24]. During exercise, the sympathetic nervous system is activated to increase heart rate and blood pressure, while the parasympathetic nervous system is activated to some extent to lower heart rate and promote digestion [25]. Notably, an exercise routine before and after meals would be compatible with this situation.

Immune cells, especially macrophages, are under the dual control of the autonomic nervous system. Macrophages express α7 nicotinic acetylcholine receptors, which are receptors for acetylcholine, a transmitter at parasympathetic nerve endings [10] [20]. In addition, they express β2 adrenergic receptors, which are receptors for adrenaline, a neurotransmitter at sympathetic nerve endings [26]. These receptors act in an anti-inflammatory manner by receiving signals from neurotransmitters.

Previous studies on the relationship between the autonomic nervous system and immune processes have largely assessed a single branch of the autonomic nervous system respectively (sympathetic or parasympathetic). However, sympathetic and parasympathetic nervous systems often function cooperatively [27]. Further evidence suggests that both branches influence immune responses. Therefore, single-branch autonomic nervous system control may not provide a comprehensive understanding

of neuroimmune connections. Thus, simultaneous sympathetic and parasympathetic influences on the immune response should be considered. However, to our knowledge, no study has focused on the effects of parasympathetic and sympathetic accentuation effects [28].

This study investigated the crucial role of the balanced coactivation of sympathetic and parasympathetic nerves in homeostasis. Using LPS-induced TNF-α production in macrophages, we showed that the autonomic receptor agonists GTS-21 and salbutamol have a concentration-dependent anti-inflammatory effect. In addition, our findings demonstrate that simultaneous administration of these agonists results in stronger anti-inflammatory effects than a single administration of each. The concurrent administration of lower concentrations of agonists suppressed inflammatory cytokines better than single doses, indicating a synergistic effect of parasympathetic and sympathetic stimulation in macrophages. Through RNA-seq analysis, we identified *Tspan13* as a gene whose expression is commonly enhanced by both parasympathetic and sympathetic stimulation. Furthermore, we found that *Tspan13* enhances synergistic anti-inflammatory effects in macrophages, suggesting that autonomic nerve stimulation works to augment the effects of each agonist in macrophages. *Tspan13* is a member of the tetraspanin family, which plays important

roles in cell-cell adhesion, interaction with the extracellular matrix, cell proliferation, and apoptosis. Although *Tspan13* is involved in the regulation of breast [29] and colon [30] cancer growth, no studies have proposed a link to immune cells or autonomic nerve stimulation. Therefore, our findings could be a key to elucidating unidentified pathways of autonomic signaling in immune cells.

Our study was limited to *in vitro* experiments, and the synergistic effects observed in macrophages may not necessarily translate to *in vivo* conditions. Additionally, this study focused solely on macrophages, and further investigation of the mechanism underlying autonomic nerve stimulation in inflammation could include the depletion of macrophages and CD4+ T cells. Our study also did not elucidate how parasympathetic and sympathetic stimulation alter macrophage characteristics or whether the synergistic effects result from intracellular signaling or cell-cell interactions. β2 adrenergic receptor stimulation activates intracellular protein kinase A (PKA) activation [31]. Activated PKA phosphorylates the nuclear transcription factor cAMP response element-binding protein (CREB), which regulates the transcription of factors involved in inflammatory responses [32]. In contrast, the α7 nicotinic acetylcholine receptor stimulation causes changes in intracellular Ca concentration and regulates CREB phosphorylation [33] [34]. Therefore, a pathway

involving CREB was postulated as a common factor in the synergistic anti-inflammatory effects. However, we could not fully elucidate the intracellular signaling involved. As *Tspan13* acts on calcium channels in the plasma membrane and regulates intracellular Ca concentration, it may play a role in intracellular signaling [35].

Our study revealed the synergistic anti-inflammatory effects of the parasympathetic and sympathetic stimulation of macrophages. Our results suggest that targeting both sympathetic and parasympathetic signaling is a promising therapeutic approach against inflammatory diseases.

Author contributions

Ryusuke Umene, Tsuyoshi Inoue, Yasuna Nakamura, and Chia-Hsien Wu designed the research studies; Ryusuke Umene conducted the main experiments, analyzed the data, and wrote the manuscript draft; Tomoya Nishino and Tsuyoshi Inoue supervised the study and revised the manuscript. All authors approved the final version of the manuscript.

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Disclosures

The authors declare that no conflict of interest exists.

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Figure legends

Figure 1. TNF-α induction by LPS was suppressed by GTS-21 or salbutamol administration. Parasympathetic or sympathetic stimulation produced a dose-dependent anti-inflammatory effect in macrophages.

(A), (B) GTS-21 (α7 nicotinic acetylcholine receptor agonist) inhibited TNF-α production in a dose-dependent manner. (C), (D) Salbutamol (β2 adrenergic receptor agonist) inhibited TNF-α production in a dose-dependent manner. TNF-α concentration was analyzed by using ELISA. ***P,0.001; ****P,0.0001.

Figure 2. Simultaneous sympathetic and parasympathetic stimulation synergistically suppressed LPS-induced TNF-α production in macrophages, even at lower drug concentrations.

(A), (B) Simultaneous administration of GTS-21 and salbutamol suppressed TNF-α more than a single administration of either. Considerable anti-inflammatory effects were also obtained with 10 μM GTS-21 + 10 nM salbutamol. The lower concentration combination was compared to 100 μM GTS-21 or 100 nM salbutamol administration. TNF-α concentrations were analyzed using ELISA. *P < 0.05; **P < 0.01; ***P,0.001; ****P,0.0001.

Figure 3. Simultaneous parasympathetic and sympathetic stimulation synergistically regulated the expression of other cytokines or chemokines.

(A), (B) The expression of *Ccl2* and *Tgfb1*, inflammatory chemokine/growth factors, was synergistically suppressed by simultaneous administration of 100 μM GTS-21 and 100 nM salbutamol. (C) The expression of *I L10*, an anti-inflammatory cytokine/growth factor, was synergistically enhanced. Gene expression is shown as a relative ratio of each group concerning the expression level of the LPS-only administration group. n.s., not significant; *P < 0.05; **P < 0.01; ***P,0.001; ****P,0.0001.

Figure 4. Gene expression profile.

(A) RNA-seq data analysis for extraction of commonly upregulated genes by simultaneous parasympathetic and sympathetic stimulation. Shown are numbers of upregulated genes 4 h after LPS, GTS-21, and salbutamol stimulation. Selection of upregulated genes was based on FPKM values in four groups: control (without LPS and agonist), LPS-treated, and LPS + agonist (GTS-21 or salbutamol). (B) A list of 72 common upregulated genes. Genes were displayed on a heatmap comparing LPS + Vehicle and LPS + agonists based on log (FPKM + 1). We showed LPS + Vehicle for salbutamol as the comparison group for LPS + salbutamol and LPS + Vehicle for GTS-21 as the comparison group for LPS + GTS-21 from

each RNA-seq data. Values are expressed as the difference from the log mean of the four groups for each gene.

Figure 5. *Tspan13* modulated synergistic anti-inflammatory effects via the autonomic nervous system.

(A), (B) The synergistic enhancement of *Tspan13* expression in LPS-induced macrophages via treatment with GTS-21 and salbutamol for 4 or 24 h, was verified by RT-qPCR. (C) Overexpression of *Tspan13* in RAW 264 cells using electroporation. No difference in efficiency was observed between the control and *Tspan13* plasmids. LPS-induced TNF-α production in RAW 264 cells overexpressing *Tspan13* resulted in decreased NF-α production after 24 h compared to macrophages transfected with the control plasmid. TNF-α concentrations were analyzed using ELISA. ***P,0.001; ****P,0.0001.

Supplementary Figure. Expression of selected genes from RNA-seq analysis in inflammatory macrophages.

RT-qPCR was performed on the top FPKM-valued genes including *Cox7a1*, *Moap1*, *Cox6a2*, *Cib2*. Administration of GTS-21 and salbutamol for 4 h did not substantially enhance any gene expression in inflammatory macrophages. n.s., not significant.

Figure

Figure 1

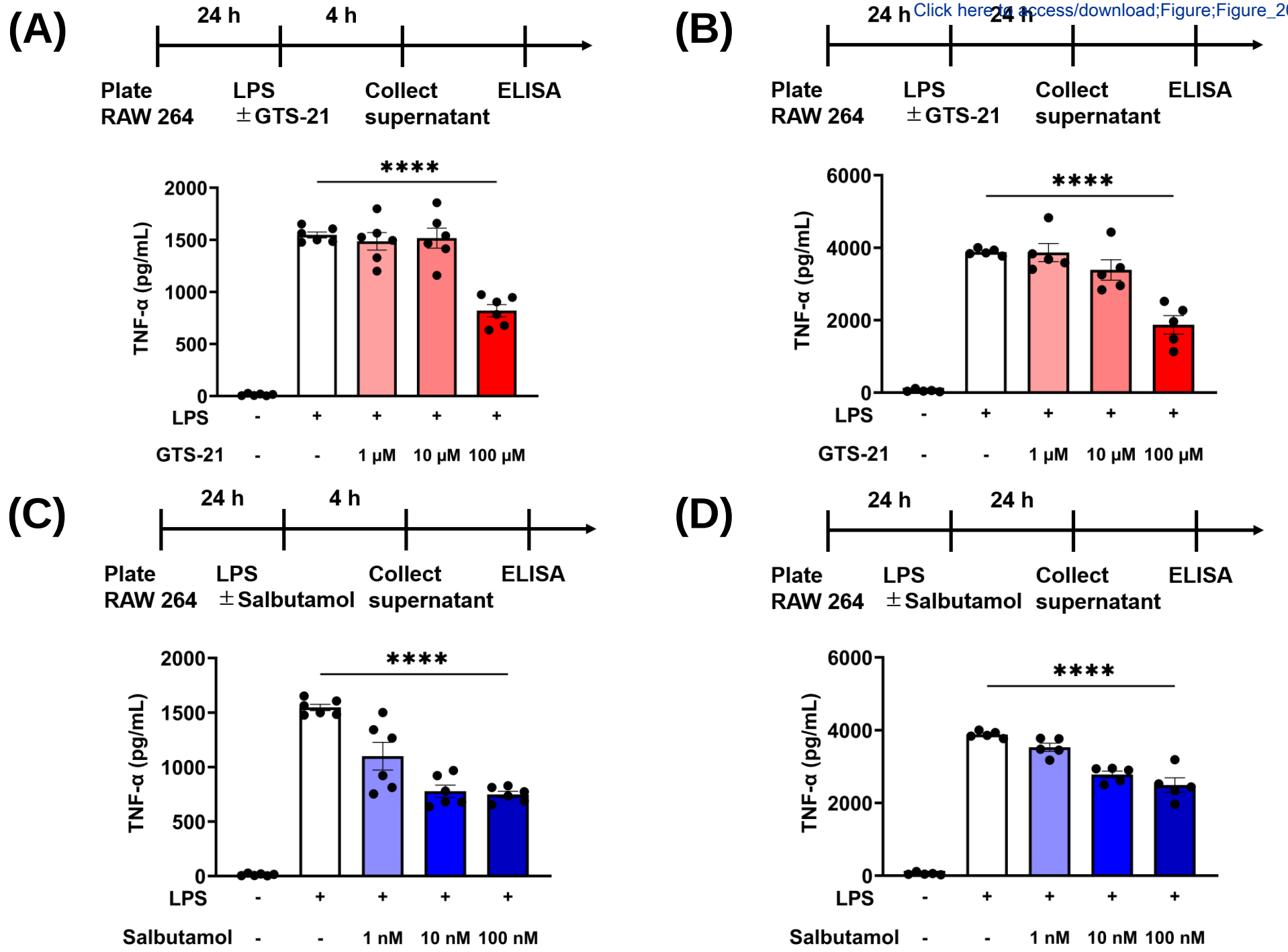


Figure 2

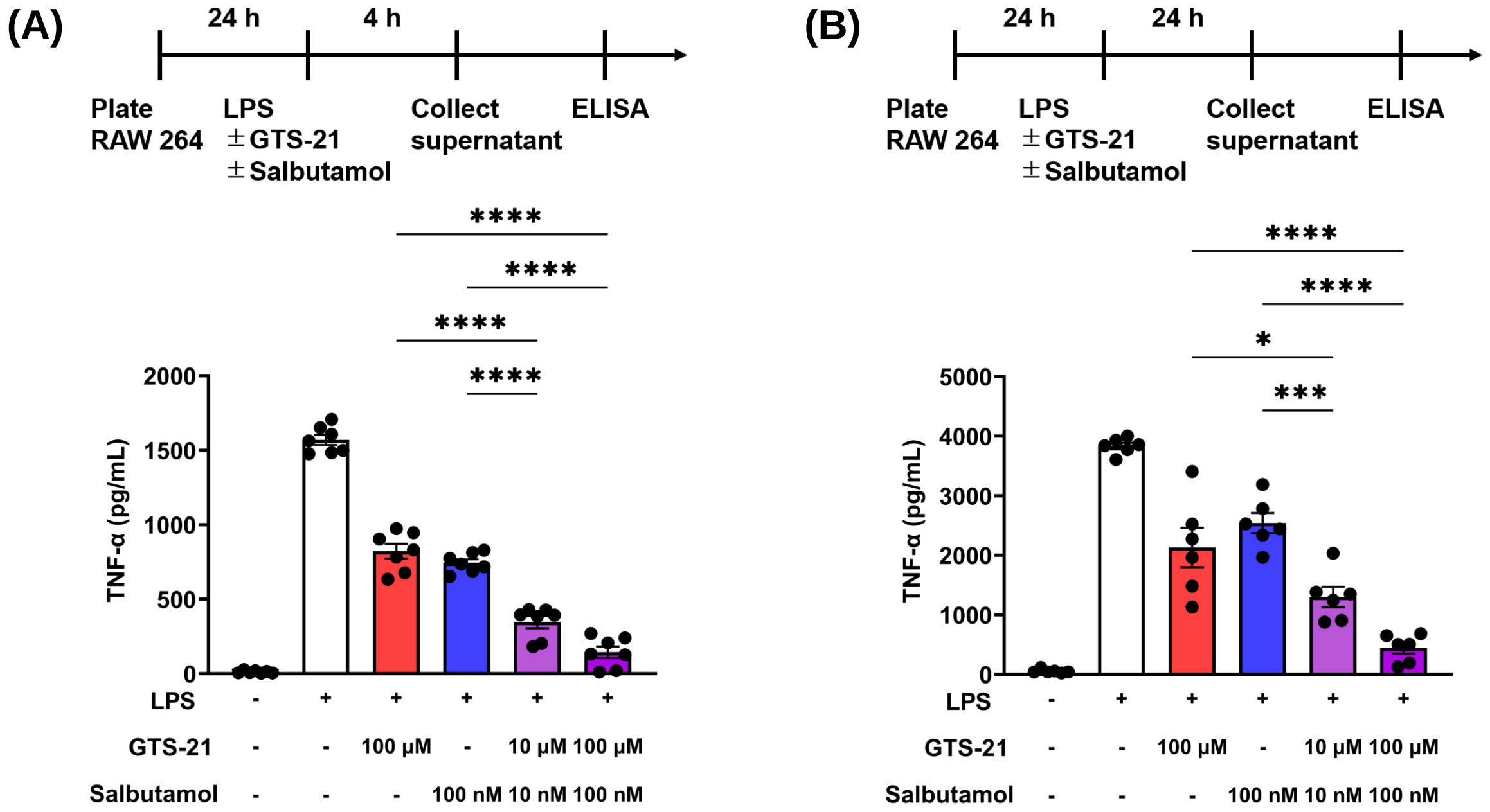
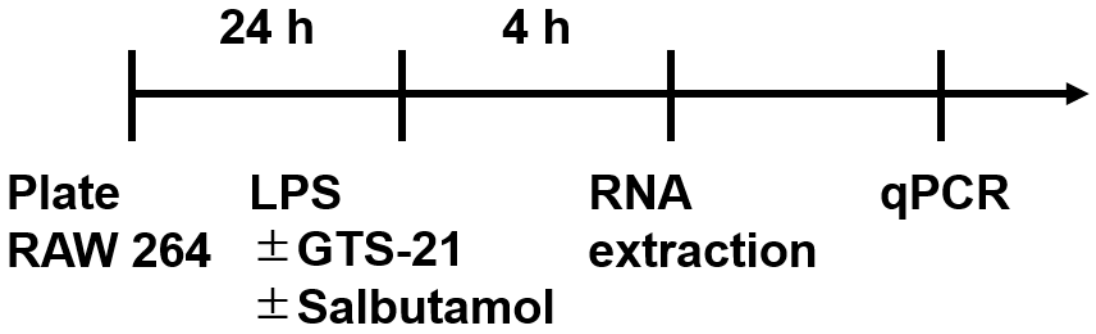

(A)
24 h
4 h
Plate RAW 264
LPS ±GTS-21 ±Salbutamol
Collect supernatant
ELISA
****
****
****
****
TNF-α (pg/mL)
2000
1500
1000
500
0
LPS - + + + + +
GTS-21 - - 100 μM - 10 μM 100 μM
Salbutamol - - - 100 nM 10 nM 100 nM
(B)
24 h
24 h
Plate RAW 264
LPS ±GTS-21 ±Salbutamol
Collect supernatant
ELISA
****
****
*
***
TNF-α (pg/mL)
5000
4000
3000
2000
1000
0
LPS - + + + + +
GTS-21 - - 100 μM - 10 μM 100 μM
Salbutamol - - - 100 nM 10 nM 100 nM

Figure 3


24 h
4 h
Plate
RAW 264
LPS
±GTS-21
±Salbutamol
RNA
extraction
qPCR

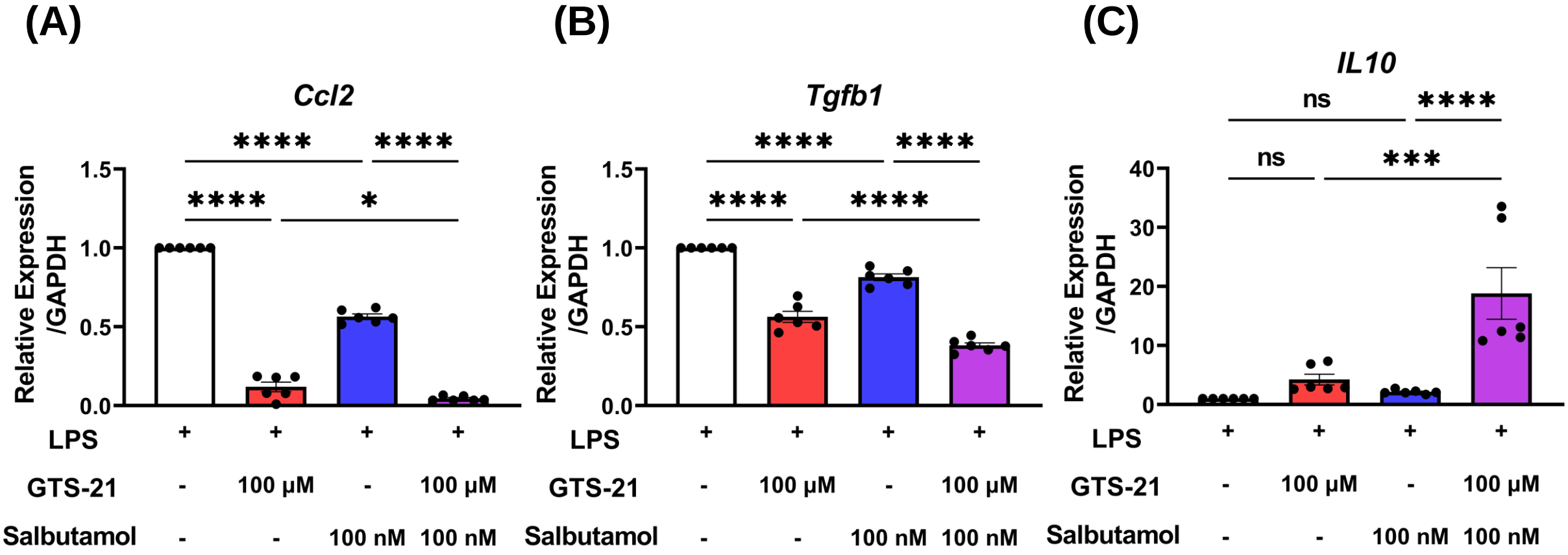
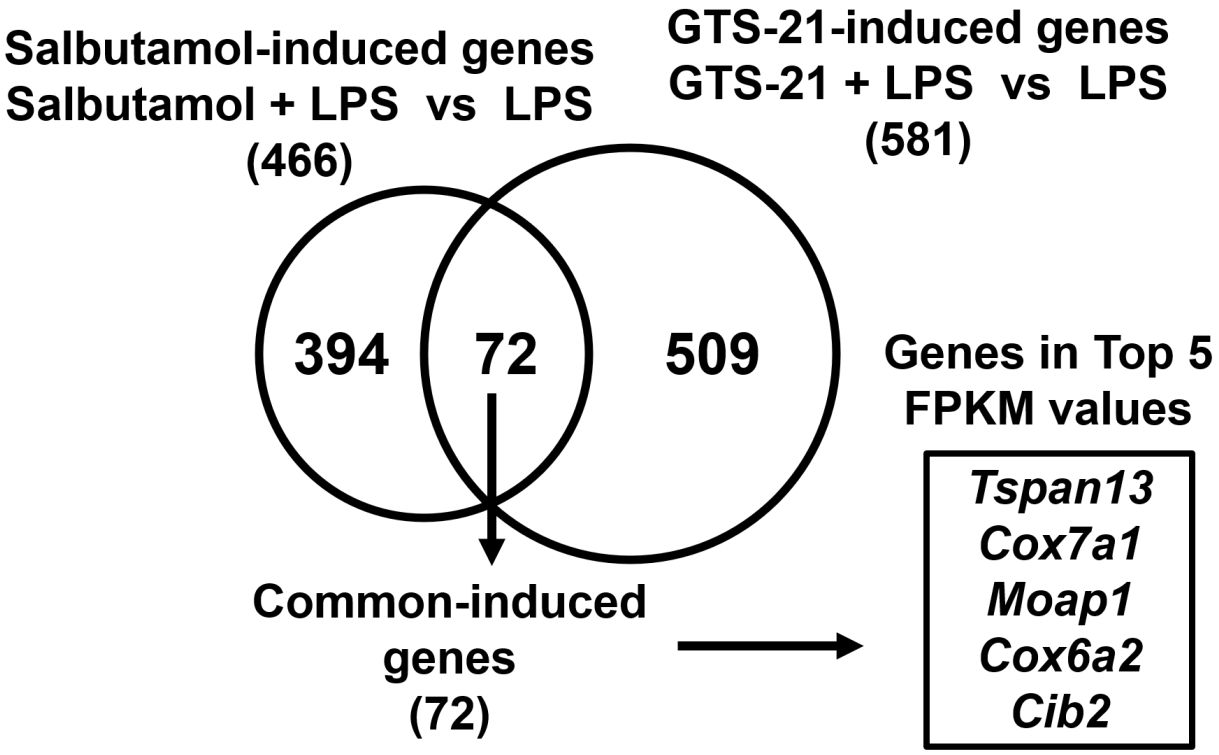

(A)
Ccl2
Relative Expression /GAPDH
1.5
1.0
0.5
0.0
****
****
****
*
(B)
Tgfb1
Relative Expression /GAPDH
1.5
1.0
0.5
0.0
****
****
****
****
(C)
IL10
Relative Expression /GAPDH
40
30
20
10
0
ns
****
ns
***
LPS + + + +
GTS-21 - 100 μM - 100 μM
Salbutamol - - 100 nM 100 nM

Figure 4

(A)


Salbutamol-induced genes
Salbutamol + LPS vs LPS
(466)
GTS-21-induced genes
GTS-21 + LPS vs LPS
(581)
394
72
509
Common-induced genes
(72)
Genes in Top 5 FPKM values
Tspan13
Cox7a1
Moap1
Cox6a2
Cib2

(B)

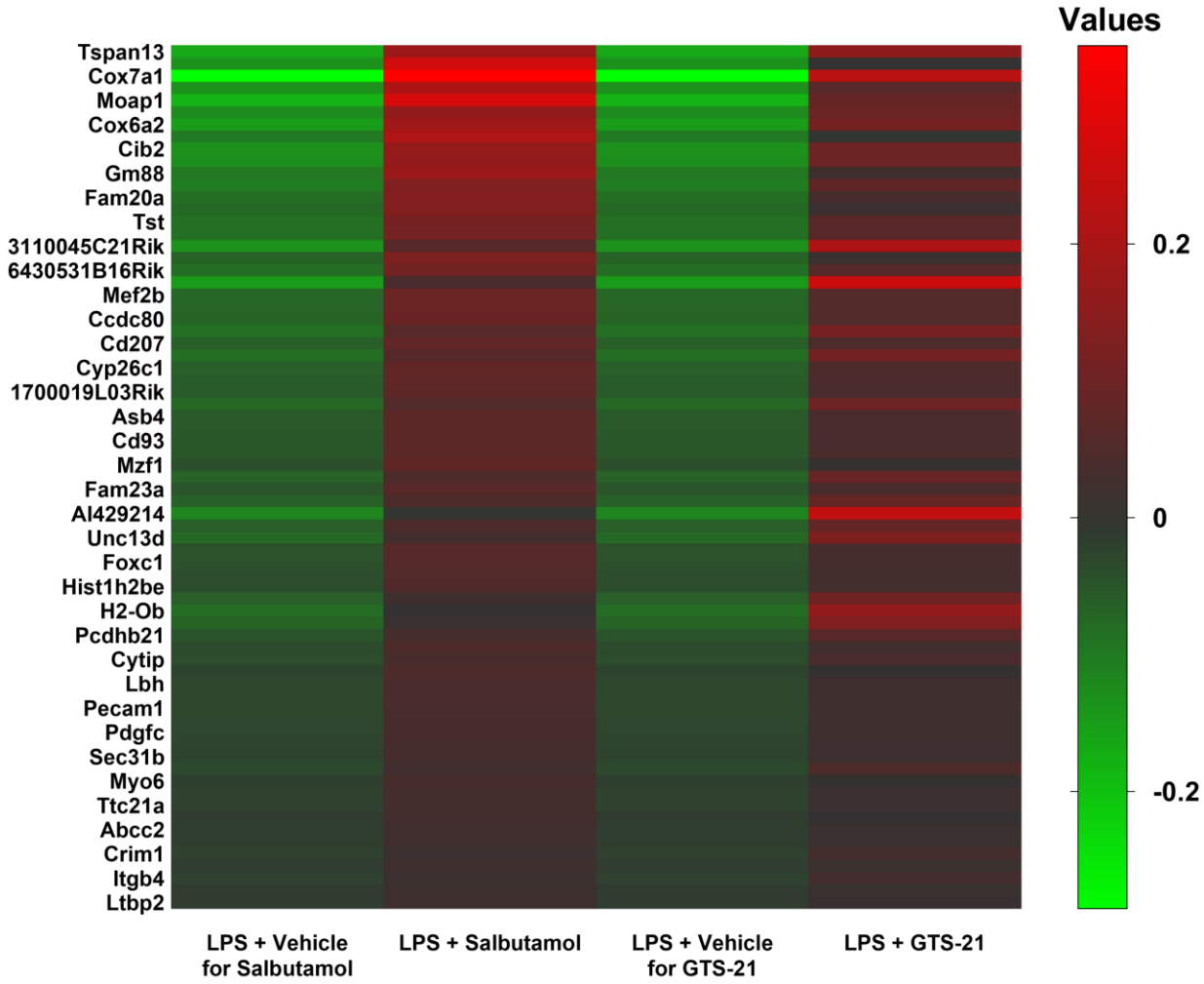
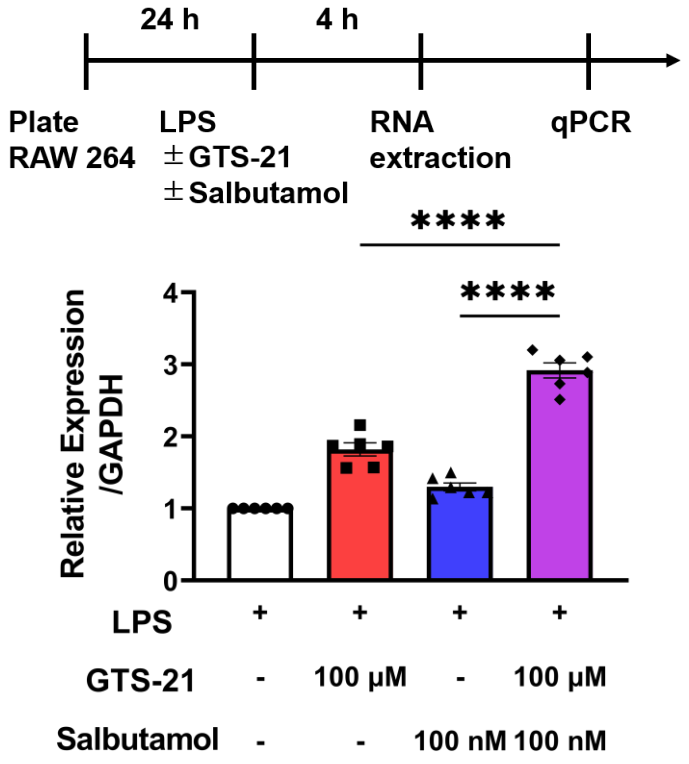
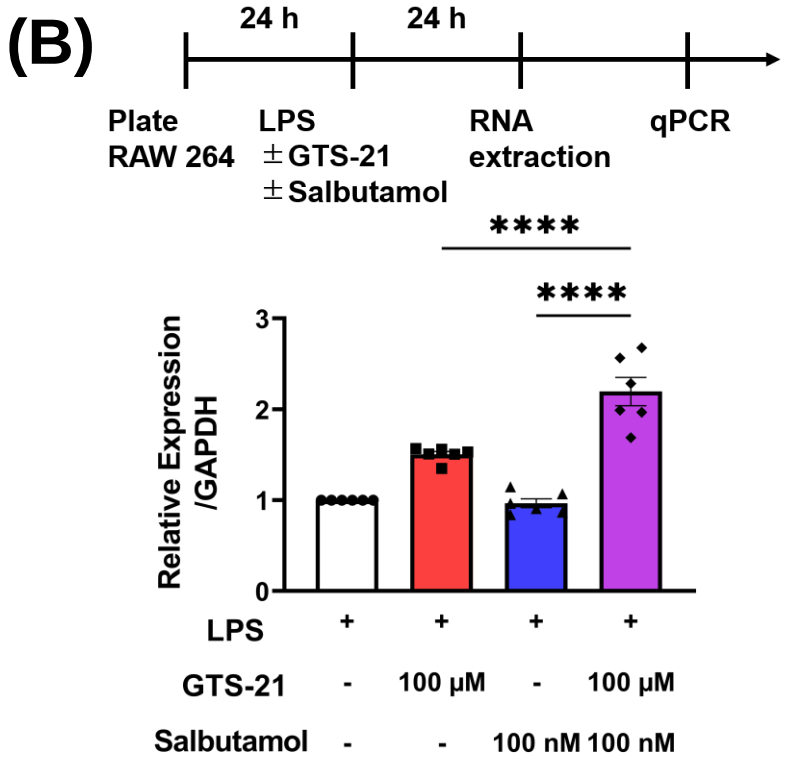
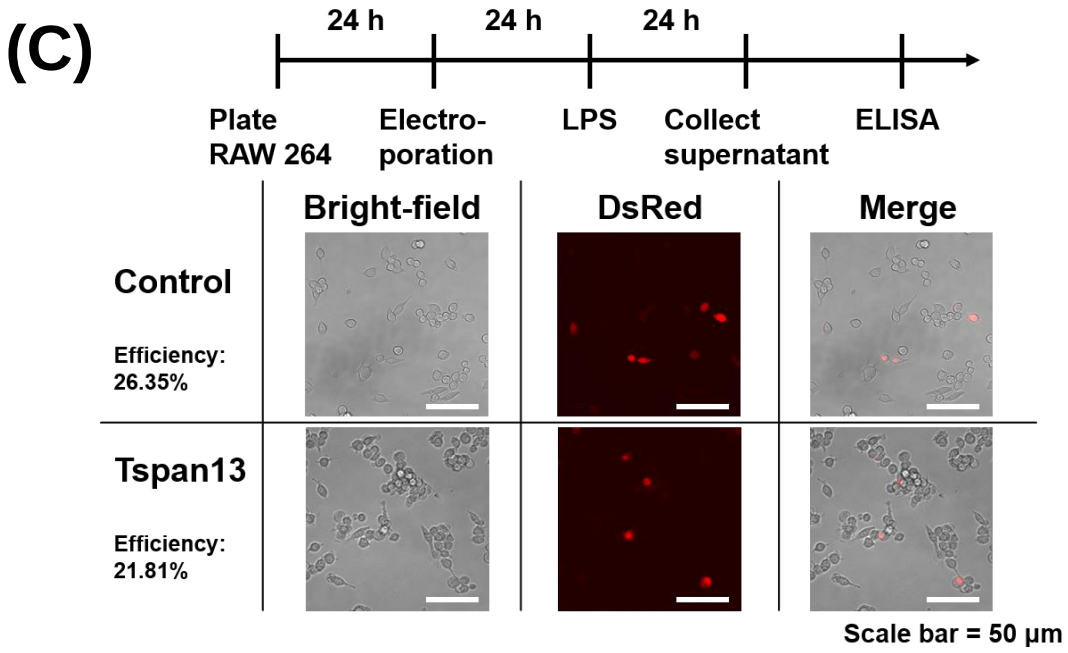
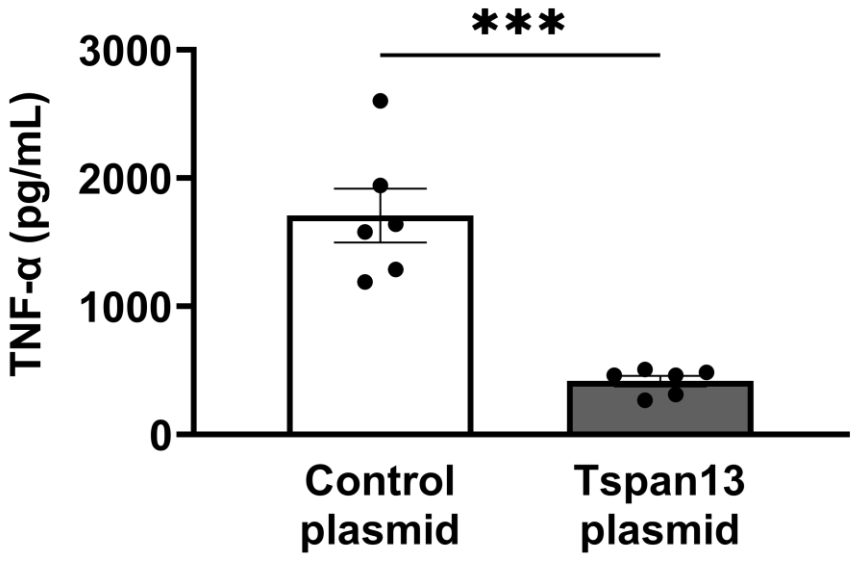

Values
0.2
0
-0.2
Tspan13
Cox7a1
Moap1
Cox6a2
Cib2
Gm88
Fam20a
Tst
3110045C21Rik
6430531B16Rik
Mef2b
Ccdc80
Cd207
Cyp26c1
1700019L03Rik
Asb4
Cd93
Mzf1
Fam23a
AI429214
Unc13d
Foxc1
Hist1h2be
H2-Ob
Pcdhb21
Cytip
Lbh
Pecam1
Pdgfc
Sec31b
Myo6
Ttc21a
Abcc2
Crim1
Itgb4
Ltbp2
LPS + Vehicle for Salbutamol
LPS + Salbutamol
LPS + Vehicle for GTS-21
LPS + GTS-21

Figure 5

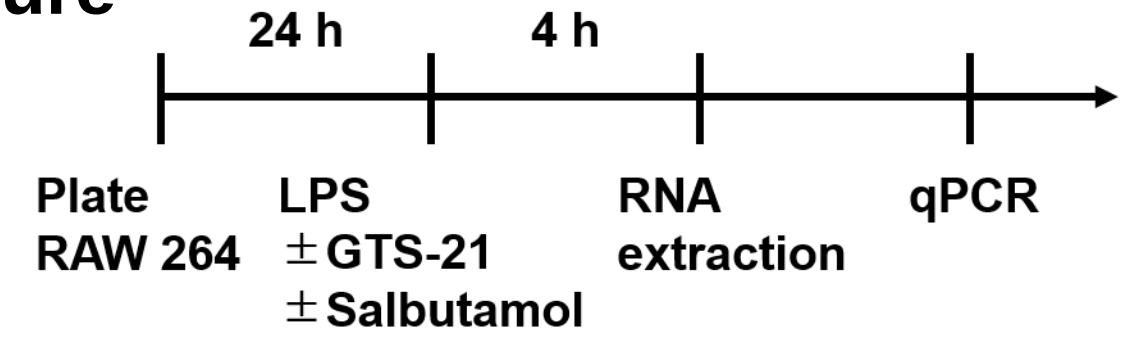

(A)
24 h
4 h
Plate RAW 264
LPS ±GTS-21 ±Salbutamol
RNA extraction
qPCR
****
****
Relative Expression /GAPDH
0
1
2
3
4
LPS + + + +
GTS-21 - 100 μM - 100 μM
Salbutamol - - 100 nM 100 nM

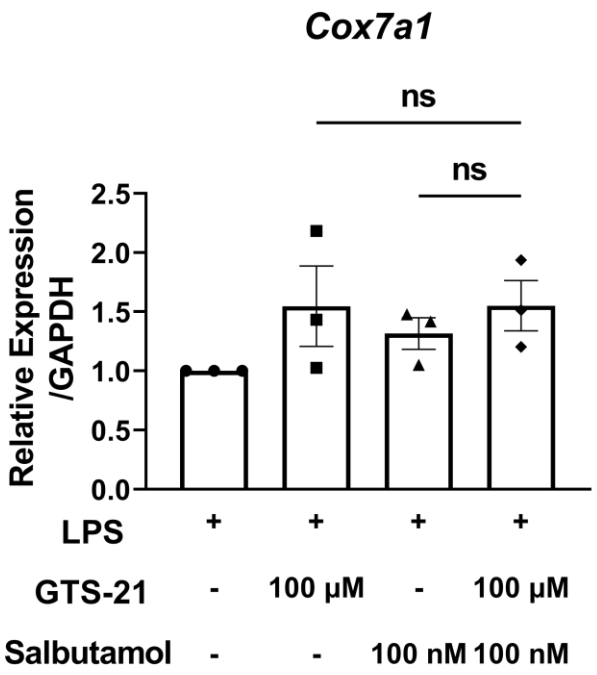

(B)
24 h
24 h
Plate RAW 264
LPS ±GTS-21 ±Salbutamol
RNA extraction
qPCR
****
****
Relative Expression /GAPDH
0
1
2
3
LPS + + + +
GTS-21 - 100 μM - 100 μM
Salbutamol - - 100 nM 100 nM

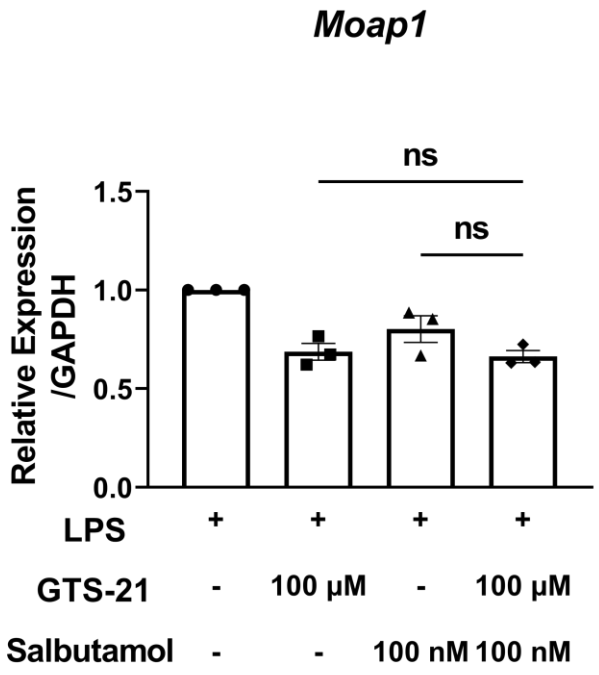

(C)
24 h
24 h
24 h
Plate RAW 264
Electro-poration
LPS
Collect supernatant
ELISA
Bright-field
DsRed
Merge
Control
Efficiency: 26.35%
Tspan13
Efficiency: 21.81%
Scale bar = 50 μm

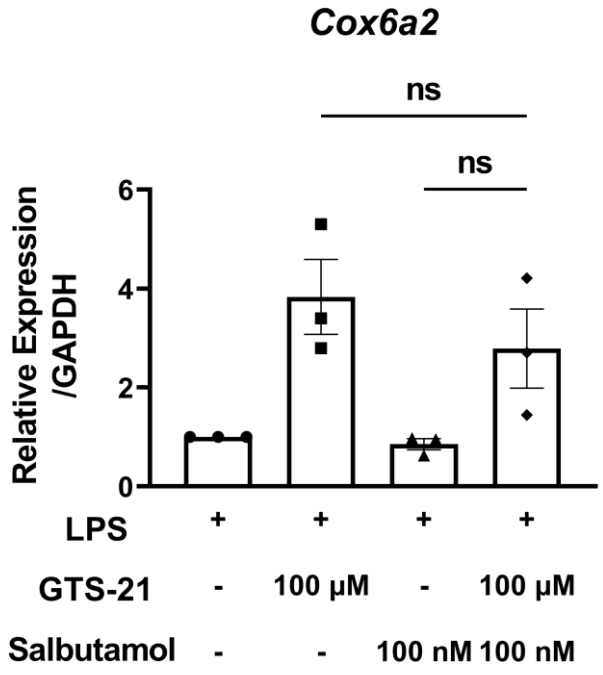

***
TNF-α (pg/mL)
0
1000
2000
3000
Control plasmid
Tspan13 plasmid

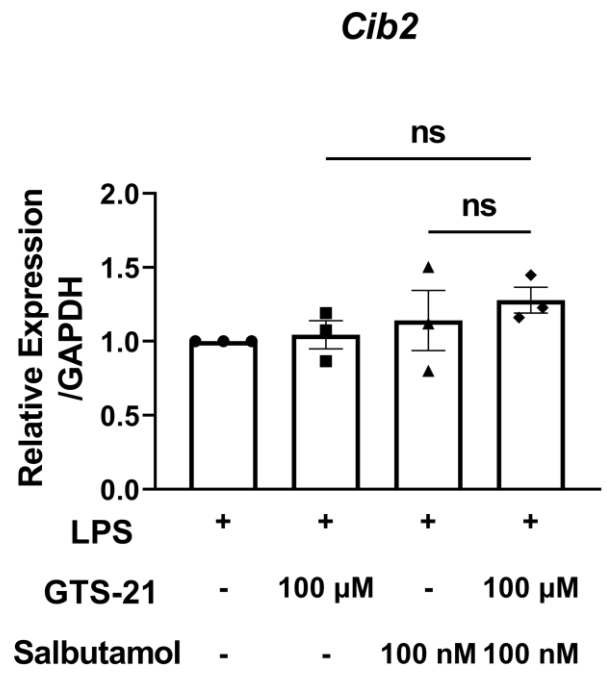
Supplementary figure











Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Tsuyoshi Inoue reports financial support was provided by Japan Society for the Promotion of Science. Tsuyoshi Inoue reports financial support was provided by Japan Agency for Medical Research and Development. Tsuyoshi Inoue reports financial support was provided by Japan Science and Technology Agency. Tsuyoshi Inoue reports financial support was provided by Public Interest Incorporated Foundation MSD Life Science Foundation. Tsuyoshi Inoue reports financial support was provided by Salt Science Research Foundation. Tsuyoshi Inoue reports financial support was provided by Smoking Research Foundation. Tsuyoshi Inoue reports financial support was provided by Takeda Science Foundation. Tsuyoshi Inoue reports financial support was provided by Astellas Foundation for Research on Metabolic Disorders. Tsuyoshi Inoue reports financial support was provided by Suzuken Memorial Foundation. Tsuyoshi Inoue reports financial support was provided by Tokyo Biochemical Research Foundation. Tsuyoshi Inoue reports financial support was provided by Japan Kidney Association and Nippon Boehringer Ingelheim Joint Research Project. Tsuyoshi Inoue reports financial support was provided by Naito Foundation. Tsuyoshi Inoue reports financial support was provided by Daiichi Sankyo Foundation of Life Science. Tsuyoshi Inoue reports financial support was provided by Uehara Memorial Foundation. Tsuyoshi Inoue reports financial support was provided by Terumo Life Science Foundation. Tomoya Nishino reports financial support was provided by Japan Society for the Promotion of Science. Kumiko Muta reports financial support was provided by Japan Society for the Promotion of Science.