

# Bromamine T, a stable active bromine compound, prevents the LPS-induced inflammatory response

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**Abstract.** Inflammation is the most common cause of most acute and chronic debilitating diseases. Towards unveiling novel therapeutic options for patients with such complications, N-bromotaurine (TauNHBr) has emerged as a potential anti-inflammatory agent; however, its therapeutic efficacy is hindered due to its relatively poor stability. To address this challenge, the present study focused on examining the effects of a stable active bromine compound, named bromamine T (BAT). The present study examined the protective properties of BAT against lipopolysaccharide (LPS)-mediated inflammation *in vitro*, by using LPS-stimulated murine J774.A1 macrophages (Mφs), as well as *in vivo*, by using a murine LPS-mediated air-pouch model. Additionally, its efficacy was compared with that of taurine, a known potent anti-inflammatory molecule. In LPS-stimulated J774.A1 Mφs, BAT and taurine were very effective in reducing the secretion of pro-inflammatory mediators. The *in vitro* experiments indicated that LPS-mediated inflammation was attenuated due to the protective properties of BAT and of taurine, probably through the inhibition of phosphorylated p65 NF-κB subunit (Ser 536) nuclear translocation. The *in vivo* experiments also revealed that BAT and taurine inhibited LPS-mediated inflammation by reducing total cell/polymorphonuclear cell (PMN) infiltration in the air-pouch and by decreasing pouch wall thickness. The analysis of exudates obtained from pouches highlighted that the inhibitory effects of BAT and taurine on the secretion of pro-inflammatory cytokines were similar to those observed *in vitro*. Notably, the effect of BAT at the highest concentration tested was superior to that of taurine

at the highest concentration. Taken together, the findings of the present study indicate that BAT prevents the LPS-induced inflammatory response both *in vitro* and *in vivo*.

## Introduction

The innate immune response is primarily elicited to protect cells from infection or tissue injury, within minutes to hours. However, when the onset of inflammation continues for longer periods of time, chronic inflammation occurs and this contributes to the pathogenesis of diseases such as arthritis, cancer, cardiovascular and neurodegenerative diseases (1). The management of inflammatory diseases is often combatted with the use of non-steroidal anti-inflammatory drugs (NSAIDs), acting through the inhibition of cyclo-oxygenase 2 (COX-2) (2,3), corticosteroids (4), or immunomodulatory drugs (5). The therapeutic benefits of NSAIDs are counteracted by multiple side-effects that arise following chronic use or high doses. The chronic use of NSAIDs is related to cardiovascular and gastrointestinal toxicities (6), while high doses of these factors may cause renal dysfunction (7). On the other hand, prolonged corticosteroid use has several side-effects, such as metabolic dysregulation, osteoporosis, glaucoma etc. (8), while immunomodulatory drugs cause immunosuppression, that is often associated with infections (9).

Taurine is a non-essential amino acid that is abundant in all mammalian tissues (10,11) and plays a significant role in homeostasis, since it is involved in the regulation of numerous vital cellular processes (osmoregulation, protein phosphorylation, membrane stability, bile acid conjugation, neuromodulation, maintenance of calcium concentration and the detoxification of xenobiotics) (12), thus ensuring the proper function of various organs. Furthermore, it has been demonstrated that taurine is effective against multiple types of inflammatory injury (13), including spinal cord injury (14), hepatic ischemia-reperfusion (15), lung injury (16,17), ischemic stroke (18), lipopolysaccharide (LPS)-induced acute lung injury (16), 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (19) and dextran sodium sulfate (DSS)-induced colitis (20,21). The anti-inflammatory effects of taurine are usually attributed to its antioxidant effects (13),

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which are evidenced by the inhibition of lipid peroxidation (22) and by its membrane-stabilizing capacity (23).

During inflammation, taurine can be conjugated with hypobromous acid (HOBr), generating *N*-bromotaurine (TauNHBr) from either activated neutrophils or eosinophils (24,25). TauNHBr has been proven to attenuate inflammatory conditions in a more efficient manner than that elicited by taurine (26). The anti-inflammatory capacity of TauNHBr is highly associated with i) its capacity to hinder phagocyte function and to impair the respiratory burst (26); ii) the reduction of various pro-inflammatory mediators [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-6, IL-1 $\beta$ , IL-8 and IL-12], nitric oxide (NO), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and chemokines in both rodent and human leukocytes (27-31); iii) the inhibition of nuclear factor  $\kappa$ B (NF- $\kappa$ B) activity (27); and iv) the induction of heme-oxygenase-1 (HO-1) expression in various cell types (3), including J774A.1 macrophages (M $\phi$ s) (32) or rheumatoid arthritis fibroblast-like synovial cells (33). From a clinical perspective, TauNHBr has been proven to be beneficial in the treatment of acne vulgaris (34), herpes zoster (35), in the local treatment of periodontal diseases, and in the elimination of oral biofilm pathogens (36).

However, TauNHBr exhibits weak therapeutic effectiveness *in vivo* due to its poor stability and its rapid degradation in the blood (37). To overcome this issue, bromamine T (BAT; chemical structure, [CH<sub>3</sub>-C<sub>6</sub>H<sub>4</sub>-SO<sub>2</sub>-N-Br]<sup>+</sup>Na<sup>+</sup> x 2H<sub>2</sub>O), which is a stable active bromine compound (38), was synthesized (39). BAT, the sodium salt of *N*-bromo-4-toluenesulfonamide, was the result of the reaction of chloramine T with elemental bromine at the Institute of Hygiene and Medical Microbiology of the Medical University of Innsbruck by Professor Dr Waldemar Gottardi according to the method of Nair *et al* (39). BAT has been illustrated to exert anti-inflammatory and anti-microbial effects to a similar extent as those of TauNHBr (38). In particular, BAT retains potent anti-microbial, anti-inflammatory and anti-cancer potential *in vitro* (38,40). The anti-inflammatory effect of BAT is mediated by reducing the protein expression levels of TNF- $\alpha$ , IL-6 and IL-12p40, with its most prominent effect emerging in the inhibition of IL-12p40 protein expression, as confirmed by enzyme-linked immunosorbent assay (ELISA) experiments (38).

BAT has been successfully used in a case of multi-bacterial scalp infection (41). Furthermore, BAT has also been used in patients with acne vulgaris, exerting comparable effects to those of clindamycin, a commonly used anti-microbial (34).

The aim of the present study was to examine the protective properties of BAT against LPS-mediated inflammation *in vitro*, by using murine J774.A1 M $\phi$ s, and *in vivo*, by using a murine LPS-mediated air-pouch model. Herein, the putative effects of BAT were compared with those of taurine.

## Materials and methods

**Agents.** Taurine (chemical structure, C<sub>2</sub>H<sub>7</sub>NO<sub>3</sub>S or NH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub>H) was purchased from AppliChem ITW Companies (Taurine BioChemica, A1140,1000, Lot 3M004589). The purity was >99% and the molecular weight was 125.15 g/mol. BAT (chemical structure, C<sub>7</sub>H<sub>7</sub>BrNNaO<sub>2</sub>S x 2H<sub>2</sub>O or [CH<sub>3</sub>-C<sub>6</sub>H<sub>4</sub>-SO<sub>2</sub>-N-Br]<sup>+</sup>Na<sup>+</sup> x 2H<sub>2</sub>O) was kindly provided by the laboratories of Professor Dr Waldemar

Gottardi and Professor Dr Markus Nagl at the Institute of Hygiene and Medical Microbiology of the Medical University of Innsbruck (Bromamine T, Lot no 29/06/2016). BAT (*N*-bromo-4-toluenesulfonamide sodium salt, BAT x 2H<sub>2</sub>O) was prepared by the reaction of chloramine T with elemental bromine according to the method of Nair *et al* (39). The specifications were a potency of 95.8%, a bromine content of 24.83% and an effective molecular weight of 322.02 g/mol. As a result, Nair *et al* (39) demonstrated the synthesis of BAT and Walczewska *et al* (38) demonstrated that BAT was a stable active bromine compound.

**Cells and cell culture.** The J774.A1 murine M $\phi$ s were obtained from the laboratories of Professor Dr Waldemar Gottardi and Professor Dr Markus Nagl at the Institute of Hygiene and Medical Microbiology of the Medical University of Innsbruck. All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 41966-029; Gibco; Thermo Fisher Scientific, Inc.). All culture media were supplemented with 10% fetal bovine serum (FBS, 16000-044; Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin) (full DMEM). The cells were grown in a humidified incubator with 5% CO<sub>2</sub> at 37°C. The J774.A1 M $\phi$ s were pre-incubated with various concentrations of the tested agents (BAT or taurine), diluted in pure DMEM without FBS for 1.5 h at 37°C. Subsequently, 100 ng/ml of LPS were diluted in full DMEM and were administered to the M $\phi$ s for an additional 24 h at 37°C, as previously described (38). Data are presented as the means  $\pm$  SEM of 3 independent experiments.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA isolation from the cultured M $\phi$ s, as well as exudates derived from the pouches of LPS-exposed mice (model described below), was carried out using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA quality was confirmed, through the presence of 28s, 18s and 5s RNA ribosomal subunits in agarose gel electrophoresis and taking into consideration the ratio of OD260/280 to be approximately 2 as well as the ratio of OD260/230 to be approximately 2.2 in all samples.

Reverse transcription was performed from 1.0  $\mu$ g of purified RNA using the SuperScript Reverse Transcriptase II (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Quantification at the mRNA level was conducted in 96-well polymerase chain reaction (PCR) plates using a Bio-Rad iCycler and the iQ5 Multicolor Real-Time polymerase chain reaction (RT-PCR) detection system (Bio-Rad Laboratories, Inc.). Each reaction contained 1X IQ SYBR-Green Supermix (Bio-Rad Laboratories, Inc.) and 150 nmol/l of each primer. All genes were tested in triplicate. The results were analyzed on the iCycler software. Values were normalized against  $\beta$ -actin. The relative quantification of complementary DNA (cDNA) was performed according to the  $\Delta\Delta$ Cq method (42). Selected primers are presented in Table SI.

**Immunofluorescence.** To detect the putative suppressive effect of BAT or taurine on the nuclear translocation of phosphorylated NF- $\kappa$ B p65 protein (Ser 536), immunofluorescence experiments were performed. In detail, 24-well plates were

seeded overnight with  $4.5 \times 10^4$  cells/well. The cells were pre-treated with 0.1–0.5 mM BAT or 100 mM taurine diluted in pure DMEM without FBS for 1.5 h at 37°C. Subsequently, LPS (100 ng/ml) diluted in full DMEM was added to each well, and the cells were incubated for 24 h at 37°C, as previously described (38). The Mφs were washed with 1X phosphate-buffered saline (PBS) for 5 min and fixed with 4% paraformaldehyde (PFA) at room temperature for 10 min. The fixed cells were permeabilized with 1X PBS/0.5% Triton X-100 for 5 min at 4°C and blocked with 1% BSA/1X PBS, diluted in 1X PBS at room temperature. The Mφs were then incubated with the primary anti-phospho-NF-κB-p65 monoclonal antibody (mAb; #3033; Cell Signaling Technology, Inc.) diluted in 1% BSA/1X PBS at 4°C overnight. The following day, the cells were incubated with (4 μg/ml) Alexa Fluor 488-labeled secondary antibody (A11008; Thermo Fisher Scientific, Inc.) diluted in 1% BSA/1X PBS for 1 h at room temperature, after washing with 1X PBS. Hoechst dye No. 33342 (B2261; Sigma-Aldrich Merck KGaA; 0.5 μg/ml) was used for cellular chromatin staining. Finally, the coverslips were mounted in Prolong Gold antifade media (Molecular Probes, Inc.) and the cells were observed under a confocal microscope (Leica Microsystems GmbH) with an excitation wavelength of 355 nm for Hoechst and with an excitation wavelength of 488 nm for phosphorylated NF-κB (Ser 536). The LAS AF program was used to acquire the images. Experiments were repeated independently 3 times and representative images are presented.

**Mice.** Male black C57BL/6 mice (20 g in weight, 6 weeks of age) were obtained from the National Hellenic Research Foundation and housed under controlled temperature ( $22 \pm 2^\circ\text{C}$ ) and photoperiod (12 h light; 12 h dark) with free access to water and food. All experiments with mice were performed in the authorized animal house of the National Hellenic Research Foundation (License no. EL 25 BIObr 031 as a breeding facility; License no. EL 25 BIOSup 032 as a supply facility; and License no. for EL 25 BIOexp 033 as a research installation). The experiments complied with the Protocol on the Protection and Welfare of Animals, as obliged by the rules of the National Hellenic Research Foundation the regulations of the National Bioethics Committee and article 3 of the presidential decree 160/1991 (in line with 86/609/EEC directive) regarding the protection of experimental animals. A total of 3 mice/group were used. The minimum number of animals was achieved according to the 3Rs (replacement, reduction and refinement), to ensure scientific and statistical validity. The health and behaviour of the animals were monitored daily. The mice were gradually euthanized, using carbon dioxide ( $\text{CO}_2$ ). The gradual flow rate of  $\text{CO}_2$  was as per the guidelines. The death of the animals was confirmed through the verification of cardiac and respiratory arrest.

**Ethics approval.** The present study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Bioethics Committee of the National Hellenic Research Foundation (date of approval: 29/3/2020). The ethic code is PN 2-3/29-3-2020.

**LPS-induced air pouch murine model of inflammation.** Air-pouches were created according to a modified method

described in the study by Sedgwick *et al* (43). An area of dorsal skin (4 cm<sup>2</sup>) was shaved, and 3 ml of sterile air were subcutaneously injected to establish a single air-pouch in 6-week-old black (C57BL/6) mice. The mice were anaesthetized by an intraperitoneal (i.p) injection of ketamine at 100 mg/kg together with xylazine at 10 mg/kg, prior to the subcutaneous injections during the LPS-induced air-pouch model of inflammation. The mice weighed approximately 20 g and were housed in filtered-air laminar-flow cabinets at a controlled temperature ( $22 \pm 2^\circ\text{C}$ ) with a 12-h light/dark cycle. A total of 3 ml of sterile air were administered on alternate days to maintain the pouch. At 10 days after air-pouch formation, the pouches were injected with a single dose of 1 ml (1 μg/ml) LPS alone (positive control) or a single dose of 1 ml (1 μg/ml) LPS plus a single dose of (3 or 6 or 9 mg/mouse) BAT concurrently or a single dose of 1 ml (1 μg/ml) LPS plus a single dose of (9 mg/mouse) taurine concurrently, inside the air pouches of the mice for 8 h, as previously described (44). Each group was composed of 3 mice. This air pouch model of inflammation has been similarly used in a wide range of studies (44–48). Inflammatory exudates were then harvested by collecting the lavage fluids after washing the air-pouch cavities with 2 ml PBS (1X, pH 7.4) followed by RNA extraction. The pouch membranes were fixed in 10% (v/v) buffered formalin for histological analysis.

**Histological analysis.** Ten days after air-pouch formation, substances were administered and after 8 h, mice were euthanized and their pouches were dissected, sampled and processed for paraffin embedding. The protocol for paraffin embedding was as follows (total 16 h): 70% ethanol (2 h), 80% ethanol (1 h), 95% ethanol (1 h), 100% ethanol (4.5 h), xylene (4.5 h), paraffin (58–60°C) (4 h), embedding tissues into paraffin blocks and trimming into the suitable 6 μm. Sections were then stained with hematoxylin (8 min) and eosin (1 min) (H&E) at 30°C, using the following protocol: The sections were deparaffinized, and treated with absolute alcohol (10 min), 95% alcohol (2 min), 70% alcohol (2 min) and stained with Harris hematoxylin solution (Thermo Fisher Scientific, Inc.) (8 min) and saturated lithium carbonate (1 min) at 30°C. After rinsing, the sections were counterstained with eosin-phloxine solution (Thermo Fisher Scientific, Inc.) (1 min) and dehydrated using 95% alcohol. Microphotographs were captured using a Nikon Eclipse 80i upright microscope with CFI60 lenses, using a Leica DFC 450 C digital camera. The magnification utilized for their capture was x200. In each section, pouch wall thickness was measured at 6 regions randomly at the upper, at the back and at the middle side of the pouch wall. The mean of six different regions in each section was determined. The thickness of the pouch and the area of the corresponding region were calculated using ImageJ software (v1.52a). The total cell number (based on nucleus count) was calculated as cells/mm<sup>2</sup>, using the ‘analyze particles’ feature of ImageJ software, in LPS-exposed mice bearing air-pouch with or without treatment, manually. The number of PMNs was calculated per mm<sup>2</sup>, in LPS-exposed mice bearing air pouch with or without treatment, manually.

**Statistical analysis.** Data are presented as the means  $\pm$  SEM. One-way ANOVA, followed by Tukey’s multiple

comparison test was used to evaluate the significance of all experiments between the LPS group with the LPS/BAT and LPS/taurine-treated groups. Two-way ANOVA was used to evaluate the statistical significance of weights between the LPS group with the LPS/BAT group and LPS/taurine group. Statistics were calculated with GraphPad Prism 6.0 (GraphPad Software, Inc.).

## Results

*BAT reduces the mRNA expression levels of pro-inflammatory mediators in LPS-stimulated J774.A1 Mφs at lower concentrations compared to taurine.* To investigate the putative inhibitory effects of BAT and taurine on the LPS-induced inflammatory response *in vitro*, the mRNA expression levels of various pro-inflammatory cytokines secreted by J774.A1 Mφs were assessed following 1.5 h of incubation with either BAT or taurine alone, prior to their stimulation with LPS for 24 h. In the present study, RT-qPCR was used to detect the mRNA expression levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-18 and IL-23 following treatment of J774.A1 Mφs with either (0.1, 0.3, 0.5, 1 and 1.75 mM) BAT or (100 and 200 mM) taurine prior to their stimulation with LPS for 24 h. Of note, BAT and taurine exerted potent protective effects against inflammation, by reducing the mRNA expression levels of pro-inflammatory cytokines in the LPS-stimulated J774.A1 Mφs (Fig. 1 and Table SII). In particular, the IL-1 $\beta$  mRNA levels were decreased by (51, 59, 59, 60, 81, 59 and 71%), the IL-23 mRNA levels by (68, 70, 73, 75, 81, 72 and 77%), the IL-18 mRNA levels by (15, 25, 50, 57, 81, 15 and 55%) and the TNF- $\alpha$  mRNA levels by (56, 79%, 83, 89, 96, 88 and 89%) in the (0.1, 0.3, 0.5, 1 and 1.75 mM) BAT-, and in the (100 and 200 mM) taurine-treated LPS-stimulated J774.A1 Mφs compared to the LPS-stimulated Mφs. A dose-dependent transcriptional downregulation in the levels of pro-inflammatory cytokines was proved in the BAT-treated LPS-stimulated J774.A1 Mφs, with the highest inhibitory effect observed at the concentration of 1.75 mM BAT. Notably, the highest suppression of IL-1 $\beta$ , IL-23 and IL-18 mRNA expression (81%) and the greatest inhibition of TNF- $\alpha$  (96%) were evidenced in the 1.75 mM BAT-treated Mφs prior to the induction of LPS-mediated inflammation. In all cases, the 1.75 mM BAT-treated LPS-stimulated J774.A1 Mφs exhibited a greater transcriptional inhibition of all pro-inflammatory cytokines than those of the 100 and 200 mM taurine-treated LPS-stimulated J774.A1 Mφs. As a result, BAT potentially exerted a more significant protective action than taurine, by hindering the LPS inflammatory stimulus to activate cytokine secretion from macrophages. These data were consistent with the reduced protein expression levels of pro-inflammatory mediators (IL-6, IL12p40, TNF- $\alpha$ ), as previously indicated by ELISA experiments in LPS-stimulated J774.A1 Mφs (38).

*BAT inhibits the nuclear translocation of the phosphorylated NF- $\kappa$ B p65 subunit in LPS-stimulated J774.A1 Mφs.* To elucidate the molecular mechanisms underlying the protective effects of BAT and taurine against LPS-mediated-inflammation, immunofluorescence experiments were employed to examine the exact localization of phosphorylated NF- $\kappa$ B p65 subunit (Ser536) in the J774.A1 Mφs. The dynamics (serine 536 phosphorylated p65 subunit of NF- $\kappa$ B) were measured in BAT- or

taurine-treated J774.A1 Mφs, prior to their stimulation with LPS. Following treatment with BAT or taurine, the nuclear translocation of phospho-NF- $\kappa$ B p65 subunit (Ser 536) was hindered in a dose-dependent manner, since it was mainly found in the cell cytoplasm (Fig. 2). In particular, the inhibition of phospho-NF- $\kappa$ B p65 (Ser 536) nuclear translocation dynamics emerged in the 0.1 mM BAT-treated J774.A1 Mφs prior to their stimulation with LPS, while the most effective inhibition of NF- $\kappa$ B p65 phosphorylation (Ser 536) was observed in the 0.5 mM BAT-treated J774.A1 Mφs prior to their stimulation with LPS, as compared to the LPS-stimulated J774.A1 Mφs. Of note, the levels of phosphorylated NF- $\kappa$ B p65 in the NC J774.A1 Mφs were similar to those of LPS-stimulated J774.A1 Mφs following BAT or taurine treatment. As a result, BAT and taurine restored not only the translocation, but also the levels of phosphorylated NF- $\kappa$ B p65 in the LPS-stimulated J774.A1 Mφs, as observed in the NC Mφs. It was hypothesized that the sequestration of p-NF- $\kappa$ B p65 subunit (Ser 536) occurs in the cytoplasm of J774.A1 Mφs following BAT or taurine treatment, prior to their stimulation with LPS, probably due to other post-translational modifications or other mechanisms that inhibit phospho-NF- $\kappa$ B p65 nuclear shuttling.

*BAT attenuates the LPS inflammatory response to a greater extent than taurine.* To confirm the protective effects of BAT and taurine against LPS-mediated inflammation *in vivo*, the LPS-induced air-pouch model of inflammation was used. This animal model has been widely used to evaluate acute inflammation due to its high sensitivity and cost-efficiency (41). In the present analysis, 4 groups of C57BL/6 mice were subjected to the following procedure: They were subjected to air-pouch formation subcutaneously; air injections were administered on specific days according to the timeline to maintain the air-pouch. At 10 days after air pouch formation, LPS inflammatory stimulus and optionally BAT or taurine were administered to the mice for 8 h. To elucidate the potential protective effect of BAT against LPS-induced inflammation *in vivo*, we compared its impact with that of a known anti-inflammatory agent, namely taurine. The 4 groups of animals were composed as follows: Group 1, mice with an air-pouch and treated with saline; group 2, LPS-exposed mice with an air pouch; group 3, BAT-treated LPS-exposed mice with an air pouch; and group 4, taurine-treated LPS-exposed mice with an air pouch. To ensure that the presence of inflammation formed inside the air-pouch, the results derived from the normal saline-negative control (NC) and the LPS-treated group (LPS) were compared. Following the induction of inflammation, the functional significance of either BAT or taurine in orchestrating the distribution of inflammatory cell populations and in determining the mRNA expression levels of pro-inflammatory cytokines was elucidated.

Air-pouch tissues were stained with H&E to evaluate the histological presence of various inflammatory cell populations. The injection of (1  $\mu$ g/ml) LPS into the air-pouch on the backs of mice for 8 h resulted in increased inflammatory cell infiltration and elevated pouch wall thickness. All the examined parameters in the LPS group were significantly increased compared with those in the NC group (Fig. 3 and Table SIII). Representative images with histological changes in the pouch wall (synovial membrane and connective tissue)

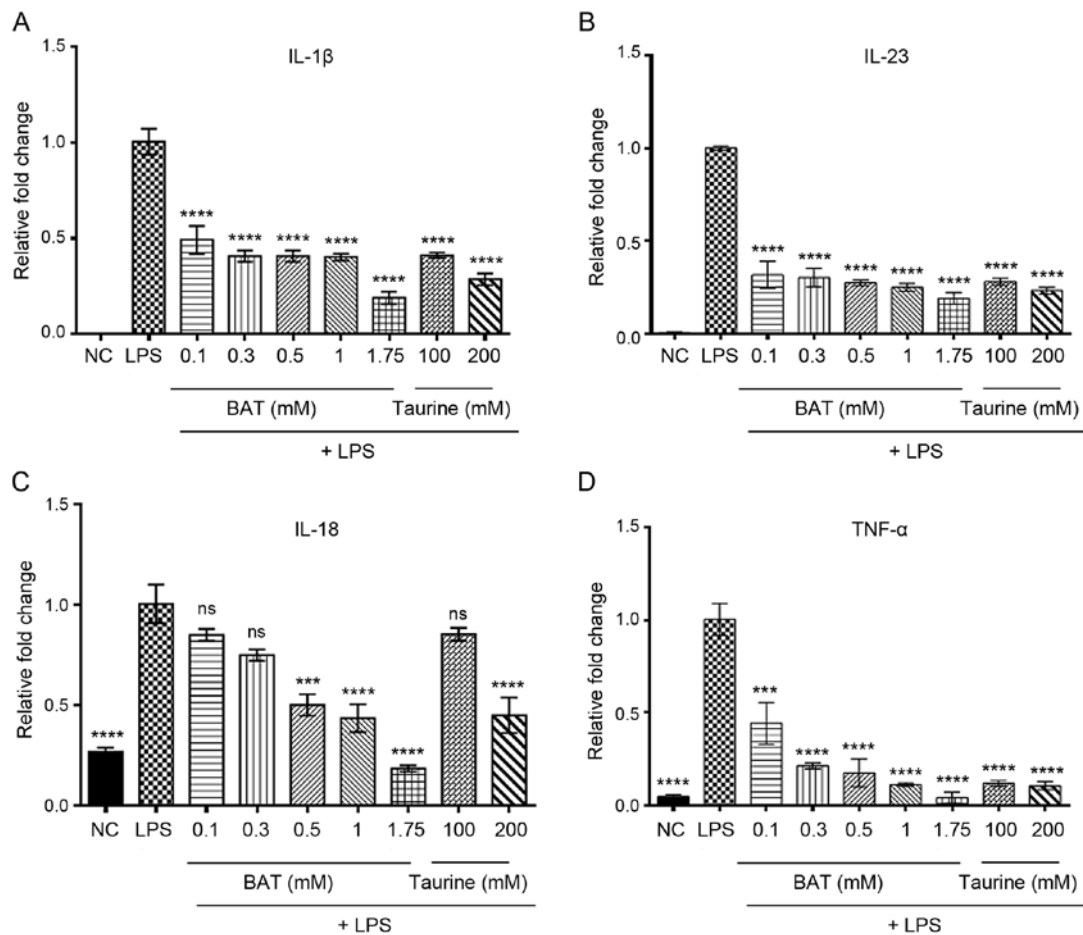


Figure 1. BAT exerts an anti-inflammatory effect *in vitro*, through the transcriptional downregulation of pro-inflammatory cytokines. RT-PCR analysis of interleukins (A) IL-1 $\beta$ , (B) IL-23, (C) IL-18, (D) TNF- $\alpha$  mRNA levels in the LPS-stimulated J774.A1 M $\phi$ s following (0.1-1.75 mM) BAT or (100-200 mM) taurine treatment for 1.5 h. In particular, the groups of J774.A1 M $\phi$ s were the following: Negative control (NC), (100 ng/ml) LPS-stimulated J774.A1 M $\phi$ s (LPS group), (100 ng/ml) LPS plus 0.1 mM BAT-treated J774.A1 M $\phi$ s (LPS + 0.1 mM BAT group), (100 ng/ml) LPS plus 0.3 mM BAT-treated J774.A1 M $\phi$ s (LPS + 0.3 mM BAT group), (100 ng/ml) LPS plus 0.5 mM BAT-treated J774.A1 M $\phi$ s (LPS + 0.5 mM BAT group), (100 ng/ml) LPS plus 1 mM BAT-treated J774.A1 M $\phi$ s (LPS + 1 mM BAT group), (100 ng/ml) LPS plus 1.75 mM BAT-treated J774.A1 M $\phi$ s (LPS + 1.75 mM BAT group), (100 ng/ml) LPS plus 100 mM taurine-treated J774.A1 M $\phi$ s (LPS + 100 mM taurine group), (100 ng/ml) LPS plus 200 mM taurine-treated J774.A1 M $\phi$ s (LPS + 200 mM taurine group). Data are presented as the means  $\pm$  SEM of 3 independent experiments. One-way ANOVA analysis followed by Tukey's multiple comparison test revealed the statistically significant differences between LPS alone-treated J774.A1 M $\phi$ s with LPS/BAT-treated M $\phi$ s and LPS/taurine-treated M $\phi$ s. Statistical analysis revealed the comparison of BAT and taurine-treated M $\phi$ s compared to LPS-treated M $\phi$ s; ns, not significant, \*\*\*P<0.001, \*\*\*\*P<0.0001 vs. LPS-stimulated M $\phi$ s. M $\phi$ s, macrophages; LPS, lipopolysaccharide; BAT, bromamine T.

in the differently treated groups compared to the LPS group are presented in Fig. 3B. In particular, the LPS-exposed mice bearing an air pouch treated with BAT or taurine had a fewer number of infiltrating cells, as well as polymorphonuclear cells (PMNs) in the pouch wall (synovial membrane and connective tissue) and thinner pouch wall formation than those injected with LPS alone (Fig. 3 and Table SIII). Notably, pouch wall thickness was similar between the LPS and 9 mg BAT-treated mice bearing an air-pouch and the LPS and 9 mg taurine-treated mice bearing an air-pouch (Fig. 3G). The analysis of the exudates obtained from the pouches also revealed that the inhibitory effects of BAT and taurine treatment on the secretion of pro-inflammatory cytokines (Fig. 4 and Table SIV) were similar to those observed *in vitro* (Fig. 1 and Table SI). In particular, the LPS-exposed mice bearing an air pouch and treated with 3 or 6 or 9 mg of BAT or 9 mg of taurine exhibited decreased IL-1 $\beta$  mRNA levels by 48, 81, 91 and 84%, decreased IL-23 mRNA levels by 19, 93, 94 and 39%, and decreased IL-18 mRNA levels by 26, 63, 86 and 87%

respectively, as compared to the untreated LPS-exposed mice bearing an air pouch. The IL-17 mRNA levels were reduced by 34, 51, 78 and 80% following treatment of the LPS-exposed mice bearing an air pouch with 3, 6 and 9 mg of BAT and 9 mg of taurine, respectively, compared to those of untreated LPS-exposed mice bearing an air pouch. Similarly, the TNF- $\alpha$  mRNA levels were reduced by 19, 53, 86 and 73% in the pouch exudates obtained from the LPS-exposed mice and treated with 3, 6 and 9 mg of BAT or 9 mg of taurine, respectively, as compared to those of untreated LPS-exposed mice bearing an air pouch. Last but not least, the mRNA levels of thymic stromal lymphopoietin (TSLP) were suppressed by 17, 61, 85 and 87% in the LPS-exposed mice with the air pouch treated with 3, 6 and 9 mg of BAT or 9 mg of taurine, respectively, as compared to those of untreated LPS-exposed mice bearing an air pouch. At the transcriptional level, IL-1 $\beta$ , IL-23 and TNF- $\alpha$  were produced at lower levels in the LPS plus 9 mg BAT-treated mice bearing an air pouch compared to those in the LPS plus 9 mg taurine-treated mice bearing an air pouch (Fig. 4A,



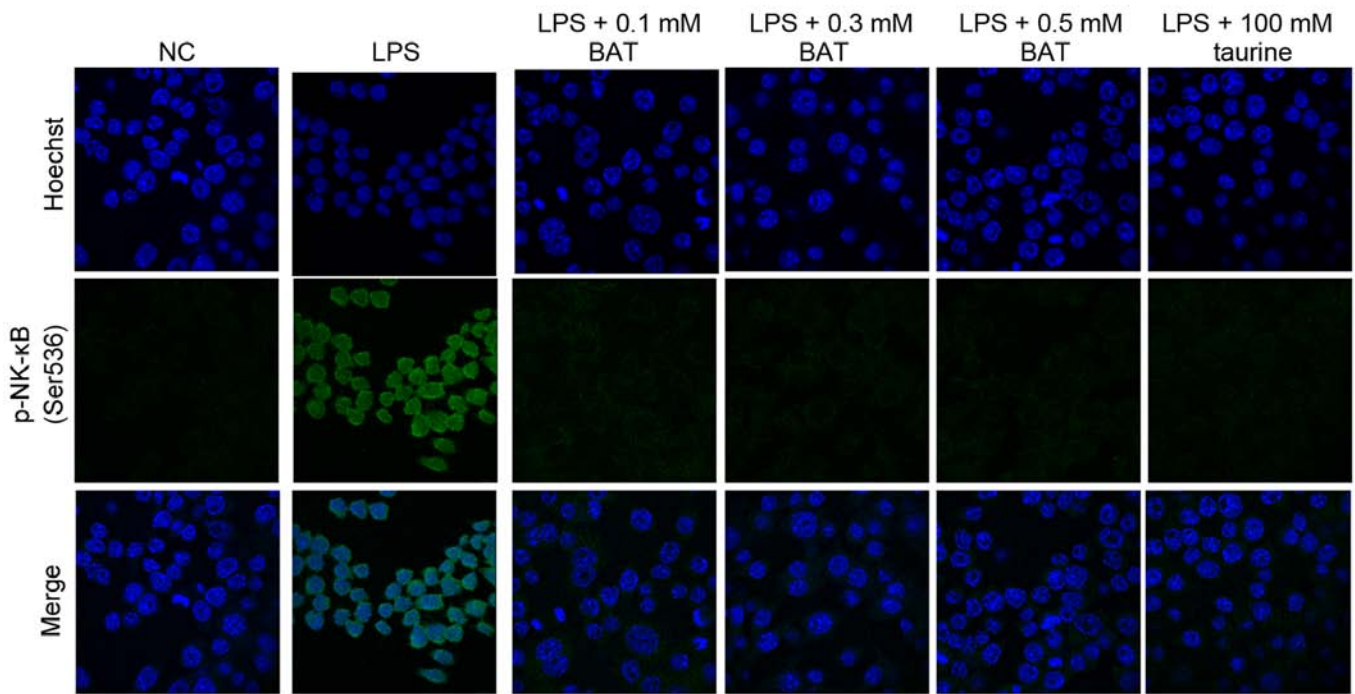


Figure 2. BAT inhibits nuclear the translocation of phosphorylated NF- $\kappa$ B p65 subunit. Immunofluorescence of phosphorylated NF- $\kappa$ B p65 subunit (Ser 536) in the LPS-stimulated J774.A1 M $\phi$ s after (0.1-0.5 mM) BAT or 100 mM taurine treatment for 1.5 h. The phosphorylated NF- $\kappa$ B p65 was detected by Alexa Fluor 488-labeled immunostaining (green); nuclei were stained with Hoechst (blue). Untreated cells were used as a negative control (NC), while cells treated only with LPS were used as a positive control for an inflammatory response (x630 magnification). In particular, the groups of J774.A1 M $\phi$ s were the following: Negative control (NC), (100 ng/ml) LPS-treated J774.A1 M $\phi$ s (LPS group), (100 ng/ml) LPS plus 0.1 mM BAT-treated J774.A1 M $\phi$ s (LPS + 0.1 mM BAT group), (100 ng/ml) LPS plus 0.3 mM BAT-treated J774.A1 M $\phi$ s (LPS + 0.3 mM BAT group), (100 ng/ml) LPS plus 0.5 mM BAT-treated J774.A1 M $\phi$ s (LPS + 0.5 mM BAT group), (100 ng/ml) LPS plus 100 mM taurine-treated J774.A1 M $\phi$ s (LPS + 100 mM taurine group). M $\phi$ s, macrophages; LPS, lipopolysaccharide; BAT, bromamine T.

B and E). Notably, the LPS plus 6 mg BAT-treated mice bearing an air pouch exhibited very low transcriptional levels of IL-23, as opposed to the LPS plus 9 mg taurine-treated mice bearing an air pouch (Fig. 4B). As regards the transcriptional levels of IL-18, IL-17 and TSLP, the 2 groups of LPS plus 9 mg of BAT and LPS plus 9 mg of taurine-treated mice bearing an air pouch presented similar values (Fig. 4C and F). Of note, the inhibitory effect of 9 mg BAT was the most effective on the IL-23 mRNA levels in the LPS-exposed mice bearing an air pouch (Fig. 4B). As a result, the LPS plus 9 mg BAT-treated mice bearing an air pouch exhibited a superior effect on the majority of the pro-inflammatory cytokines tested and on cellular infiltration, compared to the aforementioned parameters of the LPS plus 9 mg taurine-treated mice bearing an air pouch.

## Discussion

It is well established that taurine exerts a potent anti-inflammatory effect (26); however, its use in clinical practice is limited. This evidence has prompted researchers to evaluate the anti-inflammatory effect of taurine derivatives, such as TauNHBr, which comprises the reaction product of taurine with HOBr at the inflammatory site (26). It has been reported that TauNHBr is employed in the treatment of inflammatory and infectious diseases, but its clinical efficacy is obstructed by high rates of degradation (26). For this reason, BAT was designed as the stable active bromine compound (38) and its molecular mechanisms in inflammation are under

investigation. In the previous study by Walczewska *et al* (38), the anti-inflammatory activity of BAT was examined, starting from a concentration of 0.1 mM. Based on those findings, the present study examined the protective properties of BAT against LPS-induced inflammation in a concentration range of 0.1-1.75 mM. Moreover, the biocompatibility index of BAT (namely the ratio of the minimal concentration exerting *in vitro* cytotoxicity in cell culture to the minimal one exerting *in vitro* killing activity against bacteria) ranges approximately between 1 and even 100 (38). On the other hand, in the study by Sartori *et al* (49), taurine did not exhibit any significant anti-inflammatory activity in macrophages that were pre-treated with up to 10 mM of taurine; thus, in the present study, a higher dose was used to examine the potential protective activity of taurine against LPS-mediated inflammation, given that taurine is a non-essential amino-acid in the human setting (50).

The results of the present study indicated that the protective effect of BAT was superior to that of taurine *in vitro* using LPS-stimulated J774.A1 M $\phi$ s through the attenuation of pro-inflammatory mediator mRNA expression. The current *in vitro* observations were in accordance with the results of *in vivo* experiments, as discussed below.

Initially, the results proved that BAT accounted for the reduction of pro-inflammatory cytokines (IL-1 $\beta$ , IL-23, IL-18 and TNF- $\alpha$ ) at the transcriptional level, in LPS-stimulated J774.A1 M $\phi$ s to a greater extent than taurine, through the inhibition of the translocation of phosphorylated NF- $\kappa$ B p65 subunit (Ser536) in the nuclei. Accordingly, the anti-inflammatory

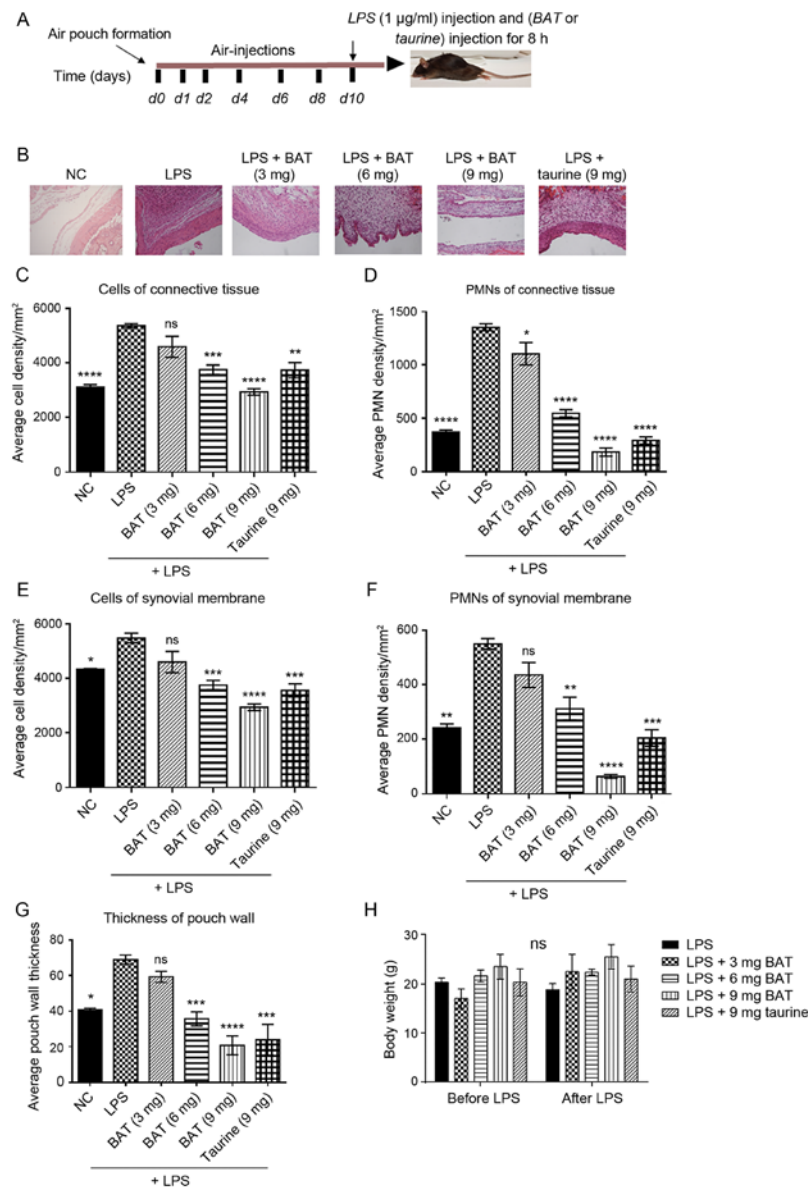


Figure 3. BAT acts as an anti-inflammatory agent in the LPS-induced air pouch model of inflammation *in vivo*, through the inhibition of total cellular infiltration and PMN recruitment. (A) Schematic experimental design. All the groups of mice were subjected to the following procedure: They were subjected to air pouch formation subcutaneously, air injections were administered on specific days according to the timeline to mice to maintain the air pouch, and (1 µg/ml) LPS inflammatory stimulus was administered to the mice at 10 days following air pouch formation. The LPS group was compared with (1 µg/ml) LPS and (3 or 6 or 9 mg) BAT-treated group and with (1 µg/ml) LPS and (9 mg) taurine-treated group for 8 h. Each group was composed of 3 mice. (B) Representative histological images of the pouch wall including synovial membrane and connective tissue in all groups of mice (x200 magnification), using H&E staining. In particular, the groups of mice were as follows: Negative control (NC), (1 µg/ml) LPS-treated mice (LPS group), (1 µg/ml) LPS plus (3 mg) BAT-treated mice (LPS + 3 mg BAT group), (1 µg/ml) LPS plus (6 mg) BAT-treated mice (LPS + 6 mg BAT group), (1 µg/ml) LPS plus (9 mg) BAT-treated mice (LPS + 9 mg BAT group), (1 µg/ml) LPS plus (9 mg) taurine-treated mice (LPS + 9 mg taurine group). (C) Graphical representation of the semi-quantified total cell distribution derived from the connective tissue of experimental groups. One-way ANOVA followed by Tukey's multiple comparison test revealed the statistically significant differences between the LPS-stimulated group with LPS/BAT-treated group and LPS/taurine-treated group; ns, not significant, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 vs. the LPS-stimulated group. (D) Graphical representation of the semi-quantified polymorphonuclear cell (PMNs) distribution derived from the connective tissue of experimental groups. Each group was composed of 3 mice. One-way ANOVA followed by Tukey's multiple comparison test revealed the statistically significant differences between the LPS-stimulated group with LPS/BAT-treated group and LPS/taurine-treated group; \*P<0.05, \*\*\*\*P<0.0001 vs. the LPS-stimulated group. (E) Graphical representation of the semi-quantified total cell distribution derived from the synovial membrane of experimental groups. Each group was composed of 3 mice. One-way ANOVA followed by Tukey's multiple comparison test revealed the statistically significant differences between the LPS-stimulated group with LPS/BAT-treated group and LPS/taurine-treated group; ns, not significant, \*P<0.05, \*\*\*P<0.001, \*\*\*\*P<0.0001 vs. the LPS-stimulated group. (F) Graphical representation of the semi-quantified PMN distribution derived from the synovial membrane of experimental groups. Each group was composed of 3 mice. One-way ANOVA followed by Tukey's multiple comparison test revealed the statistically significant differences between the LPS-stimulated group with the LPS/BAT-treated group and LPS/taurine-treated group; ns, not significant, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 vs. the LPS-stimulated group. (G) Quantification of pouch wall thickness on each section in all experimental groups of mice. Random 100 µm longitudinal pouch areas were selected to count total cells, and the mean of 6 different areas within each section was determined. LPS treatment was used as a positive control of inflammation. The results of untreated (NC) mice are also presented. Each group was composed of 3 mice. One-way ANOVA followed by Tukey's multiple comparison test revealed the statistically significant differences between the LPS-stimulated group with the LPS/BAT-treated group and LPS/taurine-treated group; ns, not significant, \*P<0.05, \*\*\*P<0.001, \*\*\*\*P<0.0001 vs. the LPS-stimulated group. (H) Graphical representation of mouse body weights, following treatment with either BAT or taurine. BAT or taurine-treated mice did not present any difference in body weight. Each group was composed of 3 mice. Two-way ANOVA did not reveal any statistically significant differences between groups; ns, not significant. LPS, lipopolysaccharide; BAT, bromamine T; PMN, polymorphonuclear cell.

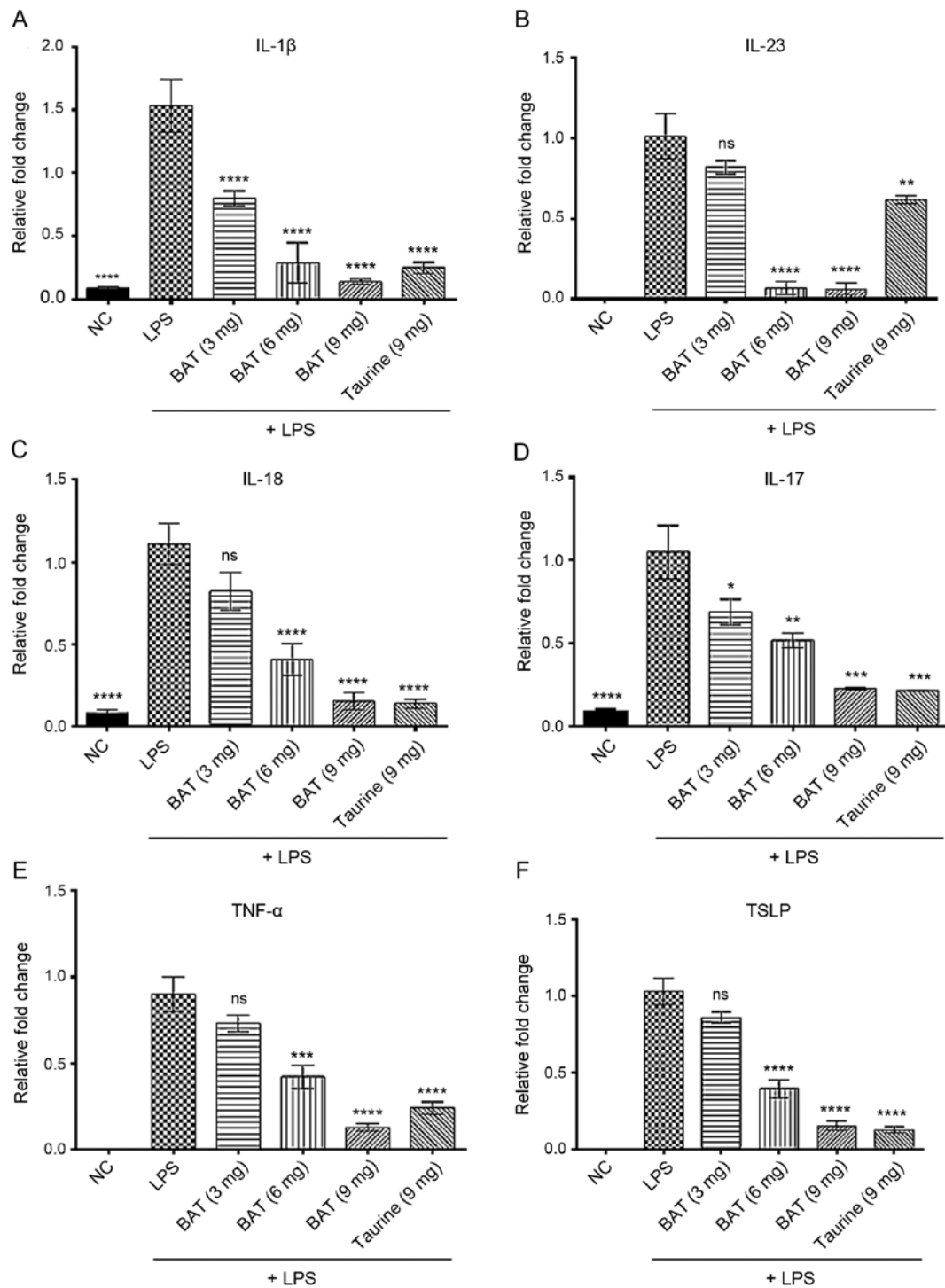


Figure 4. BAT exerts an inhibitory effect on the transcription of pro-inflammatory cytokines in the LPS-induced air-pouch model of inflammation *in vivo*. RT-PCR analysis of (A) IL-1 $\beta$ , (B) IL-23, (C) IL-18, (D) IL-17, (E) TNF- $\alpha$ , and (F) TSLP in (3 or 6 or 9 mg) BAT or (9 mg) taurine-treated LPS-exposed mice with air-pouch compared to LPS-exposed mice bearing air-pouch. In particular, the groups of mice were as follows: Negative control (NC), (1  $\mu$ g/ml) LPS-treated mice (LPS group), (1  $\mu$ g/ml) LPS plus (3 mg) BAT-treated mice (LPS + 3 mg BAT group), (1  $\mu$ g/ml) LPS plus (6 mg) BAT-treated mice (LPS + 6 mg BAT group), (1  $\mu$ g/ml) LPS plus (9 mg) BAT-treated mice (LPS + 9 mg BAT group), (1  $\mu$ g/ml) LPS plus (9 mg) taurine-treated mice (LPS + 9 mg taurine group). Each group was composed of 3 mice. One-way ANOVA analysis followed by Tukey's multiple comparison test revealed the statistically significant differences between the LPS-treated group with the LPS/BAT-treated group and LPS/taurine-treated group; ns, not significant, \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, \*\*\*\* $P$ <0.0001 vs. the LPS-stimulated group. LPS, lipopolysaccharide; BAT, bromamine T.

properties of BAT have been observed in J774.A1 M $\phi$ s, through the suppression of the protein expression of TNF- $\alpha$ , IL12p40 and PGE<sub>2</sub> (33).

Secondly, a murine air-pouch model was used to evaluate the protective effects of BAT on LPS-induced inflammation,

through relative qualitative and quantitative information about inflammatory cell infiltration. Multiple inflammatory stimuli have been used for the induction of inflammation, such as LPS, rHuPH20, HA, Hyal type I-S and IV-S, zymosan, carrageenan (44,48,51,52). The model of LPS-induced acute



inflammation differs from other established models, due to its greater reproducibility, the paucity of any visible side-effects and its dependence on the activity of TNF- $\alpha$ , IL-1 $\beta$  and myeloperoxidase (MPO) (41). In the experiments presented herein, the potential protective action of agents was examined 8 h following the LPS administration, since a significant neutrophil accumulation was only observed 8 h after the LPS injection in mice bearing an air pouch (44). A similar pattern of LPS challenge was also used in another study (53). In the present study, pouches were injected with a single dose of 1 ml (1  $\mu$ g/ml) LPS alone (positive control) or a single dose of 1 ml (1  $\mu$ g/ml) LPS plus a single dose of (3 or 6 or 9 mg/mouse) BAT concurrently or a single dose of 1 ml (1  $\mu$ g/ml) LPS plus a single dose of (9 mg/mouse) taurine concurrently, inside the air pouch of mice for 8 h, 10 days after air pouch formation (44). Each group was composed of 3 mice. In the current experimental set-up, LPS-exposed mice bearing an air pouch exhibited an increase in the inflammatory cell infiltration and the pouch wall thickness compared to the NC mice bearing an air pouch. To validate the putative protective role of BAT against LPS-induced inflammation, its effects were compared with those of taurine. The results proved that cellular density of synovial membrane or connective tissue, pouch wall thickness and PMNs of synovial membrane or connective tissue in BAT- or taurine-treated groups, were significantly lower compared to the LPS group. The highest inhibitory effect on the pro-inflammatory parameters was observed with 9 mg of BAT and was superior to that mediated by 9 mg of taurine on LPS-exposed mice bearing an air pouch. Notably, these results proved the potent protective properties of BAT against LPS-mediated inflammation *in vivo*, given that M $\phi$ s and fibroblasts are accumulated, promoting the formation of granulation tissue and subsequent collagen synthesis in the LPS-induced air pouch model of inflammation (54). In the *in vivo* experiments, higher concentrations of BAT were used relative to the *in vitro* experiments. The possible presence of compounds with amino groups (ammonium, amino acids, peptides and proteins), could allow the mixture of bromamine compounds to be formed by transfer of bromine, while in the presence of reducing substances (S-H compounds, thiosulfate), BAT could be transformed to toluenesulfonamide and, therefore, could be inactivated (personal communication with Professor Dr M. Nagl at the Institute of Hygiene and Medical Microbiology of the Medical University of Innsbruck).

BAT and taurine provided a protective environment, attenuating the possibility of inflammation caused by LPS, not only by reducing pouch wall thickness and total cellular infiltration, but also through the suppression of the mRNA expression of IL-1 $\beta$ , IL-23, IL-18, IL-17, TNF- $\alpha$  and TSLP (Fig. 4). However, the IL-6 mRNA expression levels were not measured in the *in vitro* and *in vivo* experiments in the present study, since protein expression levels of IL-6 previously appeared to be reduced in the J774A.1 M $\phi$ s following BAT treatment (38). To study the expression pattern of specific pro-inflammatory mediators according to the applied LPS stimulus, one of the most important pro-inflammatory mediators (TNF- $\alpha$ ), which is secreted from immune cells (such as M $\phi$ s, T, and B lymphocytes) in response to LPS was evaluated (55). The present study also examined the expression pattern of IL-1 $\beta$ , which is secreted by either immune or stromal cells, constituting an important mediator of inflammation (56). It is well-established that the synergistic activation

of TNF- $\alpha$ /IL-1 $\beta$  mediates acute inflammatory reactions, by inducing the expression of adhesion molecules, thus enriching the circulating leukocytes in the inflamed site (57). The inhibitory effect of either BAT or taurine on pro-inflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) should be specially considered since TNF- $\alpha$  and IL-1 $\beta$  have shown to play an important role in the activation of endothelial cells by increasing the expression of adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1), which are crucial for leukocyte recruitment (58). The results of the present study were consistent with these data, indicating that taurine is capable of reducing the expression levels of ICAM-1 and VCAM-1 in endothelial cells induced by hypoxia through the p38 mitogen-activated protein kinase (MAPK) pathway (59). Similarly, hyperglycemia-mediated endothelial dysfunction is ameliorated by taurine, through interference with adhesion molecules (60). In the present study, the analysis of the specific cytokines was conducted only in LPS-induced air-pouch model since TSLP is secreted by either synovial fibroblasts (61) or bone marrow-derived dendritic cells (DCs) (52) and IL-17 is secreted by either CD4<sup>+</sup> T helper (Th) cells (62) or neutrophils (63,64) in response to LPS inflammatory stimulus. In all cases of pro-inflammatory mediators, the protective effect of BAT was proven against LPS-mediated inflammation. In some cases, the superiority of BAT over taurine could be associated with the fact that BAT is an inducer of the anti-inflammatory molecule hemo-oxygenase-1 (HO-1) (38). Furthermore, BAT reduces COX-2 levels, thus, reducing the production of inflammatory mediators (3).

In the previous study by Walczewska *et al* (38), ELISA experiments in LPS-stimulated J774.A1 M $\phi$ s revealed reduced protein expression levels of pro-inflammatory mediators (IL-6, IL12p40 and TNF- $\alpha$ ) following pre-treatment with 0.1-0.3 mM BAT. In the present study, it was firstly demonstrated that BAT was more effective than taurine in preventing LPS-induced inflammation through a marked decline of mRNA expression levels of pro-inflammatory mediators *in vitro*. In LPS-stimulated J774.A1 M $\phi$ s, the underlying molecular mechanism relies on the activation of the transcription factor NF- $\kappa$ B, a master regulator of pro-inflammatory cytokine production. To the best of knowledge, this is the first study to demonstrate that BAT prevents the mRNA expression levels of pro-inflammatory cytokines in J774.A1 M $\phi$ s, via the inhibition of the translocation of the phosphorylated NF- $\kappa$ B p65 subunit (Ser536) in the nuclei and its subsequent cytoplasmic sequestration to similar extent as NC M $\phi$ s. In addition, the present study provides a comprehensive overview regarding the protective properties of BAT or taurine against LPS-mediated inflammation *in vivo*. In particular, the present study provides convincing evidence that BAT is superior to taurine *in vivo*, by repressing the transcription of the majority of pro-inflammatory cytokines to a greater extent, and by reducing the total cell/PMN infiltration in the air pouch of LPS-exposed mice to a greater extent. As a result, it is demonstrated that BAT, an active bromine compound, is able to inhibit LPS-mediated inflammation *in vitro* and *in vivo*. The *in vitro* findings raise the next reasonable challenge, whether primary macrophages derived from *in vivo* experiments on LPS-inflammation can get activated and comply with the dynamics of NF- $\kappa$ B signaling observed in LPS-exposed J774.

A1 Mφs. The protective effect of BAT against LPS-induced inflammation *in vivo* should be investigated in the future on other animal models of acute and chronic inflammation. The data presented herein propose the protective effect of BAT and its potential underlying molecular mechanism, suggesting that BAT could be used as a lead compound for the prevention of expanded acute inflammation. Undoubtedly, further experiments are required to address the potential anti-inflammatory effect of BAT and taurine in clinical setting, since the majority of anti-inflammatory drugs are examined after inflammation. Following LPS-mediated inflammation, further experiments will shed light on the potential anti-inflammatory effect of BAT and taurine *in vitro* and *in vivo*.

In conclusion, the present study demonstrates that BAT and taurine inhibit the nuclear translocation of the phosphorylated NF-κB p65 subunit (Ser536) in J774.A1 Mφs. To the best of our knowledge, this is the first study to demonstrate the protective properties of BAT and taurine against LPS-mediated inflammation *in vivo*, using a murine LPS-mediated air pouch model.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

### Authors' contributions

SB performed the *in vitro* and *in vivo* experiments, analyzed the data, performed the statistical analysis and prepared the manuscript. MS contributed to the H&E staining and the counting of cell distribution/membrane thickness in the samples in the *in vivo* experiments. SB, MG, DAS, PI and AMK contributed to the editing of the manuscript. SB, MG, PI and VZ contributed to the interpretation of the data. SB and PI performed the statistical analysis of data. DAS and AMK were involved in the conception and design of the study. VZ conceived the study, and also contributed to the interpretation and the editing of the manuscript. All authors have read and approved the final manuscript.

### Ethics approval and consent to participate

The present study was conducted according to the guidelines of the Declaration of Helsinki and was approved by

the Bioethics Committee of the National Hellenic Research Foundation (date of approval: 29/3/2020). The ethic code is PN 2-3/29-3-2020.

### Patient consent for publication

Not applicable.

### Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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