Mechanisms Involved in the Anti-Inflammatory Action of Inhaled Tea Tree Oil in Mice

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Tea tree oil (TTO) is well known as an antimicrobial and immunomodulatory agent. In the present study we confirmed the anti-inflammatory properties of TTO and investigated the involvement of the hypothalamic-pituitary-adrenal (HPA) axis in the immunomodulatory action of TTO administered by inhalation. Sexually mature, 6–8-week-old, $C_{57}Bl_{10}$ × CBA/H (F_1) male mice were used. One group of animals was injected intraperitoneally (ip) with Zymosan to elicit peritoneal inflammation and was then submitted to four sessions of TTO inhalation (15 mins each). Some of the mice were simultaneously injected ip with Antalarmin, a CRH-1 receptor antagonist, to block HPA axis functions. Twenty-four hours after the injections the mice were killed by CO_2 asphyxia, and peritoneal leukocytes (PTLs) were isolated and counted. Levels of reactive oxygen species (ROS) and cyclooxygenase (COX) activity in PTLs were assessed by fluorimetric and colorimetric assays, respectively. The results obtained show that sessions of TTO inhalation exert a strong anti-inflammatory influence on the immune system stimulated by Zymosan injection, while having no influence on PTL number, ROS level, and COX activity in mice without inflammation. The HPA axis was shown to mediate the anti-inflammatory effect of TTO; Antalarmin abolished the influence of inhaled TTO on PTL number and their ROS production in mice with experimental peritonitis, but it had no effect on these parameters in mice without inflammation. Exp Biol Med 232:420–426, 2007

Key words: tea tree oil; inflammation; HPA; COX; immunomodulation

Introduction

Tea tree oil (TTO) is a steam-distilled essential oil from a native Australian plant: the tea tree (*Melaleuca alternifolia*). This oil contains over a hundred different compounds, mainly monoterpenes and their derivatives. Well known for its antimicrobial properties, TTO is able to kill a wide range of bacteria, fungi, and viruses as a result of the action of terpinen-4-ol, γ-terpinen, and 1,8-cineole, the main active components of this oil (1–3).

Immunomodulatory effects of TTO have also been demonstrated. The main components of TTO exhibit anti-inflammatory activity *in vitro*, suppressing the production of proinflammatory cytokines by lipopolysaccharide (LPS)-activated human monocytes (4). The water-soluble fraction of this oil suppressed LPS-stimulated superoxide production by human monocytes, but not by neutrophils (5). An anti-inflammatory effect of TTO has also been observed in *in vivo* studies. In mice, topically applied TTO reduced the edema associated with contact hypersensitivity to a chemical hapten (6) and with intradermal injection of histamine (7), indicating that TTO has an inhibitory effect on proinflammatory cytokine production by lymphocytes. The anti-inflammatory effect of topically applied TTO in reducing the nickel-induced hypersensitivity reaction has also been shown in humans (8). On the other hand, data indicating an immuno-stimulatory effect of TTO are also available. Mice submitted to multiple sessions of TTO inhalation exhibited an increase in the level of circulating immunoglobulins and granulocyte number along with stimulation of a local graft-versus-host reaction (9).

One of the most potent regulatory loops in mammals is the hypothalamic-pituitary-adrenal (HPA) axis. Stimulated by different stressing factors, including inflammation, the HPA axis mobilizes mechanisms of adaptation to the stressor (10). Hormones of the HPA axis also possess immunomodulatory potential, mainly immunosuppressive and anti-inflammatory potential, but their effect on immune cells depends on numerous factors (11). Selective blocking of CRH-1 receptors (e.g., by Antalarmin [12]) causes inhibition of the HPA axis hormone release and therefore eliminates its regulatory functions.

The aim of this study was to continue our previous investigations on the mechanisms involved in the modifi-

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cation of the mouse immune system by TTO inhalation. Previously, we showed a weak effect of multiple sessions of TTO inhalation on the immune system of mice without inflammation plus well-expressed anti-inflammatory properties of TTO mediated by endogenous opioids (EO) (13). In the present study we investigated the involvement of the HPA axis in mediating the anti-inflammatory activities of TTO. The immune parameters evaluated were the number of peritoneal leukocytes (PTLs), their reactive oxygen species (ROS) level, and their cyclooxygenase (COX) activity. COXs are crucial enzymes involved in the production of proinflammatory mediators, the prostaglandins, which are derivatives of arachidonic acid. The expression of COX-2 is stimulated by bacterial endotoxins and proinflammatory cytokines, while several other COX isoforms are produced constitutively (14, 15). COX-2 produced by leukocytes is involved in the production of proinflammatory mediators leading to stimulation of inflammatory processes (e.g., in the cardiovascular system [16]).

Materials and Methods

Animals. Experiments were performed on 6–8-week-old $C_{57}BI_{10} \times CBA/H$ (F_1) male mice, which were randomly divided into experimental groups and housed with six to seven individuals per cage. Mice were maintained in a breeding room under standard conditions: 22–24°C, 12:12-hr light:dark cycle, with free access to standard laboratory rodent food and water. Animals were treated according to Polish regulations concerning experiments on animals, and the procedure was approved by the local ethical commission.

Experimental Design. *TTO Inhalation.* On the day of the experiment, animals were subjected to four 15-min TTO inhalation sessions every 3 hrs. The mice were transported in cages, as described previously (13), from the breeding room to the laboratory and were placed into cages dedicated to inhalation in order to eliminate spreading of TTO to the breeding room and to prevent adaptation of the olfactory neurons to the smell of TTO (17). A volume of 150 µl of TTO was applied to lightly moistened pieces of cotton wool, which were then enclosed in perforated plastic containers that were put onto the cover of the cage. For the period of inhalation, the cages were covered with a layer of material to minimize draughts and to concentrate the vapors of TTO. Concentration of TTO vapors in the inhalation chamber reached approximately 460 ppm. After each inhalation session mice were carried back to the breeding room. Mice from the control group (placebo) were submitted to sham inhalation treatment, which was identical to the procedure used for the experimental (TTO) group except that TTO was not applied to the cotton wool.

TTO was purchased from Pollena Aroma (Warsaw, Poland). Table 1 shows the major constituents of this oil, as determined by liquid gas chromatography (data from manufacturer).

Inflammation Induction. Immediately before the first inhalation session, half of the mice, assigned to both TTO and placebo groups, were injected intraperitoneally (ip) with 0.5 ml of 0.2% (w/v) Zymosan A solution (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) to induce peritoneal inflammation or were injected with the same volume of PBS as a control injection (18). Simultaneously, half of mice from each group were injected ip with 0.1 ml of 0.5% (w/v) Antalarmin (Sigma-Aldrich) solution (in PBS) or the same volume of PBS (12).

PTL Isolation. Since PTLs are easy to collect and amenable to study (19), experimental peritonitis is a good model for the investigation of systemic inflammation and modulation of this process by various factors (20, 21). Twenty-four hours after the induction of peritonitis, mice were sacrificed by asphyxiation in a CO_2–rich atmosphere.

PTLs were obtained by flushing the peritoneal cavity with 7 ml of heparinized PBS. After counting the leukocytes with a hemocytometer, the peritoneal exudates were centrifuged (250 *g*, 10 mins, 4°C), and the cell pellets were resuspended in PBS containing EDTA (0.2 mg/ml) and glucose (0.9 mg/ml), to a concentration of 2×10^6 cells/ml. PTLs were used for the measurement of ROS level and a COX activity assay.

ROS Level Measurement. ROS level was measured in PTLs pooled within groups. An aliquot of 1×10^6 cells per group was incubated at 37°C for 30 mins with the oxidation-sensitive dye dichloro-dihydro-fluorescein diacetate (H_2DCFDA; Molecular Probes, Carlsbad CA) at a concentration of 1 µg/ml (as in Van Pelt *et al.*, but modified [22]) and then distributed at 2×10^5 cells/well into a flat-bottomed, 96-well assay plate (Greiner Bio-One, Frickenhausen, Germany). Fluorescence was measured using a Victor3 fluorimeter (PerkinElmer, Wellesley, MA). Before mixing with H_2DCFDA, another aliquot of cells from each group was incubated with phorbol myristate acetate (PMA) at a concentration of 1 µg/ml to stimulate leukocyte activity (20). Autofluorescence of leukocytes was very low and similar in all experimental groups, so it was regarded as negligible.

COX Activity Measurement. A colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) was used for COX activity measurement, according to the manufacturer's instructions. PTLs were stored at –20°C before the COX assay. After thawing, cells were disrupted by sonication (three pulses of 2 secs each), centrifuged (10,000 *g*, 15 mins, 4°C), and the supernatants were collected and placed on ice. Some aliquots of cell supernatant were treated with DuP-697, a selective inhibitor of COX-2 (23). The assay reaction was stopped after 15 mins, and the colorimetric reaction product, oxygenated tetramethylphenylenediamine, was measured at 590 nm using a plate reader (Dynatech MR) (24).

Statistical Analysis. Statistical analysis was performed using the parametric Student-Newman-Keuls test in the GraphPad Instat program (San Diego, CA). Figures

Table 1. Major Constituents of Tea Tree Oil Used[a]

Component	Percentage
Terpinen-4-ol	21.90
γ-Terpinene	12.94
α-Terpinene	7.65
Cymene	7.27
α-Pinene	4.93
Terpinolene	4.19
α-Terpineol	3.63
Aromadendrene	3.34
1,8-Cineole (eucalyptol)	3.03
α-Thujene	2.48
D-Limonene	2.36

[a] Data from manufacturer.

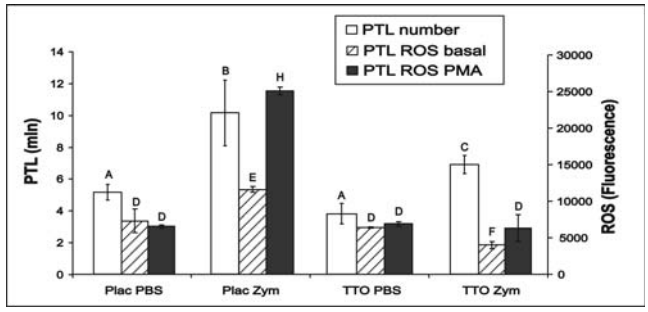

Figure 1. PTL numbers and their ROS levels in mice submitted to TTO inhalation sessions. Plac = group of mice submitted to sham inhalations and injected with PBS (Plac PBS) or Zymosan (Plac Zym); TTO = group of mice receiving TTO inhalations and injected with PBS (TTO PBS) or Zymosan (TTO Zym). Mean ± SD ($n = 4$–6). Values with different letters (e.g., A vs. B) vary significantly ($P \leq 0.01$), while values sharing letters are not significantly different. Statistical significance of PTL number and ROS level differences are compared separately.

show mean values ± standard deviation (SD). The number of mice in each group was between five and seven.

Results

PTL Number and ROS Level (Fig. 1). Intraperitoneal Zymosan injections caused an increase in leukocyte number in the peritoneal cavity of mice receiving the sham-inhalation treatment (Plac Zym). TTO inhalation did not change the number of resident PTLs (TTO PBS), but in Zymosan-injected animals, PTL numbers were significantly reduced compared with numbers in mice receiving sham-inhalations (TTO Zym vs. Plac Zym).

PTLs isolated from Zymosan-injected mice subjected to sham inhalations (Plac Zym) exhibited elevated ROS levels in comparison with animals without inflammation in the placebo (Plac PBS) and TTO (TTO PBS) groups. ROS levels in PTLs from mice without peritoneal inflammation in both the TTO and sham-inhalation groups did not differ significantly. PTLs from Zymosan-injected mice subjected to sessions of TTO inhalation had a decreased ROS level compared with those of all other groups.

In vitro stimulation with PMA had no influence on ROS levels in resident PTLs isolated both from placebo and TTO-treated groups of mice (Plac PBS vs. TTO PBS), but it resulted in a 2-fold increase in ROS level in the sham-inhalation group with peritoneal inflammation (Plac Zym). TTO inhalation sessions blocked *in vitro* stimulation of PTLs from mice with peritoneal inflammation (TTO Zym).

Modification of PTL Number and ROS Level by Antalarmin (Fig. 2). Antalarmin, a CRH-1 receptor antagonist, did not affect the number of resident PTLs in the placebo and the TTO-treated groups, as well as in the sham-inhalation mice with peritoneal inflammation (Plac Zym). In contrast, Antalarmin reversed the inhibitory influence of TTO inhalations on PTL number in mice with peritonitis, raising it to the level observed in the placebo group injected with Zymosan (Fig. 2A). Intraperitoneal injection of Antalarmin had no effect on the ROS level in PTLs of mice from the placebo and the TTO-treated groups without peritonitis. However, it completely abolished the effect of TTO inhalations on ROS level in PTLs from mice with peritonitis and blocked an increase in ROS level in Zymosan-injected sham-inhalation mice (PLAC Zym) (Fig. 2B).

PTL COX Activity (Fig. 3). TTO inhalations did not change COX activity in mice without peritoneal inflammation (injected with PBS). Zymosan injection in mice subjected to sham inhalations stimulated COX activity and therefore inflammatory mediator production, while TTO inhalations abolished the increase in COX activity caused by Zymosan injection. In addition to causing a decrease in the total activity of all COX subtypes, a selective COX-2 blocker, DuP-697, added to the PTLs *in vitro*, also abolished the differences between particular experimental groups of mice (Fig. 3). Antalarmin increased the COX activity in every experimental group but did not affect the modification of COX activity caused by TTO inhalations (results not shown).

Discussion

In a previous study we investigated the effect of prolonged inhalation of TTO (three times daily for 1 week) on immunity in mice (13). We found that this treatment had no influence on the number of PTLs and their ROS level in mice without inflammation, but in Zymosan-injected mice, TTO exerted an anti-inflammatory effect *via* an EO-mediated mechanism (13). To assess the mechanism by which TTO influences immune cells involved in inflammation, we have modified our experimental design so that peritoneal inflammation was elicited just before the start of the inhalation sessions. The results of the present study confirm the anti-inflammatory properties of TTO. PTLs isolated from mice without peritoneal inflammation were not only low in number, but they were also apparently quiescent (i.e., unable to be stimulated by PMA *in vitro*). In

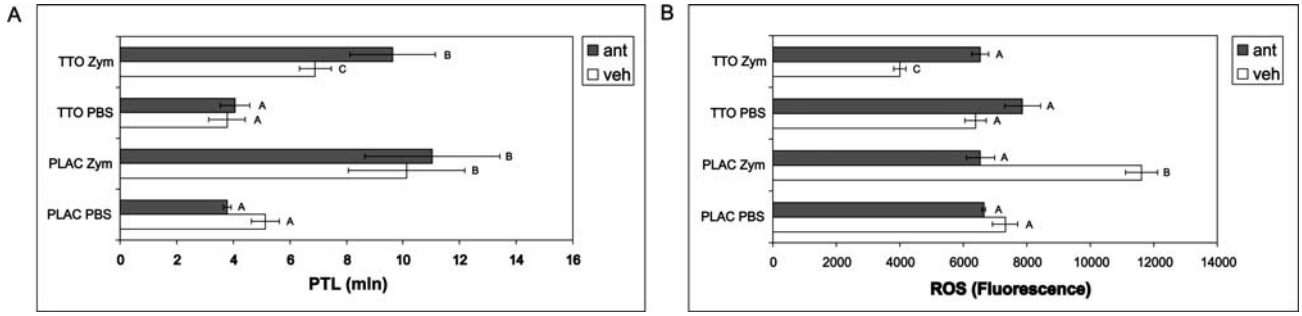

Figure 2. Antalarmin influence on inhaled TTO modification of PTL number (A) and their ROS level (B). Ant = mice injected with Antalarmin or vehicle (veh); Plac = group of mice submitted to sham inhalations and injected with PBS (Plac PBS) or Zymosan (Plac Zym); TTO = group of mice receiving TTO inhalations and injected with PBS (TTO PBS) or Zymosan (TTO Zym). Mean ± SD ($n = 4$–6). Values with different letters (e.g., A vs. B) vary significantly ($P \leq 0.01$), while values sharing letters are not significantly different.

contrast, cells obtained from Zymosan-injected animals with already-raised intracellular ROS responded to PMA stimulation with a further elevation of these levels by an almost 2-fold measure (Fig. 1). PTLs from Zymosan-injected mice subjected to sessions of TTO inhalation neither exhibited increased ROS levels nor responded to PMA stimulation *in vitro*. This confirms the anti-inflammatory action of TTO, exerted on activated immune cells. In our previous experiments (13), the PTLs' subpopulations frequency and their ROS level in control and in Zymosan–injected mice exposed to 7 days of TTO inhalation sessions were submitted to flow cytometry analysis. Since we observed the differences only in ROS level but not in the cell subpopulations frequency after TTO inhalations (see Table 2, containing unpublished data from previously described experiment), in further investigations the cytometric analysis was omitted. On the basis of these results we assume that the ROS level changes observed in the present study resulted directly from changes in PTL activity rather than from differences in cell subpopulation frequency.

The results of this study indicate the involvement of the HPA hormonal axis in the mechanism by which inhaled TTO modifies immune system function. In mammals, inflammation is one of the stressing factors causing activation of the HPA axis and, thus, mobilizing the adaptation to stressors (10). The immunomodulatory properties of HPA axis hormones are now well established (11); therefore, any alteration of their concentration in the circulation can influence immune system functions.

Antalarmin blocked an increase of the ROS level in PTLs from placebo-group mice injected with Zymosan. This is probably a result of Antalarmin blocking CRH-1 receptors on PTLs, which play an important role in signaling during the development of an inflammatory state and stimulation of immune cells (25). Antalarmin was shown to exhibit anti-inflammatory properties by blocking immune cell–derived CRH produced peripherally, rather than systemically (26). This indicates that this agent may interfere with anti-inflammatory action of TTO, but in our experiment, Antalarmin showed no anti-inflammatory influence on PTL number in placebo-treated mice (Fig. 2A). Anti-inflammatory properties of Antalarmin were also not confirmed in the experiment of Tsatsanis *et al*. (27), who showed that this substance had no influence on proinflammatory cytokine production by LPS-stimulated murine macrophages (*in vitro*). It is possible that inhaled TTO decreases production of CRH by immune cells and in this

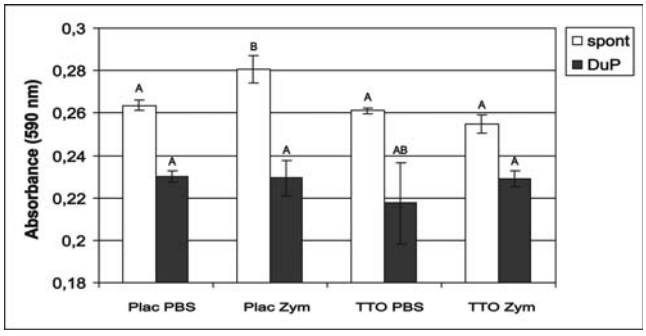

Figure 3. COX activity in PTLs from mice submitted to TTO inhalation sessions. Spont = *in vitro* PTL COX activity; DuP = *in vitro* activity of COX, DuP-697 treated; Plac = group of mice submitted to sham inhalations and injected with PBS (Plac PBS) or Zymosan (Plac Zym); TTO = group of mice receiving TTO inhalations and injected with PBS (TTO PBS) or Zymosan (TTO Zym). Mean ± SD ($n = 3$). Values with different letters (e.g., A vs. B) vary significantly ($P \leq 0.001$), while values sharing letters are not significantly different.

Table 2. Frequency (%) of Macrophages and Granulocytes in Peritoneal Exudates from PBS- and Zymosan-Injected Mice Exposed to TTO Inhalation Sessions for 7 Days[a]

	% macrophages	% granulocytes
PBS placebo	2.67 ± 0.27	5.17 ± 0.35
PBS TTO	2.76 ± 0.18	8.44 ± 5.21
Zymosan placebo	4.54 ± 0.61	37.9 ± 1.1
Zymosan TTO	4.95 ± 0.72	34.69 ± 7.31

[a] Effect of TTO Inhalations in both PBS- and Zymosan-treated groups is statistically nonsignificant.

way exerts its anti-inflammatory action. This hypothesis needs further investigation. In our previous study (13), we showed that the anti-inflammatory effect of long-term TTO inhalation was mediated by EO. It is possible that the blocking of the HPA axis by Antalarmin also reduced the production of EO in the pituitary, because corticotrophin has the same precursor (proopiomelanocortin) as some EO.

In Zymosan-injected mice, TTO inhalations decreased the activity of COXs in PTLs to the level observed in mice without inflammation (Fig. 3), which indicates that proinflammatory cytokine production by PTLs had also been reduced (15). In mice without inflammation, we observed a lack of influence of TTO inhalations on all examined parameters of PTLs as well as on COX activity. Since TTO had no influence on the assessed parameters of PTLs from mice with an unstimulated immune system, this indicates that this essential oil may be a safe means of preventing inflammation. Antalarmin injection caused an increase in the activity of COX in PTLs from every group of mice examined (data not shown), but did not abolish the differences between experimental groups, indicating that inhaled TTO modifies COX activity *via* a mechanism not involving the immune cell–derived CRH or HPA axis.

On the basis of our findings and other published data, we propose a mechanism for the action of inhaled TTO on the mouse immune system (Fig. 4). Inhaled TTO may modify the immune system directly, influencing the activity of immune cells after entering the circulation or may influence the activity indirectly *via* the central nervous system (CNS). As the components of ethereal oils are readily absorbed by the blood (28), they may exert a direct immunomodulatory effect on immune cells. Such properties of TTO have been demonstrated in *in vitro* studies, in which terpinen-4-ol, the main component of this oil, suppressed the production of proinflammatory cytokines by LPS-activated human monocytes (4). A local mediation of immune cell–derived CRH (25) should not be excluded as well.

Ethereal oils, which can be detected in the serum within 15 mins of inhalation (28), have the ability to cross the blood-brain barrier and can influence CNS function by acting directly on neurons (29, 30). A second way in which ethereal oils (including TTO) may influence CNS function is through the stimulation of olfactory receptors and neurons in the olfactory epithelium. Most ethereal oils are thought to modify CNS functions *via* this system, which senses and processes odors (31).

The chemical information recognized by the olfactory

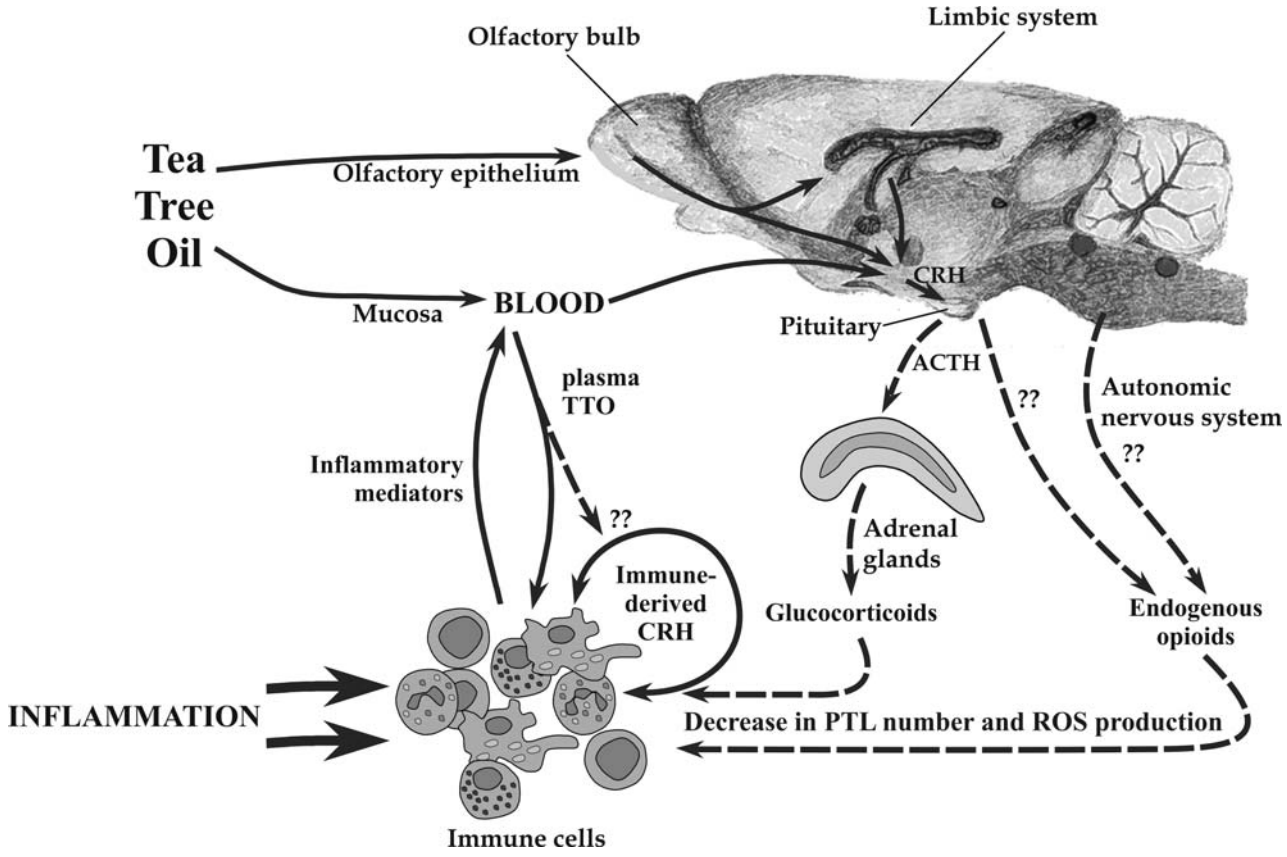

Figure 4. The proposed mechanism of action of inhaled TTO on the mouse immune system. Hard lines: literature-based data; interrupted lines: results of our investigations.

system is transmitted *via* neuronal pathways to various structures in the CNS, including the cerebral cortex and limbic system (32). Olfactory cues may influence the social interactions or sexual behavior of rodents (or humans) as well as the emotions, including the ability to induce stress responses and HPA axis activation. The potential of olfactory cues to modify the function of the immune system was shown in a study in which mice submitted to the smell of stressed individuals of the same species developed their own stress response, which influenced parameters of the immune system (33). The mice used in our experiments were probably also stressed as a result of the changes in environment (rooms and cages) during the period covering the inhalation sessions, but in previous experiments we demonstrated that sham-inhalation treatment had no influence on the PTL number (i.e., the first symptom of peritoneal inflammation in mice [13]).

In conclusion, the immunomodulatory action of TTO administered to mice by inhalation is mediated not only by the HPA axis and EO but probably also by other unidentified mechanisms that may involve novel factors and/or result from the direct action of TTO components on immune cells. Further investigation is required to dissect the complex action of inhaled TTO on the immune system.

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