Involvement of brain cytokines in zymosan-induced febrile response

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Bastos-Pereira AL, Fraga D, Ott D, Simm B, Murgott J, Roth J, Zampronio AR. Involvement of brain cytokines in zymosan-induced febrile response. J Appl Physiol 116: 1220–1229, 2014.—This study compared the involvement of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) within the central nervous system (CNS) in the febrile response induced by zymosan (zym) and lipopolysaccharide (LPS). In addition, we investigated whether zym could activate important regions related to fever; namely, the vascular organ of the laminae terminalis (OVLT) and the median preoptic nucleus (MnPO). Intraperitoneal injection of zym (1, 3, and 10 mg/kg) induced a dose-related increase in core temperature. Zym (3 mg/kg) also reduced tail skin temperature, suggesting the activation of heat conservation mechanisms, as expected, during fever. LPS increased plasma levels of TNF-α measured at 1 h, IL-1β measured at 2 h, and IL-6 measured at 3 h after injection. Zym increased circulating levels of IL-6 but not those of TNF-α or IL-1β at the same time points. In addition, an intracerebroventricular injection of antibodies against TNF-α (2.5 μg) and IL-6 (10 μg) or the IL-1 receptor antagonist (160 ng) reduced the febrile response induced by zym and LPS. Zym (100 μg/ml) also increased intracellular calcium concentration in the OVLT and MnPO from rat primary neuroglial cultures and increased release of TNF-α and IL-6 into the supernatants of these cultures. Together, these results suggest that TNF-α, IL-1β, and IL-6 within the CNS participate in the febrile response induced by zym. However, the time course of release of these cytokines may be different from that of LPS. In addition, zym can directly activate the brain areas related to fever.

zymosan; fever; cytokine; OVLT; MnPO

The number of fungal infections is increasing throughout the world (47, 72). The risk of invasive fungal diseases as coinfections in immunodeficiency situations, particularly during epidemics and immunosuppressive treatment, has increased and has led to higher rates of morbidity and mortality, and higher health costs (17, 47). However, the mechanisms involved in fungal infection pathogenicity are not completely understood. Guidelines for the prevention and treatment of invasive fungal diseases are habitually based on empirical actions (15, 17). For these reasons, further experimental studies on fungal infection and its implications, such as the febrile response, are important in choosing and developing more specific and effective therapeutic approaches.

Zymosan (zym) is a fungal-derived pathogen-associated molecular pattern (PAMP) mainly composed of polysaccharides (14, 58). It is known to act through Toll-like receptor (TLR) 2 and TLR6 (51). As a laboratory tool, zym has been used to induce arthritis (22) and other inflammatory conditions such as ear edema, peritonitis, and shock (23, 26, 46). Zym was also previously used to induce fever in laboratory animals (29, 31, 34), but compared with lipopolysaccharide (LPS), the mediators and mechanisms involved in zym-induced fever are mostly unknown.

LPS, acting on TLR4, activates the synthesis and release of endogenous pyrogens (EPs) such as the cytokines interleukin-1β (IL-1β), IL-1α, IL-6, IL-8, tumor necrosis factor-α (TNF-α), chemotactic factor for neutrophils induced by cytokines (CINC), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β, interferon-α and -γ, and receptor activator of nuclear factor-κB ligand (RANKL) (24, 33, 42, 68, 76). Some EPs, including IL-8, CINC, MIP-1α, and RANKL, seem to be generated and exert their pyrogenicity directly within the central nervous system (CNS) (24, 42, 76). Others such as IL-1β, TNF-α, and IL-6 can be produced at peripheral and central sites (9, 21, 25, 39, 63, 64). Therefore, the latter three cytokines are key players in communication between the immune system and thermoregulatory centers. There are several hypotheses that attempt to explain how these cytokines, when generated peripherally, reach the thermoregulatory centers in the hypothalamus: by entering the CNS through regions devoid of the blood brain barrier such as the vascular organ of the laminae terminalis (OVLT) (3); by binding to the vascular endothelium and generating central mediators (8); or by being transported into the CNS through specific carriers (1, 2, 52). These events would lead to the synthesis/release of mediators such as prostaglandin E2 and F2α (11, 41), corticotrophin-releasing hormone (62), endogenous opioids (5, 20), substance P (4, 56), and endothelin-1 (18), among others, resulting in fever.

Similar to the LPS receptor TLR4, the presence of the zym receptor TLR2 in inflammatory cells has been identified (16). In addition, it has been shown that zym can directly stimulate these cells (69) and, in some studies, the synthesis/release of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 has been reported in response to stimulation with zym (26, 46, 69). Several studies have shown that TLR2 is also expressed in the CNS and contributes to inflammatory diseases in the brain (28, 35, 48). Stitt and Shimada (70) also showed that zym could enhance the febrile response to semipurified EPs when microinjected directly into the OVLT. Therefore, besides the zym-induced peripheral release of cytokines, a direct activation of TLR2 in the CNS may also contribute to the febrile response to this pyrogen.

It is possible that the activation of distinct pathways by LPS (TLR4) and zym (TLR2/6) result in the release of different mediators. Indeed, it has been previously suggested that activation of the complement system is important for the initiation of LPS-induced fever, whereas zym induced fever only at
doses that were ineffective to activate this system (37, 38). In addition, the increase in intracellular calcium concentration, 
\[ [Ca^{2+}] \], and the release of inflammatory cytokines by cells from the area postrema stimulated by LPS and other TLR2 or TLR2/6 agonists (muramyl dipeptide or fibroblast-stimulating lipopeptide-1, respectively) seem to be different (74). In primary neuroglial cell cultures from the OVLT, marked differences were observed with regard to cellular calcium signaling after stimulation with LPS compared with the fibroblast-stimulating lipopeptide-1 (49). With regard to zym, no data about its direct effects on cultured brain cells are available so far.

In this study, we aimed to compare the participation of IL-1β, TNF-α, and IL-6 within the CNS in the febrile response induced by zym and LPS using specific antagonists or antibodies. We further investigated whether zym could activate important regions related to the febrile response; namely, the OVLT [as suggested in Ref. (70)] and median preoptic nucleus (MnPO), a major preoptic site involved in thermoregulation (45, 64, 75).

**MATERIAL AND METHODS**

**Animals.** Experiments were conducted in male Wistar rats (180–220 g), housed four per cage at 22 ± 1°C under a 12:12-h light-dark cycle (lights on at 7:00 A.M.) and with free access to food and tap water. A total of 30 separate groups of 5–6 animals were used in this study. Animals were used only once. All experiments were approved by the Federal University of Paraná Ethical Committee on Animal Use and were in accordance with Brazilian and international guidelines for animal care. For microculture experiments, Wistar rat pups of both sexes were used. Animal care, breeding, and experimental procedures were conducted according to the guidelines approved by the Hessian Ethical Committee, Germany.

**Intracerebral cannula implantation and microinjection.** For intracerebroventricular (icv) administration of antibodies and antagonist, a 22-gauge stainless steel guide cannula (0.8 mm outer diameter, 12 mm long) was stereotaxically implanted into the right lateral ventricle under ketamine (90 mg/kg; Vetnil Veterinary Products, Louveira, Brazil) and xylazine (7.5 mg/kg; Syntec Laboratory, Cotia, Brazil) anesthesia in aseptic conditions. The stereotaxic coordinates were as follows: 0.8 mm lateral to the midline, 1.5 mm posterior to bregma, and 2.5 mm below the brain surface, with the incisor bar lowered by 3.3 mm below the horizontal zero (54). Cannulas were fixed to the skull with jeweler’s screws that were embedded in dental acrylic cement. Animals were treated with oxytetracycline hydrochloride (400 mg/kg by intramuscular route; Pfizer Laboratories, São Paulo, Brazil) and ketoprofen (10 mg/kg by oral route; Medley Laboratories, São Paulo, Brazil) after surgery and allowed to recover for at least 5 days before experimental use. After the experiment, each rat was microinjected into the lateral ventricle with Evans blue (2.5% in saline). Brains were quickly removed under aseptic conditions and transferred to small Petri dishes that were filled with ice-cold oxygenated Gey’s balanced salt solution (HBSS). Brains were cut in coronal sections at 200 µm and incubated in a 0.1% solution of 2′,3′,5′-triiodothyroacetic acid (Triton X-100; Sigma) for 20 min at room temperature. Sections were then washed in ice-cold oxygenated HBSS and air-dried on a glass slide. The sections were then stained with cresyl violet and mounted on a glass slide using Aquamount (BDH Chemicals, Poole, UK). The sections were examined with a light microscope (40× objective; Leica SF2000; Leica Microsystems, Wetzlar, Germany) and images were captured with a digital camera (Olympus Camedia C3030 Zoom; Olympus, Tokyo, Japan) and analyzed using Adobe Photoshop v.6.0 (Adobe Systems, San Jose, CA).

**Abdominal and tail skin temperature measurement.** Abdominal core temperature (Tc) was measured in conscious unrestrained rats using data loggers (Subue, Calgary, AB, Canada). Briefly, data loggers were implanted in the peritoneal cavity at least 5 days before the experiment under the same anesthesia described earlier, and animals received the same postsurgical care. On the day of the experiment, Tc was continuously monitored at 15-min intervals from 2 h before any injection until 6 h after the injection of the pyrogenic stimulus. During the experiment, room temperature was maintained at 28 ± 1°C (within the thermoneutral zone for rats) (55, 59).

For skin temperature (Tsk) measurement, the same type of data logger was inserted into a protective covering device (Insight, Ri-

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containing EDTA (1 mM/liter; Sigma Aldrich, Munich, Germany) to inactive the enzyme. After washing thrice and gentle dispersion in 2 ml of complete medium consisting of Neurobasal medium A supplemented with 2% B 27 (Invitrogen, Carlsbad, CA), penicillin (100 U/liter), streptomycin (0.1 mg/ml), and l-glutamine (2 mM/liter) the preparations were subjected to gently repeated trituration with a fire-polished Pasteur pipette. The dissociated cells were plated onto prewarmed, poly-L-lysine-coated (1 mg/ml; Sigma Aldrich, Munich, Germany) glass coverslips (Eppendorf, Hamburg, Germany) forming the bottom of a reusable Flexiperm-micro-12-well (6-mm diameter; Greiner Bio-One, Solingen, Germany) to ensure sufficient cell density despite limited absolute cell number, and they were maintained in a humidified atmosphere of 5% CO2 and 95% air at 37°C. The medium was exchanged the next day to remove cellular debris and after that, every 2 days during the culture period. The cells were employed for intracellular calcium measurements and for a subsequent immunocytochemical characterization (anti-GFAP for astrocytes, anti-ED-1 for microglial cells, anti-MAP2a or + for neurons); supernatants were used for the determination of cytokines. Zym was dissolved in phosphate-buffered saline (PBS; pH 7.4) at concentrations of 1 mg/ml and added to the perfusion chamber by bolus application to achieve a final concentration of 100 µg/ml during a 1-min arrest of the superfusion pump (minipuls-3; Abimed Analysen-Technik, Langenfeld, Germany). The concentration was chosen according to other published reports in which other TLR agonists were tested (49, 50). A bolus control application with PBS was performed before stimulation with zym. At the end of the experiment, cells were exposed to a buffer with high potassium chloride concentration (50 mM) as a vitality test, especially for neurons (49, 74).

Measurement of intracellular calcium and cytokines in microcultures. After 5–6 days on individual glass coverslips, cells were loaded with 2 mM fur-2 AM (MoBiTec, Göttingen, Germany) in complete medium for 45 min in a humidified atmosphere of 5% CO2/95% air at 37°C. For [Ca2+]i, measurements, coverslips were placed under an inverted microscope (IMT-2; Olympus Optical, Hamburg, Germany) in a specially constructed Teflon culture chamber and superfused with buffer. Fluorescence measurements were performed using a filter-wheel-based excitation system and analyzed with MetaFluor 4.5 software (VisiRon, Puchheim, Germany). After defining regions of interest for single cells by a continuously variable aperture, the time course of emitted fluorescence (>515 nm) after alternating excitations at 340 and 380 nm, respectively, was recorded at 0.2 Hz using a Visicam 12-bit digital charge-coupled device camera (VisiRon). The 340/380 ratios proportional to [Ca2+]i were computed and analyzed. Phenotypic identification of cultured neurons and glial cells was confirmed by immunolabeling with polyclonal antisera or monoclonal antibodies directed against cell-specific marker proteins as previously described (49, 50, 74). On other glass coverslips, the OVLt or MnPO cells were incubated with zym (100 µg/ml) or vehicle in complete medium for 240 min in a humidified atmosphere of 5% CO2/95% air at 37°C. The supernatants were removed from the cells for later measurements of cytokines. Determination of TNF-α was performed by a bioassay on the basis of cytotoxic effect of this cytokine on the mouse fibrosarcoma cell line WEHI 164 subclone 13 and a murine TNF-α standard (code 88/532; National Institute for Biological Standards and Control, South Mimms, UK). Measurement of IL-6 was performed by a bioassay on the basis of dose-dependent growth stimulation of IL-6 on the B9 hybridoma cell line and a human IL-6 standard (code 89/54; National Institute of Standards and Control). The levels of TNF-α and IL-6 that were determined in our samples reflect the bioactivity in relation to established international standards (49).

Statistical analysis. Data for Tc, Tskin, and HLI responses were analyzed by two-way ANOVA for repeated measures followed by a Bonferroni test for post hoc analysis. Cytokine levels were analyzed by Student’s t-test or one-way ANOVA followed by a Bonferroni test, as appropriate. These results are reported as means ± SE. The number of zym-responsive cells of a given cellular phenotype were expressed as a percentage of all cells of this phenotype investigated and the raw frequency data analyzed by χ²-test. Stimulus-induced transient increases of [Ca2+]i were expressed as the difference between resting [Ca2+]i measured before the respective stimulation and the stimulus-induced [Ca2+]i peak (ratio [340/380 nm]). A difference in ratio [340/380 nm] of more than 0.1 was considered a stimulus-induced Ca2+ signal, which can be regarded as a strict exclusion criterion. Differences in ratio [340/380 nm] were also evaluated by one-way ANOVA followed by a Bonferroni test. All data were analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA). Differences in experiments were considered significant when P < 0.05.

RESULTS

Febrile response induced by zym. Intraperitoneal administration of vehicle (saline) did not induce any significant changes in the animals’ Tc (Fig. 1, A and B). Administration of 1 mg/kg zym also did not change the Tc of the rats (Fig. 1A). However, at doses of 3 or 10 mg/kg, zym induced an increase in Tc that started around 2 h and peaked around 3 h after the injection (Fig. 1A). Tc then decreased and was at normal levels at around 4 h after the time of injection. Administration of 3 mg/kg zym also induced a significant reduction in Tskin 1.5 h after the injection concurrently with a significant increase in Tc in these animals 30 min later (Fig. 1B). Tc of animals receiving vehicle or 3 mg/kg zym in Fig. 1A were not significantly different from the Tc of animals that had received the same dose of zym or vehicle at 24 h after halothane exposure for the adjustment of the device to measure Tskin (Fig. 1B). Figure 1C shows the HLI calculated on the basis of these results.

Circulating levels of cytokines after intraperitoneal injection of zym and LPS. Figure 2 shows the levels of cytokines measured in the plasma of animals that received LPS or zym. Circulating levels of TNF-α were undetectable in the plasma of vehicle-treated rats but increased at 1 h after the injection of LPS at a pyrogenic dose (50 µg/kg). However, TNF-α was also undetectable at 1 h after administration of zym (Fig. 2A). Circulating levels of IL-1β 2 h after injection of LPS were significantly higher than those in vehicle-treated animals (Fig. 2B). Although circulating levels of IL-1β after injection of zym were higher, they were not statistically different from those recorded in animals that received only vehicle (Fig. 2B). Conversely, levels of plasma IL-6 were significantly higher at 3 h after both LPS and zym compared with vehicle-treated animals (Fig. 2C).

Effects of IL-1RA and anti-TNF-α and anti-IL-6 antibodies in the febrile response induced by zym and LPS. Figure 3 shows the effect of anti-TNF-α and anti-IL-6 antibodies and IL-1RA on the febrile response induced by zym and LPS. Administration of the antibodies and IL-1RA by icv route plus vehicle ip did not induce significant changes in the Tc of the animals. Zym at 3 mg/kg induced a febrile response that started at 2 h and lasted until approximately 4 h after the injection (Fig. 3, A, C, and E), whereas LPS induced a febrile response that started at the same time but lasted until the end of the experiment (6 h) (Fig. 3, B, D, and F). Pretreatment of animals with anti-TNF-α, IL-1RA, and anti-IL-6 via the icv route significantly reduced the febrile response induced by both LPS and zym by about 50% (Fig. 3).

Effects of zym and LPS on OVLt and MnPO cell activation. Figure 4, A and B, depicts the result based on the analysis of
233 neurons, 385 astrocytes, and 46 microglial cells derived from 5 different OVLT preparations. Out of these cells, 9 neurons (3.86%), 138 astrocytes (35.84%), and 20 microglial cells (43.48%) were responsive to zym application (Fig. 4A). The number of astrocytes and microglial cells responsive to zym application was significantly higher than that of neurons ($\chi^2$-test 81.90, df = 1, $P < 0.0001$; and $\chi^2$-test 64.73, df = 1, $P < 0.0001$, respectively). There was no significant difference between astrocytes and microglia from OVLT preparations ($\chi^2$-test 1.031, df = 1). The mean strength of the zym-induced Ca\textsuperscript{2+} signals was different in the three cellular phenotypes, being stronger in astrocytes and microglia compared with neurons. The average $\Delta$ratio [340/380 nm] values were 0.13 for neurons, 0.24 for astrocytes, and 0.3 for microglia (Fig. 4B).

Four different MnPO preparations were investigated, and 290 neurons, 163 astrocytes, and 36 microglial cells were analyzed (Fig. 4C). Out of these cells, 8 neurons (2.75%), 33 astrocytes (20.24%), and 16 microglia cells (44.44%) re-
sponded to zym stimulation, with a pronounced elevation in [Ca^{2+}]. (Fig. 4C). The number of astrocytes and microglial cells from MnPO responsive to zym application was significantly higher than that of neurons (χ²-test 38.76, df = 1, P < 0.0001; and χ²-test 81.60, df = 1, P < 0.0001, respectively). However, a significant difference between astrocytes and microglia from MnPO preparations was also observed (χ²-test 9.30, df = 1, P < 0.0023). The mean strength of zym-induced Ca^{2+} signals was similar for the three cellular phenotypes, with average Δratio [340/380 nm] values of 0.16–0.25 (Fig. 4D).

Figure 4E shows a typical example of Ca^{2+} tracings recorded from a small group of Fura 2-loaded OVLT cells, illustrating the responsiveness of three astrocytes and the lack of responsiveness of three neurons to a bolus application of zym. Zym induced an increase in [Ca^{2+}], within seconds after administration. Bolus injections of an equivalent volume of buffer had no effect on [Ca^{2+}], excluding possible nonspecific mechanical stimulation. All nonresponsive neurons responded to KCl perfusion as a vitality test.

Effects of zym and LPS on levels of TNF-α and IL-6 in the supernatants of OVLT and MnPO microcultures. In addition, we tested the capacity of OVLT and MnPO microcultures to release cytokines in response to zym stimulation (100 μg/ml). Two hundred forty minutes after zym stimulation, an increase in the concentration of TNF-α was observed in supernatants from both OVLT and MnPO cells compared with PBS treatment (Fig. 5A). The concentration of TNF-α in supernatants from OVLT-stimulated cells was approximately twofold higher than in the supernatants from MnPO-stimulated cells. In
addition, stimulation of OVLT cells for 240 min also increased IL-6 concentration in the supernatant (Fig. 5B). For MnPO cultures, the increase in IL-6 levels was smaller compared with increases in the OVLT and were not statistically different from the PBS-treated group (Fig. 5B).

DISCUSSION

The results of the present study confirm the capacity of zym to induce a febrile response in rats and suggest the involvement of TNF-α, IL-1β, and IL-6 in this process. Moreover, this PAMP increased [Ca\(^{2+}\)]\(_i\) in a primary rat neuroglial culture in two important areas related to central thermoregulation and fever induction.

Zym induced a febrile response at the higher doses used but no statistically significant difference was observed between 3 and 10 mg/kg. For this reason we chose to continue the experiments using a lower dose that caused a significant febrile response. We did not observe hypothermia, as observed by Li et al., at any dose used (37). Instead, a remarkable febrile response with a peak of 1.5°C, which lasted 2 h, was observed at the higher dose. Hubschle et al. using the same dose (10 mg/kg) observed a febrile response that lasted 4 h (29). In another study, Kanashiro et al. observed that intraarticular administration of 4 mg of zym led to a pronounced and long-lasting febrile response (31). These differences in intensity and duration of fever may be related to animal models, route of administration, and ambient temperatures in which the experiments were performed (29, 31, 37). Our results also corroborate previous studies showing that an increase in \(T_c\) caused by zym is in fact a febrile response. We showed here that an injection of zym caused a decrease in \(T_{skin}\), which preceded the increase of \(T_c\) by some minutes, suggesting that the increase in \(T_c\) observed after zym is partially due to the activation of heat conservation mechanisms such as peripheral vasoconstriction. Similar results were obtained with the rat arthritis model of zym (31). The increase in \(T_c\) promoted by zym was also associated with the presence of sickness behavior, also characterizing a febrile response (29). These results together confirm that zym can activate central mechanisms to induce fever.

Nevertheless, the EPs released after zym injection are not known. The role of cytokines such as TNF-α, IL-1β, and IL-6...
in an LPS-induced febrile response has already been reported (36, 60, 61). In our study, levels of TNF-α, IL-1β, and IL-6 were also increased in the plasma of animals that received LPS, reinforcing the participation of these cytokines in the febrile response induced by this PAMP. However, at the time points evaluated in this study, only IL-6 showed significantly increased levels in plasma after zym administration, although the plasma levels of IL-1β were apparently high.

It should be noted that the febrile response induced by LPS lasted longer than that induced by zym, even at a higher dose of this stimulus. In addition, it has been suggested that the first cytokines to be released after LPS injection are TNF-α and IL-1β followed by IL-6, which is released with some delay (10, 36). In our study, anti-TNF-α and IL-1RA preferentially reduced the later phase of LPS-induced fever, whereas they reduced the early stage of the zym-induced febrile response. In contrast, the anti-IL-6 antibody seemed to reduce the LPS-induced fever from the beginning, whereas it reduced only the final phase of the zym-induced fever without altering the peak of the response. The early effect of anti-TNF-α and IL-1RA in the zym-induced fever guided us to evaluate the presence of TNF-α and IL-1β in the plasma at 1 and 2 h after the injection of this PAMP. Our results suggest that these time points are not ideal to detect TNF-α and IL-1β in the plasma after zym injection. It seems to us that the evidence obtained with the antibodies and IL-1RA is robust enough to suggest that these cytokines actually participate in the febrile response induced by zym. A more accurate measurement at earlier time points should be performed to determine the kinetics of these cytokines in plasma after zym injection. Together, these results indicate that distinct profiles of cytokine release may be caused by LPS and zym.

The most probable source for IL-6 in plasma after both stimuli is the population of peritoneal macrophages, because TLR4 and TLR2/TLR6 are present in these cells (16). The involvement of both peripheral and central cytokines in manifestation of a febrile response induced by various stimuli has been extensively shown (9, 21, 25, 39, 63, 64). Whether the increased levels of cytokines in the brain are derived from the periphery is still a matter of debate, although the most tempting hypothesis suggests that these cytokines act on the blood vessels of the circumventricular organs and induce central mediators of fever. However, it has also been demonstrated that these cytokines can be generated in the CNS, particularly in the hypothalamus and nearby areas after LPS injection (30, 43, 71). A peripheral effect of centrally injected IL-1RA seems improbable because the doses of IL-1RA administrated intraperitoneally necessary to reduce LPS-induced fever are 600 to more than 1,000 times higher than the dose we used here (40, 66). For IL-6 and TNF-α, no studies about the effects of purified antibodies administered peripherally in fever have been published. Some studies used high doses of antisera (12, 64). Anti-TNF-α and anti-IL-6 at doses 20 to 800 times higher than the one used on this study were necessary to block this cytokine activity in other inflammatory conditions (53, 57, 65). In addition, the dose of anti-IL-6 was based on previous studies that also suggested a central effect of the antibodies (19, 67). Therefore, our results suggest that during zym-induced fever, IL-1RA and the antibodies are blocking cytokines within the CNS, although the precise origin of these cytokines cannot be precisely identified by the experimental approach used in this study.

The OVLT and MnPO are important and connected hypothalamic areas that are related to illness and febrile responses (3, 27, 64, 75). The OVLT is reported to possess a less tight blood-brain barrier, thus receiving more information from the peripheral sites of the body, and sending signals to closer areas such as MnPO (49, 50). Moreover, expression of TLR2 and TLR6 was reported in endothelial cells of the CNS, and zym was associated with an increase in the permeability of the vessels by damage of the intercellular junctions (44). In addition, as mentioned earlier, zym injected into the OVLT potentiated EP-induced fever (70). Altogether, these studies indicated that zym could also have a direct effect on the CNS, particularly in areas related to fever. In primary neuroglial microcultures, we were able to identify a substantial number of OVLT and MnPO cells that were responsive to a single zym application. This phenomenon was significantly observed in astrocytes and microglial cells compared with neurons in both areas analyzed. Another important characteristic of our results was that the ∆ratio [340/380 nm] values were significantly different among the zym-responsive cellular phenotypes in OVLT microcultures, but not in MnPO. The response in astrocytes and microglia cells was stronger than in neurons. This phenomenon can be associated with the specific functions of glial cells and the expression of TLR2/TLR6 in microglia, and of TLR2 in astrocytes (6, 32, 48). These issues, in addition to our results, suggest that even though the calcium responses
were observed separately in OVLT and MnPO, the responses induced by zym in OVLT could be transmitted to MnPO.

In both OVLT and MnPO, a rapid increase in [Ca$^{2+}$]i (a few seconds) followed by a slower decrease of about 2 min was observed in the majority of responsive cells, which suggests a direct effect of this PAMP on these cells. For LPS stimulation of OVLT cultures, four distinct patterns of calcium signaling have been described (49). Zym exhibited a pattern of calcium signaling similar to an acute response to a short-term stimulation with LPS.

One of the consequences of the stimulation of TLRs in the CNS with activation of glial cells is the production of inflammatory mediators such as cytokines, (6, 48). We observed increased levels of the proinflammatory cytokines TNF-α and IL-6 in OVLT and MnPO microcultures after stimulation with zym in the same concentration that promoted increases in [Ca$^{2+}$]i. Similar investigations were previously performed with LPS, in OVLT, and area preoptica (49, 74). Even if we cannot affirm that the concentration used to stimulate the microculture cells is proportional to a pyrogenic peripheral stimulus, we can presume an association with the febrile response observed in rats with partial blockade when the antibodies and IL-1RA were administered. Although we did not evaluate this issue, it is possible that increased levels of IL-1β in microcultures might have been present as well, considering that microglia cells, the most stimulated cells in our experiments, are important sources of IL-1β production (13). It is possible that generation of these cytokines within the CNS by zym provide a mechanistic explanation for the effect of zym in the febrile response observed in vivo by Stitt and Shimada (70).

These cytokines are known to induce cyclooxygenase-2 (COX-2) in the brain vasculature and, consequently, prostaglandin synthesis, which is important for fever induction (7, 8). On the basis of our results, it is therefore plausible to hypothesize that the febrile response induced by zym could depend on prostaglandin synthesis. Indeed, Kanashiro et al. observed that the febrile response induced by intraarticular zym was blocked by different COX inhibitors (31).

In conclusion, our study has shown for the first time that the zym-induced febrile response is at least in part dependent on the action of cytokines such as TNF-α and IL-6, and possibly IL-1β, within the brain. This febrile response can also be associated with the direct activation by zym of important areas related to fever and sickness behavior; in our case, the OVLT and MnPO. These results are helpful in establishing a model for a fungal-induced febrile response that will enable us to study pathological mechanisms and search for pharmacological targets. The specific roles of other cytokines such as macrophage-inflammatory protein-1α, CINC-1, and other mediators (prostaglandins, endothelin-1, and opioids) require further investigation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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