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Full-length Article

Cytokine contributions to alterations of the volatile metabolome induced by inflammation

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ABSTRACT

Several studies demonstrate that inflammation affects body odor. Volatile signals associated with inflammation induced by pyrogens like LPS are detectable both by conspecifics and chemical analyses. However, little is known about the mechanisms which translate detection of a foreign molecule or pathogen into a unique body odor, or even how unique that odor may be. Here, we utilized C57BL/6J trained mice to identify the odor of LPS-treated conspecifics to investigate potential pathways between LPS-induced inflammation and changes in body odor, as represented by changes in urine odor. We hypothesized that the change in volatile metabolites could be caused directly by the pro-inflammatory cytokine response mediated by TNF or IL-1β, or by the compensatory anti-inflammatory response mediated by IL-10. We found that trained biosensors generalized learned LPS-associated odors to TNF-induced odors, but not to IL-1β or IL-10-induced odors. Analyses of urine volatiles using headspace gas chromatography revealed distinct profiles of volatile compounds for each treatment. Instrumental discrimination relied on a mixture of compounds, including 2-*sec*-butyl-4,5-dihydrothiazole, cedrol, nonanal, benzaldehyde, acetic acid, 2-ethyl-1-hexanol, and dehydro-exo-brevicomin. Although interpretation of LDA modeling differed from behavioral testing, it does suggest that treatment with TNF, IL-1β, and LPS can be distinguished by their resultant volatile profiles. These findings indicate there is information found in body odors on the presence of specific cytokines. This result is encouraging for the future of disease diagnosis via analysis of volatiles.

1. Introduction

Body odors often contain information about the physiological state of the producer. In many species, including mice, body odor contains information on age, sex, genetics, diet, reproductive status, dominance, and health (Kimball et al., 2014, 2016a; Kwak et al., 2008, 2010; Novotny, 2003; Olsson et al., 2014; Osada et al., 2008; Penn and Potts, 1998). The ability to detect illness or infection in conspecifics has obvious potential advantages. Evidence indicates odor cues allow this detection in multiple species, especially rodents (Arakawa et al., 2011; Boillat et al., 2015; Penn and Potts, 1998). For example, female mice demonstrate reduced attraction to the odors of male conspecifics infected with parasites or a respiratory virus (Kavaliers and Colwell, 1995; Kavaliers et al., 1998; Penn et al., 1998). Treatment of rats with lipopolysaccharide (LPS) or IL-1β results in avoidance by healthy conspecifics (Arakawa et al., 2011). However, pretreatment with the anti-inflammatory cytokine IL-10 blocked production of the aversive odor (Arakawa et al., 2010). These findings suggest that cytokines serve as mediators of disease-induced odors. It is unclear, however, which cytokines specifically contribute to disease-related odors and how they impact the volatile metabolome as a whole.

Cytokine responses to inflammatory stimuli such as pathogens often represent a complex series of events. When faced with a common pathogen associated molecular pattern (PAMP) like LPS, there is rapid production of pro-inflammatory cytokines, most notably TNF and IL-1β (Bradley, 2008; Zuckerman et al., 1991). LPS rapidly induces TNF, which induces its own production in macrophages (Vassalli, 1992). TNF also induces production of IL-1β in leukocytes (Dinarello et al., 1986), which must be processed by an activated inflammasome before it can be secreted (Burm et al., 2015). Both cytokines activate the vascular epithelium, induce the febrile response, and aid in the recruitment of effector immune cells. TNF also activates the respiratory burst in phagocytes and increases vascular permeability (Bradley, 2008). Following shortly after the inflammatory response is a compensatory anti-inflammatory response, primarily regulated by the cytokine IL-10 (Bone, 1996; Moore et al., 2001). IL-10 inhibits production of IL-1β, TNF, IL-6, and numerous other inflammatory cytokines

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(Fiorentino et al., 1991; Moore et al., 2001; Opal and DePalo, 2000; Malefyt et al., 1991). This limits the damage done by inflammatory mediators, promotes tissue repair, and promotes restoration of homeostasis (Ariel and Timor, 2013).

Given the wide ranging effects of cytokine signals to immune and metabolic tissue (Chen et al., 2009; Doerrler et al., 1994; Grimble, 1996; Hardardottir et al., 1992; Moldawer et al., 1988; Oberholzer et al., 2002), we hypothesized that cytokine signaling mediates changes in body odor occurring in response to treatment with LPS. To test this, we first demonstrated LPS induced a robust inflammatory and anti-inflammatory response, as reported many previous studies (Bone, 1996; DeForge and Remick, 1991; Mezayen et al., 2007; Fiorentino et al., 1991; Lu et al., 2008, Tateda et al., 1996). Next, we tested the hypothesis that pro-inflammatory cytokines TNF and IL-1β would each elicit changes in body odor (determined behaviorally) and changes in the volatile metabolome (determined chemometrically) similar to those induced by treatment with LPS. We used urine as our source of body odor for several reasons, most importantly the central role of urine odors in regulating mouse behavior and physiology (Beauchamp and Yamazaki, 2003). Urine also serves as a useful source of body odors due to its relative ease of collection, ability to be collected from the same animal multiple times without harming the animal, and the fact urine odor contains most of the volatile chemicals found in mouse body scent (Rock et al., 2006). We also tested the alternate hypothesis that IL-10 produces a similar urine odor to LPS. Although IL-10 has an opposing inflammatory effect, we hypothesized its downstream effect may be responsible for urine odor alteration. To test these hypotheses, we used odor trained biosensor mice, as well as GC/MS headspace chemical analysis. Both of these techniques have been successfully employed to study persistent changes in volatile profiles occurring as a consequence of disease, inflammation, and injury (Kimball et al., 2014, ;Kimball et al., 2016b,a; Hanai et al., 2012; Singer et al., 1997; Yamazaki et al., 2002) but they seldom have been used in tandem. In doing so, we tested a third hypothesis that these two techniques would provide consistent and complimentary outcomes.

2. Methods

2.1. Subjects

Inbred male C57BL/6 mice were commercially purchased (Jackson Laboratories, Bar Harbor, ME) for use as urine, blood, and tissue donors as well as trained biosensors. Mice received LPS or cytokine treatments at 6–8 weeks of age, consistent with previous studies (Kimball et al., 2014). This study did not investigate the effects of age on inflammation-induced urine odor. Donor mice were provided ad libitum access to food (Teklad Rodent Diet) and water. Biosensor mice were give ad libitum access to food but underwent water restriction for 23 h prior to Y-maze experiments. Biosensors received water during and after experiments. Health and bodyweight of biosensors was monitored daily during water restriction. Mice were taken out of the training panel and given access to water if their body weight ever dropped 10% below the previous reading, or if biosensors showed any signs of illness. All procedures involving animals were approved by the Monell Institutional Animal Care and Use Committee, protocols #1123 and #1174.

2.2. Treatments

Donors used to generate urine for Y-maze training were treated with 300 μL i.p. injections of either 0.01 M sterile phosphate-buffered saline (PBS) or 0.2 mg/mL, yielding an approximate dose of 2 mg/kg LPS in sterile PBS (Sigma-Aldrich, St Louis, MO). Previous experiments have demonstrated this dosage is sufficient to generate a change in subject urine odor (Kimball et al., 2014). We note that our dose of LPS was much milder than other values used in related studies that have been as high as 12 mg/kg (Vuaden et al., 2011). Mice treated with cytokines received 100 μL i.p. injections of 30 μg/mL TNF, IL-1β, or IL-10 (BioLegend, San Diego, CA) in sterile PBS (Cook et al., 2006). These cytokine doses triggered cascades with final concentrations close to those seen in serum following LPS injections (Figs. 1 and 2).

2.3. Urine collection

Urine was collected nearly daily from treated donors by application of gentle abdominal pressure as described previously (Yamazaki et al., 1983). Urine volumes collected were typically 10–50 μL per mouse per day, although efforts to collect from individual mice frequently resulted in no volume collected. Collection continued for 30 days. Vials of urine were stored at −20 °C for later behavioral and chemical analyses.

2.4. Blood collection

Blood was collected in one of two ways. Mice were anesthetized with 2% isoflurane using a small-animal inhalation anesthesia apparatus. While unconscious, ~100 μL blood was collected from the tail vein. Blood was only collected once per mouse in this fashion. Alternatively, as a terminal procedure, up to 1.5 mL blood was collected from anesthetized mice via cardiac puncture. Isoflurane was then increased to euthanize animal. Blood was immediately processed as described below.

2.5. RNA extraction

After isolation from the mouse, blood was centrifuged in 1.5 mL tubes at 2500×*g* for 7.5 min to pellet red blood cells. Serum was removed and stored at −40 °C. Remaining cells underwent hypertonic lysis for 20 s before normal saline levels were restored. Samples were then rinsed with PBS and remaining cells were lysed with TRIzol reagent (Thermo Fisher Scientific, Wilmington, DE). RNA extraction proceeded as per manufacturer's instructions. Briefly, cell pellets were lysed in TRIzol and extracted with chloroform. The aqueous phase was combined with isopropanol to precipitate RNA. RNA samples were purified through additional precipitation with 5 M ammonium acetate and ethanol. Purity of samples was verified using a Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE).

2.6. Quantitative PCR

After RNA purification, RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster City, CA). Resulting cDNA was then quantified using the Fast SYBR Green system using the StepOne Real-Time PCR System. B-actin was also run as a loading control. The following primers were used: TNF, Forward-TGGCCTCCCTCTCATCAG, Reverse-ACTTGGTGGTTTGCTACGAC; IL-1β, Forward-CCCAACTGGTACAT CAGCACCTC, Reverse-GACACGGATTCCATGGTGAAGTC; IL-10, For ward-GCCCTTTGCTATGGTGTCCTTTC, Reverse-TCCCTGGTTTCTCTT CCCAAGAC; B-actin, Forward- TGTTACCAACTGGGACGACA, Reverse- CTGGGTCATCTTTTCACGGT.

2.7. ELISA

ELISA assays of serum for TNF, IL-1β, and IL-10 were performed using DuoSet ELISA kits, according to manufacturer's instructions (R&D Systems, Minneapolis, MN).

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