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Brain, Behavior, and Immunity 69 (2018) 312–320

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Cytokine contributions to alterations of the volatile metabolome induced by inflammation

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ARTICLE INFO

Article history:
Received 26 September 2017
Received in revised form 28 November 2017
Accepted 9 December 2017
Available online 11 December 2017

Keywords:
Inflammation
Body odor
Cytokines
Volatile metabolome

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.

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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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https://doi.org/10.1016/j.bbi.2017.12.005
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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-CCCAACTGGTACATCAGCACCTC, Reverse-GACACGGATTCCATGGTGAAGTC; IL-10, Forward-GCCCTTTGCTATGGTGTCCTTTC, Reverse-TCCCTGGTTTCTCTTCCCAAGAC; 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).

2.8. Behavioral assay

A Y-maze apparatus was used for odor training and generalization assays as previously described (Kimball et al., 2016a; Yamazaki et al., 2002). Six biosensor mice were first trained to identify the odor associated with LPS by providing the choice of urine odor derived from an LPS-treated donor and urine odor derived from a PBS-treated donor. To generate these odors, urine samples (~0.25 mL) were placed in petri dishes and loaded into separate odor chambers. Urine samples collected from individual mice over multiple days were pooled by donor for this purpose. Pooled urine included samples collected from days 4 to 25 after treatment. Previous unpublished work in our laboratory indicated that persistent urine odor differences are still present 4 weeks after treatment. Each pooled urine sample was used for a single day of testing and then discarded. Air was blown over the samples and into either arm of the Y-maze. A biosensor mouse was place in the central arm of the Y-maze and allowed to choose between the two arms containing the two odors. Gates were manually lowered and raised to permit training or testing of biosensor mouse. During training, mice choosing the arm of the Y-maze containing LPS odor were rewarded with a drop of water. Samples were randomly assigned to left or right arms of the Y-maze for each trial of a session. During training, sessions consisted of approximately 35 water-rewarded trials. Biosensor training and experimentation took place in 3 stages.

2.8.1. Training

Rewarded training trials are employed to teach biosensors to discriminate the urinary volatiles resulting from treatment with LPS and PBS. The reward is associated with LPS odor to promote generalization of this response in later sessions. To reduce the possibility that biosensors were learning individual identities of donors rather than the donor's treatment (i.e. PBS, LPS), each training set consisting of urine from three different donors (for each treatment). Dishes were offered singly or in combinations of two to three (keeping treatment consistent), resulting in 19 unique pairs of odor stimuli. This procedure sought to acclimatize the biosensors to not receiving a reward on every trial, even if they behaved "correctly".

2.8.2. Extinctions

Extinction trials are introduced so that biosensors become acquainted with unrewarded trials. Such intermittent reinforcement also serves to strengthen the operant response (Bodyak and Slotnick, 1999). Once biosensors chose the LPS-treated mouse urine odor $\geq$75% of the time (in 2 consecutive sets of 12 trial blocks), extinction trials were implemented. These trials consisted of unrewarded trials interspersed among rewarded trials of LPS and PBS urines. The training samples were used as stimuli during extinctions.

2.8.3. Generalizations

Unrewarded generalization trials are used to test the biosensors. Because LPS odor was associated with the reward in training, these trials can be used to determine which test stimuli are most similar to LPS. Once biosensors demonstrated they could maintain their identification rate when faced with unrewarded trials, biosensors faced generalizations, wherein novel samples were used as stimuli. The first generalization set was to novel donors receiving either LPS or PBS. Urine collected from a subset of animals in the training cohort was set aside and not used in any initial training trials. Novel donor generalizations were the first time biosensor animals were exposed urine collected from that individual animal. After generalization, any remaining urine from that individual was used as training urine. Generalizations to donors treated with novel test stimuli (i.e. cytokines) were only initiated after biosensors demonstrated their ability to correctly identify novel LPS-treated donors. These novel treatment generalizations were run multiple days apart. Generalization trials were run on a minimum of 3 separate days per treatment, each day alternating between different treatments.

2.9. GC/MS headspace

Twenty five microliter urine samples fortified with 100 ng of L-carvone by addition of 10 μL aqueous solution of as an internal reference were analyzed using a HT3 dynamic headspace analyzer (Teledyne Tekmar, Mason, OH) outfitted with Supelco Trap K Vocarb 3000 thermal desorb trap (Sigma-Aldrich, St Louis, MO), attached to a Thermo Scientific ISQ GC-MS equipped with a single quadrupole mass spectrometer (Restek, Bellefonte, PA), as described elsewhere (Kimball et al., 2016a,b). Urine samples were maintained at 40 °C and swept with helium for 10 min at a flow rate of 75 mL/min. Volatiles were collected directly on the thermal desorption trap. The trap was desorbed at 260 °C directly into gas chromatograph. Split injections (5:1) were made using a 2.0 mL/min column flow and 10.0 mL/min split vent flow. The GC oven began at 40 °C for 3 min, increased temperature at a rate of 7 °C/min until reaching 260 °C. The mass spectrometer was operated in scan mode from 33 to 400 m/z. Peak identifications were assigned based on the spectral library search of the NIST Standard Reference Database. Vials containing 100 ng of L-carvone without urine and entirely empty vials were periodically analyzed as well.

2.10. Behavioral data analysis

Generalization data from the six biosensors were pooled across sessions into total number correct and total number of trials. Data were then subject to two-tailed binomial proportion hypothesis testing using the PROC FREQ function of SAS Studio. Each pool of data contained $\geq$75 trials. Significance was determined by exact test of binomial proportion.

2.11. Chemometric data analysis

Chromatographic data were exported for baseline correction, noise elimination and peak alignment by Metalign software (Lommen, 2009). MSClust was then used on resulting multivariate data files for mass spectral extraction (Tikunov et al., 2012). Individual peak responses were standardized to the L-carvone response in that particular sample. Sample outliers were identified using influence plots in Unscrambler (CAMO Software) to identify samples with abnormally high residual variance and leverage. Subject means of all carvone-standardized peak responses were calculated for individual mice. Sample means consisted of 3–5 samples, which for LPS and PBS samples were collected from days 4 to 16, during which time trained biosensors can detect a difference in odor (Kimball et al., 2014). Samples from days 1 to 8 from cytokine-treated animals were used for chemometric analysis. Donors were then assigned to specific data sets for model building and prediction using linear discriminant analysis (LDA). Data set 1 consisted of donors receiving LPS and PBS treatments generated for Y-maze training. To bolster samples size, data set 1 also included samples from mice that were restricted from food for 48 h immediately following injections (LPS-injected mice normally fast for 48 h even with ad libitum access). Data set 2 consisted of donors receiving IL-10, IL-1β, TNF, and PBS treatments generated for Y-maze generalizations. Data set 3 consisted of a second cohort of LPS treatments generated for Y-maze training.

LDA models were first constructed with donor means from data set 1 using PROC STEPDISC in SAS to identify those chromatographic peaks which contribute to discrimination of the LPS-

treated mouse urine vs. PBS-treated mouse urine. Contingency tables for LPS and PBS classifications resulting from the model were constructed using PROC DISCRIM and cross-validation was employed to evaluate LDA results. Individual samples from data sets 2 and 3 were then subjected to LDA classification (PROC DISCRIM in SAS) using the previously generated LDA model for LPS vs. PBS. The significance of these LDA classifications was then determined through an exact test of binomial proportion with a null hypothesis of 0.5.

These analyses were then repeated, except that in this instance model building was conducted with donor means from data set 2 to identify those chromatographic peaks which contributed to discrimination among IL-10, IL-1β, TNF, and PBS treatments. Individual samples from data sets 1 and 3 were then subjected to LDA classification using this model generated from data set 2. The significance of these LDA classifications was then determined through an exact test of binomial proportion with a null hypothesis of 0.25.

Pairwise comparisons were made between treatment groups using one-way ANOVA assays for those peaks contributing to the LDA models. One-way ANOVA analysis was performed using individual means for each mouse, correcting for the false discovery rate using multiple tests (Benjamini and Hochberg, 1995).

3. Results

3.1. LPS induces cytokine response

As anticipated, mice treated with LPS exhibited numerous signs of illness for the first 24–48 h. Symptoms included lethargy, reduced food intake, and weight loss. We analyzed cytokine mRNA expression in peripheral blood leukocytes (PBLs) (Fig. 1A–C). We saw significant increases in TNF, IL-1β, and IL-10 mRNA in LPS vs. saline treated animals. These increases were present 24 h following injection, but returned to baseline by day 5. No significant increases in IL-4, IL-6, or IFN-g were observed (data not shown). Additionally, we observed significant levels of TNF, IL-1β, and IL-10 protein in the serum during the first 24 h (Fig. 1D–F). IL-1β and IL-10 levels were relatively consistent during that period. Circulating levels of TNF quickly dropped, consistent with prior reports of the cytokine's short half-life in vivo (Beutler et al., 1985; Flick and Gifford, 1986).

After verifying the TNF, IL-1β and IL-10 cytokine responses in our model, our next step was to treat mice with these individual cytokines. Levels of circulating cytokines were measured at 4 h and 24 h following treatment (Fig. 2).

3.2. Biosensors generalize to specific pro-inflammatory cytokines

Trained biosensors identified LPS urine at a rate of 93% during rewarded training trials (Fig. 3). This rate fell to 75% when asked to identify novel conspecific odors with the same treatment. This type of decline in recognition is typically observed in Y-maze trials when biosensors are faced with odors from novel conspecifics, even ones that are presumably genetically identical to the training mouse urines (Kimball et al., 2014). Despite our best efforts, it is possible that biosensors incorporate some information on the individual identities of the donor animals into their discrimination task learning during rewarded trials. Testing with novel donors receiv-

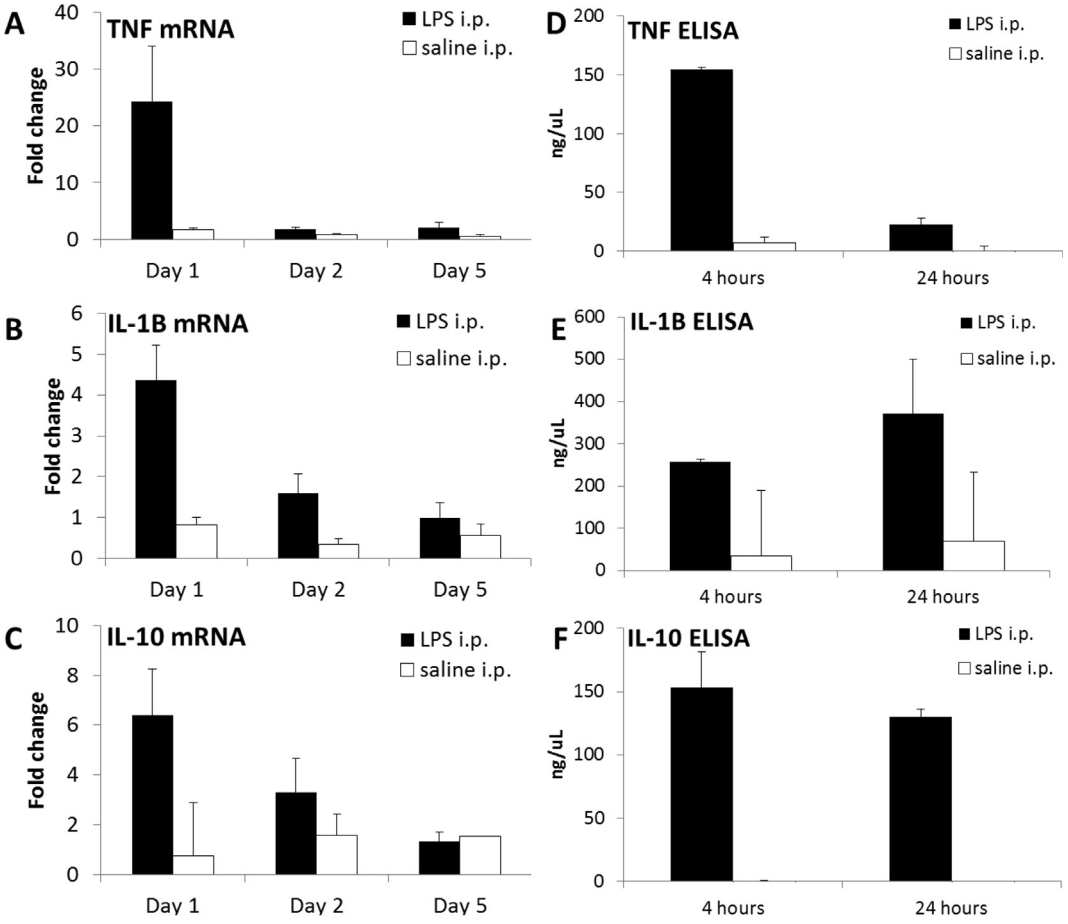

Fig. 1. A–C) qPCR analysis of isolated PBLs following i.p. injections of saline, 0.2 mg/kg LPS. Columns represent fold change in A) TNF; B) IL-1β; C) IL-10 mRNA, relative to Day 1 Saline sample. D–F) ELISA analysis of serum concentrations of D) TNF, E) IL-1β, and F) IL-10 protein levels following i.p. injections. Bars represent SEM (n = 3).

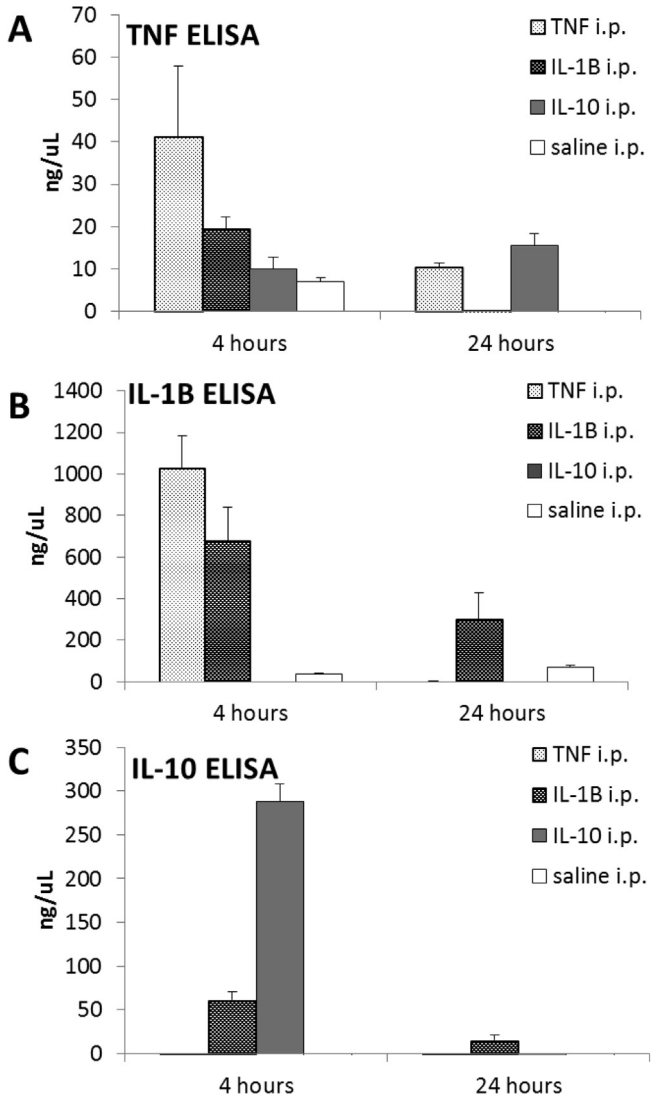

Fig. 2. ELISA analysis of serum samples following i.p. injections of TNF, IL-1β, IL-10, saline, or 0.2 mg/kg LPS. Columns represent ng/μL in A) TNF; B) IL-1β; C) IL-10 cytokine at 4 and 24 h after injection. Bars represent SEM (n = 3).

ing the same treatments used for training permits is used to demonstrate that biosensors are making discriminations on the basis of treatment, not individual donor identity. Accordingly, this identification rate was significantly greater than 50% identification expected by chance (75%; p = .006) demonstrating that the trained mice differentiated LPS-induced odor, not individual donors.

As we hypothesized, biosensors generalized their training to TNF-induced odor (71%; p = .0003). However, this training did not generalize to IL-1β vs. PBS-induced odor (57%; p = .2461). This indicated the biosensors perceived a similarity between LPS and TNF-induced odor, but not IL-1β-induced odor. This distinction was not predicted by our hypothesis.

3.3. Biosensors do not generalize to anti-inflammatory cytokine

Biosensors trained to LPS-induced urine odor were also asked to generalize their training to IL-10 induced urine odor (Fig. 3). Here, biosensors generalized to the cytokine signal only 38% of the time. Lack of generalization to IL-10 induced odor, as evidenced by selection of control odor at a rate significantly greater than chance (62%; p = .05), indicated the biosensors did not perceive a similarity between IL and 10 and LPS-induced odor. It further suggests IL-10 induces its own distinctive odor which biosensors perceived as different from training odors.

3.4. Chemometric discriminations differ from those from biosensors

Urine samples from the behavioral experiments underwent headspace GC/MS analyses. We used donors from data set 1 (LPS and PBS treatments) to construct an LDA model that best separated the treatments while minimizing the number of chemical predictors. The model consisted of five chemical predictors (nonanal, benzaldehyde, dehydro-exo-brevicomin (DHB), acetic acid, and 2-ethyl-1-hexanol). The overall cross-validation error rate was 11.1%, meaning that error was low even when individual samples were held out of model building and rerun. We then used this model to make predictions for a different cohort of mice receiving LPS (data set 3) and for donors receiving cytokine treatments (data set 2). No data from analyses of these mice were used in model building. The majority (17 of 24) of samples from donors in data set 3 (LPS-treated) were classified as LPS with our discriminant model based solely on data set 1. All of the samples from IL to 1β-treated donors (15 of 15) were categorized as LPS by this model (Table 1), however samples from TNF- and IL-10-treated donors

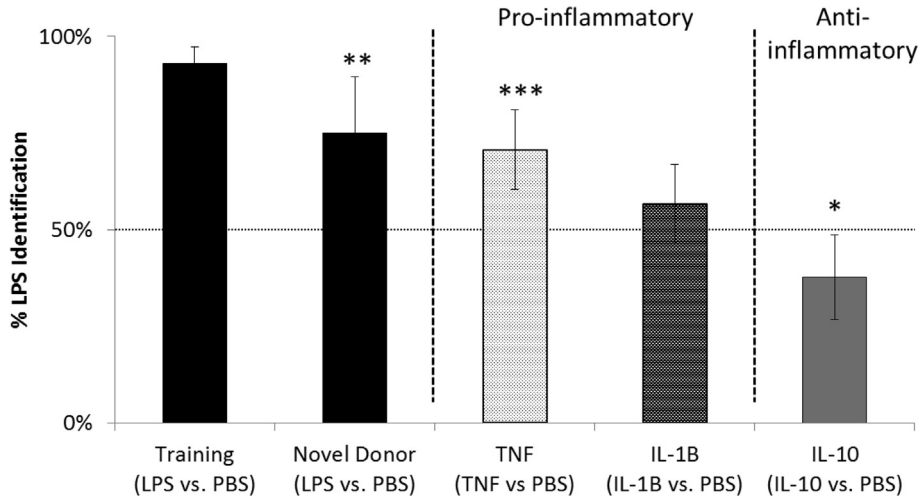

Fig. 3. Mean responses of trained biosensors. Bars represent 95% confidence intervals. Training trials (shaded) were rewarded, while generalization trials (white) were unrewarded. Labels indicate (*) p < .05, (**) p < .01 or (***) p < .001 for a two-tailed exact binomial test of the hypothesis that identification differs from 50%.

Table 1
Cross-categorization of TNF, IL-1β, IL-10 and LPS samples into the LPS vs PBS LDA model. LPS samples categorized here represent a different data set (data set 3) not used to build the model. *: p < .05, ***: p < .001 by exact test of binomial proportion.

Cross-Categorization		
	LPS	PBS
LPS*	70.8% (17 of 24)	29.2% (7 of 24)
IL10	53.3% (8 of 15)	46.7% (7 of 15)
IL-1β***	100% (15 of 15)	0% (0 of 15)
TNF	51.6% (16 of 31)	48.4% (15 of 31)

were evenly split between LPS and PBS categorizations. In contrast to the behavioral assay, the chemometric model suggested LPS-induced volatiles were more similar to IL-1β- than TNF-induced volatiles.

We next constructed a model to discriminate among IL-10, IL-1β, TNF, and PBS using data set 2. Three predictors (2-*sec*-butyl-4,5-dihydrothiazole (SBT), acetic acid, and cedrol) yielded a model with an overall cross-validation error rate of 24.4%. When this model was used to classify LPS donors from data sets 1 and 3, 52.9% of LPS donors were classified as TNF (Table 2). The remaining LPS samples were evenly distributed among the other 3 categories: IL-1β (11.8%), IL-10 (17.7%) and PBS (17.7%). Finally, we conducted pairwise analysis on all metabolites identified by our models (Table 3). Only one metabolite, SBT, showed a significant pairwise reduction in response to a specific treatment, which in this case was IL-1β.

4. Discussion

In this study, we sought to explore the immune signaling events involved in the generation of LPS-mediated urine odor change. Previous work found that treatment with either LPS or IL-1β induced an acute odor aversive to conspecifics during the first 4 h after intracerebroventricular (icv) infusion (Arakawa et al., 2010). This study follows the novel discovery that urine odor alteration due to treatment with LPS or head injury persists for many weeks (Kimball et al., 2014, 2016a). This surprising effect occurs even though the acute physiological effects of inflammation (cytokine expression in particular) subside within hours or days of the inflammatory event. It is entirely plausible that there are acute changes in body odor during the early hours of inflammation when cytokines are still circulating that our study would not detect. However, the work presented here focusses primarily on urine odor signatures several days to weeks following LPS administration during which time the odor signal remains stable based on previous work. We consider not only the involvement of pro-inflammatory cytokines TNF and IL-1β on this persistent odor change, but the compensatory anti-inflammatory response mediated by IL-10.

Table 2
Cross-categorization of LPS samples into the TNF, IL-1β, IL-10 vs PBS LDA model. ***: p < .001 by exact test of binomial proportion.

Cross-Categorization				
	TNF***	IL1B	IL10	PBS
LPS	44.2% (23 of 52)	9.6% (5 of 52)	26.9% (14 of 52)	19.2% (10 of 52)

Table 3
Pairwise comparisons of significance for all metabolites contributing to either discriminant model. **: p < .01.

Metabolite	Pairwise
2-*sec*-butyl-4,5-dihydrothiazole (SBT)	IL-1β lower**
Cedrol	
Nonanal	
Benzaldehyde	
Acetic Acid	
2-Ethyl-1-hexanol	
Dehydro-exo-brevicomin (DHB)	

To study the role of these cytokines in persistent urine odor changes, we began by confirming these cytokine responses were present in our experimental system. We then proceeded to treat mice with these individual cytokines. While the cytokine appeared to clear from the serum more quickly when injected individually rather than induced through LPS administration, we were able to confirm similar levels of cytokines were present in both LPS and individual cytokine treatment systems.

Our first behavioral experiment tested whether mice trained to enter the arm of the Y-maze scented by LPS-induced odor would generalize this learned response to TNF and IL-1β. We hypothesized that biosensors would generalize their training with LPS to both inflammatory cytokines. Surprisingly, biosensors generalized to TNF-induced odor, but not IL-1β-induced odor, confirming only one part of our first hypothesis. We also tested the alternate hypothesis that LPS-induced urine odor resulted from the compensatory anti-inflammatory response, rather than the inflammatory response itself by determining whether LPS-trained biosensors generalized to IL-10-induced urine odor. We rejected this second hypothesis, as LPS-odor trained biosensor mice did not generalize to the IL-10-induced odor.

In parallel, we used GC/MS headspace to analyze the volatile metabolites present in the urine. Mirroring our training and generalization approach of the Y-maze, we first constructed a LDA model to discriminate between LPS and PBS treatments. Then, based on the generalization pattern of the Y-maze experiments, we made predictions with donors receiving specific cytokine treatments. We hypothesized that doing so would yield results consistent with our behavioral data. However, our chemometric model did not yield the same discrimination that the biosensors did. We next reversed the data sets used for model building and prediction. This enabled us to perform an experiment that would not be possible in the Y-maze, namely, to "train" the model on the volatiles produced by treatment with several different cytokines and then make predictions based on the compounds identified in the model for the discrimination between LPS and PBS donors. This approach categorized the LPS samples as most similar to TNF, which is consistent with our behavioral data.

Biosensor mice were trained to differentiate between urine odors from saline and LPS-treated conspecifics collected no earlier than four days post-treatment. This is well after the cytokine response has peaked and the plasma has been largely cleared of detectable levels of circulating inflammatory and anti-inflammatory cytokines (Fig. 1). This effect does not appear to be dependent on IL-10. While IL-10 is produced in response to TNF and IL-1β and is generally found later in the course of acute inflammation (Moore et al., 2001; Opal and DePalo, 2000), our data indicate that it does not produce an odor profile to which LPS-trained biosensor mice generalize. Inflammation-induced changes in odor and the volatile metabolome is therefore a downstream effect of inflammation and cytokine signaling. Our data suggest that TNF is the upstream mediator of LPS-induced changes in odor.

Our finding that TNF- and IL-1β-induced odors do not elicit the same generalization responses from the biosensors was particularly surprising since TNF and IL-1β generally have very similar

cellular effects in immune tissue. Both trigger degradation of IkBα, translocation of NF-kB to the nucleus, and activation of inflammation (Medzhitov et al., 1998; Zhang et al., 2011). The physiological effects of TNF and IL-1β are also very similar. Both induce fever, stimulate expression of C-reactive protein, and upregulate expression of adhesion molecules (Dinarello et al., 1986; Gabay and Kushner, 1999; Oberholzer et al., 2000). Their effects, however, are not identical. One possible explanation for the biosensors generalization to TNF but not IL-1β is sequence of the inflammatory cytokine cascade. TNF is the primary stimulator of IL-1β (Dinarello et al., 1986; Feldmann et al., 1996). IL-1β, however, induces IL-6, which downregulates TNF (Aderka et al., 1989; Dinarello, 2009). While IL-1β has some capacity to induce TNF, TNF signaling is generally upstream of IL-1β expression.

Our behavioral data suggest that one or more of the downstream effects of TNF not mediated by IL-1β may be responsible for LPS-induced urine odor. What specific effect or effects are involved is still unclear. Aside from its role in activating fever and inflammation, a role it shares with IL-1β, TNF has several functions all its own. TNF blocks fatty acid synthesis, increases glycogenolysis, lipolysis, and lactate production (Lee et al., 1987; Moldawer et al., 1988). Rats injected with TNF show increased hepatic citrate levels, leading to more free fatty acid synthesis in the liver and higher free fatty acids in serum (Chen et al., 2009; Grunfeld et al., 1988). Within 2 h, TNF administration to NMRI mice causes hypoglycemia and around 80% loss of liver glycogen (Mahony and Tisdale, 1990). The mechanism by which TNF mediates these effects is unclear. While it is possible that some metabolic effects of TNF are in actuality the result of another downstream cytokine like IL-1β, the two inflammatory mediators seem to have different metabolic effects in adipocytes (Doerrler et al., 1994). In 3T3-F442A adipocytes, TNF, IFN-α, and IFN-γ each decrease levels of acetyl-CoA carboxylase (ACC), while IL-1β slightly increased expression of ACC. Both cytokines are capable of blocking lipoprotein lipase (LPL) in adipocytes, but only TNF can promote the production of prostaglandin E2, a potent inhibitor of lipolysis (Hardardottir et al., 1992).

The third hypothesis tested in this study was that results from behavioral tests and from the chemometric analyses would produce similar or consistent results. Underlying this hypothesis is the hope that the volatile compound changes that allow animals to identify a diseased animal's odors overlap with those that are identified in chemical analyses. However, there are good reasons to be skeptical that this is the case and the results from this study are equivocal. While the behavioral assays indicated that TNF-induced odor was similar to LPS-induced odor, the first set of chemometric assays (Table 1) suggested that cytokine-induced changes in volatiles are difficult to separate from one another. The initial data suggest while the pattern of TNF-induced volatiles resembled those of LPS-treated animals, the pattern of IL-1β-induced volatiles was more alike. When a different chemometric approach was used (Table 2), it provided evidence that LPS volatiles shared more with TNF volatiles than with PBS, IL10 or IL-1β volatile patterns, as we predicted from the behavioral results. We might therefore infer that the metabolites identified in our second model are more likely to be involved in the biosensor perception of inflammation-induced body odor.

Many of the metabolites identified by our LDA modeling have been observed in previous studies of murine volatiles and pheromones. Two murine pheromones, SBT and DHB were identified in our modeling. Both pheromones have previously been demonstrated to increase male aggression and to be drastically reduced in castrated mice (Novotny et al., 1985; Schwende et al., 1984). Previous work has demonstrated SBT decreases in response to stress (Schaefer et al., 2010), a response which seems to occur during inflammation as well. Benzaldehyde levels are also known to change with stress and with genetic background (Kwak et al., 2008). Cedrol, a sesquiterpine compound found in cedar, is undoubtedly a byproduct of the red cedar bedding used in our housing. We speculate that the decrease in cedrol observed in animals treated with an inflammatory agent reflects a decline in the amount of bedding animals consumed, or possibly a change in mouse metabolism following injection.

Whether the microbiome is responsible for any of the changes in urine odor and volatile metabolites is unknown. The co-metabolome arising from metabolic interactions of the host and its microbiome is known to cause alterations in the metabolite signature of host fluids such as urine (Nicholson et al., 2005). Furthermore, the gut microbiome is directly altered by certain inflammatory irregularities or conditions with chronic inflammation (Slack et al., 2009; Faber and Baumler, 2014). Thus, inflammatory events may alter the volatile metabolome via a similar interaction. For example, TLR5 deficient mice show altered gut microbiota, as well as the hallmark symptoms of metabolic syndrome (Vijay-Kumar et al., 2010). The metabolic syndrome phenotype appears to arise directly from the alteration of gut microbiota. For example, when germ-free wild-type mice receive a transfer of gut microbiota from the TLR5 deficient mouse, the metabolic syndrome phenotype is transferred as well. Chronic conditions which cause inflammation, such as chronic kidney disease and non-alcoholic fatty liver disease, can alter the products of intestinal flora, as measured by volatile organic compounds in fecal samples (Meinardi et al., 2013; Reid et al., 2016). Inflammation associated with inflammatory bowel disease (IBD) is also known to impact the gut microbiome and the volatile metabolites from fecal samples (Arasaradnam et al., 2009). While these findings implicate the microbiome as a contributor to alterations of the volatile metabolome, we are not aware of any studies that demonstrate acute inflammation affects the gut microbiome and the associated volatile metabolome. Determining if, or how, acute inflammation affects the microbiome could potentially be a rich area of research. On the surface, our results appear to contradict the findings of the Arakawa group that demonstrate IL-1β treatment in rats induced an odor that was aversive to conspecifics (Arakawa et al., 2010). Our data indicate that IL-1β treatment generates an odor which biosensors do not perceive as similar to LPS-induced odor. The reason for these differences likely stems from the differences in methodology between that study and our own. Previous research by Boillat et al. (2015) demonstrated the essential role played by the vomeronasal organ (VNO) in sick conspecific avoidance. Considerable evidence suggests the VNO responds primarily to non- or low-volatile compounds (Brennan and Kendrick, 2006). Much of the work demonstrating conspecific avoidance has used models where the involvement of low- and non-volatile compounds was more plausible (Arakawa et al., 2010; Boillat et al., 2015; Zala et al., 2015). The design of the Y-maze ensures that biosensor mice come nowhere near the sample itself, implying that their decision is based entirely or at least primarily on volatile compounds which are most likely detected by the main olfactory system, not the VNO. Interestingly, we informally observed that in the context of the Y-maze, biosensor animals have no particular aversion to the odors of LPS-treated conspecifics, even during early stages of training.

Behavioral studies can demonstrate that an odor differences exists. For those interested in the diagnostic value of odor, we argue that this is a logical first step. The olfactory system is the most sophisticated detector of patterns of volatile variation and thus provides prima facie evidence that a signal is present. However, it is unlikely that animals will be used for actual diagnostic purposes. Hence, it is crucial to devise chemometric approaches that will accomplish this. Indeed, one might argue that behavioral experiments could be dispensed with entirely and that all work

with diagnostics in mind should employ chemical analysis approaches much as it does in searches for other biochemical and molecular biological biomarkers of disease. We disagree with this approach and continue to believe that a combination of behavioral and chemical approaches will prove most fruitful in the long run in identifying sensitive and specific signals of disease. As advances are made in our understanding of how the mammalian brain processes complex odor mixtures, we anticipate that novel techniques of chemometric analysis will follow. Time will tell if this speculation is correct.

Acknowledgments

We would like to thank Dr. Jim Albrecht and the USDA-APHIS-WS-National Wildlife Research Center for their financial support of this project. This work was done at the Monell Center animal facility, which received support from the infrastructure Grant G20OD020296, and at the Monell Genomics Core, which received support from the P30 Grant DC011735.

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