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Immunization Alters Body Odor

Bruce A. Kimball
USDA APHIS Wildlife Services, bruce.kimball@ars.usda.gov

Maryanne Opiekun

Kunio Yamazaki

Gary K. Beauchamp

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Immunization alters body odor

Bruce A. Kimball [a,*], Maryanne Opiekun [b], Kunio Yamazaki [b,#], Gary K. Beauchamp [b]

[a] United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, 3500 Market Street, Philadelphia, PA 19104, USA
[b] Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104, USA

HIGHLIGHTS

- Biosensors discriminated between urines collected from donors receiving a rabies vaccine (RV) versus controls
- Biosensors discriminated between the urine odors of mice treated with lipopolysaccharide (LPS) from the urine of control mice
- LPS-trained biosensors could distinguish between the odors of LPS-treated mouse urine and RV-treated mouse urine.
- Discriminations by the biosensors were made on the basis of odor alone.
- Volatile metabolites arising from immunization may be products of an innate immune response.

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ABSTRACT

Infections have been shown to alter body odor. Because immune activation accompanies both infection and immunization, we tested the hypothesis that classical immunization might similarly result in the alteration of body odors detectable by trained biosensor mice. Using a Y-maze, we trained biosensor mice to distinguish between urine odors from rabies-vaccinated (RV) and unvaccinated control mice. RV-trained mice generalized this training to mice immunized with the equine West Nile virus (WNV) vaccine compared with urine of corresponding controls. These results suggest that there are similarities between body odors of mice immunized with these two vaccines. This conclusion was reinforced when mice could not be trained to directly discriminate between urine odors of RV- versus WNV-treated mice. Next, we trained biosensor mice to discriminate the urine odors of mice treated with lipopolysaccharide (LPS; a general elicitor of innate immunological responses) from the urine of control mice. These LPS-trained biosensors could distinguish between the odors of LPS-treated mouse urine and RV-treated mouse urine. Finally, biosensor mice trained to distinguish between the odors of RV-treated mouse urine and control mouse urine did not generalize this training to discriminate between the odors of LPS-treated mouse urine and control mouse urine. From these experiments, we conclude that: (1) immunization alters urine odor in similar ways for RV and WNV immunizations; and (2) immune activation with LPS also alters urine odor but in ways different from those of RV and WNV.

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1. Introduction

Chemical signals are the primary form of social communication for many species [1–3]. Although most research has been devoted to communication of social messages such as sex, age, and individual identity, volatile odorants may also communicate information about an animal's health status [4–6]. Odors associated with illness are often avoided by members of the same species, presumably acting to reduce the probability of disease spread [7]. Conversely, it might be adaptive for the infective agent to induce chemosensory changes in the host that increase both inter- and intraspecific interactions, thereby increasing transmission rates. An example of attraction has been demonstrated for interspecific interactions between human hosts and an insect vector [8].

The mechanisms underlying changes in body odor caused by disease are poorly understood and the specificity of odor changes to a specific disease has rarely been explored. For some diseases the mechanism underlying a body odor change is obvious and very non-specific. For example, diseases that alter feeding and drinking behavior or gut function are likely to alter odors associated with waste products such as urine and feces in non-specific ways. A more interesting potential pathway for diseases to alter body odor is via immune function — which is known to be intimately related to body odor composition [9–11]. Based on this reasoning, we hypothesized that vaccination may induce a significant alteration of body odor.

To test this hypothesis, we first conducted experiments asking whether immunization with rabies (RV) or West Nile virus (WNV)

* Corresponding author. Tel.: +1 267 519 4930.
E-mail addresses: bruce.a.kimball@aphis.usda.gov (B.A. Kimball), mopiekun@monell.org (M. Opiekun), beauchamp@monell.org (G.K. Beauchamp).
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vaccines alters body odors as determined by trained sensor mice in Y-maze odor training paradigm [12]. This behavioral assay is well-equipped to identify differences among various stimuli on the basis of odor. The Y-maze has successfully been used to demonstrate that rodents can discriminate between many different sources of odor variation, including: fetal odortype [13,14], disease [12,15], age [16], and diet [17]. We used urine as a representative source of body odor in these experiments because it is a potent source of mouse body odors [12,18].

Finding that urine odors were altered by treatment with the vaccines, we next examined whether odor changes are also elicited from exposure to lipopolysaccharide (LPS), a component of bacterial cell walls. Treatment with LPS induces a robust pro-inflammatory cytokine response [19] and previous experiments have indicated that body odors from LPS-treated animals elicit aversive behavioral responses from conspecifics [20]. The Y-maze odor training paradigm was used to evaluate similarities in body odor alterations produced by these different immunogens.

2. Materials and methods

2.1. Subjects

Inbred C57BL/6 mice were used as both biosensors and urine donors receiving treatments. Three biosensor panels (a unique panel for each experiment) each consisted of 5–7 individuals of mixed sex ranging in age from 6 to 14 months (Table 1). Urine donors were males 7–10 weeks of age at the time of treatment. The number of donors in each treatment group depended on the number of samples needed for bioassay trials (Table 1). Treated urine donors were provided ad libitum access to water and pelleted rations. Biosensors were provided ad libitum feed (Teklad Rodent Diet 8604; Harlan, Madison, WI), but were restricted from water for 23 h daily. Procedures involving animals were approved by the Institutional Animal Care and Use Committees of Monell (#1123) and the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center (QA-2021).

2.2. Treatments

The inactivated rabies vaccine RabVac-3® (RV; Fort Dodge Animal Health, Fort Dodge, IA, USA) was diluted (1:15) in 0.01 M sterile phosphate-buffered saline (PBS) for i.p. delivery of 300 μL. The live, attenuated West Nile virus (WNV) vaccine, PreveNile® (Intervet, Inc., Millsoboro, DE, USA) was prepared by dissolving 15 mg of the vaccine in 100 mL PBS for i.p. delivery of 200 μL. Vaccine dilution rates (based on relative mass ratios among species of intended vaccine use) resulted in ~50× reduction of RV dose as indicated for pets and ~1500× reduction of WNV vaccine dose as recommended for horses. The lipopolysaccharide (LPS) solution was prepared in PBS at a concentration of 0.20 mg/mL for i.p. delivery of 300 μL (60 μg per mouse or ~2 mg/Kg). LPS doses exceeding 12 mg/Kg (~400 μg per mouse) have been used in C57BL/6 inflammation models [21]. Donors in control groups received matching volume i.p. injections of PBS solution. Different groups of donors were employed for each of the three experiments (Table 1).

Table 1
Overview of trials conducted in a Y-maze with number of donors by treatment and urine collection days (post-treatment). Rabies (RV) = treatment with RabVac-3® vaccine; West Nile virus (WNV) = treatment with PreveNile® West Nile virus vaccine; LPS = treatment with lipopolysaccharide; and control = treatment with phosphate-buffered saline.

Experiment	Training[a]	Generalization[b]	Donors
1a	**RV** vs. control (7)[c]	RV vs. control (validation)	70 RV
		WNV vs. control	30 control
		WNV vs. RV	16 WNV
1b	**RV** vs. WNV (7)[d]	None	
2a	**LPS** vs. control (3)	LPS vs. control (validation)	40 LPS
	LPS vs. **control** (3)	LPS vs. control (validation)	20 control
			10 RV
2b	**LPS** vs. control (7)[e]	LPS vs. RV	
3	**RV** vs. control (5)	RV vs. control (validation)	21 RV
		LPS vs. control	17 control

[a] Odors associated with treatments in bold were the rewarded choice in training trials.
[b] Identities of stimuli were blind to the operator during generalization trials and were unrewarded.
[c] Number of subjects in the biosensor panel is indicated in parentheses.
[d] Experiment 1b donors are the same as Experiment 1a.
[e] Experiment 2b donors are derived from Experiment 2a.

2.3. Urine stimuli

Urine collection from treated donors commenced on day four post-treatment by application of abdominal pressure as previously described [22]. Urine was collected daily for several weeks. Expressed urine from each subject was collected in a vial and frozen until used for behavioral assays. To attain the necessary volumes for bioassay, samples collected from the same individual on multiple collection days were often combined. Unless otherwise specified, training trials were conducted with urine collected 4–13 days post-treatment with RV, WNV, or LPS.

2.4. Apparatus

A Y-maze was used for biosensor training and testing as previously described [12]. Air was conducted through two odor chambers, containing urine (0.5 mL) exposed in 35 mm Petri dishes (11 mL) to the two arms of the maze (Table 1). Samples were randomly assigned to the left or right odor boxes of the Y-maze. Gates were manually raised and lowered in timed sequence to permit the training or testing of each mouse in a session of up to 48 consecutive trials. The reward for a correct response was a drop of water (the mouse having been restricted of water for 23 h).

2.5. Experiment 1

Experiment 1 was conducted to determine if the administration of RV altered body odor and to investigate if this learned response was generalized to a different vaccine treatment. For Experiment 1a, daily training sessions consisted of pair-wise offerings of urine from individual control and RV donors and the reward was always associated with RV-urine. Training continued daily until each biosensor achieved greater than 80% concordance (correct response to stimulus associated with reward). Throughout training, unrewarded trials were interspersed (on average 25% of the time) among rewarded trials.

Following training, testing sessions consisted of rewarded training trials with interspersed unrewarded training trials (extinction) and unrewarded generalization trials (total of four per session). Experiment 1a consisted of three different generalization experiments (Table 1). The first, and most important, generalizations constituted the validation experiment designed to demonstrate that the trained response was related to the vaccine treatment. Validation trials employed stimuli from the same treatment groups as used in training sessions with the important exception that urine samples were collected from novel individual mouse donors (not used during training). Five sessions were completed in the validation experiment, resulting in 78 total validation trials among the seven biosensors. Additional generalization tests were paired comparisons of WNV-urine versus control urine (69 trials in four sessions) and WNV-urine versus RV-urine (59 trials in six sessions).

Experiment 1b was conducted when it was concluded that treatment with the two vaccines resulted in similar odor alterations. Training sessions were initiated with the Experiment 1a biosensor panel to

directly discriminate RV-urine and WNV-urine. The water reward was associated with urine odors from RV vaccinated mice. Because biosensors showed no evidence of discrimination between urines collected from donors receiving the two vaccines, no generalization trials were run in Experiment 1b.

2.6. Experiment 2

Investigation of LPS-induced odor change was conducted in two phases to determine if LPS-trained mice could discriminate LPS- and RV-urines in a paired comparison. In Experiment 2a, mice were trained to discriminate between urine odor pairs from control and LPS-treated donors collected 4–8 days post-treatment. Three biosensor mice were rewarded for choosing the maze arm associated with LPS urine odor and three were rewarded for association with control urine odor (Table 1). There were six validation sessions (control versus LPS-urine collected days 4–8 from novel donors) resulting in 69 trials. To facilitate generalization testing with RV, a new panel of trained mice in Experiment 2b consisted of the three biosensors that had been rewarded for selection of LPS during Experiment 2a as well as four naive subjects. Training was similar to Experiment 2a except that all mice were rewarded for selection of odor associated with LPS treatment. One biosensor died during Experiment 2b. The single generalization experiment (LPS-urine versus RV-urine collected on days 7 and 8) consisted of six sessions resulting in 46 trials.

2.7. Experiment 3

The final experiment was conducted to determine if mice trained to recognize urine from RV-mice generalized the learned response to urine from LPS-mice. Employing a new biosensor panel, training was conducted identically to Experiment 1a (paired comparison of RV-urine and control urine with reward associated with RV-urine). There were 9 validation sessions (control versus RV-urine collected 4–22 days post-treatment from novel donors) resulting in 97 trials. The other generalization experiment (pairing control versus LPS-urine collected 4–14 days post-treatment) resulted in 56 trials in 3 sessions.

2.8. Data analysis

Correct responses for individual biosensors during training were subjected to chi-square tests of independence. When training responses did not differ among individuals, cumulative responses of the full panel were calculated for training and subsequent generalization trials. Success rates (number of correct trials divided by total trials) for specific generalization trials were subjected to statistical tests of binomial proportion using the continuity correction for low incidence events [23]. No comparisons were made among generalization trial types. For Experiment 2a, an additional test (chi-square test of independence) was conducted to determine if training responses differed according to the stimulus associated with the water reward (LPS or control).

3. Results

Following treatment, subjects receiving LPS treatment demonstrated visible indicators of sickness — i.e., lethargy and dehydration. Reduced urinary output, while not explicitly measured, was evident during urine collection for up to 5 or 6 days following LPS administration. No similar effects were observed among subjects treated with the vaccines or control.

3.1. Experiment 1

Trained mice could discriminate RV-urine from control urine by smell. Overall, biosensor concordance was 90% in rewarded training trials in Experiment 1a. The proportion of correct responses did not differ among individual biosensors during training ($p = 0.10$). In validation trials, biosensors correctly chose the maze arm associated with urine of novel RV-treated donors in 51 out of 78 (65%) unrewarded trials versus control urines ($p = 0.0046$; Fig. 1). In a second set of generalization trials, biosensors trained to respond to RV vaccine-treated urine selected the maze arm associated with the urine of West Nile virus (WNV) vaccine-treated donors in 49 out of 69 (71%) unrewarded trials versus controls ($p = 0.0004$) suggesting that RV and WNV treatments result in similar volatile odor profiles. Biosensors offered RV and WNV urines in direct comparisons did not discriminate between the two different immunization types 36 out of 68 times (53%; $p = 0.36$; Fig. 1).

Training trials employing direct pairing of RV-urine and WNV-urine in Experiment 1b were not successful. No progress toward concordance was evident after six consecutive training sessions. Whereas the novel biosensor panel trained in Experiment 1a correctly identified RV-urine (paired with control urine) in 62% of trials ($p < 0.0001$ for $H_0 = 50\%$) after six sessions; the Experiment 1b panel identified RV-urine in only 44% of the trials when paired WNV-urine donors in rewarded trials. As a result of this performance, the experiment was terminated.

3.2. Experiment 2

LPS-trained mice discriminated between LPS- and control mice as well as between LPS-urine and RV-urine in paired comparisons. The new biosensor panel discriminated LPS and control urine in rewarded training trials (82% concordance). Response rates did not differ among individuals during training ($p = 0.15$) and were not impacted by which urine was reinforced (LPS or control) in Experiment 2a ($p = 0.20$). In validation trials, biosensors selected the maze arm associated with LPS urine when paired with control urine 47 out of 69 times (68%; $p = 0.0019$; Fig. 2). In generalization trials with RV-urine, discrimination was observed when LPS-urine was compared with RV-urine 36 out of 46 times (78%; $p = 0.00011$).

3.3. Experiment 3

RV-trained mice did not generalize the response to LPS-urine. Five new biosensors were successfully trained to discriminate urine of RV vaccine-treated donors versus controls (87% concordance). The proportion of correct responses did not differ among individual biosensors during training ($p = 0.58$). In validation trials with urine of novel donors, biosensors selected the maze arm associated with urine of RV

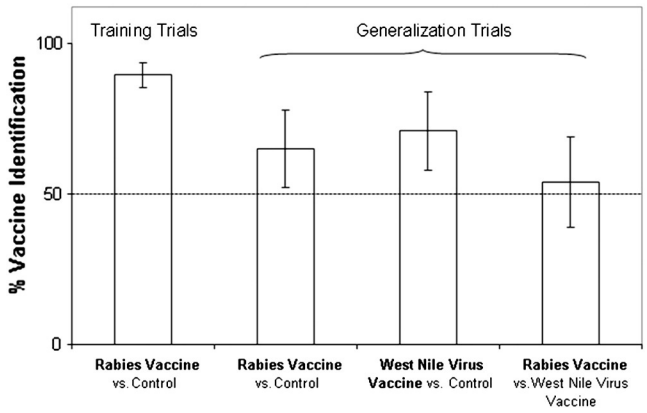

Fig. 1. Biosensor results from Experiment 1a. Mean responses are provided with 95% confidence intervals. The urine sample type associated with % response is indicated in bold. Training trials were rewarded, while generalization trials with novel urine sources were unrewarded. Rabies = urine of donors treated with RabVac-3® vaccine; West Nile virus = treatment with PreveNile® vaccine; and control = treatment with phosphate-buffered saline.

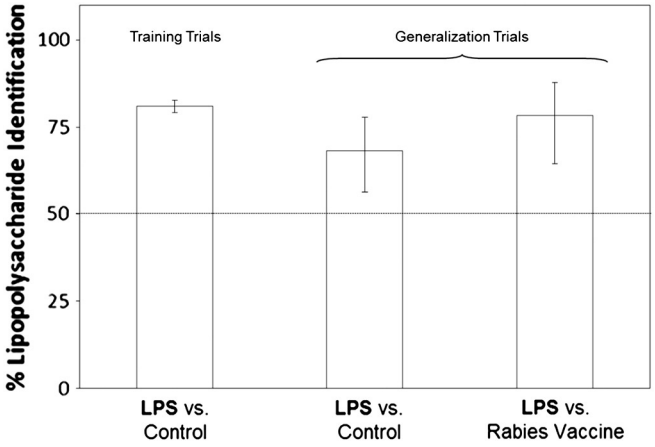

Fig. 2. Biosensor results from Experiment 2. Mean responses are provided with 95% confidence intervals. The urine sample type associated with % response is indicated in bold. Training trials were rewarded, while generalization trials with novel urine sources were unrewarded. Rabies = urine of donors treated with RabVac-3® vaccine; lipopolysaccharide (LPS) = treatment with LPS solution in buffered saline; and control = treatment with phosphate-buffered saline.

vaccinated donors in 65 out of 97 unrewarded trials ($p = 0.0006$; Fig. 3). However, RV-trained mice did not discriminate between urines of LPS-treated and control donors in generalization trials (24 out of 56; $p = 0.18$; Fig. 3). These results suggest that volatile cues associated with LPS treatment were different from the volatiles used by trained biosensors to identify RV-urine.

4. Discussion

Mice were successfully trained to discriminate urines from RV-treated and control mice (Figs. 1 and 3) as well as urines from LPS-treated and control mice (Fig. 2). The training response was validated with generalization trials employing urine samples from donors that were unfamiliar to the trained mice. The purpose of these validation trials was to exclude the possibility that individual cues, rather than treatment effects, could have been used to characterize urine samples used during training. Information regarding the identity of samples used in these generalization trials could be withheld from the maze operator (thereby making her blind to the expected results) which was possible because the generalization trials were unrewarded. Validation results demonstrated that biosensor discrimination was made on the basis of odors related to LPS or RV treatments, not cues learned about the urine donors or the training paradigm.

In Experiment 1a, RV-trained biosensors generalized their response to RV-urine by discriminating urines from WNV-treated and control donors. However, RV-trained biosensors did not discriminate RV- and WNV-urines when presented in unrewarded head to head comparisons (Fig. 1); neither was discrimination observed after six training sessions with RV- and WNV-urines in Experiment 1b. Although we cannot be certain that training with RV- and WNV-urines would not have been successful with many additional trials, these data (along with considerable past experience with the Y-maze training paradigm) led us to conclude that odor changes induced by these two vaccines were perceptibly very similar.

To our knowledge, these are the first data to demonstrate that immune activation causes the expression of a distinctive odor. Because the design of the Y-maze ensures that biosensors cannot make contact with the odor stimuli, it is certain that the cues responsible for biosensor discrimination are volatile compounds. Yet, the mechanisms underlying these odor changes remain in question. Volatile metabolites may arise at any point in the complex processes of innate or adaptive immunity. Major histocompatibility complex (MHC) variation has been identified as a source of odor differences [22]. However, aspects of the current study may exclude adaptive immunity as a source of metabolites indicative of vaccination. First, discrimination of vaccination status is apparent in as few as four days, presumably too early to implicate adaptive immunity which occurs over several weeks. For example, peak antibody production in humans has been observed between 14 and 28 days for RV [24] and WNV vaccines [25]. Second, inactivated vaccines (e.g., RV) do not participate in the cellular MHC class I pathway analogous to live vaccines such as WNV [26]. The perceptual similarities between RV and WNV-produced odors suggest that innate immunity processes may be responsible for the volatile metabolites [27]. Innate immunity triggers inflammation and initiates adaptive immunity via the complement system within hours of antigen presentation.

As a first step toward examining the possible role of an innate immunity activation pathway to odor change, we examined odor changes elicited by exposure to the potent immune activator, LPS, the prototypical bacterial endotoxin [28]. This was chosen, in part, because LPS has been used to induce "sickness-related odors" in mice [29]. In particular, rats exhibited avoidance behaviors of soiled bedding material collected from conspecifics four hours after receiving 100 μg/Kg doses of LPS; which could be blocked by the administration of anti-inflammatory cytokine IL-10 [29]. Validation trials in Experiment 2a indicated not only that the biosensor response was related to treatment, but also that discrimination was possible regardless of which choice (LPS- or control urine) was associated with the reward. Because Experiment 2b required the biosensors to generalize their response to LPS-urine odors, a new panel consisting of individuals reinforced to LPS-urine odor was assembled. Biosensor discrimination of LPS urine and RV urine demonstrated that LPS and RV administration produced distinct alterations in volatile urine metabolites (Fig. 2).

These results also suggest that the odor alterations resulting from treatment with LPS or RV are not merely quantitatively different. If the salient odor cues of RV-urine were quantitatively greater than the odors resulting from LPS treatment, biosensors would have likely generalized the control response to the weaker LPS signal. Rather, they maintained the response to LPS-related odors and discriminated LPS- and RV-urines. Likewise, biosensors would have discriminated LPS-urine and control-urine in Experiment 3 if the odor cues of LPS-urine were merely quantitatively greater than RV-urine odors.

A new panel was directly trained with LPS urine and control urine in Experiment 3. Biosensors trained to discriminate RV-urine and control urine did not generalize this response to LPS urine in Experiment 3 (Fig. 3), further indicating that these two activators of the immune

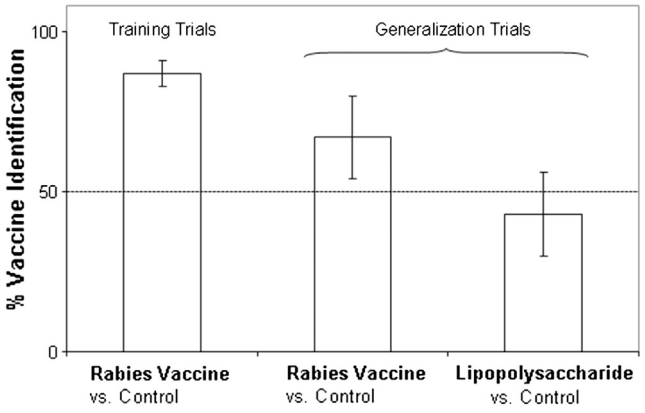

Fig. 3. Biosensor results from Experiment 3. Mean responses are provided with 95% confidence intervals. The urine sample type associated with % response is indicated in bold. Training trials were rewarded, while generalization trials with novel urine sources were unrewarded. Rabies = urine of donors treated with RabVac-3® vaccine; lipopolysaccharide (LPS) = treatment with LPS solution in buffered saline; and control = treatment with phosphate-buffered saline.

system induced different alterations of urine odor. Although outward indicators of acute inflammation (e.g., lethargy, dehydration) were evident for subjects treated with approximately 2 mg/Kg LPS in the current study, it is unlikely that inflammation per se was responsible for the differences in RV- and LPS-related odors. Peak inflammatory cytokine levels in mice administered with 3 mg/Kg LPS are commonly observed approximately four hours after treatment and return to baseline levels after 24 h [21]. Furthermore, RV and control odors were readily discriminated by the biosensors (Fig. 1) despite the fact that inactivated viruses (such as RV) lack the capacity to elicit potent inflammatory responses [30].

Volatile cues present in urine many days after treatment with LPS or vaccine likely represent the "memory" of an innate immunity process that differs between these immunogens. Interaction with Toll-like receptors (TLRs) is one example of how different classes of immunogens may differ in innate immunity. Recognition by the innate immune system is accomplished by a number of pattern recognition receptors. For example, cell surface recognition of LPS is accomplished by TLR4 [19]. Conversely, a variety of TLRs, RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) are responsible for cytoplasmic and endosomal recognition of viruses [31]. Discrimination between LPS- and RV-urine odors (Fig. 2) and inability to discriminate between odors resulting from the two vaccines (Fig. 1) are consistent with the concept that immunogens interacting with similar pattern recognition receptors elicit similar volatile cues.

Following the detection of an immunogen, a number of innate immunity processes are initiated: antimicrobial enzymes and peptides are released, inflammatory processes may be triggered, and attachment of antigens to phagocytes is promoted. Among the biochemicals released to achieve these tasks are lipases, cytokines, and complement protein complexes. The volatile cues detectable by trained mice may be influenced by any or all of these cellular events. Specifically, differences between LPS and the vaccines may be related to the complement system. Complement proteins participate in innate immunity and initiate adaptive immunity. Because complement participates in the humoral response via interactions with immunoglobulins [19], it might be expected that the complement protein cascade responding to a bacterium (e.g., LPS) would differ from a viral response (e.g., RV or WNV). The finding that the volatile signatures of RV and WNV vaccines are similar, although different from LPS, may be related to this aspect of innate immunity.

Metabolic inputs to innate immunity may be another source of the volatiles used by the biosensors to discriminate treatments. Using LPS and keyhole limpet hemocyanin (KLH; a very large immunogenic protein often employed in vaccines) treatments in mice, a tradeoff between innate immunity and maximal metabolic rate was observed — but no such cost was evident for adaptive immunity [32]. Differences in volatiles arising from LPS treatment as compared with the vaccines may reflect differences in the costs of innate responses to these immunogens. Therefore, anabolic and/or catabolic pathways required for meeting the energy requirements of innate immunity may themselves be the source of the volatile cues. For example, prolonged fasting produces markers indicative of amino acid catabolism in humans, including volatile metabolites [33]. Non-volatile metabolites of folate catabolism persist up to eleven days in rats following administration of radio-labeled folic acid [34]. Liver functions, such as cytochrome P-450 metabolism, may also be impacted by inflammation. Reduced P-450 metabolism was observed in mice 24 h after i.p. treatment with LPS [35]. In summary, volatile cues of immunization detectable by trained mice may result directly from immunogen recognition or response pathways; or indirectly via persistent metabolites associated with the metabolic cost of innate immunity. Perhaps the mechanism could be further elucidated in a series of bioassays employing urine collected from receptor knock-out transgenic mouse strains and/or donors treated directly with cytokines.

An important question raised by these studies is the potential role that vaccination-induced odor changes may have in the context of normal social behavior of the species. Generally speaking, disease-based odors are avoided by conspecifics [36–38] which probably functions to limit the transmission of disease. If some or all of the odor changes indicative of disease are consequences of immune activation, one would predict that vaccination-induced odors such as those we have studied here may also be avoided. This hypothesis is now under investigation. If this is so, large-scale immunization projects (e.g., to limit spread of rabies in raccoons [39]) could have unintended consequences for social and reproductive behaviors.

In conclusion, we have demonstrated that volatile metabolites indicative of some aspect of innate immune activation are present in the urine several days following i.p. administration of two different vaccines or LPS. Odor differences induced by the two vaccines (RV and WNV) were apparently imperceptible. However, it is still an open question as to whether other vaccination-induced odors can be differentiated. This is significant because the more specific the odor change, the more useful these odors could be for monitoring specific immunization status. Also, further research is necessary to identify the metabolic pathway(s) responsible for these odor and odorant changes, their underlying chemical bases, their potential role in modifying social behavior, and their diagnostic capabilities.

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