Objective: Immune and inflammatory pathways play important roles in the pathogenesis of metabolic disorders. This study investigated the role of toll-like receptor 4 (TLR4) in orosensory detection of dietary lipids and sugars.

Methods: Taste preferences of TLR4 knockout (KO) and wild-type (WT) male mice under a standard and a high-fat, high-sugar diet were assessed with two-bottle tests. Gene expression of taste signaling molecules was analyzed in the tongue epithelium. The role of TLR4 in food intake and weight gain was investigated in TLR4 KO and WT mice fed a high-fat and high-sugar diet for 12 weeks.

Results: Compared to WT mice, TLR4 KO mice showed reduced preference for lipids, sugars, and umami in a two-bottle preference test. The altered taste perception was associated with decreased levels of key taste regulatory molecules in the tongue epithelium. TLR4 KO mice on a high-fat and high-sugar diet consumed less food and drink, resulting in diminished weight gain.

Conclusions: TLR4 signaling promotes ingestion of sugar and fat by a mechanism involving increased preference for such obesogenic foods.

Introduction

The gustatory system allows animals to discriminate among foods in order to select nutritious diets and maintain energy balance. Although a broad range of economic, social, and behavioral factors influences food choices in humans, the immediate pleasantness generated by taste is still, for most individuals, the driving force behind food consumption. The hedonic response to high-caloric sugars and fats, and consequent overconsumption of such foods, likely plays a role in the increasing prevalence of obesity worldwide (1). However, the cellular and molecular mechanisms underlying such eating behavior are largely unknown.

Toll-like receptors (TLRs) recognize structurally conserved molecular patterns expressed in pathogens, triggering inflammatory and immune responses. Since the discovery that subjects with obesity, type 2 diabetes, and metabolic syndrome have increased levels of toll-like receptor 4 (TLR4) expression in various tissues, many studies have been conducted to elucidate TLR4 functions in the metabolic consequences of diet-induced obesity (2). Interestingly, while most studies of TLR4 have focused on its role in macrophages and other immune system cells, recent findings have demonstrated that TLR4 is expressed in neurons, where, in the absence of pathogens, it plays important roles in regulating the development and plasticity of neuronal circuits (3). Notably, hypothalamic activation of TLR4 signaling by fatty acids has been shown to influence anorexigenic signals (4). It has also been shown that multiple TLRs are expressed in the tongue gustatory papillae, where they may initiate immune responses to pathogens (5). In this study, we investigated the possibility that TLR4 could be involved in taste perception and regulation of food preference and intake.

Methods

Animals and diets

Adult male TLR4−/− (TLR4 knockout, KO) and TLR4 +/+ wild-type (WT) mice (25-30 g) were kept in a 12-hour light/12-hour dark cycle on a standard diet (Harlan Teklad 2018). For the diet-induced obesity model, mice of each genotype were randomly assigned to a control diet (diet #101845; Dyets Inc., Bethlehem, Pennsylvania) with water or a high-fat diet (diet #101842; Dyets Inc.) with water containing 12% sterile fructose. The macronutrient composition of the different diets utilized in the study is shown in Table 1. This research was approved by the National Institute on Aging Animal Care and Use Committee and was performed according to guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RNA extraction and real-time PCR

The tongue epithelium containing foliate and circumvallate papillae was dissociated from the underlying muscle, snap frozen, and stored...
TABLE 1 Macronutrient composition of the diets utilized in the study (all diets supplemented with minerals, amino acids, and vitamin mixtures)

| Macronutrient | Standard diet | Control diet | High-fat diet |
|---------------|---------------|--------------|--------------|
|               | (Harlan Teklad), 3.1 kcal/g | (Dyets 101845), 3.8 kcal/g | (Dyets 101842), 5.0 kcal/g |
| Crude protein | 18.6 | 20.0 | 20.0 |
| Fat (soybean oil) | 6.2 | 7.0 | 27.1 |
| Saturated Fat | 0.9 | - | - |
| Unsaturated Fat | 4.7 | - | - |
| Carbohydrate | 44.2 | 64.7 | 47.6 |
| Fiber | 18.2 | 5.0 | 5.0 |

at −80°C. RNA was isolated using Trizol (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts) and purified with an RNA Micro Kit (Qiagen, Valencia, California). Following treatment with DNase I, RNA was quantified and equal amounts were reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR analysis was performed with a PTC-200 Peltier Thermo Cycler and Chrom4 fluorescent detector (Bio-Rad, Hercules, California), and SYBR Green PCR Master Mix according to the manufacturer’s instructions (Applied Biosystems, Foster City, California). The comparative Ct method was used to determine the normalized changes of the target gene relative to a calibrator reference (β-actin).

Two-bottle preference test
The test was performed in the home cage of individually housed mice given free access to food. Two sipper bottles (modified 15 mL polypropylene conical tubes with a sipper tube) containing water were provided for the first 2 days to habituate the mice to the two bottles. After the mice were acclimatized to the experimental setting, they were presented with a bottle containing a tastant solution and a bottle containing water plus the proper vehicle for 2 days. The order of taste tests was as follows: water, saccharin (0.1%), fructose (12%), quinine (0.1-1 mM), denatonium benzoate (3 mM), citric acid (10 mM), inosine-5-monophosphate (10 mM), calcium chloride (75 mM), sodium chloride (300 mM), linoleic acid (0.1%-2% vs. 0.3% xanthan gum to minimize textural cues), and capsaicin (0.1%). To eliminate the potential effect of body weight on solution intake preference ratios, we calculated the ratio of 48-hour tastant intake over total liquid (water + tastant) consumption.

Cell culture, lipid uptake, and colocalization experiments
HEK 293 cells (ATCC) were cultured in DMEM containing 10% HyClone FetalClone III and 1% penicillin-streptomycin at 37°C in a humidified 5% CO2 atmosphere. Based on the experimental end point, cells plated on cover slips were transfected with either vector, pcDNA3-TLR4-YFP (gift from Doug Golenbock, Addgene plasmid #13018) or a mixture 1:1 of pcDNA3-TLR4-YFP and pcDNA3-CD36-Flag (gift from Nada Abumrad) using FuGENE 6. After 24 hours, cells were incubated with fresh, complete media containing 1 μM 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diazocyclobuta-2,1-indene-3-dodecanoid acid (BODIPY #3832) for lipid uptake experiments or 100 μM linoleic acid (C18:2) for colocalization experiments. At different time points following treatment, the cells were washed with phosphate buffered saline and fixed with 4% paraformaldehyde. CD36 expression was detected by immunocytochemistry using an anti-Flag M2 antibody (Sigma). DNA was counterstained with DAPI. Z-stack photomicrographs were acquired on a Zeiss LSM 510 confocal using a 40X objective. BODIPY uptake was measured in 3D reconstructed images using Fiji. Data are expressed as total cell fluorescence.

Statistical analysis
Statistical analysis was performed by Student’s t test or two-way analysis of variance (ANOVA) as appropriate for the experimental design using Graph Pad Prism 6.0. The results are expressed as mean + standard error of the mean (SEM). P < 0.05 was considered statistically significant.

Results
TLR4 is expressed in the tongue epithelium
To study the impact of TLR4 signaling on taste perception, we first analyzed the expression of several different TLRs in WT and TLR4 KO mice maintained on a standard diet by real-time PCR. In agreement with previous reports (5), we found that cells in the tongue sensory epithelium of WT mice expressed TLR2, -3, and -6 (Figure 1A, test) and had comparable levels of mRNAs encoding TLR2, -3, and -4 (Figure 1B, t test). While the level of the mRNA encoding the fat taste receptor G-protein-coupled receptor 120 (GPR120) was unchanged (Figure 1B, P > 0.05, unpaired t test), we surprisingly found a significant decrease in the expression of CD36 in TLR4 KO
mice (Figure 1B, $P < 0.001$, unpaired $t$ test). No changes were observed for the G-coupled proteins $z$-gustducin ($z$Gus) and transducin (Gnat) (Figure 1B, $P > 0.05$, unpaired $t$ test), but levels of mRNAs encoding TRPM5 (Figure 1B, $P < 0.001$, unpaired $t$ test) and the G-protein-dependent phospholipase C $\beta_2$ (PLC$\beta_2$) (Figure 1B, $P < 0.001$, unpaired $t$ test) were also significantly decreased in TLR4 KO mice compared to WT mice. Overall, these results indicate that TLR4 deficiency is associated with altered taste signaling transducer expression.

**TLR4 KO mice are less responsive to sweet, umami, spicy, and fat tastants**

To determine the taste preferences of TLR4 KO mice, we performed a 48-hour two-bottle test. Naive animals were allowed to acclimate to single housing in cages with two sipper bottles for 2 days and were then tested for the various tastants following the order stated in Methods. All mice had unrestricted access to standard diet (carbohydrate, 44.2%; fat, 6.2%; protein, 18.6%). Preference scores showed that TLR4 KO mice consumed less artificial (saccharine, $P = 0.01$, unpaired $t$ test) and natural (fructose, $P = 0.044$, unpaired $t$ test) sweet, as well as less umami (inosine-5'-monophosphate, $P = 0.04$, unpaired $t$ test) and fat (linoleic acid [LA], $P = 0.05$, unpaired $t$ test) solutions (Figure 2). Notably, TLR4 KO mice were also less sensitive to capsaicin ($P = 0.001$, unpaired $t$ test). No difference between the genotypes was observed for calcium, bitter, salty, and sour tastes (data not shown).

**TLR4-dependent altered orosensory perception influences diet-induced obesity**

The reduced preference for sweet and fat molecules shown by the TLR4 KO mice suggested that TLR4 may impact the propensity of mice to consume high amounts of obesogenic diets rich in fat and simple sugars. We therefore tested high-fat diet palatability in WT and TLR4 KO naive mice. Similar to the two-bottle taste preference experiment, singly housed mice were given access to two hoppers containing a known amount of control diet (carbohydrate, 64.7%; fat, 7%;...
protein, 20%) or high-fat diet (carbohydrate, 47.6%; fat, 27.1%; protein, 20%) and water. The position of the hoppers was changed after 24 hours to account for position bias. The amount of food consumed was measured after 48 hours, and a preference score for the high-fat diet was calculated as grams of high-fat diet over total food consumed. Not surprisingly, we found that WT mice displayed a preference for the high-fat diet (Figure 3A), with about 43% of the mice exclusively consuming the high-fat food. TLR4 KO mice showed a reduced preference for the high-fat diet, with none of the animals exclusively consuming it (Figure 3A, P = 0.01, unpaired t test). We next tested how the observed changes in orosensory perception influenced weight gain in WT and TLR4 KO mice fed with a high-fat, high-sugar diet. Mice of both genotypes were randomly assigned to either a control diet supplemented with water or a high-fat diet supplemented with 12% fructose (average sugar content in sodas), and their weight, food, and liquid intake were monitored over a 3-month period. In mice on the control diet, there were no significant differences between the genotypes in the average food (Figure 3C, P > 0.05, two-way ANOVA) and water (Figure 3D, P > 0.05 two-way ANOVA) consumption, with identical caloric intake and weight gain at the end of the study (Figure 3B-3E). In contrast, compared to WT mice, TLR4 KO mice consumed less high-fat diet (P < 0.05, two-way ANOVA) and fructose solution (P < 0.05, two-way ANOVA) throughout the study (Figure 3C-3D), resulting in an overall diminished caloric intake (Figure 3E, P < 0.01, two-way ANOVA) and, ultimately, in diminished weight gain (Figure 3B).

### Influence of TLR4 on high-fat diet-induced taste preferences

A growing body of evidence suggests that obesity modifies taste sensitivity (6). We tested whether TLR4 signaling influences diet-induced taste changes, subjecting WT and TLR4 KO mice to a two-bottle preference test using the same control diet and high-fat diet they had consumed for 12 weeks. Similar to what we observed for naive mice on the standard diet, TLR4 KO mice on the control diet showed decreased saccharin (P = 0.039, two-way ANOVA), fructose (P = 0.037, two-way ANOVA), and inosine-5′-monophosphate (P = 0.005, two-way ANOVA) preference scores compared to WT mice (Figure 4A). Consumption of high-fat diet significantly decreased the preference scores for sweet (saccharin, P = 0.039; fructose, P = 0.044, two-way ANOVA) and umami (P < 0.0001, two-way ANOVA) in WT mice (Figure 4A) but had no effect in TLR4 KO mice (P > 0.05, two-way ANOVA). We also performed a dose-response experiment using increasing concentrations of LA. Compared to mice kept on a standard diet, all mice showed higher preference scores for LA (compare Figure 2 and Figure 4B). Multiple exposures to increasing concentrations of LA resulted in increased preference scores in WT mice on the control diet but not in those on the high-fat diet (Figure 4B, P = 0.01, two-way ANOVA). TLR4 KO mice showed lower preference for LA regardless of the diet regimen (Figure 4B). The analysis of the relative expression of taste receptors and transducers in the tongue epithelium revealed decreases in levels of mRNAs encoding CD36 (P = 0.041, two-way ANOVA), TRPM5 (P = 0.027, two-way ANOVA), and PLCβ2 (P = 0.002, two-way ANOVA) in TLR4 KO mice on the control diet compared to WT mice, similar to the mice fed a standard diet (Figure 4C). Surprisingly, a significant reduction of T1R2 (P < 0.001, two-way ANOVA) expression was also found in TLR4 KO mice on the control diet compared to WT animals (Figure 4C). High-fat diet consumption resulted in a decrease in levels of mRNAs encoding T1R2 (P = 0.005, two-way ANOVA) and TRPM5 (P = 0.035, two-way ANOVA) in WT but not in TLR4 KO mice (Figure 4C).

**TLR4 mediates lipid uptake and colocalizes with CD36 during LA cellular internalization**

Fatty acids have been shown to activate the TLR4 signaling cascade through the leucine rich repeat region in the extracellular domain,
which allows the association with hydrophobic ligands (7,8). CD36 and TLR4 cooperation is important for the recognition and internalization of bacteria (9) as well as oxidized lipoproteins (10). We tested the possibility that TLR4 could mediate lipid cellular uptake using the TLR-deficient cell line HEK 293. As shown in Figure 5A, the expression of TLR4-YFP increased about 3.4-fold the cellular uptake of the fluorescent fatty acid analog (C12) compared to vector transfected cells \( (P < 0.001 \text{ unpaired } t \text{ test}) \). Qualitative differences

Figure 3 TLR4-deficient mice display reduced preference for high-fat plus fructose diet (HFD) and are resistant to diet-induced obesity. (A) Naive WT and TLR4 KO mice were offered a choice between control and HFD. Compared to WT mice, TLR4 KO mice showed decreased preference for HFD. While 42.9% of the WT mice exclusively fed on HFD, none (0%) of the TLR4 KO mice showed a similar behavior. Data are mean and standard error of the mean for five to seven animals. ** \( P < 0.01 \) vs. WT animals. WT and TLR4 KO mice were fed control or HFD supplemented with water or 12% fructose, respectively, and their (B) body weight, (C) food intake, (D) drink intake, and (E) caloric intake were monitored over a 12-week period. Values are mean and standard error of the mean for 17 to 19 animals per group. * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \) vs. TLR KO HFD. * \( P < 0.001 \) vs. control diet. ** \( P < 0.001 \) vs. TLR4 KO HFD.
in the localization patterns were also observed. In vector transfected cells, within 30 minutes of treatment the fluorescent C12 appeared to have a diffused distribution with few punctate perinuclear structures (Figure 5A). However, more defined lipid droplets colocalizing with TLR4 were observed in the TLR4-YFP transfected cells (Figure 5A). We next assessed the possibility that TLR4 and CD36 may cooperate in lipid internalization transfecting HEK 293 with both TLR4-YFP and CD36-Flag expressing plasmids and incubating the cells with LA (C18:2). In untreated cells, TLR4-YFP and CD36 had a diffuse distribution with minimal colocalization (Figure 5B). In cells expressing both proteins, within 15 minutes of incubation with LA, increased colocalization of both receptors in perinuclear areas was seen (Figure 5B). The absence of similar clustering in cells expressing only CD36 (Figure 5B) suggests that TLR4 expression provides the cells with additional mechanisms to mediate lipid uptake. Overall, our results indicate that TLR4 can mediate the internalization of saturated fatty acids and of unsaturated fatty acids in the presence of CD36.

### Discussion

Under conditions in which a choice of food is possible, hedonic signals will override homeostatic physiological needs (11). Similar to addictive drugs, palatable foods activate reward-learning regions and dopamine signaling (12), leading to the possibility that overeating and obesity could be the result of food addiction. Given the huge social and economic burden of obesity-related comorbidities, it is crucial to improve our understanding of the mechanisms underlying pleasure and aversion to food. Because food ingestion represents a major route of pathogen entry, it is not surprising that many components of immune and inflammatory signaling pathways are expressed in the tongue epithelium (5,13). Interestingly, in recent years, functions for innate immune molecules unrelated to immuno-surveillance...
have begun to emerge. For example, the proinflammatory cytokine tumor necrosis factor alpha (TNFα) (13) and the anti-inflammatory cytokine interleukin-10 (14) are secreted in gustatory buds, and TNFα KO mice are less sensitive to bitter compounds (15). Conversely, elements of the taste transduction system are expressed in tissues of the body not traditionally associated with nutrient sensing. For example, the bitter receptor T2R5 and its entire taste transduction cascade are expressed in testis, where they regulate spermatogenesis (16). With regards to obesity, the role of TLR4 in the establishment of insulin resistance and in the metabolic consequences of diet-induced obesity is well characterized (17). To date, however, no study has analyzed the potential impact of TLR4 signal in food and beverage preference. Here we show that TLR4 KO mice display reduced preference for sweet, fat, and savory tastants, decreased intake of a high-fat and high-sugar diet, and, ultimately, reduced weight gain.

The changes in tastant and food preferences of the TLR4 KO mice are associated with decreased expression of the key taste molecules CD36, PLC2β, and TRPM5 in tongue epithelium. Our findings are consistent with previous reports showing that the production of the cytokine TNFα in tongue epithelium is TLR4 dependent, and limited to a subset of gustatory cells that express T1R3 receptors (umami and sweet) and PLC2β (13). Although the functional consequences of a partial decrease in PLC2β and TRPM5 are unknown, their complete ablation in mice results in loss of sweet, umami, fat, and bitter taste responses (18-20). Substantiating the importance of palatability in feeding behavior, TRPM5 KO mice have attenuated weight gain when fed diets with high-caloric carbohydrates and fats as a result of reduced overeating (21,22). In addition to changes in sweet, fat, and savory perception, we also found that TLR4 KO are less

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**Figure 5** TLR4 enhances cellular lipid uptake. (A) HEK 293 cells transfected with vector or TLR4-YFP expressing plasmids were incubated with fluorescent fatty acid analog C12 (BODIPY). The lipid uptake quantification expressed as total cell fluorescence (TCF), and representative images following 30-min incubation are shown. Data are mean and standard error of the mean for three independent experiments in triplicate (n = 46-48 cells). ***P ≤ 0.001 vs. vector transfected cells. (B) Representative images showing the distribution of TLR4-YFP and CD36-Flag in absence (−) and presence of 100μM linoleic acid. [Color figure can be viewed at wileyonlinelibrary.com]
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