Title: Regulation of bitter taste responses by tumor necrosis factor

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Abstract

Inflammatory cytokines are important regulators of metabolism and food intake. Over production of inflammatory cytokines during bacterial and viral infections leads to anorexia and reduced food intake. However, it remains unclear whether any inflammatory cytokines are involved in the regulation of taste reception, the sensory mechanism governing food intake. Previously, we showed that tumor necrosis factor (TNF), a potent proinflammatory cytokine, is preferentially expressed in a subset of taste bud cells. The level of TNF in taste cells can be further induced by inflammatory stimuli. To investigate whether TNF plays a role in regulating taste responses, in this study, we performed taste behavioral tests and gustatory nerve recordings in TNF knockout mice. Behavioral tests showed that TNF-deficient mice are significantly less sensitive to the bitter compound quinine than wild-type mice, while their responses to sweet, umami, salty, and sour compounds are comparable to those of wild-type controls. Furthermore, nerve recording experiments showed that the chorda tympani nerve in TNF knockout mice is much less responsive to bitter compounds than that in wild-type mice. Chorda tympani nerve responses to sweet, umami, salty, and sour compounds are similar between TNF knockout and wild-type mice, consistent with the results from behavioral tests. We further showed that taste bud cells express the two known TNF receptors TNFR1 and TNFR2 and, therefore, are potential targets of TNF. Together, our results suggest that TNF signaling preferentially modulates bitter taste responses. This mechanism may contribute to taste dysfunction, particularly taste distortion, associated with infections and some chronic inflammatory diseases.

Key words: cytokine, TNF, inflammation, taste-related behavior, taste buds

1. Introduction

Taste is the sensory system for detecting nutrients and potentially harmful substances in food and drink and, therefore, plays important roles in guiding food intake. Among the five basic taste modalities, sweet and umami tastes detect sugars and amino acids, respectively, and are generally preferred. Bitter taste recognizes toxins and noxious compounds and elicits avoidance behavior. Acids and salts are detected by sour and salt taste mechanisms. Recent research has made rapid progress in understanding taste receptors and signaling pathways, particularly for sweet, umami, and bitter tastes (Breslin and Huang, 2006; Chandrashekar et al., 2006; Liman et al., 2014). What remain largely unclear, however, are the regulatory mechanisms that modulate taste responses or taste bud structure under diverse physiological and pathological conditions.

Inflammation is likely one of such regulatory mechanisms. Many diseases with underlying inflammation, such as infections and autoimmune ailments, are associated with taste alterations (Bromley and Doty, 2003; Pribitkin et al., 2003; Schiffman, 1983). Taste alterations can occur as taste loss (lacking or reduced taste reception) or taste distortion (e.g. persistent bitter or metallic taste in the mouth) (Brand, 2000; Bromley, 2000). In animal models, induced inflammation has been shown to affect taste responses and taste bud structure (Cavallin and McCluskey, 2005; Cohn et al., 2010; Phillips and Hill, 1996). How inflammation exerts its effects on taste reception or taste bud structure has not been fully elucidated. Inflammation is an immune response to infection, tissue damage, and stress. In addition to its roles in regulating immunity and tissue repair, inflammation can strongly affect metabolism and food intake (Forsythe et al., 2008; Hotamisligil, 2006). The various effects of inflammation are often mediated by inflammatory cytokines, a group of signaling proteins that are highly induced during inflammatory responses. A number of inflammatory cytokines, such as tumor necrosis factor

(TNF), interleukin (IL)-6, and interferons, are pleiotropic and play important parts in regulating immunity, metabolism, and food intake (Cannon, 2000; Dantzer, 2001; Plata-Salaman, 1998).

Our studies have found that several inflammation-associated cytokines are preferentially expressed in taste bud cells compared to nontaste lingual epithelial cells (Cohn et al., 2010; Feng et al., 2014a; Feng et al., 2014b; Feng et al., 2012; Kim et al., 2012; Wang et al., 2007), suggesting that these cytokines may have special functions in the peripheral taste system. In particular, we have found that TNF is specifically expressed in a subset of taste bud cells even in healthy mice (Feng et al., 2012; Kim et al., 2012). Immunocolocalization experiments showed that TNF is colocalized with the sweet and umami taste receptor subunit T1R3, indicating that TNF is expressed specifically in the sweet and umami taste bud cells (Feng et al., 2012). Moreover, TNF expression level and its secretion in taste buds can be further augmented by inflammation, such as lipopolysaccharide (LPS)-induced inflammation (Cohn et al., 2010; Feng et al., 2012; Kim et al., 2012).

TNF was thought to be produced primarily by macrophages, but it is also produced by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, fibroblasts, adipocytes, and neurons (Hotamisligil et al., 1993; Niu et al., 2009; Walsh et al., 1991). TNF is known to activate a variety of cellular signaling pathways that are important not only for fighting against certain pathogens but also for regulating stress responses and metabolism (Cabal-Hierro and Lazo, 2012; Silke, 2011; Wajant et al., 2003). TNF contributes to behavioral changes associated with various illnesses (i.e. sickness behavior) which include fatigue, malaise, depression, and anorexia. It has been shown that administration of recombinant

TNF induces significant reduction in food intake both in rodents and in humans (Bernstein et al., 1991; Michie et al., 1989; Spiegelman and Hotamisligil, 1993). How TNF regulates food intake remains incompletely understood. Both peripheral and brain mechanisms are likely involved (Bernstein et al., 1991; Dantzer, 2001; Plata-Salaman, 1998).

The taste system plays an important role in regulating food intake. Considering the specific expression of TNF in taste bud cells, it is conceivable that TNF may be involved in modulating taste responses under physiological and pathological conditions. In this study, we used TNF knockout mice and their wild-type controls to investigate the role of TNF in the taste system. We conducted gustatory nerve recording and taste behavioral testing using these mice. Our results show that TNF-deficient mice are significantly less responsive to bitter compounds than control mice, whereas their responses to sweet, umami, sour, and salty compound did not differ significantly from those of control mice. Our results suggest that TNF is involved in the regulation of bitter taste reception.

2. Materials and methods

2.1 Animals

TNF knockout mice (stock number 005540) and wild-type control mice (C57BL/6J, stock number 000664) were purchased from the Jackson Laboratory (Bar Harbor, ME) and then bred and maintained at the Monell Chemical Senses Center. Generation of TNF knockout mice was described by Pasparakis et al. (1996) (Pasparakis et al., 1996). In these TNF knockout mice, the first coding exon (including the ATG translation initiation codon) and a portion of the first intron

of the TNF gene were deleted. The mutant mice have been backcrossed to C57BL/6J genetic background for ten generations. All mice were housed at the Monell Chemical Senses Center animal facility under a 12 h/12 h light/dark cycle. Mice were given free access to standard rodent food (8604 Teklad rodent diet, Harlan Laboratories) and water except during the periods of taste behavioral tests (described below). 4-10 months old mice were used for all the experiments described below. Age and gender matched wild-type and TNF-knockout mice were included for the experiments. All procedures were performed according to protocols approved by the Monell Chemical Senses Center Institutional Animal Care and Use Committee.

2.2 Reagents

All taste compounds used in behavioral and electrophysiological tests were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody against mouse ecto-nucleoside triphosphate diphosphohydrolase 2 (ENTPDase2) was purchased from Centre de Recherche (Quebec, Canada) (Bartel et al., 2006). Rabbit polyclonal antibodies against phospholipase C- β 2 (PLC- β 2, sc-206) (Clapp et al., 2001) and gustducin (sc-395), goat polyclonal antibodies against the voltage-gated potassium channel KCNQ1 (sc-10646) (Wang et al., 2009) and TNFR1 (sc-1069), and a blocking peptide (sc-1069p) for the anti-TNFR1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A purified rabbit polyclonal antibody against neural cell adhesion molecule (NCAM) was purchased from Millipore (Billerica, MA). Purified goat polyclonal antibodies against carbonic anhydrase 4 (Chandrashekar et al., 2009) and TNFR2 and a blocking antigen (recombinant mouse sTNFR2, 426-R2-050) for the anti-TNFR2 antibody were purchased from R&D Systems (Minneapolis, MN). Dylight-649 (or Dylight-488)-

conjugated donkey anti-rabbit or donkey anti-goat antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.3 Taste behavioral tests

Two-bottle preference tests were conducted as previously described (Bachmanov and Beauchamp, 2008; Bachmanov et al., 2002; Bachmanov et al., 2001; Wang et al., 2009). Briefly, TNF-deficient and wild-type mice were individually caged. For the first 2 days, mice were familiarized with the two drinking bottles, both containing deionized water. For the following days, mice were presented with two drinking bottles: one contained deionized water and the other a taste solution. The positions of the bottles were switched after 24 h to minimize positional effect. The volume of consumed liquid from each bottle was recorded at 24 and 48 h. Each concentration of a taste compound was tested for 48 h. The taste compounds were tested in the following order: NaCl (37.5, 75, 150, 300, and 600 mM), quinine hydrochloride (QHCl) (0.003, 0.01, 0.03, 0.1, and 0.3 mM), Saccharin (0.0625, 0.25, 1, 4, and 16 mM), inosine-5'-monophosphate (IMP) (0.3, 1, and 3 mM), and citric acid (1, 3, and 10 mM). Each mouse in the experiment was tested with all the above listed compounds. Between the testing of two different compounds, mice received deionized water in both drinking tubes for at least three days. During the experiment mice had free access to food. TNF-deficient mice and wild-type mice were tested at the same time in parallel. Taste preference scores were calculated for each animal by dividing the volume of consumed taste solution during the 48 h test period by the total volume of fluid intake during the same 48 h test period (i.e. preference score = intake of taste solution/(intake of taste solution + intake of water)). The average preference scores were then calculated for each group. By this calculation formula, preference scores between 0.5 and 1 indicate that mice prefer

the taste solution over water, whereas preference scores between 0 and 0.5 indicate that mice avoid the taste solution. 8-16 male mice (4-8 months old) in each group were used.

Brief-access tests were performed using the Davis MS-160 mouse gustometer (Dilog Instruments, Tallahassee, FL) as previously described (Kim et al., 2012). Briefly, mice were water-deprived for 22.5 h before 30 min training sessions and test sessions for aversive taste compounds (bitter, salty, and sour compounds). Mice were food- and water-restricted (1 g of food and 1.5 ml of water) for 23.5 h before test sessions for appetitive taste compounds (sweet and umami compounds). Water and food restrictions were used to motivate mice to lick the taste solutions presented during the short test periods and were comparable to published protocols for this type of tests (Glendinning et al., 2002). In each test session, mice were tested with three different concentrations of each taste compound along with a water control. Water and taste compounds were randomly presented to mice following random presentation schemes generated by the computer software. Inter-presentation interval was 10 s. The maximum wait for the first lick was 120 s. Lick time limit was 5 s, which was the time from the first lick until the shutter closed. So, for each presentation, mice had maximum 5 s direct contact (or lick) time with the presented solution or water. The session time limit was 30 min. The following taste compounds were tested: QHCl (0.03, 0.3, and 3 mM), NaCl (0.1, 0.6, and 1 M), and citric acid (3, 10, and 100 mM), sucrose (0.1, 0.2, and 0.6 M), IMP (1, 10, and 30 mM). Each mouse was tested with all the compounds. TNF-deficient mice and wild-type mice were tested at the same time in parallel. After each session, mice received a recovery day with free access to food and water for 24h. Taste stimulus to water lick ratios were calculated by dividing the number of licks for taste compounds by the number of licks for water presented in the same test session. Lick ratios less

than 1 indicate avoidance behavior to the taste solution, and lick ratios more than 1 indicate preference behavior. 8-16 TNF-deficient mice and wild-type control mice (all male, 6-10 months old) were included in this experiment. The same sets of mice were used for two-bottle preference tests and then for brief-access tests with a 3-week interval between the two testing procedures to minimize the possible effects of prior experience on subsequent testing.

2.4 Gustatory nerve recording

Chorda tympani nerve recording was carried out as previously described (Kim et al., 2012). Briefly, under pentobarbital anesthesia (50-60 mg/kg of body weight, i.p.), the trachea of each animal was cannulated, and the mouse was then fixed in the supine position with a head holder. The right chorda tympani nerve was exposed at its exit from the lingual nerve, cut near its entrance to the bulla, and was placed on a platinum wire recording electrode. An indifferent electrode was positioned nearby in the wound. Neural responses resulting from chemical stimulations of the tongue were fed into an amplifier (Grass Instruments, West Warwick, RI), monitored on an oscilloscope and an audio monitor. Whole-nerve responses were integrated with a time constant of 1.0 s and recorded using a computer for later analysis using a PowerLab system (PowerLab/sp4; AD Instruments, Colorado Springs, CO). For taste compound stimulation, the tongue was enclosed in a flow chamber, and solutions were delivered into the chamber by gravity flow. The following solutions were used as stimuli: 0.1-20 mM QHCl, 0.1-20 mM denatonium benzoate, 0.003-0.5 mM cycloheximide, 3-300 mM MgSO₄, 10-1000 mM sucrose, 0.3-20 mM saccharin, 10-1000 mM monopotassium glutamate (MPG) with or without 0.5 mM IMP, 0.1-10 mM IMP, 1-100 mM citric acid, 0.01-10 mM HCl, 10-1000 mM NaCl with or without 100 μ M amiloride, and 0.1M NH₄Cl. To analyze nerve responses to each stimulus,

the magnitudes of integrated responses at 5, 10, 15, 20, and 25 s after stimulus onset were measured and averaged. The relative response magnitude for each stimulus was calculated against the response magnitude to 0.1 M NH₄Cl, and this value was used for statistical analysis and for plotting dose-response curves. Eight wild-type mice (6 males and 2 females) and seven TNF-deficient mice (5 males and 2 females), 4-8 months old, were used for nerve recordings.

2.5 mRNA *in situ* hybridization

Digoxigenin (DIG)-labeled sense and antisense cRNA probes corresponding to the coding region of mouse TNFR1 and TNFR2 were synthesized using the DIG RNA labeling kit (Roche Applied Science). Fresh-frozen taste sections (10 µm/section) from C57BL/6J mice (4-6 months old) were attached to clean glass slides. Sections were then fixed with 4% paraformaldehyde and processed for *in situ* hybridization as previously described (Wang et al., 2007). Hybridizations were performed at 72°C overnight with DIG-labeled probes in 50% formamide, 5× SSC, 5× Denhardt's solution, 250 µg/ml yeast RNA, and 500 µg/ml sperm DNA. Sections were washed three times at 72°C with 0.2× SSC. Hybridized DIG-labeled cRNA was detected immunologically with an alkaline-phosphatase-conjugated anti-DIG antibody and standard chromogenic substrates 4-Nitro Blue tetrazolium chloride (NBT, Roche Applied Science). Images were taken using a Nikon fluorescence microscope. In all the experiments, hybridizations to antisense and sense probes were performed in parallel to verify the specificity of hybridization signals.

2.6 Immunohistochemistry

Tissue preparation and immunofluorescent staining procedures were described previously (Feng et al., 2012; Wang et al., 2007). All mice used for this procedure were 4-6 months old. Briefly, excised mouse tongue tissues were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h on ice and then cryoprotected in 20% sucrose/PBS solution at 4°C overnight and embedded in mounting medium. Tissues were sliced into 10- μ m-thick sections using a Microm HM 500 OM cryostat (Thermo Scientific Microm, Walldorf, Germany). Purified goat polyclonal antibodies against TNFR1 and TNFR2 (see Section 2.2) were used to detect the expression of TNFR1 and TNFR2 in taste tissues. Two control experiments for TNFR1 and TNFR2 immunostaining were conducted. In one control experiment, primary antibodies against TNFR1 and TNFR2 were omitted in the procedure. In the second control experiment, antibodies against TNFR1 and TNFR2 were preincubated with their corresponding blocking antigens before adding to tissue sections. To investigate what types of taste bud cells express TNFR1 or TNFR2, double immunostaining was carried out using rabbit antibodies against taste-cell-type markers and goat antibodies against TNFR1 or TNFR2. Antibodies to the following taste-cell-type markers were used: ENTPDase2 (1:500), PLC- β 2 (1:1000), and NCAM (1:300). Dylight-649-conjugated donkey anti-rabbit and Dylight-488-conjugated donkey anti-goat secondary antibodies were used. For immunostaining of taste tissue sections from wild-type and TNF knockout mice, antibodies to KCNQ1 (1:1000), PLC- β 2 (1:1000), gustducin (1:1000), and carbonic anhydrase 4 (1:500) were used. Secondary antibodies were Dylight-649-conjugated donkey anti-goat and Dylight-488-conjugated donkey anti-rabbit antibodies. Fluorescent images were acquired using Leica Sp2 confocal microscope.

2.7 Statistical analysis

Data from nerve recording experiments and taste behavioral tests were first compiled using Microsoft Excel. For statistical analyses, repeated measures two-way ANOVA with *post hoc* t tests were performed using Statistica (Dell Software, Aliso Viejo, CA) or Statcel (OMS, Tokyo, Japan). *P*-values < 0.05 were considered significant.

3. Results

3.1 TNF-deficient mice display reduced behavioral responses to the bitter compound quinine.

Our previous studies have shown that TNF is expressed in a specific subset of taste bud cells (Feng et al., 2012; Kim et al., 2012), but its roles in the peripheral taste system remains unclear. To explore the function of TNF in the taste system, we investigated whether TNF deficiency affects taste responses. We first examined behavioral responses in TNF knockout mice and their wild-type controls by two-bottle preference tests and brief-access gustometer tests. In the 48 h two-bottle preference tests, mice were given free access to water and a taste solution. The volumes of the consumed water and taste solution were recorded and preference score for the taste solution was calculated. As shown in Fig. 1A, both wild-type and TNF knockout mice showed avoidance behavior towards increasing concentrations of quinine. However, statistical analyses showed that wild-type mice avoided all concentrations of quinine tested (0.003-0.3 mM), whereas TNF knockout mice only avoided 0.03-0.3 mM of quinine. This result suggests that TNF knockout mice have higher avoidance threshold to quinine than wild-type mice (0.03 mM vs. 0.003 mM). In addition, at 0.03 and 0.1 mM of quinine, TNF knockout mice displayed significantly reduced avoidance behavior (i.e. higher preference scores) compared to wild-type mice. These results suggest that TNF knockout mice are significantly less sensitive to the bitter compound quinine.

To further confirm that TNF knockout mice are less responsive to bitter compounds in behavioral tests, we also conducted brief-access gustometer tests. In contrast to two-bottle preference tests, gustometer tests reduce post-ingestive effects of taste compounds, because mice are only given brief access to taste solutions during the test sessions. As shown in Fig. 1B, wild-type mice significantly avoided all concentrations of quinine tested (0.03-3 mM), whereas TNF knockout mice only avoided 0.3-3 mM of quinine. Additionally, TNF knockout mice are significantly less responsive to quinine than wild-type mice at all three concentrations tested. These results suggest that the reduced responses to quinine in TNF knockout mice are likely due to defects in the taste sensory system. It should be pointed out that because mice were only allowed brief access to the taste solutions in brief-access tests, higher concentrations of taste compounds were needed to achieve the same degree of avoidance to that observed in 48 h two-bottle preference tests. Together, these behavioral experiments indicate that TNF knockout mice are less responsive to quinine due to reduced sensitivity to the bitter compound.

In contrast, TNF knockout mice and their wild-type controls had similar behavioral responses to sweet (saccharin, Fig. 1C), umami (IMP, Fig. 1D), sour (citric acid, Fig. 1E), and salty (sodium chloride, Fig. 1F) compounds in two-bottle preference tests.

3.2 TNF-deficient mice show decreased gustatory nerve responses to bitter compounds.

Our previous studies have shown that TNF is expressed in the taste buds of fungiform, foliate, and circumvallate papillae (Feng et al., 2012). However, TNF is also expressed in the brain and can act on various regions in the brain (Bernstein et al., 1991; Plata-Salaman, 1998).

To confirm that the reduced behavioral responses to quinine are due to defects in taste sensing and to investigate whether peripheral or central nervous system mechanisms are involved, we conducted gustatory nerve recording experiments. We examined electrophysiological responses from the chorda tympani nerve which innervates taste buds in fungiform papillae and the anterior part of foliate papillae (Mangold and Hill, 2007). Chorda tympani nerve recordings show that TNF-deficient mice are less responsive to bitter compounds (Fig. 2), consistent with behavioral test results (Fig. 1A, B). Significant differences between TNF knockout mice and wild-type controls were observed in nerve responses to all four bitter compounds tested, including quinine (Fig. 2A and E), denatonium benzoate (Fig. 2B and E), cycloheximide (Fig. 2C and E), and magnesium sulfate (Fig. 2D and E).

We did not observe any differences between TNF knockout mice and wild-type controls in chorda tympani responses to sucrose, a natural sugar, (Fig. S1A and E) and saccharin, an artificial sweetener (Fig. S1B and E). To test umami taste responses, we chose to use MPG instead of monosodium glutamate (MSG) as an umami stimulus to avoid the effect of sodium ion which activates salt taste signaling. IMP synergizes with MPG to boost umami signaling, especially at low concentrations of MPG (Fig. S1D). Again, we did not observe any differences in chorda tympani responses to MPG and MPG with IMP between TNF knockout and wild-type mice (Fig. S1C, D, and E). TNF knockout mice also responded normally to sour and salt taste stimuli in chorda tympani nerve recordings. The tested sour compounds included citric acid (Fig. S2A and E) and hydrochloric acid (Fig. S2B and E), representing organic and inorganic acids, respectively. For salt taste, current research suggests that it contains amiloride-insensitive and amiloride-sensitive components. The epithelial sodium channel (ENaC) is involved in amiloride-

sensitive salt taste reception, whereas the receptor for amiloride-insensitive salt taste remains unclear (Bachmanov et al., 2014; Bosak et al., 2010; Chandrashekar et al., 2010; Lindemann, 2001; Oka et al., 2013). TNF knockout mice showed normal chorda tympani responses to sodium chloride without (Fig. S2C and E) and with amiloride (Fig. S2D and E), suggesting that both amiloride-sensitive and amiloride-insensitive salt taste components are normal in TNF-deficient mice.

These nerve recording data are in complete agreement with behavioral test results (Fig. 1). These data show that TNF deficiency decreases peripheral taste responses to bitter compounds but does not affect peripheral responses to sweet, umami, sour, and salty compounds. Together, these results suggest that TNF is preferentially involved in the regulation of bitter taste.

3.3 TNF receptors are broadly expressed in taste bud cells

TNF signaling is mediated through two receptors, TNFR1 and TNFR2. These two receptors, especially TNFR1, are expressed in many types of tissues and play important biological functions (Cabal-Hierro and Lazo, 2012; Silke, 2011). We suspect that either or both of these receptors are expressed in the peripheral taste system and mediate the effects of TNF on taste. Thus, next we investigated the expression of TNFR1 and TNFR2 in taste tissues.

We performed mRNA *in situ* hybridization using sense and antisense probes to TNFR1 and TNFR2. As shown in Fig. 3A, the antisense probe to TNFR1 gave strong hybridization signals in the circumvallate epithelium, especially in taste buds (right panel), whereas sense

probes did not produce any specific signal (left panel). Antisense probes to TNFR2 also displayed robust hybridization signals in circumvallate taste buds (Fig. 4A, right panel), while sense probes to TNFR2 did not show specific hybridization signal (Fig. 4A, left panel). Some scattered cells in the connective tissues surrounding circumvallate epithelium also showed hybridization signals to antisense probes of TNFR1 and TNFR2 (Fig. 3A and 4A, right panels). These cells are likely resident immune cells in the taste papillae (Feng et al., 2010; Kim et al., 2012).

Next, we did immunohistochemical studies using antibodies against TNFR1 and TNFR2. Fig. 3B and 4B (left panels) show that the immunoreactivities to TNFR1 and TNFR2 are preferentially localized to taste buds in tongue tissue sections, consistent with *in situ* hybridization data (Fig. 3A and 4A). No specific signals were observed in control immunostainings in which the anti-TNF receptor antibodies were either preincubated with the blocking antigens (Fig. 3B and 4B, middle panels) or omitted from the staining procedure (Fig. 3B and 4B, right panels). Immunostaining experiments detected the expression of TNFR1 and TNFR2 in the taste buds of all lingual taste papillae, including circumvallate (Fig. 3B, D, E and 4B, D, E), foliate (Fig. 3C and data not show), and fungiform papillae (Fig. 4C and data not shown).

There are three major types of mature taste cells in taste buds (Murray, 1971). Each of them can be identified by specific molecular markers that are related to their biological functions. To investigate what types of taste bud cells express TNF receptors, we performed double-immunostaining using antibodies against TNFR1 or TNFR2 and antibodies against

various taste cell-type markers. As shown in Fig. 3C-E, TNFR1 is colocalized with ENTPDase2 (type I taste cell marker), PLC- β 2 (type II taste cell marker), and NCAM (type III taste cell marker), suggesting that TNFR1 is expressed in types I-III taste bud cells. TNFR1 immunoreactivities are also detected in some nerve fibers in close proximity to circumvallate taste buds, some of which are also detected by the anti-NCAM antibody (Fig. 3E, arrows). However, the identity of these nerve fibers is unclear. Double immunostaining experiments show that TNFR2 is also colocalized with ENTPDase2, PLC- β 2, and NCAM (Fig. 4C-E). Together, these results show that TNFR1 and TNFR2 are both broadly expressed in taste bud cells, including types I-III taste cells. This expression pattern of TNF receptors suggests that the effects of TNF on taste responses are likely conducted through TNFR1 and/or TNFR2 mediated signaling pathways in taste bud cells.

3.4 The gross structure of taste buds appears normal in TNF-deficient mice.

TNF plays important roles in the regulation of cell survival and death in various types of cells (Cannon, 2000; Gaur and Aggarwal, 2003). To investigate whether TNF deficiency affects taste bud structure, we performed immunostaining using a specific antibody to the voltage-gated potassium channel KCNQ1, a taste bud marker that is expressed in nearly all taste bud cells (Wang et al., 2009). Immunofluorescent staining of KCNQ1 shows general morphology of individual taste buds. As shown in Fig. 5A, taste buds from TNF knockout mice appear normal. We also performed immunostaining using antibodies to several taste-cell-type markers, including PLC- β 2 (a marker for all type II taste cells), gustducin (a marker that preferentially labels type II bitter cells in circumvallate and foliate taste buds), and carbonic anhydrase 4 (a marker for type III sour cells). These experiments show that type II cells labeled by antibodies to PLC- β 2 and

gustducin and type III cells labeled by the antibody to carbonic anhydrase 4 appear normal in TNF knockout mice (Fig. 5B-D).

4. Discussion

Many inflammatory cytokines are involved in the regulation of energy metabolism and food intake (Hotamisligil, 2006; Plata-Salman, 1998). During bacterial and viral infections, elevated levels of inflammatory cytokines lead to profound changes in physiology and related behaviors, including anorexia and reduced food intake (Dantzer, 2001; Dantzer and Kelley, 2007). Taste sensing is an important mechanism governing food preference and intake. However, few studies have investigated whether inflammatory cytokines are involved in the regulation of taste reception.

On the other hand, it is known that taste abnormalities can develop in various diseases with underlying inflammation, such as upper respiratory infections, periodontal infections, autoimmune diseases, and chronic inflammatory diseases (Bromley, 2000; Pribitkin et al., 2003; Schiffman, 1983). Our recent studies show that taste abnormalities also develop in LPS-induced inflammation model and in MRL/lpr mice with autoimmune disease (Cohn et al., 2010; Kim et al., 2012). Intriguingly, we found that several inflammation-associated cytokines are expressed in specific subsets of taste bud cells. TNF is preferentially produced by T1R3-positive sweet/umami cells (Feng et al., 2012). Interferon- γ is expressed in a subset of type II and III taste cells (Kim et al., 2012). The anti-inflammatory cytokine IL-10 is expressed in type II bitter cells (Feng et al., 2014a). Whether these cytokines are involved in taste sensing remains largely unknown.

In this study we investigate the roles of TNF in the peripheral taste system using TNF knockout mice. We performed taste behavioral tests and gustatory nerve recordings. In both sets of experiments we found that TNF-deficient mice are significantly less responsive to bitter compounds (Fig. 1 and 2). In contrast, TNF-deficient mice respond normally to sweet, umami, sour, and salty taste compounds in chorda tympani recordings and in two-bottle preference tests (Fig. 1, S1, and S2). Taste behavioral tests, especially long-term (e.g. 48 h) preference tests, may be confounded by post-ingestive effects of the stimuli. This effect can be reduced by using brief-access tests which quantifies immediate lick responses in much shorter testing sessions (e.g. 30 min) to extremely small volumes of taste solutions. In this study, we performed both types of behavioral tests. It should be pointed out that brief-access tests were performed with the same sets of mice used for two-bottle preference tests. It is possible that the results from brief-access tests might be influenced by prior exposure to taste compounds, although a 3-week interval between the two procedures was installed to minimize this effect. However, the consistent results from two-bottle preference tests, brief-access tests, and taste nerve recordings suggest that the reduced responses to bitter compounds in TNF-deficient mice are most likely due to defects in bitter taste sensing.

We then examined the expression of TNFR1 and TNFR2 in the peripheral taste tissues. mRNA *in situ* hybridization showed that both TNF receptors are preferentially expressed in taste bud cells (Fig. 3A and 4A). Immunostaining experiments confirmed the expression of these receptors in taste buds (Fig. 3B and 4B). Double immunostaining using antibodies to TNFR1 or TNFR2 and antibodies to taste-cell-type markers showed that the two TNF receptors are both

broadly expressed in taste bud cells. TNFR1 and TNFR2 are both colocalized with markers of types I-III taste cells (Fig. 3C-E and Fig. 4C-E). These results suggest that most, if not all, taste bud cells express TNFR1 and TNFR2 and thus are potential targets of TNF. Yet, based on gustatory nerve recordings and behavioral tests, TNF deficiency only significantly reduces the responses to bitter taste out of the five basic taste modalities (bitter, sweet, umami, sour and salt tastes). It is possible that bitter taste cells contain cell-type-specific factors that make the cells more sensitive to TNF actions under physiological conditions when relatively small amount of TNF is produced by T1R3-positive cells (Feng et al., 2012) (Fig. 6, solid arrows). This pattern of regulation may change under inflammatory conditions when the production of TNF in taste buds is induced (Cohn et al., 2010; Feng et al., 2012; Kim et al., 2012). Stronger TNF signaling may also affect other taste modalities (Fig. 6, dashed arrows). We propose that under physiological conditions TNF plays a role to sensitize bitter taste responses, while under inflammatory conditions, TNF may further augment taste responses in bitter cells and in addition affect the function of other types of taste bud cells (Fig. 6). The roles of TNFRs in taste buds deserve further investigation. It is likely that the effects of TNF in taste buds are mediated through TNFR1 and/or TNFR2. TNFR-deficient mice may have similar impairment in bitter taste responses. However, TNFR1 and TNFR2 are also involved in lymphotoxin (LT)- α signaling. We have found that LT- α is also expressed in the taste tissue (unpublished results). It is possible that TNFR1 and TNFR2 are not only involved in TNF signaling but also in LT- α signaling in taste buds. Therefore, it may also be possible that the phenotypes of TNFR(s) knockout mice are different from those of TNF knockout mice. Future experiments will investigate taste responses in TNFR1 and TNFR2 knockout mice.

It remains to be determined how TNF modulates bitter taste responses. TNF signaling pathways are complex, and depending on the cellular context and the microenvironment, TNF signaling may lead to distinct functional outcomes. In many types of cells, TNF is a regulator of cell survival and death (Cabal-Hierro and Lazo, 2012; Mathew et al., 2009). Our data suggest that the gross structure of taste buds appears normal in TNF-deficient mice (Fig. 5A). Immunostaining using antibodies to PLC- β 2 (all type II taste cells), gustducin (mostly bitter cells in circumvallate and foliate taste buds), and carbonic anhydrase 4 (type III sour cells) shows similar numbers of type II and III taste cells in TNF-deficient mice compared to wild-type control mice (Fig. 5B-D). These results suggest that the reduced responses to bitter compounds in TNF knockout mice is not likely due to a reduced number of bitter cells in taste buds. Another effect of TNF signaling is to regulate gene expression through activation of transcription factors such as NF- κ B and AP1 (Cabal-Hierro and Lazo, 2012). Currently, it is unclear whether any genes involved in bitter taste signaling are regulated by NF- κ B or AP1. TNF signaling can also affect cytoskeleton structure and cell barrier permeability (Mathew et al., 2009). Whether these cellular properties are regulated by TNF in bitter taste bud cells is unknown. Taste preference could also be influenced by other sensory systems such as the olfactory system and the visceral sensory system when animals ingest foods. TNF may play a role in olfactory impairment associated with rhinosinusitis (Lane et al., 2010). Whether TNF deficiency affects the olfactory system remains unknown. The subdiaphragmatic vagus nerve plays important roles in sensing visceral cues and regulating inflammation, including TNF production (Olofsson et al., 2012; Pavlov and Tracey, 2012). It would be interesting to investigate whether TNF could affect taste preference by altering olfactory, vagal, and/or other functionally related tissue systems. Future experiments will investigate these possibilities.

TNF is a potent inflammatory cytokine and an important marker for inflammation. Its level is induced during acute infections and in various chronic diseases. Our results suggest that TNF plays a role to sensitize bitter taste responses. It is conceivable that elevated levels of TNF during inflammation contribute to heightened or persistent bitter taste sensation in some patients with taste abnormalities.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. TNF-deficient mice show significantly decreased behavioral responses to the bitter compound quinine. Both two-bottle preference tests (A) and brief-access gustometer tests (B) show that TNF-deficient mice (TNF KO) display significantly weaker aversive responses to the bitter compound quinine hydrochloride than wild-type mice. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ (significant difference between preference score or lick ratio of quinine solutions and that of water within each group of mice). * $p < 0.05$; ** $p < 0.01$ (significant difference between preference score or lick ratio of quinine solutions of TNF knockout mice and that of wild-type mice). TNF-deficient mice show normal behavioral responses to sweet (C), umami (D), sour (E), and salt (F) taste compounds in two-bottle preference tests. Preference scores in two-bottle preference tests were calculated by dividing the volume of consumed taste solution by the total volume of fluid intake (i.e. preference score = volume of consumed taste solution / (intake of taste solution + intake of water)). Lick ratios in gustometer tests were calculated by dividing the number of licks of a taste solution by the number of licks of water in each test session. Data are mean $\pm$ SEM. Data were analyzed with two-way ANOVA with post hoc t tests. 8-16 mice were included in each group.

Fig. 2. TNF-deficient mice show significantly decreased gustatory nerve responses to bitter compounds. (A-D) Average chorda tympani nerve responses to bitter compounds quinine hydrochloride (A), denatonium benzoate (B), cycloheximide (C), and MgSO_4 (D). Nerve responses to taste compounds were normalized against responses to 0.1 M NH_4Cl . Data are mean $\pm$ SEM. Data were analyzed with two-way ANOVA with *post hoc* t tests. * $p < 0.05$; ** $p < 0.01$ (significant difference between TNF knockout mice and wild-type mice). Eight wild-type and

seven TNF-deficient (TNF KO) mice were included in the experiment. (E) Representative chorda tympani nerve responses to taste compounds. Nerve responses to 0.1 M NH₄Cl are shown as reference. QHCl, quinine hydrochloride; DB, denatonium benzoate; Cyc, cycloheximide.

Fig. 3. TNFR1 expression in taste tissues. (A) mRNA *in situ* hybridization using sense and antisense probes to TNFR1. Taste tissue sections containing circumvallate taste buds are shown. Strong hybridization signals were observed in taste buds when antisense probes to TNFR1 were used (right panel). No specific signals were observed when sense probes to TNFR1 were used (left panel). Scale bars, 50 μ m. (B) Confocal images of immunofluorescent staining using a purified antibody against TNFR1 (left panel) or control immunofluorescent staining in which the antibody against TNFR1 was preincubated with its blocking peptide (middle panel) or was omitted from the immunostaining procedure (right panel). Tissue sections containing circumvallate taste buds are shown. Immunoreactivities to TNFR1 were observed in taste buds (dashed circles, left panel). No positive signals were observed in control staining (middle and right panels). Scale bars, 35 μ m. (C-E) Confocal images of double immunofluorescent staining using antibodies against TNFR1 (left panels) and taste-cell-type markers (middle panels). Merged images are shown in right panels. Tissue sections containing foliate (C) or circumvallate (D, E) taste buds are shown. TNFR1 show colocalization with ENTPDase2, PLC- β 2, and NCAM. Arrows in E indicate nerve fibers. All tissue sections were from wild-type mice. Scale bars, 35 μ m.

Fig. 4. TNFR2 expression in taste tissues. (A) mRNA *in situ* hybridization using sense and antisense probes to TNFR2. Taste tissue sections containing circumvallate taste buds are shown.

Strong hybridization signals were observed in taste buds when antisense probes to TNFR2 were used (right panel). No specific signals were observed when sense probes to TNFR2 were used (left panel). Scale bars, 50 μ m. (B) Confocal images of immunofluorescent staining using a purified antibody against TNFR2 (left panel) or control immunofluorescent staining in which the antibody against TNFR2 was preincubated with its blocking antigen (middle panel) or was omitted from the immunostaining procedure (right panel). Tissue sections containing circumvallate taste buds are shown. Immunoreactivities to TNFR2 were observed in taste buds (dashed circles, left panel). No positive signals were observed in control staining (middle and right panels). Scale bars, 35 μ m. (C-E) Confocal images of double immunofluorescent staining using antibodies against TNFR2 (left panels) and taste-cell-type markers (middle panels). Merged images are shown in right panels. Tissue sections containing fungiform (C) or circumvallate (D, E) taste buds are shown. TNFR2 show colocalization with ENTPDase2, PLC- β 2, and NCAM. All tissue sections were from wild-type mice. Scale bars, 35 μ m.

Fig. 5. The gross structure of taste buds appears normal in TNF-deficient mice. Confocal images of immunofluorescent staining using antibodies against taste bud cell markers on tissue sections from wild-type and TNF knockout (TNF KO) mice. Purified goat polyclonal antibodies against KCNQ1 and carbonic anhydrase 4 (CA4) were paired with a Dylight-649-conjugated donkey anti-goat secondary antibody (A, D). Purified rabbit polyclonal antibodies against PLC- β 2 and gustducin (Gust) were paired with a Dylight-488-conjugated donkey anti-rabbit secondary antibody (B, C). Scale bars, 40 μ m.

Fig. 6. TNF signaling in taste buds. TNF receptors are expressed in most taste bud cells, including types I-III taste cells. TNF, on the other hand, is produced predominantly by T1R3-positive sweet and umami cells. Our results suggest that under physiological conditions, TNF preferentially sensitize bitter taste responses (solid arrows). TNF-deficiency results in decreased neural and behavioral responses to bitter compounds. Under inflammatory conditions, when the production of TNF increases in taste buds, TNF may affect other types of taste cells in addition to its effect on bitter cells (dashed arrows).

Fig. S1. TNF-deficient mice show normal gustatory nerve responses to sweet and umami compounds. (A-D) Average chorda tympani nerve responses to sweet compounds sucrose (A) and saccharin (B) and umami taste compound MPG without or with 0.5 mM IMP (C, D). Nerve responses to taste compounds were normalized against responses to 0.1 M NH₄Cl. Data are mean $\pm$ SEM. Data were analyzed with two-way ANOVA with *post hoc* t tests. Eight wild-type and seven TNF-deficient (TNF KO) mice were included in the experiment. (E) Representative chorda tympani nerve responses to taste compounds. Nerve responses to 0.1 M NH₄Cl are shown as reference. Suc, sucrose; Sac, saccharin.

Fig. S2. TNF-deficient mice show normal gustatory nerve responses to sour and salt taste compounds. (A-D) Average chorda tympani nerve responses to sour taste compounds citric acid (A) and hydrochloric acid (B) and salt taste compound sodium chloride without or with 100 μ M amiloride (C, D). Nerve responses to taste compounds were normalized against responses to 0.1 M NH₄Cl. Data are mean $\pm$ SEM. Data were analyzed with two-way ANOVA with *post hoc* t

tests. (E) Representative chorda tympani nerve responses to taste compounds. Nerve responses to 0.1 M NH_4Cl are shown as reference. Ami, amiloride.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Cell Type	Type I	Type II		Type III
Function	Supporting	Sweet/ Umami	Bitter	Sour/ Salty
Markers	ENTPDase2	PLC- β 2/T1R3	PLC- β 2/T2Rs	NCAM
TNF	-	+	-	-
TNFR	+	+	+	+