Bitter taste receptors stimulate phagocytosis in human macrophages through calcium, nitric oxide, and cyclic-GMP signaling

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Abstract

Bitter taste receptors (T2Rs) are GPCRs involved in detection of bitter compounds by type 2 taste cells of the tongue, but are also expressed in other tissues throughout the body, including the airways, gastrointestinal tract, and brain. These T2Rs can be activated by several bacterial products and regulate innate immune responses in several cell types. Expression of T2Rs has been demonstrated in immune cells like neutrophils; however, the molecular details of their signaling are unknown. We examined mechanisms of T2R signaling in primary human monocyte-derived unprimed (M0) macrophages (MΦs) using live cell imaging techniques. Known bitter compounds and bacterial T2R agonists activated low-level calcium signals through a pertussis toxin (PTX)-sensitive, phospholipase C-dependent, and inositol trisphosphate receptor-dependent calcium release pathway. These calcium signals activated low-level nitric oxide (NO) production via endothelial and neuronal NO synthase (NOS) isoforms. NO production increased cellular cGMP and enhanced acute phagocytosis ~3-fold over 30-60 min via protein kinase G. In parallel with calcium elevation, T2R activation lowered cAMP, also through a PTX-sensitive pathway. The cAMP decrease also contributed to enhanced phagocytosis. Moreover, a co-culture model with airway epithelial cells demonstrated that NO produced by epithelial cells can also acutely enhance MΦ phagocytosis. Together, these data define MΦ T2R signal transduction and support an immune recognition role for T2Rs in MΦ cell physiology.

Keywords: live cell imaging, innate immunity, quorum sensing, airway epithelium, G-protein-coupled receptors

Introduction

Taste family 2 receptors (T2Rs) are GPCRs involved in bitter taste perception on the tongue [1, 2]. However, T2Rs are expressed throughout the body, including the nose and sinuses [3, 4] and lung [5]. T2Rs canonically decrease cAMP via $G\alpha_{\text{gust}}$ or $G\alpha_i$ [6, 7] and $G\beta\gamma$ activation of phospholipase C (PLC), production of IP_3 , and elevation of calcium (Fig 1B) [3]. There are 25 T2Rs on the human tongue [1]. Nasal ciliated epithelial cells express T2Rs 4, 14, 16, 38, and possibly others. In addition to their canonical role in taste, mounting evidence suggests T2Rs are *bona fide* immune recognition receptors for chemosensation of bacteria. Cilia T2Rs detect bacterial products and activate local defense responses within seconds [7, 8], including Ca^{2+} -dependent nitric oxide (NO) production that increases ciliary beating and has direct antibacterial effects [8, 9]. T2R38, T2R10, and T2R14 respond to bacterial acyl-homoserine lactone (AHL) quorum-sensing molecules [9-11]. T2Rs 4, 16, and 38 respond to *Pseudomonas* quinolone signal (PQS), and T2R14 responds to heptyl-hydroxyquinolone (HHQ) secreted by *Pseudomonas* [7].

Clinical importance of extraoral T2Rs in immunity is supported by correlation of polymorphisms rendering T2R38 non-functional with susceptibility to chronic rhinosinusitis CRS [9, 12-17]. A high number of T2R polymorphisms exist, contributing to individual taste preferences, but possibly also contributing to susceptibility to infection. It is critical to elucidate mechanisms of extraoral chemosensation by T2Rs to understand if and how to target T2Rs to activate innate immune responses.

Dedicated immune cells also express T2Rs [18-21]. T2R38 detects AHLs in neutrophils [18, 19] and is expressed in both resting and activated lymphocytes [20]. Circulating human monocytes, natural killer (NK) cells, B cells, T cells, and polymorphonuclear (PMN) leukocytes also express T2Rs [21]. Stimulation of PMNs with saccharin, which activates both T2Rs and T1R2/3 sweet receptors, enhanced leukocyte migration [21]. However, our understanding of the details of T2R signaling and physiological consequences in immune cells are limited.

Our goal was to determine if T2Rs in primary human macrophages (MΦs) are coupled to calcium and if this activates NO as in airway epithelial cells. MΦs are important players in early innate immune responses, and unprimed (M0) MΦs express Ca^{2+} -activated endothelial (e) and/or neuronal (n) nitric oxide synthase (NOS) isoforms, though studies of e/nNOS in MΦ function are limited. The majority of studies of NO in MΦs focuses on higher level NO production via upregulation of inducible iNOS in LPS ± $IFN\gamma$ -activated (classically-activated or M1) MΦs and the relationship of the NO to bacterial killing or metabolism [22]. However, iNOS is not expressed or is expressed at very low levels in monocytes and unstimulated M0 MΦs [22], while eNOS and/or nNOS are expressed [23-28] and can be activated by calcium [23, 26, 28].

Although relatively few papers examine e/nNOS activation in MΦ function, evidence suggests a potential role for NO in phagocytosis. Stimulation of $Fc\gamma$ receptor ($Fc\gamma R$, not a GPCR) in M0 human MΦs activates both eNOS and nNOS to enhance phagocytosis [23], and the NO produced upregulates nNOS expression [23]. $IFN\gamma$ also up-regulates phagocytosis in an NO-dependent manner in mouse MΦs [29]. Thus,

we hypothesized that T2R activation of eNOS or nNOS may play a role in very early responses of MΦs encountering secreted “bitter” bacterial metabolites.

Results

MΦs express functional T2Rs tied to calcium signaling

Human monocytes obtained from healthy apheresis donors were differentiated to MΦs by adherence culture for 12 days, confirmed by functional expression of H1 receptors (**Supplementary Fig. 1**). We observed expression of several T2Rs in unprimed (M0) MΦs. Immunofluorescence using previously validated antibodies [7, 8] directed against T2R4 (**Fig. 1a**) and T2R46 (**Fig. 1b**), but not T2R16 (**Fig. 1c**), exhibited plasma membrane staining similar to GLUT1, a major MΦ glucose transporter [30]. Reverse transcription (rt) PCR for T2Rs 4, 14, 38, and 46 demonstrated expression in MΦs from 3 different individuals (**Fig. 1d**).

We tested a variety of T2R agonists (listed in **Supplementary Table 1**) using live cell calcium imaging. We observed low-level but sustained calcium responses to denatonium benzoate, which activates eight T2Rs including T2R4 and T2R46 (**Fig. 1e**) [31, 32], and to quinine, which activates 11 T2Rs including T2R14 and 46 (**Fig. 1f**) [31, 32]. Bacterial T2R agonists PQS and HHQ also increased calcium (**Fig. 1g-h**). A panel of bitterants revealed responses to T2R14 agonists thujone and flufenamic acid (FFA), T2R38 agonist phenylthiocarbamide (PTC), T2R14/38 agonist 3-oxo-dodecanoylhomoserine lactone (3oxoC12HSL), and T2R14/46 agonist parthenolide [31, 32]. Peak responses are summarized in **Fig. 1i** and representative traces in **Supplementary Fig. 2**. No response was observed with T2R16 agonist salicin, fitting with observed lack of expression (**Fig. 1c**). Stimulation of $G\alpha_q$ -coupled purinergic receptors with ATP was a positive control.

The responses to PTC were blocked by pertussis toxin (PTX; **Fig. 1i**), which ADP ribosylates and inactivates both $G\alpha_{\text{gust}}$ and $G\alpha_i$. Calcium responses to FFA and HHQ were inhibited by PTX and PLC inhibitor U73122, which inhibits T2R responses in airway cells [7, 8], $G\beta\gamma$ inhibitor gallein, which inhibits T2R responses in airway smooth muscle [33], and inositol trisphosphate receptor (IP_3R) inhibitor xestospongine C (**Fig. 1j**). Moreover, responses to thujone, HHQ, or FFA were eliminated after intracellular calcium store depletion (thapsigargin in calcium-free solution) (**Fig. 1j**). Thus, T2R agonists activate calcium release from intracellular stores through GPCR signaling. Representative traces from experiments in **Fig. 1i-j** are in **Supplementary Fig. 2** and **3**. We found no differences in responses in MΦs differentiated by adherence + cytokine M-CSF (**Supplementary Fig. 4**).

To more concretely tie these results specifically to T2Rs, we used known T2R inhibitors; 4'-fluoro-6-methoxyflavanone is a T2R14 and T2R39 antagonist [34], while probenecid inhibits T2R16 and T2R38 [35]. Calcium responses to apigenin, which activates T2R14 and T2R39, were inhibited after pretreatment with 4'-fluoro-6-methoxyflavanone (**Fig. 1k**). Calcium responses to PTC were inhibited after pretreatment with probenecid (**Fig. 1l**). Representative experiments are in **Supplementary Fig. 5**. Knockdown of T2R14 with pooled siRNAs inhibited responses to T2R14 agonists niflumic acid (NFA; [31, 32]; **Fig. 1m**) and HHQ (**Fig. 1m**).

MΦ T2R activation reduces baseline and stimulated cAMP signaling

As described above, T2R-stimulated G_{α_i} or $G_{\alpha_{gust}}$ can reduce cAMP through inhibition of adenylyl cyclase or activation of phosphodiesterase, respectively (**Fig. 2a**). Therefore, we also tested if bitter agonist stimulation reduced cAMP in MΦs. We utilized an mNeonGreen-based cAMP biosensor (cADDiS [36]) in a baculovirus modified for mammalian cells (BacMam) to monitor cAMP in real time (**Fig. 2b**). Stimulation with β -adrenergic agonist isoproterenol increased cAMP (**Fig. 2c**), whereas stimulation with 3oxoC12HSL, FFA, or PQS reduced cAMP (**Fig. 2d**). The cAMP reductions were eliminated by PTX (**Fig. 2d**). Moreover, cAMP increases with isoproterenol were reduced in the presence of PQS or 3oxoC12HSL (**Fig. 2e**); this was likewise eliminated by PTX (**Fig. 2f**). We confirmed these results using another cAMP biosensor, the mTurquoise2-Venus FRET-based EPAC-S^{H187} [37]. MΦs expressing EPAC-S^{H187} exhibited PTX-sensitive cAMP decreases during stimulation with quinine, FFA, or 3oxoC12HSL (**Fig. 2g**).

Data demonstrate that T2Rs affect both basal and stimulated cAMP levels in MΦs. Using a ratiometric Cerulean-Venus FRET-based PKA biosensor, AKAR4 [38], we observed that PKA activity was also reduced during stimulation with PQS or FFA, and this was eliminated by PTX (**Fig. 2h**). Together, these data indicate that both arms of canonical T2R signaling appear to be active in MΦs when stimulated with bitter compounds.

MΦ T2R calcium responses drive NO production

These T2R-activated low-level calcium responses were reminiscent of airway cells [3, 7, 8]. We tested if NO production was also downstream. M0 MΦs express calcium-sensitive eNOS and/or nNOS [23-28]. We confirmed eNOS transcript by rtPCR (**Fig. 3a**) and detected eNOS and nNOS by Western (**Fig. 3b**). We used the fluorescent indicator DAF-FM to monitor production of reactive nitrogen species (RNS) in living MΦs in real time. DAF-FM terminally reacts with NO and RNS derivatives, increasing fluorescence. Non-specific NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) was used as a positive control.

Several T2R agonists, but not T2R16 agonist salicin, caused increases in DAF-FM fluorescence (**Fig. 3b**) that were blocked by PTX (**Fig. 3c**) or U73122 (**Fig. 3d**), suggesting they are downstream of T2R calcium signaling. DAF-FM fluorescence increases in response to T2R stimulation, but not SNAP, were blocked by pretreatment with NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME), but not inactive D-NAME, and were reduced in the presence of NO scavenger carboxy-PTIO (cPTIO; **Fig. 3e**). DAF-FM results were confirmed by measuring NO decomposition products NO₂⁻ and NO₃⁻ in culture media (**Supplementary Fig. 6**). Thus, DAF-FM fluorescence increases reflect NOS activation.

MΦ T2R signaling increases cGMP

To test the hypothesis that NO production would increase cGMP, we utilized a fluorescent cGMP biosensor (Green GENie). We observed increases in cGMP production with denatonium benzoate or parthenolide (**Fig. 4a**), which activate calcium and NO responses, but not with salicin or sodium benzoate, which do not activate calcium or NO. The cGMP responses to FFA or PQS were blocked by L-NAME or

cPTIO (**Fig. 4b**), PTX (**Fig. 4c**), or elimination of calcium (**Fig. 4d**). The cGMP increase was enhanced when MΦs were co-infected with BacMam to over express soluble guanylyl cyclase (sGC), further tying these responses to NO-induced cGMP.

We used siRNA to confirm involvement of T2Rs. MΦs treated with control siRNA exhibited cGMP responses to HHQ, PQS, thujone, and niflumic acid (NFA; **Fig. 4f**). Treatment with a cocktail of eNOS and nNOS siRNA blocked the response to each agonist. T2R14 siRNA, but not T2R10 siRNA significantly reduced the cGMP responses to T2R14 agonists HHQ and NFA, but the response to PQS, which does not activate T2R14 [7], remained intact (**Fig. 4f**). The response to thujone, which activates both T2R14 and T2R10 [31, 32], was slightly inhibited by either T2R14 or T2R10 siRNAs, and more fully inhibited with a cocktail of both siRNAs (**Fig. 4f**).

MΦ T2R activation may alter MΦ metabolism through combined elevations of calcium and NO.

We briefly examined if T2R stimulation had acute effects on MΦ metabolic state, tied to MΦ activation [39], by live-cell imaging of NAD(P)H autofluorescence [40]. Ultraviolet autofluorescence increased with bitter stimulation in a PTX- and L-NAME-sensitive manner (**Supplementary Fig. 7**), suggesting an increase in NAD(P)H levels. Increases in autofluorescence were not observed with either thapsigargin or SNAP separately to non-specifically increase Ca^{2+} or NO, respectively. However, addition of thapsigargin plus SNAP robustly increased NAD(P)H autofluorescence, suggesting this is an effect of raising both Ca^{2+} and NO simultaneously (**Supplementary Fig. 7**). Together, data suggest that T2R stimulation may acutely alter MΦ metabolism, at least short term, supporting a role for T2Rs in activation of MΦ immune responses.

MΦ T2R-driven NO signaling enhances phagocytosis

Activation of T2Rs in MΦs increases intracellular calcium to activate e/nNOS and produce NO and cGMP. What consequences does this have for MΦ cell physiology? As mentioned above, NO was implicated in regulation of phagocytosis. We tested if T2R stimulation increased acute phagocytosis of FITC-labeled *E. coli* bioparticles. Incubation of MΦs with FITC-*E. coli* in the presence of T2R bitter agonists that activated calcium and NO also increased phagocytosis by ~300% (**Fig. 5a-c**). Responses to FFA were inhibited by L-NAME, cPTIO, PKG inhibitor KT5823, or PTX (**Figure 5C**). Responses to denatonium benzoate were inhibited by PLC inhibitor U73122 or $\text{G}\beta\gamma$ inhibitor gallein (**Fig. 5d**). A cocktail of pooled T2R14 siRNA inhibited responses to NFA or HHQ but not denatonium benzoate, which does not activate T2R14 (**Fig. 5e**). Responses to all three agonists were reduced with e/nNOS siRNAs (**Fig. 5e**).

We confirmed that we were observing phagocytosis using *Staphylococcus aureus* bioparticles labeled with pHrodo, a dye that fluoresces at the low pHs existing in lysosomes and phagosomes (**Fig. 6a**). MΦs phagocytosed pHrodo *S. aureus*, evidenced by increased fluorescence, when incubated at 37 °C but not 4 °C; this was enhanced by denatonium benzoate but not sodium benzoate (**Fig. 6b and d**). Similar results were

observed with FFA (**Fig. 6c and e**). Increased phagocytosis with FFA was inhibited by L-NAME, cPTIO (**Fig. 6c and e**), U73122, or PTX (**Fig. 6f**). Increased phagocytosis with denatonium benzoate or quinine was also inhibited by PKG inhibitor KT5823 but not PKA inhibitor H89 (**Fig. 6g**).

Others have suggested that cAMP inhibits MΦ phagocytosis or activation [41-44]. We hypothesized that T2R-mediated cAMP decreases also facilitate phagocytosis. Baseline phagocytosis was inhibited by direct adenylyl cyclase activation by forskolin or cell permeant cAMP analogs, including 8-Br-cAMP and 8-CPT-cAMP, which activate both PKA and EPAC, and to a lesser extent by PKA-specific 6-Bnz-cAMP or EPAC-specific 8-CPT-2-O-Me-cAMP (**Fig. 6h**). In contrast, cell permeant cGMP analog 8-Br-cGMP increased phagocytosis via PKG; this was inhibited by KT5823. Thus, increased cAMP likely reduces phagocytosis partially through PKA and partially through EPAC, unlike cGMP which increases phagocytosis through PKG. Denatonium benzoate-induced phagocytosis was inhibited by forskolin or permeant cAMP analogs used above, but were not inhibited by 8-Br-cGMP (**Fig. 6i**). Together, these data suggest both the calcium/NO and cAMP arms of the T2R pathway facilitate MΦ phagocytosis.

NO crosstalk from airway epithelial cells may also enhance MΦ phagocytosis

Airway epithelial cells also make NO in response to various stimuli including T2R [3, 7] and estrogen receptor activation [45]. We hypothesized that MΦs close to the airway surface may be influenced by local intercellular NO production. We designed an assay to test epithelial-MΦ crosstalk using PTX-treated MΦs and H441 small airway epithelial cells, which produce NO in response to 17β-estradiol (E2) (**Supplementary Fig. 8**). Although MΦs exhibited low-level calcium transients to 10 nM E2, these were eliminated by PTX pretreatment (**Supplementary Fig. 9**).

DAF-FM-loaded, PTX-treated MΦs were incubated with unloaded H441s separated by a transwell filter in close proximity (≤ 1 mm; **Fig. 7a**). Stimulation of H441 cells with E2 resulted in increased MΦ DAF-FM fluorescence (**Fig. 7b**), suggesting NO produced by the H441s translated to an increase in RNS within the MΦs. Thus, epithelial NO/RNS could act as an intercellular signal. Inclusion of cPTIO or pretreatment of H441 cells with PTX or L-NAME blocked the MΦ DAF-FM response (**Fig. 7c**), but when MΦs were pretreated with L-NAME, there was no inhibition (**Fig. 7c**). Thus, the NO/RNS originated in the H441 cells. E2 had no effect when MΦs were incubated in the absence of H441s (**Fig. 7c**). Together, these data support the potential of airway epithelial cells to produce enough NO to be sensed by MΦs in close proximity.

To test if airway epithelial NO production could act as a signal to stimulate MΦ phagocytosis, we measured changes in pHrodo *S. aureus* phagocytosis by PTX-treated MΦs when H441s were stimulated with E2 (**Fig. 7d**). E2 stimulation of H441s increased MΦ phagocytosis (**Fig. 7e and f**), and this was blocked by H441 pretreatment with PTX or L-NAME or inclusion of cPTIO in the media (**Fig. 7f**). E2 had no effect when PTX-treated MΦs were incubated in the absence of H441 cells (**Fig. 7f**).

Discussion

We only understand the “tip of the iceberg” of extraoral T2Rs and their signaling. We demonstrated that T2Rs in MΦs may enhance phagocytosis through calcium-driven NO-activation of guanylyl cyclase to increase cGMP (**Fig. 8**). Interestingly, this is a similar pathway to airway epithelial cells [3, 7-9, 46], but the physiological output is different. In ciliated cells, the pathway controls cilia beating; in MΦs, it regulates phagocytosis. Both processes are critical for innate defense, supporting a role for T2Rs as immune receptors. In MΦs encountering bitter bacterial agonists at sites of infection, activated T2Rs may “rev up” acute phagocytic activity while likely simultaneous toll-like receptor (TLR) stimulation up-regulates expression of iNOS and antimicrobial proteins such as lysozyme to further combat infection. Of note, T2Rs also respond to a variety of natural plant compounds associated with complementary medicines, including flavonoids [8, 47-49], as well as common clinical drugs [50, 51]. Activation of extraoral T2Rs in MΦs may underlie some effects of homeopathic plant-based treatments or off-target drug effects.

This study also suggests that NO produced by airway epithelial cells could increase MΦ phagocytosis in a paracrine fashion (**Fig. 8**). NO produced during activation of T2Rs in airway epithelial cells [3, 7] may influence immune cells or amplify immune responses. NO may regulate phagocytosis via several mechanisms, including ADP-ribosylation of actin to regulate cytoskeletal polymerization, and pseudopodia formation, and phagocytosis in mouse peritoneal MΦs [52]. In Raw 264.7 mouse MΦ-like cells, NO acutely regulates actin organization through cGMP in combination with Ca^{2+} /calmodulin [53], and cGMP/PKG may be important in phagocytic responses during TLR2 stimulation in THP-1 monocytic leukemia cells [54].

It is important to better understand e/nNOS and cGMP signaling in MΦs beyond phagocytosis. Production of cGMP was linked to protection against toxicity of peroxynitrite (ONOO-) or oxidized LDL [55], reduction of cytokine release during LPS stimulation [56] or high fat exposure [57, 58], and promotion of wound healing [59]. NO itself may be anti-inflammatory by activation of PPAR γ , antagonizing NOX2 production of reactive oxygen species [60], or attenuation of NF- κ B and activation of Nrf2 transcription factors [61]. However, MΦs from eNOS knockout mice exhibit reduced NO production, NF- κ B activation, and iNOS upregulation during LPS stimulation [28].

Interestingly, T2R-induced lowering of cAMP levels may be another mechanism to enhance phagocytosis; cAMP elevation through prostaglandins decreases phagocytosis in human MΦs [41] and other inhibitory effects of cAMP have been reported [42-44]. Canonical T2R signaling is driven by Ca^{2+} downstream of G $\beta\gamma$ activation of PLC. The decrease in cAMP by G_{gust} was proposed to accentuate calcium signaling via type III IP₃Rs in taste cells [62, 63], but data supporting this mechanism are limited. Increased cAMP and PKA phosphorylation of IP₃R is more often reported to enhance calcium signaling [64], though PKA phosphorylation of type III IP₃R has been suggested to slow kinetics of calcium release in pancreatic acinar cells [65]. If taste cell signaling is primarily driven by calcium [66], why have T2Rs not evolved to couple to G_q instead of G_{gust} or

G_i? This may be because, as observed here, decreases in cAMP have important biological effects in extraoral tissues.

Materials and Methods

Reagents and solutions

Unless indicated, all reagents and solutions were as described [7, 8, 67, 68]. Full details of materials, reagents, and solution compositions are in the **Supplementary Material**. Bitter agonists used and known cognate T2Rs are in **Supplementary Table 1**. Immunofluorescence (IF) microscopy was carried out as described [7, 8, 67, 68], with more details in the **Supplementary Material**.

MΦ culture

Monocytes were isolated by the University of Pennsylvania Human Immunology Core from healthy apheresis donors with institutional review board approval by RosetteSep™ human monocyte enrichment cocktail (Stem Cell Technologies, Vancouver, Canada), and MΦs were differentiated by 12-days adherence culture in RPMI 1640 media containing 10% human serum and 1x cell culture pen/strep antibiotic mix (Gibco). All cells were deidentified before receipt. No investigator had contact with personal identifying information or demographics. Between 5-15 million cells were obtained from each individual. Cells from 29 individuals were used; some individuals donated multiple times during the course of the study. Details of siRNA protocols are in the **Supplementary Material**.

Live cell imaging of intracellular calcium, reactive nitrogen species (RNS) production, and cAMP/cGMP signaling

Intracellular calcium and RNS were imaged using Fura-2 or Fluo-4 and DAF-FM, respectively, as described [7, 8, 67]. MΦs were infected with BacMams containing green downward cADDis or green downward GENie (both from Montana Molecular, Bozeman MT). For AKAR4 or Epac-S^{H187}, MΦs on 8-well chambered coverglasses were transfected with 0.5 µg/well plasmid using Effectene (MilliporeSigma, Burlington, MA) per the manufacturer's protocol. Full details are in the **Supplementary Material**.

Phagocytosis assays

For microscopy, MΦs on chambered coverglasses were incubated with heat-killed FITC-labeled *E. coli* bioparticles at 250 µg/ml (strain K-12; Vybrant phagocytosis assay kit; ThermoFisher Scientific) in phenol red-free, low glucose DMEM ± bitter agonists or inhibitors for 15 min at 37°C. For microscopy, extracellular FITC was quenched with trypan blue and cells were washed ≥5x in PBS to remove residual extracellular FITC-*E. coli*. Remaining adherent MΦs were fixed in 4% formaldehyde (Electron Microscopy Sciences) for 10 min followed by DAPI staining. For pHrodo Red-labeled *S. aureus* (strain Wood 46; ThermoFisher A10010), living MΦs on chambered coverglass were visualized by fluorescence microscopy at room temperature (which drastically reduces any further phagocytosis) immediately after the assay incubation at 37°C.

For plate reader assays, MΦs on microplates were incubated similarly as above with 500 µg/ml FITC-*E. coli*. As phagocytosis was eliminated at 4 °C and almost completely eliminated at room temperature, we recorded fluorescence from living cells at room temperature immediately after the 15 min incubation with FITC-*E. coli*. Extracellular FITC was quenched with trypan blue, and fluorescence recorded on a Spark 10M plate reader (Tecan; 485 ex, 535 em). Phagocytosis assays were carried out similarly using 125 µg/ml pHrodo-*S. aureus* for 30 min at 37°C.

Airway epithelial cell culture and coculture

H441 cells (ATCC HTB-174) were cultured in MEM with Earle's salts (Gibco) containing 10% FetalPlex (Gemini Biosciences) in T75 flasks, lifted with trypsin when 75% confluent and transferred to 24 well plates and grown to confluence for MΦs co-culture experiments or seeded onto glass chamber slides and imaged for acute calcium and NO measurements.

For phagocytosis and NO co-culture, MΦs were seeded separately on transparent Falcon filters (#353095; 0.3 cm²; 0.4 µm pores), which come very close (0.8 mm) to the bottom of standard 24 well plates. Filters containing 100 µL HBSS on the apical side were transferred into the 24 well plates containing H441 cells and a small amount of HEPES-buffered HBSS (200 µL) at the time of the assay. MΦs were pretreated with PTX (100 ng/ml) for ~18 hrs in media (37°C, 5% CO₂), followed by copious washing with HBSS to remove residual PTX before co-incubation with H441s. Similarly, DAF-FM loading of MΦs was performed prior to co-incubation with H441s. Addition of fluorescently-labeled bacteria to MΦs on transwells was done at the time of assay start. Immediately after, 100 µL of a 3x E2 solution in HBSS was added to the basolateral side to stimulate H441 NO production and the 24 well plate was then incubated at 37°C.

Data analysis and statistics

Statistical significance was determined in GraphPad Prism as indicated in figure legends; $p < 0.05$ was considered statistically significant. Other analyses were performed in Excel. Data are presented as mean $\pm$ SEM. Data points in bar graphs are ≥ 3 independent experiments using cells from ≥ 2 -3 donors.

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Figures

Fig. 1 Unprimed (M0) monocyte-derived MΦs express functional bitter taste receptors (T2Rs). **a-c** Plasma membrane staining observed for T2R4 (**a**) and T2R46 (**b**) reminiscent of plasma membrane-localized glucose transporter GLUT1 (**c**). No immunofluorescence was observed with secondary only control or T2R16 antibody (**c**). **d** T2Rs detected in MΦs by rtPCR. Airway lines were used as control. **e-h** Representative low-level Ca²⁺ responses (fura-2) observed in response to denatonium benzoate (**e**), quinine (**f**), HHQ (**g**), and PQS (**h**). Subsequent stimulation with purinergic receptor agonist ATP used as control for viability. **i** Peak change in Ca²⁺ (fura-2 340/380 ratio) during 5 min stimulation with water soluble, DMSO-soluble, and liquid bitter compounds. ATP response shown for comparison. Also shown is inhibition of responses to PTC and 3oxoC12HSL by pertussis toxin (PTX; 100 ng/ml, 18 hrs pretreatment). Significance by one-way ANOVA with Bonferroni posttest with preselected paired comparisons; ***p* < 0.01 between bracketed bars and #*p* < 0.05 and ##*p* < 0.01 denote significance compared with DMSO only for DMSO-soluble bitterants. **j** Peak Ca²⁺ responses (fluo-4 F/F₀) with T2R14 agonists FFA and HHQ (concentrations are μM) in the presence of inhibitors of GPCR signaling. Significance by one-way ANOVA, Dunnett's posttest comparing each value to DMSO only. **k** Peak Ca²⁺ responses (fluo-4) to T2R14/39 agonist apigenin ± T2R14/39 antagonist 4'-fluoro-6-methoxy-flavanone (50 μM). Significance by Student's *t* test; ***p* < 0.01. **l** Bar graph showing peak Ca²⁺ (fluo-4) in response to T2R38 agonist PTC ± antagonist probenecid (1 mM). Significance by Student's *t* test; ***p* < 0.01. **m** Peak Ca²⁺ (fluo-4) during stimulation with T2R14 agonists NFA or HHQ in MΦs pre-treated with ON-TARGET plus SMARTpool siRNAs. Significance by one-way ANOVA with Bonferroni posttest; ***p* < 0.01 vs control siRNA condition for each respective agonist. Representative traces are averages of 10-30 MΦs from single experiments. Data points in bar graphs are independent experiments using cells from ≥6 experiments (≥3 separate donors; ≥2 experiments per each donor)

Fig. 2 T2R signaling in MΦs decreases cAMP. **a** Diagram (created using Biorender.com) showing canonical T2R transduction pathway by which $G_{\alpha i}$ (G_i) or $G_{\alpha \text{ gust}}$ (G_{gust}) decreases cAMP. **b-c** Representative image series from 3 separate experiments showing MΦs expressing green downward cADDIs (**b**) and graphs (**c**) showing reproducibility of fluorescence changes in response to cAMP-elevating isoproterenol. Decrease in fluorescence equals an increase in cAMP, thus shown as an upward deflection (note inverse y axis). **d** Representative traces (left) and bar graph (right) showing cAMP decreases with 3oxoC12HSL, FFA, and PQS in control MΦs (blue) but not PTX-treated MΦs (pink); adenylyl cyclase-activating forskolin used as control. Traces are mean $\pm$ SEM of ≥ 6 independent experiments. **e** Traces of cADDIs fluorescence (mean $\pm$ SEM; ≥ 6 independent experiments) during stimulation with isoproterenol $\pm$ PQS or 3oxoC12HSL. **f** Bar graph of peak cAMP increases with 100 nM isoproterenol $\pm$ PQS or 3oxoC12HSL (AHL) $\pm$ PTX. Graph shows mean $\pm$ SEM; each data point equals one independent experiment ($n = \geq 6$ total from ≥ 3 donors). Significance by one-way ANOVA with Dunnett's posttest (control is isoproterenol only, no PTX). **g** Traces (left and middle) of changes EPAC-SH¹⁸⁷ CFP/FRET fluorescence emission ratio with quinine $\pm$ PTX. Each representative trace is a single experiment. Downward deflection equals decrease in cAMP. Right is bar graph of peak cAMP decreases with quinine, FFA, and 3oxoC12HSL $\pm$ PTX (mean $\pm$ SEM; ≥ 6 experiments each condition from ≥ 3 donors). **h** Trace of AKAR4 FRET/CFP fluorescence emission ratio (left; mean $\pm$ SEM of independent experiments) with PQS (100 μM) $\pm$ PTX. Downward deflection equals a decrease in PKA activity. Bar graph (right) shows peak PKA decrease with PQS or FFA $\pm$ PTX. Data points in bar graphs use cells from ≥ 3 donors (≥ 6 experiments total, ≥ 2 per donor). Significance in **d**, **g**, and **h** by one-way ANOVA with Bonferroni posttest. Significance in **f** by one-way ANOVA with Dunnett's posttest comparing values to control (no PTX); * $p < 0.05$ and ** $p < 0.01$.

Fig. 3 T2R stimulation activates NO production in MΦs. **a** Reverse transcription (rt) PCR of MΦs from 3 donors showing expression of eNOS (NOS3). **b** Westerns of eNOS and nNOS using MΦ lysates from 3 donors. **c** Traces and bar graph showing DAF-FM fluorescence increases during stimulation with FFA (100 μM), quinine (500 μM), PQS (100 μM), HHQ (100 mM), or salicin (3 mM). Non-specific NO donor SNAP (10 μM) shown as a control. **d** Traces and bar graph showing DAF-FM fluorescence increase in response to FFA (100 μM) or denatonium benzoate (1 mM) ± PTX. **e** Traces and bar graph showing DAF-FM fluorescence increases in response to FFA (100 μM), denatonium benzoate (1 mM), or PQS (100 μM) in the presence of PLC inhibitor U73122 or inactive analog U73343 (30 min pretreatment, 10 μM). **f** Traces and bar graph showing DAF-FM fluorescence increases in response to FFA (100 μM) or denatonium benzoate (1 mM) in the presence of L-NAME or inactive D-NAME (100 μM; 30 min pretreatment) as well as with NO scavenger cPTIO (10 μM). **g** DAF-FM increases in response to 100 μM NFA or 1 mM denatonium benzoate in MΦs treated with Accell SMARTpool siRNAs as indicated. Traces are mean ± SEM from 20-30 MΦs from single representative experiments. Bar graphs are mean ± SEM with data points shown from independent experiments using cells from ≥3 donors (≥6 experiments total, ≥2 per donor). Significance determined by one-way ANOVA with Bonferroni posttest; **p>0.01 vs control, #p<0.05 vs bracketed group, n.s. = no statistical significance

Fig. 4 T2R-induced NO production increases cGMP. **a** Traces and bar graph showing changes in Green GENIE cGMP indicator with T2R agonists. Decrease in fluorescence equals increase in cGMP, plotted as upward deflection (note inverse y axis). **b** Traces and bar graph of cGMP increases with FFA or PQS (100 μ M each) $\pm$ L-NAME or D-NAME (100 μ M 30 min pretreatment) or cPTIO (10 μ M). **c** Traces and bar graph of cGMP increases with 3oxoC12HSL or FFA (100 μ M each) $\pm$ PTX (100 ng/ml pretreatment for 18 hrs). **d** Traces and bar graph of cGMP increases with quinine, FFA (100 μ M), or 3oxoC12HSL (100 μ M) $\pm$ Ca^{2+} signaling; 0- Ca^{2+} conditions are 1 μ M BAPTA-AM loading for 60 min with stimulation in HBSS containing no added Ca^{2+} and 1 mM EGTA. **e** Traces and bar graph of cGMP increases with HHQ or PQS (100 μ M each) $\pm$ co-infection with BacMam expressing soluble guanylyl cyclase (sGC). **f** Bar graph of cGMP increases in response to HHQ (100 μ M), PQS (100 μ M), thujone (600 μ M), or NFA (100 μ M) in M Φ s pre-incubated with ON-TARGET plus SMARTpool siRNAs directed against T2R14 or T2R10, a cocktail of eNOS and nNOS, or control siRNA. All traces are mean $\pm$ SEM of ≥ 6 independent experiments. Bar graphs are mean $\pm$ SEM with data points shown from independent experiments using cells from at least 2 separate donors (≥ 5 experiments total, ≥ 2 experiments per donor). Significance determined by one-way ANOVA with Bonferroni posttest; * or # $p < 0.05$ and ** $p < 0.01$.

Fig. 5 T2R-induced NO/cGMP acutely increases M Φ phagocytosis of FITC-labeled *E. coli*. **a** Representative image of fixed M Φ s after 15 min with FITC *E. coli* $\pm$ FFA. **b** Bar graphs of normalized phagocytosis (quantified by microscopy) $\pm$ various T2R agonists. **c** Normalized phagocytosis (quantified by microscopy) $\pm$ FFA (100 μ M) $\pm$ D/L-NAME (100 μ M, 30 min pretreatment), cPTIO (10 μ M), KT5823 (1 μ M), or PTX (100 ng/ml; 18 hrs pretreatment). **d** Bar graphs showing normalized phagocytosis $\pm$ denatonium benzoate (1 mM) $\pm$ U73122 (10 μ M, 30 min pretreatment), U73343 (10 μ M, 30 min pretreatment) or gallein (100 μ M). **e** Bar graph of phagocytosis $\pm$ NFA (100 μ M), HHQ, (100 μ M), or denatonium benzoate (1 mM) in M Φ s treated with Accell SMARTpool siRNAs as indicated. Results in **b**, **c**, and **d** were quantified by microscopy; each independent experiment is average of 10 fields from a single well. Results in **e** were quantified by plate reader; each independent experiment is average of 2 wells. Bar graphs are mean $\pm$ SEM with data points shown from independent experiments using cells from ≥ 3 separate donors (≥ 2 independent experiments per donor)

Fig. 6 T2R-induced NO and cGMP acutely increases MΦ phagocytosis of pHrodo-labeled *S. aureus*. **a** pHrodo-labeled *S. aureus* were resuspended in PBS buffered to various pHs as indicated to confirm increase in fluorescence with decreasing pH. Experiment in top graph representative of 3 independent replicates, plotted on bottom graph. **b** Representative images (20x; scale bar 20 μm) of pHrodo labeled *S. aureus* and MΦs showing phagocytosis occurring only when both were combined and only at 37°C. **c** Representative images of increased phagocytosis with FFA and inhibition with L-NAME (100 μM, 30 min pretreatment), cPTIO (10 μM), U73122 (10 μM, 30 min pretreatment), and PTX (100 ng/ml; 14 hrs pretreatment). **d** Quantification of experiments performed as in **b**. Significance by Bonferroni posttest; ** $p < 0.01$. **e-f** Quantification of experiments performed as in **c**. **g** Similar experiments were performed with denatonium benzoate (2 mM) and quinine (500 μM); phagocytosis increase was inhibited by KT5823 but not H89 (5 μM, 10 min preincubation each). Significance by one-way ANOVA with Dunnett's posttest; ** $p < 0.01$. **h** Quantification of pHrodo *S. aureus* phagocytosis assays $\pm$ 10 μM forskolin or cell permeant cAMP or cGMP analogs as indicated. Forskolin or cAMP analogs inhibited phagocytosis; however 8-Br-cGMP increased phagocytosis via PKG, as it was blocked by KT5823 (5 μM, 10 min preincubation). **i** Quantification of pHrodo *S. aureus* assays with 1 mM denatonium benzoate $\pm$ forskolin or cAMP analogs or cGMP analog. Significance by 1-way ANOVA with Bonferroni posttest. Data points in **d-i** are independent experiments (≥ 6 from ≥ 2 individual donors, all taken with identical microscope settings), each experiment is average of 10 fields from a single well. Incubations for **b-g** were 20 min. Incubations for **h** and **i** were 60 min.

Fig. 7 Airway epithelial cells can acutely increase MΦ phagocytosis via intercellular NO. **a** Diagram of experimental design for *b-c*. PTX-pretreated DAF-FM-loaded MΦs were placed in close proximity (<1 mm) to unloaded H441s separated by a permeable plastic filter. **b** Stimulation of H441s with 10 nM 17β-estradiol (E2) resulted in an increase in MΦ DAF-FM fluorescence. SNAP (10 μM) added at the end as a positive control. **c** Bar graph of DAF-FM increases from experiments performed as in *a-b*. DAF-FM increases were inhibited by treatment of H441s with PTX (100 ng/ml; 18 hrs) or L-NAME (10 μM, 30 min) or in the presence of cPTIO (10 μM). Treatment of MΦs with L-NAME did not alter responses, and there was no response to E2 in the absence of H441s. **d** Diagram of experimental design for *e-f*. PTX-pretreated MΦs incubated with pHrodo-labeled *S. aureus* were placed in close proximity (<1 mm) to H441s. **e** Representative images of fluorescence increases with PTX-pretreated MΦs with H441s stimulated as indicated for 30 min. **f** Bar graph of MΦs fluorescence increases quantified by microscopy with stimulations as indicated. E2 increased MΦ phagocytosis that was blocked by PTX, L-NAME, cPTIO, or absence of H441s. Bar graphs are mean ± SEM with data points shown from independent experiments using cells from at least 2 individual donors (≥2 independent experiments per donor). Significance by one-way ANOVA with Bonferroni posttest with each bar compared with its respective HBSS only control; ** *p*<0.01. Figures in *a* and *d* created with Biorender.com

Fig. 8 Model of T2R signaling in M0 MΦs. Activation of T2Rs increases Ca^{2+} , lowers cAMP, activates eNOS and nNOS, increases cGMP, and enhances phagocytosis. Acute increase in phagocytosis may also be activated by airway epithelial cell NO production. Figure created with Biorender.com

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