**Bitter taste receptor T2R14 detects quorum sensing molecules from cariogenic
Streptococcus mutans and mediates innate immune responses in gingival epithelial cells**

Medapati Manoj Reddy¹, Singh Nisha¹, Bhagirath Anjali Yadav^{1,2}, Kangmin Duan^{1,2}, Barbara
Triggs-Raine^{2,3}, Batista Eraldo Jr⁴ and Chelikani Prashen^{1,2*}

¹Manitoba Chemosensory Biology Research Group and Department of Oral Biology, Dr. Gerald
Niznick College of Dentistry, Rady Faculty of Health Sciences, 780 Bannatyne Avenue,
University of Manitoba, Winnipeg, Canada.

²Children's Hospital Research Institute of Manitoba (CHRIM), 715 McDermot Avenue,
University of Manitoba, Winnipeg, Canada.

³Department of Biochemistry and Medical Genetics, Rady Faculty of Health Sciences, 745
Bannatyne Avenue, University of Manitoba, Winnipeg, Canada.

⁴Department of Diagnostics and Surgical Sciences, Dr. Gerald Niznick College of Dentistry,
Rady Faculty of Health Sciences, 790 Bannatyne Avenue, University of Manitoba, Winnipeg,
Canada.

*To whom the correspondence should be addressed: Dr. Prashen Chelikani, D319, Manitoba
Chemosensory Biology Research Group and Department of Oral Biology, 780 Bannatyne
Avenue, University of Manitoba, Winnipeg, MB R3E 0W4. Canada. Tel: 204-789-3539; Fax:
204-789-3913; Email: Prashen.Chelikani@umanitoba.ca

Abstract

Host-pathogen interactions play an important role in defining the outcome of a disease. Recent studies have shown bacterial quorum sensing molecules (QSM) can interact with host cell membrane proteins, mainly G protein-coupled receptors (GPCRs), and induce innate immune responses. However, few studies have examined QSM-GPCR interactions and their influence on oral innate immune responses. In this study, we examined the role of bitter taste receptor T2R14 in sensing competence stimulating peptides (CSPs) secreted by cariogenic bacterium *Streptococcus mutans* and in mediating innate immune responses in gingival epithelial cells (GECs). Transcriptomic and western blot analyses identify T2R14 to be highly expressed in GECs. Our data shows that only CSP-1 from *S. mutans* induces robust intracellular calcium mobilization compared to CSP-2 and CSP-3. By using CRISPR-Cas9, we demonstrate that CSP-1 induced calcium signaling and secretion of cytokines CXCL-8/IL-8, TNF- α and IL-6 is mediated through T2R14 in GECs. Interestingly, the NF- κ B signaling activated by CSP-1 in GECs was independent of T2R14. CSP-1 primed GECs attracted differentiated HL-60 immune cells (dHL-60) and this effect was abolished in T2R14 knock down GECs and also in cells primed with T2R14 antagonist 6-Methoxyflavone (6-MF). Our findings identify *S. mutans* CSP-1 as a peptide ligand for the T2R family. Our study establishes a novel host-pathogen interaction between cariogenic *S. mutans* CSP-1 and T2R14 in GECs leading to an innate immune response. Collectively, these findings suggest T2Rs as potential therapeutic targets to modulate innate immune responses upon oral bacterial infections.

KEYWORDS: *Gingival epithelial cell (GEC), Bitter taste receptor 14 (T2R14), S. mutans, Competence stimulating peptide (CSP), Host-pathogen interaction, Innate immune responses.*

46 **Abbreviations**

47 Gingival Epithelial Cell (GEC)

48 G protein-coupled receptors (GPCRs)

49 Bitter Taste Receptors (T2Rs)

50 Competence Stimulating Peptide (CSP)

51 Quorum Sensing Molecules (QSMs)

52 Short chain fatty acids (SCFAs)

53 Lipopolysaccharide (LPS)

54 Phospho Lipase C (PLC)

55 Differentiated Human leukemia 60 cells (dHL-60)

56 2-heptyl-3-hydroxy-4-quinolone (PQS)

57 4-dihydroxyquinolone or 4-hydroxy-2-heptylquinolone (HHQ)

58 N3-oxododecanoyl homoserine lactone (3-O-CS12-HSL)

Introduction

The oral epithelium forms a physical barrier and acts as a primary line of defense against oral pathogens by generating innate immune responses. Epithelial cells recognize several bacterial molecules, including lipopolysaccharide (LPS) and flagellin, using pattern recognition receptors (PRRs) such as the family of Toll-like receptors (TLRs) [1, 2]. Although TLRs are recognized as sentinels of the innate immune system there is still ambiguity in the mediators of host-pathogen cross talk. Studies have suggested that human G protein-coupled receptors (GPCRs) including the formyl peptide receptors (FPRs), free fatty acid receptors (FFAR) and mast-cell specific GPCR (MRGPRX2) can recognize bacterial signaling peptides and are involved in host defense against pathogens [3-6]. Recent studies have shown that the 25 bitter taste receptors (T2Rs) in humans which form the third largest GPCR group, act as important sensors for the bacterial quorum sensing molecules (QSMs) [7-10].

T2Rs are predominantly involved in taste chemosensing though studies have identified their extra-oral localization [11-14]. Emerging evidence shows T2R interaction with bacterial QSMs leads to innate immune responses in diseases such as Cystic Fibrosis (CF) and Chronic Rhinosinusitis (CRS) [15, 16]. In CF, the *Pseudomonas aeruginosa* derived QSMs, mainly acyl homoserine lactones (AHLs), interact with T2Rs in airway epithelial cells [8, 9]. In our previous study, we characterized the binding sites on T2R4, T2R14 and T2R20 for the QSMs 3-Oxo-C12-AHL and C8-AHL [17]. Other quinolone derived QSMs such as HHQ and NHQ from *P. aeruginosa* activate T2R14, leading to innate immune responses such as nitric oxide (NO) generation and increased ciliary beat frequency (CBF) in upper airway epithelial cells [9]. A recent study suggested T2Rs are immune sentinels in the gingival solitary chemosensory cell (GSC) of a mouse periodontitis infection model [18]. Thus, T2Rs have emerged as important targets in host-pathogen interaction studies. However, most of the T2R-QSM interaction studies

are limited to Gram-negative *P. aeruginosa* QS. One of the main remaining questions is how the host immune system detects and responds to the QSMs secreted by Gram-positive oral pathogenic bacteria such as *S. mutans*.

S. mutans a cariogenic bacterium, secretes QSMs known as competence stimulating peptides (CSPs) in the early growth phases which are crucial for interspecies interaction, biofilm formation, acid production, genetic transformation and caries progression [19-21]. Although CSP mediated QS is well studied with respect to *S. mutans* microbe-microbe interactions, its effect on host cells response is not well established. The interaction of *S. pneumoniae* CSP-1 with MRGPRX2 leading to mast cell degranulation was recently reported [6]. Hence, there exists evidence that CSPs are recognized by host cell GPCRs.

We hypothesize that CSPs secreted by *S. mutans* interact with T2Rs on oral epithelial cells and their activation leads to innate immune responses. Our study characterizes the interaction between peptide QSMs and T2Rs and the results add to the growing body of evidence suggesting T2Rs as sentinels of the innate immune system in oral tissues.

Materials and methods

Reagents used in the study

Synthetic *S. mutans* QS peptides CSP-1, CSP-2, CSP-3, CSP-1 (Δ K21), CSP-1 (Δ G20, Δ K21) of $\geq 98\%$ purity were purchased from GenScript USA Inc. (Piscataway, NJ). RNeasy Mini kit, Fluo-4NW, Gallein and Pepinh-MyD inhibitory peptide were purchased from Qiagen (Hilden, Germany), Thermo Scientific (Carlsbad, CA), Tocris (Oakville, ON) and abcam (Toronto, ON) respectively. Apigenin, N-Formylmethionine-leucyl-phenylalanine (fMLP), U73122, U73343, 6-Methoxyflavone (6-MF), BAY 11-7082, Adenosine triphosphate (ATP), Phorbol 12-myristate 13-acetate (PMA), protease and phosphatase inhibitor cocktails were

purchased from Sigma Aldrich (Oakville, ON). Pre-coated cytokine multiplex ELISA 96-well plates were purchased from Meso Scale Diagnostics (MSD) (Rockville, MD). xCelligence migration or CIM plates were purchased from ACEA Biosciences, Inc. (San Diego, CA). Keratinocyte growth medium -2 (KGM-2) for GEC culture and Iscove's modified dulbecco's medium (IMDM) for promyeloblast (HL-60) cells were purchased from promo cell (Heidelberg, Germany) and ATCC (Gaithersburg, MD) respectively. The following antibodies were purchased: mouse monoclonal anti- β -actin (#A5441) from Sigma Aldrich (Oakville, ON), rabbit polyclonal anti-T2R14 (#OSR00161W), rabbit polyclonal anti-T2R39 (#PA5-39711) from Invitrogen (Carlsbad, CA), goat anti- rabbit IgG-HRP conjugate (#17-6515) from Bio-Rad (Mississauga, ON), goat anti- mouse IgG- HRP conjugate (#A-10668) from Thermo Scientific (Carlsbad, CA), rabbit monoclonal anti phospho-IKK α/β (Ser176/180) (#2697), phospho-IkBa^{Ser}³² (#2859), IKK α (#11930), IKK β (#8943), IkB α (#4814) from Cell signaling (Danvers, MA), FITC anti-human CD11b (#301330), FITC mouse IgG1, k Isotype Ctrl (Fc) (#400110), APC anti-human CD14 (#367118), APC mouse IgG1, k Isotype Ctrl (Fc) (#400122) from BioLegend (San Diego, CA).

Cell lines used in the study

The human gingival epithelial cell line (GEC) TIGK-hTERT (CRL-3397TM) and HL-60 cells (CCL-240TM) were purchased from ATCC (Gaithersburg, MD). The oral keratinocyte cell line OKF6 was kind gift from Dr. Gill Diamond, University of Florida [22, 23]. All the GECs were cultured in serum free KGM-2 supplemented with 0.6mM CaCl₂. HEK293T and HEK293T cells overexpressing T2R14 and T2R39 receptor were generated as previously described [24]. The promyeloblast (HL-60) cells were cultured in IMDM supplemented with 20% FBS. The HL-60 cells were differentiated toward neutrophils using previously established protocols [25].

Briefly, 2×10^5 cells/ml were seeded in 25 ml and were treated with 1.3% DMSO. The cells were then maintained in culture for 7-8 days. All the growth media were supplemented with 100-units/ml penicillin, and 0.1mg/ml streptomycin.

Methods

RNA extraction and transcriptomic analysis

The GECs (OKF6 and TIGK) were seeded in a 6-well plate at a density of 1×10^6 cells/wells. After reaching 80% confluency the cells were lysed and RNA was extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. The concentration of total RNA was measured and 5 μ g of RNA was shipped on dry ice to ThermoFisher Scientific (Santa Clara, CA) for processing. The samples were processed using GeneChip® WT pico kit (WT Pico) on an Affymetrix GeneChip® human gene 2.0 ST array platform using the GeneTitan™ Microarray system. The whole transcriptome (21448 genes) expression data sets for the GECs were downloaded from Egnyte server as .CEL files. The .CEL files correspond to whole transcriptomes of the GECs. These files were analyzed for differential expression using Transcriptome Analysis Console (TAC) from ThermoFisher Scientific. The Clariom™ S array human analysis library files downloaded from Affymetrix were used for analysis. The analysis was carried out using default analysis tool: Expression (Gene) and SST-RMA algorithm. The following criteria were used in the SST-RMA algorithm: Gene-Level Fold change ≤ -2 or ≥ 2 , Gene-Level-P-value ≤ 0.05 . The signal intensity values were used to generate heat maps for transcripts in the Rstudio using ggplot2 package (Version 3.2.1) [26].

Generation of T2R14 knock down OKF6 cells using CRISPR-Cas9 system

According to the HUGO nomenclature the T2R coding genes are represented as *TAS2R* [27]. A total of 100,000 OKF6 cells/well were seeded in a 6 well plate. After 24h, the cells were transfected with 30 nm of Alt®-R-CRISPR-Cas9 crRNA targeting the *TAS2R14* gene TTTAGTGTCTGGTTAGCTAC (5'-3') (Hs.Cas9.TAS2R14.1.AB), fluorescent labeled (ATTO 550) Alt®-R-CRISPR-Cas9 tracrRNA or a Alt®-R-control CRISPR-crRNA (#1072554, IDT, Iowa) and recombinant *S. pyogenes* Cas9 nuclease V3 (#1081058, IDT, Iowa) using CRISPRMAX™ transfection reagent (#CMAX00008, Invitrogen, CA) according to the manufacturer's protocol as previously described [28]. The efficiency of transfection was analyzed using cytoflex flow cytometer on the basis that only positive cells contain ATTO 550 dye. After this, the single cell clones were isolated using serial dilution in 96-well plates and were observed for colony formation. Only wells containing a single colony were isolated and further propagated. The single cell colonies were harvested by trypsin/EDTA and were split into two and plated in a 96-well plate and after reaching 90% confluency the cells were split to 24-well plate to increase the cell number. For each clone, half the cells were stocked and the remaining half were used to extract genomic DNA according to manufacturer's protocol (Qiagen). The extracted DNA was quantified and 100 ng of DNA was amplified using Phusion Flash High-Fidelity PCR Master Mix (#F-548S, Thermo Scientific, Carlsbad, CA) and *TAS2R14* forward primer TGGGTGGTGTGCATAAAGAGCAT (5'-3'), reverse primer GAGGGCTCCCCATCTTTGAA (5'-3') that bound the region targeted by the guide RNA. The following protocol was used: 95°C/10s, 35 cycles of 95°C/30s, 62°C/30s, 72°C/30s and a final extension at 72°C/1min. The amplified PCR product was used for T7 endonuclease I (T7EI) mismatch assay. The T7EI assay was performed according to the manufacturer's protocol (#1075932, IDT, Iowa). Only clones that showed complete digestion of *TAS2R14* PCR product compared to wild type (WT) and MOCK

were used for protein analysis. The knock down of T2R14 protein was confirmed by western blot analysis and flow cytometry.

Intracellular calcium mobilization assay

The intracellular calcium mobilization (Ca^{2+})_i upon agonist stimulation was measured using calcium binding fluorescent dye Fluo-4NW as described previously [17, 24]. Briefly, GECs (3×10^4 /well) and HEK293T cells stably over-expressing T2R14 or T2R39 (1×10^5 /well each) were seeded in a black well 96-well BD optilux plate and grown overnight to full confluency. The cells were then incubated with Fluo-4NW at 37°C for 30 min followed by another incubation at room temperature for 30 min. The cells were treated using *S. mutans* CSP-1, CSP-2 and CSP-3 (50 μM) each. For concentration response curves the following were used: CSP-1 and its variants CSP-1 (ΔK21), CSP-1 (ΔG20 , ΔK21) (10 –70 μM), T2R14 agonist DPH (0.03-2 mM) and apigenin (6.25-400 μM). For T2R signaling inhibition the cells were pretreated using G $\beta\gamma$ inhibitor gallein (10 μM), Phospholipase C (PLC) inhibitor U73122 (5 μM) and its analogue U73343 (5 μM), as described previously[13]. ATP (1 μM) a well-known activator of PLC signaling induced by purinergic receptors was used as a control [29]. The release in (Ca^{2+})_i upon agonist stimulation was measured using FlexStation 3 plate reader (Molecular devices, CA). Fluorescence was measured for 120s at emission of 525 nm and excitation of 494 nm wavelengths. The fluorescence values obtained were subtracted from the buffer treated cells and represented as Δ relative fluorescent units (RFU).

Intracellular cAMP assay:

The cAMP assay was carried out in OKF6 cells using a commercially available cAMP assay kit (cAMP-Glo max Assay, Promega Corporation, Madison, WI) according to

manufacturer instructions [30]. Briefly, 20,000 OKF6 cells were diluted in 20 μ l of PBS and were seeded in 96-well clear bottom white plate (VWR, Canada). These cells were incubated with different doses of CSP-1 (0-70 μ M) and isoproterenol (0-10 μ M) as a positive control for 10 mins at room temperature. Later, luminescence was measured using a FlexStation 3 plate reader (Molecular Devices, CA). The assays were carried out 2-3 times in triplicate, and the data were analyzed using graph pad prism 7 (GraphPad Software Inc., San Diego, CA).

Western blot analysis

For western blot analysis of T2R14 the GEC (OKF6 and TIGK) cell total fractions were lysed using 10 mM Tris-HCL, 50 mM NaCl (pH 7.4), and 10% glycerol containing protease inhibitor cocktail. The lysates were then centrifuged at 12,000 g for 15 mins and the protein supernatant was stored at -80°C until further use [31]. To analyze phosphorylated proteins the cells were lysed using 2x Laemmli buffer (Tris pH 6.8, 2-mercaptethanol, 20% SDS) containing 1x protease and phosphatase inhibitors. The total protein concentration was measured using DC protein assay kit (Bio-Rad). 20 μ g of protein sample was resolved on a 12% SDS-PAGE gel followed by wet transfer onto the nitrocellulose membrane. The membranes were blocked using 5% milk for 1 hour at room temperature followed by incubation with corresponding primary antibodies: rabbit polyclonal anti-T2R14 (1:500 dilution), rabbit monoclonal anti phospho-IKK α / β ^{Ser176/180}, phospho-IkB α ^{Ser 32}, IKK α , IKK β , IkB α (1:1000 dilution), mouse monoclonal anti- β -actin (1:25000 dilution) overnight at 4°C followed by incubation with HRP conjugated secondary antibodies: goat anti- rabbit IgG-HRP (1:5000), goat anti- mouse IgG- HRP (1:5000). The chemiluminiscence was measured by Bio-Rad Chemidoc-MP imaging system and the blot densities were quantified using Image lab 5.2.1 (Bio-Rad).

Measurement of secreted cytokines

OKF6 WT, MOCK and T2R14 KD cells were seeded in 6 well plates at a density of 5×10^5 cells/ well and were cultured for 24 hours. The cells were then treated with CSP-1 (50 μ M), DPH (200 μ M), PMA (100 nM) [32], NF- κ B signaling inhibitor BAY 11-7082 (10 μ M) [33], peptide inhibitor for TLR adaptor protein MyD88 Pepinh-MyD (40 μ M) [33], U73122 (5 μ M) and gallein (10 μ M) for 18 hours. Following incubation, the cell culture supernatants were collected and a multiplex ELISA was performed in 96-well plates pre-coated with IFN γ , IL-2, IL-4, IL-6, IL-8, IL-10 and Tumor necrosis factor-alpha (TNF- α) antibodies using the customized V-Plex 7 proinflammatory panel 1 (Meso Scale Discovery, Rockville, MD) [34]. The detection limit for each of the seven cytokines is as follows: IFN- γ (0.37-938 pg/ml), IL-2 (0.09-938 pg/ml), IL-4 (0.02-158 pg/ml), IL-6 (0.06-488 pg/ml), IL-8 (0.07-375 pg/ml), IL-10 (0.04-233 pg/ml) and TNF- α (0.04-248 pg/ml) respectively.

Flow cytometry

Undifferentiated and differentiated HL 60 (dHL 60) cells were used to detect surface expression of CD 11b and CD 14 using BD FACS canto analyzer. Briefly, 100,000 cells were washed using ice-cold FACS buffer (0.5% BSA in 1x PBS) and incubated with FITC-CD 11b (1:100 dilution) and APC-CD 14 (1:100 dilution) for 1 hour on ice. The cells were then washed thrice with FACS buffer and were re-suspended in 200 μ l of the same. The fluorescent intensity was measured using FACS canto analyzer and quadrant gating was used to identify the percentage of cells expressing the markers.

xCELLigence® real-time chemotaxis assay

dHL 60 cells were seeded on the top chamber of the CIM plate at a density of 4×10^5 cells/ well in HBSS containing 0.5 % BSA. The bottom chamber was loaded with conditioned medium from OKF6 WT, MOCK and T2R14 KD cells treated with CSP-1 (50 μ M), DPH (200 μ M), apigenin (75 μ M), PMA (100 nM), fMLP (100 nM) alone or in combination with U73122 (5 μ M), gallein (10 μ M) and T2R14 antagonist 6-MF (30 μ M). The changes in cellular impedance was measured in real-time every 5 minutes over a period of 24 hours and represented as cell index (CI). The slope over 24 hours is obtained using RTCA software as described before [31].

Statistical analysis

The data shown are the $\pm$ SEM from at least three independent experiments (n). One-way ANOVA and Tukey's multiple comparisons test were used to determine statistical significance for more than two sample groups. A $*p < 0.05$ was considered significant. Statistical analysis was performed using graph pad prism 7.0 (San Diego, CA).

Results

1. Analysis of expression of TAS2Rs (T2R) in GECs.

Recent studies using transcriptomic arrays have identified differences in GPCR expression profiles in smooth muscle cells, endothelial cells, cancer-associated fibroblasts and brain tissues [35-37]. We isolated RNA from different GECs and the samples were processed using the Affymetrix GeneChip® human gene 2.0 ST array platform and analyzed by the Clariom™ S array human analysis library to identify the abundant transcripts. The signal intensities of transcripts obtained ranged from 3 (low-expression) to 17 (high-expression). Given the large number of transcripts (21448 genes), we used a signal cut-off of 50% (or 8) to

investigate only the medium and high expression GPCR transcripts in GECs. The transcripts thus obtained were normalized using the ubiquitous 40S ribosomal protein S23 (RPS23) transcript signal. We have used keratin (KRT) 5 and KRT14 as epithelial cell markers. The T2R coding genes are represented as *TAS2Rs* [27]. Our analysis identified 26 GPCR transcripts that are medium to highly expressed in both GECs and *TAS2R14* is present among these (Fig 1A). The normalized signal intensity values were compared using one-way analysis of variance (Anova) to examine the differential gene expression. The expression of *TAS2R14* was observed to be significantly higher as compared to the other 24 *TAS2R* genes ($***p<0.001$), and 8 *TLR* genes ($***p<0.001$), with the exception of *TLR3* and *TLR6* where there was no difference in expression ($p<0.9$) (Fig 1B). To verify abundant expression of the T2R14 protein in these GECs, we used western blot analysis (Fig 1C). The detected T2R14 was much higher than that of another T2R, T2R39 (Fig 1D). These findings were consistent with the mRNA expression profiles shown in Fig. 1B where *TAS2R14* mRNA levels were significantly higher than that of *TAS2R39*.

2. Calcium responses in GECs induced by *S. mutans* QSM peptides.

The cariogenic bacterium *S. mutans* is known to secrete three CSPs (CSP-1, CSP-2 and CSP-3). CSP-1 contains 21 amino acids and is produced by *S. mutans* UA159 strain [21]. CSP-1 is cleaved at the C-terminus by membrane protease sepM into an 18 amino acid CSP-2 [38]. CSP-3 is secreted by JH1001 strain of *S. mutans* and is an 18 amino acid CSP with a S3T variation at the N-terminus [39]. The *S. mutans* strains secreting CSP-1 were shown to have higher transformation efficiency compared to strains containing c-terminal truncated CSP [21]. To further study CSP-1, CSP-2 and CSP-3, we had these peptides synthesized commercially with >98% purity (Fig 2A). The GECs (OKF6 and TIGK) were treated with increasing concentrations of CSP-1 (10-70 μ M) and analyzed for responses of the second messengers cAMP and Ca^{2+} . We

observed a concentration dependent increase in intracellular calcium mobilization in the GEC cell lines upon treatment with CSP-1 with an EC₅₀ of ≈ 50 μ M (Fig 2B, C). Next, we treated the GECs with CSP-2 and CSP-3 using the EC₅₀ value of CSP-1. CSP-2 and CSP-3 treated cells had significantly lower intracellular calcium mobilization ($p < 0.0231$) as compared to CSP-1 treated cells (Fig 2D-G).

The robust calcium response that CSP-1 induced in GECs compared to CSP-2 and CSP-3 suggested that the 3 terminal amino acids of these peptides may be important to CSP function. We analyzed two truncated variants of CSP-1, with truncation of C-terminal amino acids K21 and/or G20 resulting in CSP-1 (Δ K21) and CSP-1 (Δ G20, Δ K21) (Fig 2H). CSP-1 (Δ K21) showed an EC₅₀ of 45-51 μ M, similar to cells treated with full length CSP-1. The double variant CSP-1 (Δ G20, Δ K21) did not induce a concentration dependent calcium response (Fig 2I-L). We also examined the release of another important secondary messenger cAMP. The OKF6 cells treated with CSP-1 did not exhibit any cAMP responses whereas a strong response was imparted by the known β_2 -AR agonist isoproterenol (Fig 2M and N).

3. CSP-1 induced calcium response in GECs occurs through T2R14.

To test whether CSP-1 induced calcium responses are dependent on T2R14 we knocked down (KD) T2R14 in OKF6 cells using CRISPR-cas9 genome editing as described in the methods. Clonal isolates with mutations in the *TAS2R14* gene were verified using the T7EI assay. The data from T7EI assay showed complete editing of *TAS2R14* amplicon (908 bp) into smaller fragments of 769 bp and lower lengths in the T2R14 KD cells but not in WT and MOCK cells (Fig 3A). Next, western blot analysis was performed to confirm T2R14 KD. T2R14 protein expression is not detectable in CRISPR-Cas9 cells as compared to the WT and MOCK cells (Fig

3B). Next, calcium mobilization assays were performed using DPH (0.03-2 mM) and CSP-1 (10-70 μ M) to characterize T2R14 activity. These assays show that DPH and CSP-1 mediate intracellular maximal calcium mobilization responses (E_{max}) that are significantly decreased in the T2R14 KD cells compared to the WT and MOCK cells ($***p<0.001$) (Fig 3C-F). We also assayed CSP-1 induced calcium mobilization in the presence of the T2R14 antagonist 6-MF [40]. The OKF6 cells co-treated with 6-MF (1.2 –100 μ M) and CSP-1 (50 μ M) showed a concentration dependent decrease in calcium mobilization with an IC_{50} of 30 μ M for 6-MF (Fig 6G and H). Previous studies have shown 6-MF as an antagonist for T2R39 [41]. However, our western blot analysis revealed T2R39 expression was minimal in GECs (Fig. 1D) and CSP-1 did not induce intracellular calcium mobilization in HEK293T cell overexpressing T2R39 (Fig S1A and B). In contrast, HEK293T cells overexpressing T2R14 (Fig. 3I) showed a concentration dependent increase in calcium mobilization upon treatment with CSP-1 and DPH (Fig 3J-M). These data indicate CSP-1 induced calcium responses are predominantly mediated through T2R14 in GECs.

4. CSP-1 induces calcium response in GECs through the canonical T2R14- $G_{\alpha\beta\gamma}$ -PLC β signaling pathway.

To characterize the canonical signaling pathway activated by CSP-1 and T2R14 interaction the following inhibitors were used: PLC inhibitor (U73122) that inhibits the coupling of G protein subunit with PLC β and $G_{\beta\gamma}$ inhibitor (gallein) which inhibits the dissociation of G_{α} from the $G_{\beta\gamma}$ subunit. Pre-treatment of GECs with U73122 (5 μ M) and gallein (10 μ M) inhibited CSP-1, DPH and ATP induced calcium mobilization compared to agonist treated GECs alone ($***p<0.001$). As expected this inhibitory effect is not observed upon pre-treatment with PLC inhibitor analogue U73343 (5 μ M) (Fig 4A-D).

5. CSP-1 activates NF-kB signaling pathway independent of T2R14 in GECs.

Given that the two GEC lines showed similar responses to CSPs we focused on OKF6 cells for further analysis, as this is a well characterized oral keratinocyte cell line [22]. The PLC signaling is shown to induce PKC isoforms [42] which can activate the NF-kB signaling pathway [43]. We determined the effect of CSP-1 on the kinetics and intensity of phosphorylation of NF-kB signaling intermediates IKK α/β and I κ B α (Fig 5A-C). The western blot analysis of total cell protein lysates collected at different time points (0-60 min) revealed a significant increase in phosphorylation of IKK α/β (0 min vs 5 min $*p<0.05$, 0 min vs 15 min $*p<0.05$) and of I κ B α (0 min vs 5 min $**p<0.006$, 0 min vs 15 min $***p<0.001$, 0 min vs 30 min $**p<0.01$ and 0 min vs 60 min $**p<0.01$) was observed upon short-term exposure with CSP-1 (Fig 5B, C). The phosphorylation of IKK α/β reached basal levels after 30 mins (Fig 5B), while phosphorylation of I κ B α did not reach basal levels (Fig 5C). From the time kinetics data, we have chosen 15 minutes as a treatment time for further analysis using T2R14 agonist DPH, PMA and BAY 11-7082 (inhibitor of I κ B α phosphorylation). The data from inhibition studies showed that BAY 11-7082 did not effect the phosphorylation of IKK α/β induced by CSP-1 and PMA (Fig 5D and E) but inhibited phosphorylation of I κ B α (Fig 5D and F). The T2R14 agonist DPH also induced phosphorylation of IKK α/β and I κ B α and BAY 11-7082 inhibited the phosphorylation of I κ B α (Fig 5G-I). To characterize CSP-1/T2R14 mediated activation of NF-kB signaling pathway, we have used MOCK and T2R14 KD cells. The data show that upon treatment with CSP-1, DPH and PMA no significant change in phosphorylation of IKK α/β and I κ B α levels were observed in MOCK and T2R14 KD cells (Fig 5J-L). We further used inhibitors for the T2R downstream signaling targets PLC and G $\beta\gamma$. The data from these studies show that CSP-1 and DPH induced

phosphorylation of NF-kB signaling intermediates were inhibited by U73122 (PLC blocker); however, gallein (Gβγ inhibitor) did not inhibit the phosphorylation of NF-kB signaling intermediates in OKF6 cells (Fig S2 A and B).

6. CSP-1 induces cytokine secretion in GECs.

Recent studies have shown increased secretion of cytokine IL-8 in corneal epithelial cells in response to *P. aeruginosa* QSMs and TNF-α in mast cells in response to *S. pneumoniae* CSP-1 [6, 44]. To analyze cytokine secretion in OKF6 cells they were treated with CSP-1, DPH, and PMA alone or in combination with U73122, gallein, BAY 11-7082 and peptide inhibitor of MyD88. After 18 hours of treatment with the above-mentioned compounds, the cell culture supernatants were collected, and the levels of seven cytokines were determined using V-PLEX 7 Meso Scale multiplex ELISA. The levels of IFN-γ were below the detection limit in our cell system (data not shown). We observed increased levels of IL-6 (** $p<0.0034$) (Fig 6C), IL-8 (** $p<0.0014$) (Fig 6D) and TNF-α (* $p<0.0177$) (Fig 6F) in CSP-1 treated cells. However, no significant change in levels of IL-2 (Fig 6A), IL-4 (Fig 6B) and IL-10 (Fig 6E) were observed upon CSP-1 treatment. The treatment with T2R14 agonist DPH, and PKC activator PMA induced secretion of IL-2 (* $p<0.05$, **** $p<0.0001$) (Fig 6A), IL-4 (* $p<0.05$, *** $p<0.0008$) (Fig 6B), IL-6 (** $p<0.002$, *** $p<0.0009$) (Fig 6C), IL-8 (** $p<0.002$, **** $p<0.0001$) (Fig 6D), IL-10 (* $p<0.05$, *** $p<0.0008$) (Fig 6E) and TNF-α (** $p<0.002$, **** $p<0.0001$) (Fig 6F). To examine the role of TLRs in cytokine secretion, OKF6 cells were treated with CSP-1, *E.coli* LPS alone or in combination with Pepinh-MyD inhibitor. Only TNF-α was increased upon LPS treatment that was inhibited by Pepinh-MyD inhibitor (* $p<0.05$) (Fig 6F). None of the other cytokines were affected by Pepinh-MyD inhibition of CSP-1 or LPS treatment. Interestingly, DPH induced

secretion of IL-2 ($*p<0.05$); IL-4 ($**p<0.003$) and IL-10 ($*p<0.05$) were significantly reduced upon treatment using Pepinh-MyD inhibitor. The IL-8 and TNF- α levels remain unchanged in cells treated with CSP-1 alone or in combination with Pepinh-MyD inhibitor.

To characterize the role of CSP-1/T2R dependent and independent cytokine secretions cells were co-treated using T2R downstream signaling inhibitors. The IL-8 ($***p<0.001$) (Fig 6D) and TNF- α ($*p<0.05$) (Fig 6F) levels were significantly decreased in cells co-treated with CSP-1 and gallein compared to CSP-1 treated cells. However, IL-8 and TNF- α levels remain unaffected in cells co-treated with CSP-1 and U73122 ($p<0.99$). Next, we analyzed T2R14 specific cytokine responses in MOCK and T2R14 KD cells (Fig 6G-L). A significant decrease in basal IL-2, IL-6, IL-8, TNF- α levels were observed ($*p<0.05$) upon T2R14 KD (Fig 6G-L). Strikingly, CSP-1 or DPH treatment did not recover the majority of the cytokine secretions in T2R14 KD as compared to MOCK cells (IL-2 $**p<0.01$, IL-4 $*p<0.05$, IL-6 $**p<0.01$, IL-8 $**p<0.01$, IL-10 $**p<0.01$, TNF- α $**p<0.01$) (Fig 6G-L). Furthermore, PMA treatment recovered cytokine secretions in T2R14 KD cells albeit not to the same levels in MOCK cells (IL-2 $**p<0.01$, IL-4 $**p<0.01$, IL-6 $***p<0.001$, IL-8 $p>0.05$, IL-10 $*p<0.05$, TNF- α $*p<0.05$) (Fig 6G-L).

7. Effect of CSP-1 on dHL-60 cell migration.

To characterize the effect of CSP-1 induced GEC secretion on immune cell migration, we used differentiated HL-60 cells (dHL-60) as a model (Fig 7A) [25]. FACS analysis showed an increased expression of CD11b and CD14 cell surface markers in dHL-60 cells as compared to the undifferentiated cells (Fig S3A). The 18 hour conditioned medium (CM) of OKF6 cells was used to induce migration in dHL-60 cells over a period of 24 hours. The data revealed that CM

from CSP-1 treated OKF6 cells increased migration of dHL-60 cells by 2.5-fold as compared to the CM from untreated cells ($***p<0.002$) (Fig 7B, C). In contrast, CM of OKF6 cells co-treated with CSP-1 and T2R signaling inhibitors U73122, gallein and T2R14 antagonist 6-MF significantly decreased dHL-60 migration compared to CM from CSP-1 treated cells ($****p<0.0001$, $**p<0.01$, $***p<0.001$) (Fig 7B, C). We observed an early increase in the migratory cell index, which was similar between untreated, and CSP-1 treated cells; however, the cell index in CSP-1 treated OKF6 cells continued to increase over a period of 24 hours but not in the untreated cells. A similar trend was observed in cells co-treated with CSP-1 and U73122 where the cell index reached lower than basal levels. In cells co-treated with CSP-1 and gallein there was an early spike in the cell index, which then reached below the level of CSP-1 treated cells but was not restored to basal levels. The inhibitors alone did not influence dHL-60 migration and neither did the dHL-60 cells treated with CSP-1 peptide. This shows that CSP-1 primed OKF6 cells were able to attract immune cells and this effect is dependent on T2R14 and its canonical signaling proteins $G_{\beta\gamma}$ and PLC_{β} . We also used the CM from MOCK and T2R14 KD cells. The data from these experiments show a significant decrease in dHL-60 migration upon treatment with CM from T2R14 KD cells primed with CSP-1 ($**p<0.01$) and DPH ($**p<0.01$) as compared to MOCK cells (Fig 7D, E). However, the migratory effect was the same when PMA treated CM from MOCK and T2R14 KD cells was used (Fig 7D, E). These results confirm that CSP-1 activation of T2R14 in OKF6 cells creates a microenvironment that can act as an attractant for neutrophils.

Discussion

Over the past decade several studies have examined the role of host – pathogen interactions. The T2R family, the third largest class of GPCRs with 25 members [7] has an

exceptionally broad ligand spectrum. Although they are primarily identified as targets for bitter compounds, recent studies have shown their importance in host-pathogen interactions [9, 45]. Most of these studies focused on Gram-negative *P. aeruginosa* and QSMs, specifically AHLs, secreted by it. Our study was the first to examine the effect of oral QSMs (CSPs) secreted by cariogenic *S. mutans* on host GECs. Together, these studies suggest a novel role of T2Rs as sentinels of the innate immune system in our body.

Oral epithelial cells act as the primary barrier against microbial pathogens. Our transcriptomic analysis suggests 26 GPCRs are expressed at higher levels than the well-recognized pattern recognition receptors, TLRs (Fig 1A and B). Among 25 T2Rs that were examined, T2R14 expression is highest in GECs, indicating a potential role for this T2R in host-pathogen interactions (Fig 1B). Several studies have shown that the activation of T2Rs by bacterial QSMs leads to an increase of intracellular calcium mobilization in different cell types such as upper airway epithelial cells, nasal epithelial cells and in heterologous cell systems [9, 13, 17, 46]. In oral epithelial cells, we observed a robust increase in intracellular calcium mobilization upon treatment with *S. mutans* CSP-1 compared to CSP-2 and CSP-3 (Fig 2B and C). The cariogenic *S. mutans* is shown to secrete CSP-1 that is cleaved by membrane bound protease sepM to produce CSP-2 [21, 38]. Interestingly several strains of *S. mutans* (BM71, GB14, LT11, NG8 and UA159) are known to secrete CSP-1 with an exception for JH1005 that secretes CSP-2 [21] and NCTC10449 strain secreting 23-mer CSP containing LGKIR at the C-terminus [47]. This variability of C-terminal amino acids is known to effect genetic transformation efficiency in these strains [21]. Hence the C-terminal amino acids play important role in *S. mutans* quorum sensing. Interestingly, our results suggest the importance of the last three C-terminal amino acids in CSP-1 in inducing calcium mobilization in GECs. Removal of the terminal lysine (K21) did not affect the calcium response, while removal of the two C-

terminal residues G20 and K21 significantly reduced intracellular calcium mobilization in GECs (Fig 2I and K). These data suggest that G20 at the C-terminus is an important amino acid in *S. mutans* CSP-1. Interestingly, a recent study has also shown an increased calcium response in mast cells upon incubation with *S. pneumonia* CSP-1 [6]. The same study also suggested that peptide QSMs above a net charge of 2 were able to activate calcium responses in mast cells. The *S. mutans* CSP-1 has a net charge of 3.0 as compared to CSP-2 and 3, which have a net charge of 1.8 each. Previous structural analysis showed CSP-1 to assume a random α -helix conformation in aqueous and membrane-mimicking solutions [48]. A recent study utilizing *S. mutans* CSP analogues suggested a slight decrease in α -helix conformation and consistent decrease in the activity of CSP-2 [49]. Although this decrease in QS activity cannot be compared to the activation of host-signaling pathways, the change in the α -helix conformation between CSP-1, CSP-2 and CSP-3 might affect the dynamics of CSP interaction with the host membrane proteins.

Our results suggest the CSP-1 induced calcium response in GEC occurs primarily via the T2R14- $G_{\beta\gamma}$ - PLC_{β} pathway. The knock down of T2R14 in OKF6 cells led to a significant decrease in maximal (E_{max}) calcium mobilization upon treatment with CSP-1 and DPH. However, the EC_{50} values remain unchanged (Fig 3C and E). These results suggest CSP-1 as potent agonist for T2R14. Our findings are in alignment with the recent findings, in which *P. aeruginosa* QSM 4-dihydroxyquinolone or 4-hydroxy-2-heptylquinolone (HHQ) and 3-oxo-C12 HSL were shown to induce T2R14- $G_{\beta\gamma}$ - PLC_{β} dependent intracellular calcium mobilization in airway epithelial cells and platelets [9, 50]. In addition to characterizing T2R14 as a major target for *S. mutans* CSP-1 in GECs it is important to study the downstream signaling pathways affected by CSP-1 and T2R14 interaction. Thus, we examined activation of NF- κ B pathway by CSP-1. We observed time-dependent increase in the phosphorylation of NF- κ B signaling intermediates $IKK\alpha\beta$ and $IKB\alpha$ (Fig 5A-C). Interestingly the NF- κ B signaling induced by either CSP-1 or DPH is not inhibited

upon T2R14 knock down (Fig 5J-L). The results using signaling inhibitors of T2Rs also show a decrease in phosphorylation only upon PLC inhibition but not with $G_{\beta\gamma}$ inhibition (Figure S2A and B). Several studies have shown PKC dependent activation of NF- κ B pathway and PKC is a known downstream target for PLC β [42, 43, 51]. Studies have also reported Gq-PLC-PI3K mediated activation of NF- κ B signaling [52, 53] and a recent study involving rat pulmonary nociceptors showed T2R-PLC-PKC signaling mediated sensitization of transient receptor potential vanilloid receptor 1 (TRPV1) [54]. Hence, we believe CSP-1 mediated NF- κ B signaling in GEC might be regulated by PLC-DAG-PKC pathway or by Gq coupled GPCRs.

To examine the role of CSP-1 on oral innate immunity, we quantified the secretion profile of several cytokines in GEC supernatants. We observed an increase in cytokine/chemokine IL-6, IL-8 and TNF- α upon CSP-1 and DPH treatment (Fig 6C, D and F). The cytokines IL-2, IL-4, IL-10 were not affected by CSP-1. Our data reflects on recent studies in which higher levels of TNF- α and IL-8 were observed in children affected with caries as well as in oral epithelial cell cultures [55, 56]. TNF- α is highly expressed in taste buds and its signaling in these cells preferentially modulate bitter taste responses [57]. Interestingly, IL-8 and TNF- α secretion are shown to be dependent on $G_{\beta\gamma}$ subunit activation but not PLC β , NF- κ B or TLR signaling pathways. Further, the knock down of T2R14 resulted in decreased basal levels of IL-6, IL-8 and TNF- α . These are recovered only with PMA but not with CSP-1 or DPH. Previously, regulation of IL-8 is shown to be dependent on several transcriptional factors such as NF- κ B, CREB, AP-1, NF-1L6 in human bronchial epithelial cells treated with *P. aeruginosa* [58]. Currently there exists no direct evidence of T2R mediated transcriptional regulation of AP-1, CREB or NF- κ B; however, a recent study on mice suggested that AP-1 transcription factors (c-Fos, Fosb and c-Jun) play a key role in maintaining taste cell proliferation and taste bud structures [59]. The data from our study suggest CSP-1 and T2R14 mediated cytokine secretions can occur via AP-1,

NFAT or CREB and PMA induced cytokine secretions are mediated by NF- κ B signaling. We assume that the knock down of T2R14 might alter one or many of the above mentioned AP-1 transcription factors leading to the impairment of IL-6, IL-8 and TNF- α secretion. Similar to IL-8 and TNF- α we also observed an increased secretion of IL-6 with CSP-1, DPH and PMA (Fig 6I). Intriguingly, the knock down of T2R14 completely abolished IL-6 levels and these were not recovered upon treatment with any of the above-mentioned compounds. We believe IL-6 secretion to be independent of T2R14 since its secretion is primarily dependent on NF- κ B signaling; however a knock down of T2R14 might interfere with signaling pathways that are responsible for IL-6 secretion. One possibility is the interference of T2R14 knock down on transcription factor NF-IL6, which along with NF- κ B, is a known transcriptional regulator for IL-6 [60]. This adds another layer of complexity in deciphering T2R14 mediated immune responses. These data indicate activation of multiple signaling pathways leading to a broad spectrum of innate immune responses in GECs. Studies have shown that alternate $G_{\beta\gamma}$ signaling led to an activation of PI3K and its downstream signaling molecules such as PKB/Akt, src-kinase, ERK [61] and Rho family GTPase [62]. These findings collectively suggest that in GECs CSP-1 mediated activation of T2R14 can lead to several signaling outcomes such as T2R14- $G_{\beta\gamma}$ – PLC β -IP $_3$ -Ca $^{2+}$ and T2R14- $G_{\beta\gamma}$ –PI3K-PKB/Akt/MAPK pathway. The later signaling pathway however, is yet to be studied.

Gingival tissue inflammation is followed by the infiltration of immune cells such as neutrophils and macrophages [63]. To investigate whether CSP-1 primed GECs recruit immune cells, we used differentiated HL-60 cells (dHL-60) as a model for examining immune cell infiltration. Our results indicate that CSP-1 treated GEC cells were better at attracting dHL-60 cells than the untreated cells (Fig 7B). This effect was abolished when GECs were treated with $G_{\beta\gamma}$ or PLC β inhibitors and the T2R14 antagonist 6-MF (Fig 7B). We also examined the

migratory effect of CSP-1 treatment on dHL-60 cells. Our results indicate that CSP-1 alone did not influence dHL-60 cell migration. We believe that the migratory effect of dHL-60 cells is caused by CSP-1 conditioned GEC secretions. Our cytokine analysis showed an increased secretion of IL-8 upon treatment with CSP-1, DPH and PMA only in MOCK cells (Fig 6J). Previously, IL-8 is shown to be a potent chemoattractant for neutrophils [64]. It is possible that CSP-1 mediated T2R14 activation facilitates IL-8 secretion, which in turn influences neutrophil migration. Our data supports a recently published study in which mouse treated with *S. pneumonia* CSP-1 have increased number of mast cells and this effect is dependent on mouse GPCR (Mrgprb2) [6]. Our results suggest a novel role for T2R14 in oral mucosa that involves recruitment of immune cells in response to chemoattractants.

In summary, our findings identify *S. mutans* CSP-1 as first bacteria derived peptide ligand for the T2R family. The novel CSP-1/T2R14 interaction is responsible for mediating innate immune responses in oral epithelial cells. The results from this study add to a very limited pool of knowledge regarding the role of QSM-host interaction in defining the outcome of oral diseases. Future studies using infection models will be useful to decipher the nature of CSP-1 and T2R14 interactions to enable use of T2Rs as potential therapeutic targets.

Supplementary material

This manuscript contains supplementary information consisting of three figures.

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Author contributions

P.C. and M.M.R designed and directed the study; M.M.R, N.S and A.B.Y conducted the experiments and analyzed the data. B.T.R designed CRISPS/Cas9 probes. All authors contributed to writing of the manuscript.

Figure legends:

Figure 1: Expression analysis of GPCRs, TAS2Rs and TLRs in GECs. A: Heat map of

highly expressed GPCRs in OKF6 and TIGK cells. **B:** Heat map of *TAS2Rs* and *TLRs*. The signal intensities were normalized using *RPS23* transcript. Epithelial cell markers (*KRT5* and *KRT14*) were used as a reference in the analysis. The arrow represents *TAS2R14*. The heat maps were generated using ggplots in Rstudio version 1.2.5001. One-way Anova using multiple comparison was performed to calculate the statistical significance (** $p < 0.001$). **C and D:** The OKF6 and TIGK total cell lysates were used to quantify T2R14 and T2R39 protein expression by western blot analysis as described in the methods. Briefly, the lysates were probed using primary rabbit polyclonal anti-T2R14, anti-T2R39 antibody (1:1000), mouse monoclonal anti β -actin antibody (1:25,000), secondary anti-rabbit HRP antibody (1:5000) and anti-mouse HRP antibody (1:25000). The corresponding molecular weights of T2R14, T2R39 and β -actin were shown in parenthesis.

Figure 2: Intracellular calcium mobilization in GECs in response to *S. mutans* CSPs.

A: Multiple sequence alignment of *S. mutans* CSPs used in this study. **B and C:** OKF6 and TIGK cells are treated with increasing concentrations of synthetic CSP-1 (10 μ M-70 μ M) and the intracellular calcium mobilization was measured using Fluo-4NW fluorescent dye. The EC₅₀ values were obtained by generating a non-linear regression dose response curve for each cell line using graph pad prism 7.0 **D-G:** The intracellular calcium mobilized is measured in GECs treated with a single concentration (50 μ M) of *S. mutans* synthetic peptides CSP-1, CSP-2 and CSP-3 with the corresponding raw calcium traces for each of the treatments are shown alongside. Cells treated with the assay buffer were used as a baseline

for comparison. One-way Anova using Tukey's multiple comparison analysis was performed to measure the significance between buffer and CSP treated samples (* $p < 0.05$, and **** $p < 0.0001$). **H:** Multiple sequence alignment of CSP-1 and C-terminal deleted peptides. **I-L:** The concentration (10 - 70 μM) responses of CSP-1 (red curve), C- terminal amino acid deletion peptides CSP-1 (ΔK21 , green curve) and CSP-1 (ΔG20 , ΔK21 blue curve) treated OKF6 and TIGK cells. The intracellular calcium mobilized was measured and EC_{50} values were calculated by non-linear regression curves. Cells treated with buffer were used as a baseline and the corresponding raw calcium traces are shown. **M and N:** The intracellular cAMP production is measured in OKF6 cells upon treatment with CSP-1 (10 - 70 μM) and β_2 -AR agonist isoproterenol. The absolute cAMP levels are measured using a cAMP standard and were analyzed using non-linear regression dose response curves. The data represent SEM of ≥ 3 experiments performed in triplicates.

Figure 3: Characterization of CSP-1 and T2R14 dependent calcium signaling in OKF6 and in heterologous cells overexpressing T2R14. A: Characterizing CRISPR-Cas9 mediated T2R14 knock down (KD) by T7EI mismatch assay. 100 ng of genomic DNA from OKF6 WT, MOCK, and T2R14 KD cells was amplified using *TAS2R14* primers. The PCR products were denatured and re-annealed in a thermal cycler. The re-annealed products were digested using 2U of T7EI for 60 min at 37°C and analyzed using 1.7% agarose gel electrophoresis. The size of the *TAS2R14* amplicon (908 bp) in WT, MOCK and the smaller fragments of lower bp in the *TAS2R14* KD cells are indicated by arrows. **B:** T2R14 protein knock down is confirmed by western blot analysis using total protein lysates isolated from OKF6 WT, MOCK and, T2R14 KD cells as described in methods. **C-F:** Concentration

dependent intracellular calcium mobilization for DPH (0.03 – 2 mM), CSP-1 (10 – 70 μ M) in WT (green) MOCK (blue) and T2R14 KD (red) cells with representative calcium raw traces shown. **G and H:** Characterization of 6-MF as inhibitor of CSP-1 induced T2R14 signaling in OKF6 cells. Competition calcium mobilization assay is performed in OKF6 cells co-treated with CSP-1 (50 μ M) and T2R14 antagonist 6-MF (1.25 – 100 μ M). The IC₅₀ value was calculated by nonlinear regression analysis and representative raw calcium traces were shown. **I:** Western blot analysis of total protein lysates of HEK293T cells overexpressing T2R14. The lysates were probed using primary rabbit polyclonal anti-T2R14, mouse monoclonal anti β -actin antibody (1:25,000), secondary anti-rabbit HRP antibody (1:5000) and anti-mouse HRP antibody (1:25000). **J-M:** Concentration dependent intracellular calcium mobilization for CSP-1 (10-70 μ M) and T2R14 agonist DPH (0.03- 2 mM) in HEK293T cells overexpressing T2R14. The EC₅₀ values were calculated using non-linear regression analysis using graph pad prism 7.0 with representative raw calcium traces shown. The data represents SEM of ≥ 3 independent experiments performed in triplicates.

Figure 4: Characterization of effect of PLC β and G $\beta\gamma$ inhibitors on calcium mobilization in CSP-1 and T2R14 agonist DPH treated GECs. A and C: OKF6 and TIGK cells are treated with CSP-1 (50 μ M), DPH (500 μ M) and ATP (1 μ M) alone or in combination with 5 μ M of U73122 (PLC β inhibitor) and its analogue U73343 and 10 μ M gallein (G $\beta\gamma$ inhibitor). The intracellular calcium mobilized was measured using Fluo-NW fluorescent dye. **B and D:** The representative raw calcium traces for the respective treatments in A and C. The bar graphs were generated using graph pad prism 7.0. One-way Anova using Tukey's multiple comparison was performed to calculate the statistical

significance (** $p < 0.001$). The data represents SEM of ≥ 3 independent experiments performed in triplicates.

Figure 5: *S. mutans* CSP-1 induced activation of NF- κ B signaling pathway in WT, MOCK and T2R14 KD OKF6 cells. Western blot analysis of total protein extracts collected and separated on 10% SDS-PAGE. The blots were probed with phospho and total antibodies for IKK α / β , I κ B α (1:1000) and HRP- Goat anti-rabbit or mouse (1:3000). **A:** OKF6 cells treated with CSP-1 (50 μ M) for different time points (0-60 mins). **B and C:** Densitometry analysis of phosphorylation of IKK α / β ^{Ser176/180} and I κ B α ^{Ser32} are represented by quantifying the band intensity using imageJ analysis software. **D and G:** Cells treated with CSP-1 (50 μ M), DPH (500 μ M), PMA (100 nM) alone or in combination with I κ B α phosphorylation inhibitor BAY 11-7082 (10 μ M). **E, F, H and I:** Densitometry analysis of phosphorylation of IKK α / β ^{Ser176/180}, I κ B α ^{Ser32} normalized to corresponding total proteins. **J:** MOCK and T2R14 KD OKF6 cells treated with CSP-1 (50 μ M), DPH (500 μ M) and PMA (100 nM). **K and L:** Densitometry analysis of phosphorylation of IKK α / β ^{Ser176/180} and I κ B α ^{Ser32} normalized to corresponding total proteins. The band intensities were measured using imageJ analysis software. The respective bar graphs were generated using graph pad prism 7.0. One-way Anova using Tukey's multiple comparison was performed to calculate the statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The data represents SEM of ≥ 3 independent experiments performed in triplicates.

Figure 6: Quantification of cytokines secreted in OKF6 WT, MOCK and T2R14 KD OKF6 cells by Meso Scale multiplexing ELISA. **A-F:** OKF6 WT cells were treated for 18 hours using *S. mutans* CSP-1 (50 μ M), U73122 (5 μ M), Gallein (10 μ M), BAY 11-7082 (10 μ M), Pepinh-MyD inhibitor (40 μ M), DPH (200 μ M), PMA (100 nM) and LPS (10 μ g/ml). **G-L:** The MOCK and T2R14 KD OKF6 cells are treated with CSP-1, DPH and PMA using the above-mentioned concentrations. The supernatants were collected, filtered using 0.25 μ M nylon filter to remove cell debris and the following cytokines were analyzed (IL-2, IL-4, IL-6, IL-8, IL-10 and TNF- α) using a recombinant standard by a Meso Scale reader. The representative graphs were generated using graph pad prism 7.0. One-way Anova using Tukey's multiple comparison was performed to calculate the statistical significance and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ was considered significant. The data represents SEM of ≥ 3 independent experiments performed in triplicates.

Figure 7: Effect of migration of differentiated HL-60 (dHL-60) cells treated with conditioned medium from OKF6 cells. **A:** Schematic display of xCELLigence migration chamber showing direction of migration of dHL-60 cells towards OKF6 cell culture supernatant. **B:** The graphs shows the increase in migration of dHL-60 cells over a period of 24 hours upon incubation with conditioned medium from OKF6 cells treated with CSP-1 (50 μ M) alone or in combination with U73122 (5 μ M), gallein (10 μ M), 6-MF (30 μ M) and fMLP (100 nM). **C:** The representative raw migration traces depicting the cell index over a period of 24 hours. **D:** dHL-60 cells migration upon incubation with CM from MOCK and T2R14 KD OKF6 cells treated with CSP-1 (50 μ M), DPH (200 μ M) and PMA (100 nM). **E:** The representative raw migration traces depicting the cell index over a period of 24 hours. One-

way Anova using Tukey's multiple comparison was performed to calculate the statistical significance and a * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ was considered significant. The data represents SEM of ≥ 3 independent experiments performed in triplicates.

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Figure 1

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Figure 1

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Figure 7

Supplementary figures

Figure S1

Figure S2

Figure S1: Characterization of T2R39 activity using heterologous cells. **A:** Total cell lysates from HEK293T cells overexpressing T2R39 were used to quantify T2R39 protein expression by western blot analysis. The lysates were probed using primary rabbit polyclonal anti-T2R39 antibody (1:1000), mouse monoclonal anti β -actin antibody (1:25,000), secondary anti-rabbit HRP antibody (1:3000) and anti-mouse HRP antibody (1:25000). **B:** The intracellular calcium mobilization is measured in HEK293T-T2R39 cells treated with CSP-1 (50 μ M) and known T2R39 agonists, quinine (1 mM) and pentagalloyl glucose (100 μ M). Cells treated with the assay buffer

Supplementary figures

were used as a baseline for comparison. One-way Anova using Tukey's multiple comparison analysis was performed to measure the significance between buffer and CSP treated samples (** $p < 0.02$).

Figure S2: Characterization of NF- κ B signaling pathway upon inhibition of T2R downstream signaling. Western blot analysis of total protein extracts separated on 10% SDS-PAGE. The blots were probed with phospho and total antibodies for IKK α/β , I κ B α (1:1000) and HRP- Goat anti-rabbit or mouse (1:3000) to quantify IKK α/β ^{Ser176/180} and I κ B α ^{Ser32} protein phosphorylation. **A and B:** OKF6 cells were treated with CSP-1 (50 μ M), DPH (500 μ M) alone or in combination with inhibitors U73122 (5 μ M), gallein (10 μ M), and quinazoline (10 μ M).

Supplementary figures

Figure S3

Figure S3: Cell surface expression of CD14 and CD11b in differentiated and undifferentiated HL-60 cells. HL-60 cells are treated with 1.3% DMSO for 7 days and were allowed to differentiate into neutrophil-like cells. The cells were labeled with fluorescent-tagged CD14 (APC) and CD11b (FITC) antibodies and IgG. **A:** The graph represents the percentage of cells expressing the CD surface marker in each gate obtained by using quadrant gating. **B:** Quadrant gating method was used to determine the single-positive and double-positive cells. One-way Anova using Tukey's multiple comparison was performed to calculate the statistical significance and a $*p < 0.05$ was considered significant. The data represents SEM of ≥ 3 independent experiments performed in triplicates.

Supplementary figures