

**Bitter taste receptors (T2Rs) role in oral innate immunity and their
characterization in extracellular vesicles (EVs)**

By

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Doctor of Philosophy

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DISCLAIMER

This modified sandwich thesis comprises five multi-authored manuscripts. Four of these manuscripts contain a majority of original work contributed by the author of this thesis, as demonstrated by his first-authorship. The author of this thesis was listed as second author in the book chapter titled “Chemistry and Biological Mechanisms of Peptides that Modulate Taste”. Individual author contributions are listed at the beginning of each chapter.

THESIS ABSTRACT

Bitter taste receptors (T2Rs) belong to the G protein-coupled receptor family. There are 25 T2Rs, which are primarily expressed in oral tissues and mediate bitter taste perception. Recent studies identified bacterial quorum-sensing molecules (QSMs) as ligands for T2Rs expressed in airways. Most of the known QSM ligands of T2Rs were derived from *Pseudomonas aeruginosa*. These QSM ligands are mainly acyl homoserine lactone (AHL) such as C12-AHL, C8-AHL and quinolones such as HHQ and NHQ. However, till now there are no studies on T2R's interaction with Gram-positive bacterial QSMs in gingival epithelial cells (GECs). The cariogenic bacterium, *Streptococcus mutans*, secretes competence stimulating peptides (CSPs) as QSMs. The first part of my thesis elucidates the role of T2R and *S. mutans* CSPs interaction in mediating GEC innate immune responses. The second part of my study, characterizes the physiological role of T2R14 in Gram-positive bacterial internalization and survival. The final chapter, reports the characterization of T2Rs expressed in extracellular vesicles (EVs) isolated from cultured cells and saliva.

Transcriptomic and western blot analyses identify T2R14 to be highly expressed in GECs. My results suggest that only *S. mutans* CSP-1 induces robust intracellular calcium mobilization in GECs, compared to CSP-2 and CSP-3. The knock down of T2R14 by CRISPR-Cas9 revealed CSP-1 mediated cytokine regulation (IL-6, IL-8 and TNF- α) and modulation of dHL-60 migration. Interestingly, CSP-1 and bitter agonist mediated T2R14 activation in GECs inhibits growth of *Staphylococcus aureus* but not for *S. mutans*. Further, immuno transmission electron microscopy (immuno-TEM) and ELISA show presence of T2R4 and T2R38 in EVs. In conclusion, this work provides novel insights into the roles of T2Rs in oral tissues and in host-microbe interactions.

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There is a saying that “*a man travels the world in search of what he needs, and returns home to find it*”. I am blessed with an awesome family, who has supported me in every stretch of life. First I would like to express my love and thanks to my mom and dad for their unconditional love. I am blessed with an amazing wife (Anjali) who happened to be my colleague as well. Her constant support and encouragement are an immense strength to me and kept me afloat throughout the program. I feel that words might fall short to describe her; she has literally been my strength and stay all these years.

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DEDICATION

I would like to dedicate this thesis to Apple & Charlie (we miss you).

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LIST OF ABBREVIATIONS

2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein-Acetoxymethyl Ester (BCECF-AM)

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

2-nonyl-4(1H)-quinolone (NHQ)

4,5-dihydroxy-2,3-pentanedione (DPD)

4-hydroxy-2-alkylquinolines (HAQs)

4-hydroxy-2-heptylquinoline (HHQ)

6-Methoxyflavone (6-MF)

Adenosine triphosphate (ATP)

Adenylyl cyclase (AC)

Amiloride-sensitive epithelial sodium channel (ENaC)

Angiotensin II type 1 receptor (AT1R)

Antimicrobial peptide (AMP)

ATP-binding cassette (ABC)

Beef protein alcalase hydrolysates (BPAH)

Beef protein chymotrypsin hydrolysates (BPCH)

Bitter taste receptors (T2Rs)

Brain heart infusion (BHI)

Calcium (Ca^{2+})

Carboxymethyl-lysine (CML)

Chemokine receptors 4 and 7 (CXCR4, CCR7)

Cholecystokinin (CCK)

Chronic rhinosinusitis (CRS)

Ciliary beat frequency (CBF)

Cluster of differentiation 36 (CD36)

Competence stimulating peptides (CSPs)

Conditioned medium (CM)

Constitutively active mutant (CAM)

Cyclic AMP (cAMP)

Cyclic nucleotide (cNMP)

Cystic Fibrosis (CF)

Dextromethorphan (DXM)

Diacyl glycerol (DAG)

Dimethyl sulphoxide (DMSO)

Diphenhydramine (DPH)

Dulbecco's modified Eagle medium (DMEM)

Endoplasmic reticulum (ER)

Enteroendocrine cells (EEC)

Enterohaemorrhagic *E. coli* (EHEC)

Epidermal growth factor (EGF)

E-tongue (Alpha MOS)

Eukaryotic translation initiation factor 2 α (eIF2 α)

Extracellular loop 2 (ECL2)

Extracellular signal-regulated kinase (ERK)

Extracellular vesicles (EVs)

Fetal bovine serum (FBS)

Fluorescent Imaging Plate Reader (FLIPR)

Formyl peptide receptors (FPRs)

Free fatty acid receptors (FFAR)

G protein-coupled receptors (GPCRs)

G $\beta\gamma$ inhibitor (Gallein)

G α t (transducin)

Gingival epithelial cells (GECs)

Gingival solitary chemosensory cell (GSC)

Glucagon-like peptide 1 (GLP-1)

Glyoxal-derived lysine dimer (GOLD)

GPCR kinases (GRKs)

G α gustducin (G α gust)

Ham's F12 (F12)

Human β -defensin-2 (hBD-2)

Inositol-1,4,5-triphosphate (IP3)

Intracellular calcium mobilization (Ca²⁺)_i

IP₃ binds to type III IP₃ receptor (IP3R)

Keratinocyte growth medium -2 (KGM-2)

Lipopolysaccharide (LPS)

Luria-Bertani (LB)

Mast-cell specific GPCR (MRGPRX2)

Methylpiperidino pyrazole (MPP)

Microvesicles (MVs)

Mitogen activated protein kinase (MAPK)

N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL or C12-AHL)

N-acyl homoserine lactone (AHL)

Natural killer cells (NK)

Nitric oxide (NO)

N α , N α -bis (carboxymethyl)-L-Lysine (BCML)

Pattern recognition receptors (PRRs)

Peptide YY (PYY)

Phorbol 12-myristate 13-acetate (PMA)

Phosphate buffered saline (PBS)

Phospho lipase C inhibitor (U73122)

Phospholipase C- β 2 (PLC β 2)

Polymorphonuclear leucocytes (PMNs)

Promyeloblast cells (HL-60)

Protein kinase A (PKA)

Quorum-sensing molecules (QSMs)

Solitary chemosensory cells (SCCs)

Somatostatin receptor 3 (SSTR3)

Taste receptors cells (TRCs)

Transmission electron microscopy (TEM)

Toll-like receptors (TLRs)

Transient receptor potential melastatin 5 channel (TRPM5)

Tris acetate EDTA (TAE)

Tris-buffered saline (TBS)

Wild type (WT)

γ -aminobutyric acid (GABA)

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Chapter 1: Includes work from the two book chapters:

Pharmacology of T2R Mediated Host-Microbe Interactions.

Medapati MR, Bhagirath A, Singh N and Chelikani P.

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Chapter 4: Bitter taste receptor T2R14 detects quorum sensing molecules from cariogenic *Streptococcus mutans* and mediates innate immune responses in gingival epithelial cells.

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Chapter 6: Characterization of GPCRs in Extracellular Vesicle (EV).

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CHAPTER 1

1. INTRODUCTION

1.1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) form the largest druggable receptor superfamily in the human genome. Chemosensory GPCRs that include both olfactory and taste receptors account for over half of the GPCR superfamily (Isberg et al., 2017). Humans are capable of sensing five basic tastes: salt, sour, umami, sweet and bitter (Adler et al., 2000; Baumer-Harrison et al., 2020; Chandrashekar et al., 2000; Tu et al., 2018). Fat and kokumi (thickness of food) tastes are considered as possible additions (DiPatrizio, 2014; Laugerette et al., 2005; Lewandowski et al., 2016). Sweet, umami and bitter taste are mediated by GPCRs whereas ion channels ENaC and OTOP1 mediate the salt and sour tastes (Baumer-Harrison et al., 2020; Gilbertson et al., 1992; Heck et al., 1984; Tu et al., 2018).

1.2. Taste sensation

Taste is an important determinant of food choices. Both the quality and the perceived intensity of sensory experiences help to determine the palatability of the food. Taste sensation is initiated when a tastant molecule (taste inciting substance) comes in contact with taste proteins (receptors and ion channels) located on taste cells in the oral cavity. The perception of taste is complex involving both sensory and neural mechanisms. Further, taste perception varies between individuals and influences food preferences and hence nutritional choices (Bartoshuk, 2000). While it is understood that the spectra of molecular detection via these taste proteins is quite broad, they also exhibit significant diversity in structure and functional roles, making them important therapeutic targets. The pharmacology, physiology and signaling of different taste proteins

(receptors and ion channels) are extensively reviewed (Behrens, 2021; Dutta Banik et al., 2021; Hichami et al., 2021; Servant et al., 2021; Sharma et al., 2021)

The taste receptor cells (TRCs) are predominantly located in taste buds on the papillae of the tongue. The papillae on the tongue appear as small pink bumps, and are classified into four main types: the mushroom like fungiform papillae, which are concentrated on the front of the tongue; the leaf like foliate papillae, which are concentrated on the sides of the tongue; the randomly distributed small filiform papillae and the large circumvallate papillae located at the back of the tongue (**Figure 1.1**). The papillae contain the taste buds and are supplied by one of the three afferent nerves: the chorda tympani from the facial nerve, the glossopharyngeal nerve and the vagus nerve. These nerves relay the sensory input for the taste buds to the brain. The information obtained is interpreted in the form of the nutrient content of food.

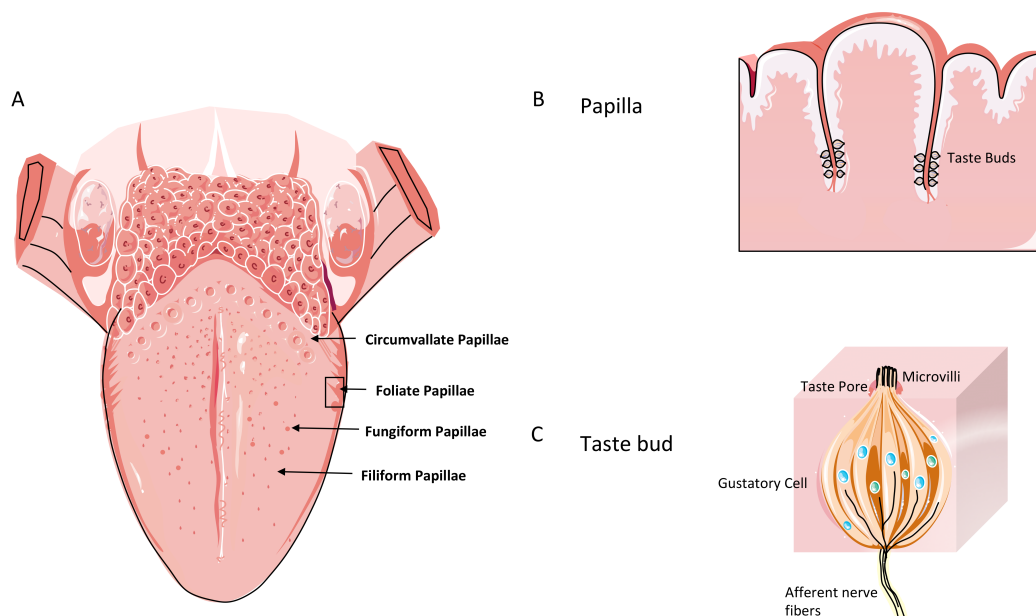


Figure 1. 1: Morphology of taste structures on the human tongue.

A. The human tongue consists of four main types of papillae that appear as raised bumps on the surface of the tongue. Depending on their location and shape they are classified as circumvallate, fungiform, filiform, and foliate papillae. **B.** Each papilla harbors several taste buds which can

sense taste qualities. C. A taste bud is made up of several taste receptor cells. The apical end of the taste bud consists of microvilli and opens into the oral cavity through the taste pore. Taste receptor cells are specialized cells that can function as chemoreceptors and transduce chemical stimuli into signals for the afferent nerve fibers that supply it. The figure was drawn using the Adobe Illustrator software. Reproduced with permission from Royal Society of Chemistry.

1.2.1. Mechanism of taste perception

Taste perception is a complex process and involves both sensory and neural mechanisms. At the receptor level, sweet taste is sensed by a heterodimer of taste type I receptors referred to as T1R2 and T1R3 receptors (Assadi-Porter et al., 2010), whereas umami is sensed by a heterodimer of T1R1 and T1R3 (X. Li et al., 2002; Nelson et al., 2002). Bitter taste is sensed by bitter taste receptors also referred to as taste type II receptors or T2Rs (Chandrashekar et al., 2000) (**Figure 1.2**). Originally, two models have been proposed based on biochemical and electro-physiological studies for sweet transduction in taste cells. The first is a GPCR-G α s-cAMP pathway (Houpt et al., 1996; Striem et al., 1991) in which, sucrose and other sugars lead to activation of G α s-T1R heterodimers. Receptor-activated G α s activates adenylyl cyclase to generate cAMP. Increased cAMP increases cation influx through cyclic nucleotide (cNMP) gated channels directly or acts indirectly to activate protein kinase A. Protein kinase A phosphorylates a basolateral K⁺ channel, leading to closure of the channel, depolarization of the taste cell, voltage-dependent Ca²⁺ influx, and neurotransmitter release (Striem et al., 1991). The second mechanism is a GPCR- G α - gustducin /G $\beta\gamma$ -IP₃ pathway (Bernhardt et al., 1996). It is suggested that artificial sweeteners can also bind and activate T1R heterodimers followed by activation of either the α subunit of G-gustducin or by G $\beta\gamma$ subunits.

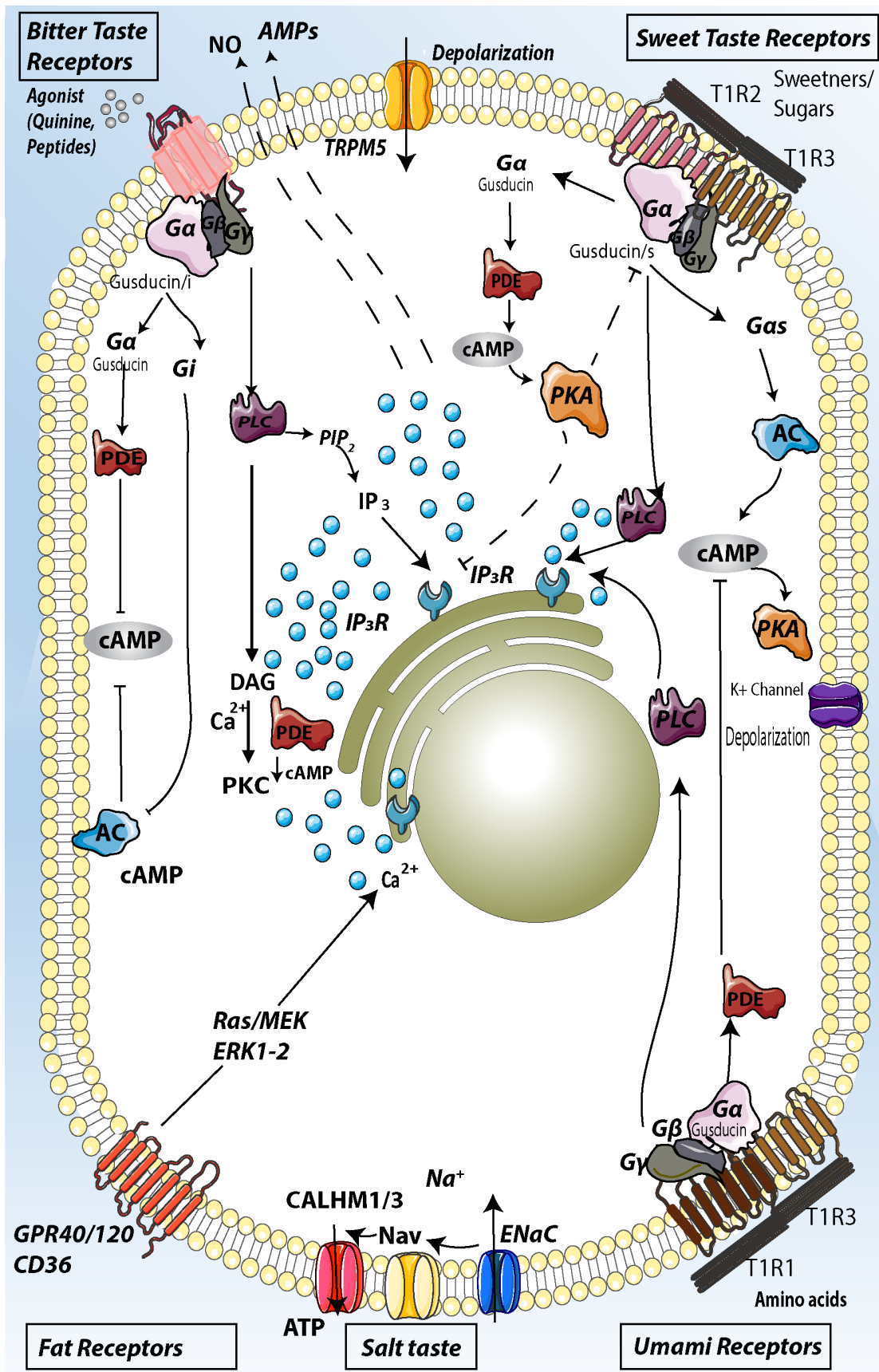


Figure 1. 2: Cellular mechanisms of signaling by taste sensing proteins.

Bitter, sweet and umami tastes are perceived by GPCRs on predominantly type II taste receptor cells. Sweet taste signaling is mediated by a heterodimeric complex of T1R2 and T1R3. Activation of sweet receptors (T1R2 + T1R3) via sweetener and sugars leads to activation of multiple downstream effectors in a cell and tissue specific manner. These effectors include phospholipase C isoform β_2 (PLC β_2) and adenylyl cyclase (AC) the activation of which leads to release of calcium (Ca^{2+}) and cyclic AMP (cAMP) generation. Activation of AC can inhibit basolateral K^+ channels through cAMP activated protein kinase A (PKA). Umami taste signaling requires heterodimerization of T1R1 and T1R3. Upon binding of an agonist such as amino acids, $\text{G}_{\beta\gamma}$ dissociates from the heterotrimeric G-protein complex to activate PLC β_2 . PLC β_2 then generates the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to type III IP_3 receptor (IP_3R) to elicit release of Ca^{2+} from intracellular stores and subsequent activation of transient receptor potential melastatin 5 (TRPM5) channel, which results in taste cell depolarization. Alternatively, G_α -gustducin activates phosphodiesterase (PDEs) to decrease intracellular cAMP. Bitter taste sensation is mediated by binding of bitter ligands to T2Rs, triggering downstream activation of PLC β_2 . PLC β_2 then causes IP_3 production, activating the IP_3R on the endoplasmic reticulum to stimulate the release of Ca^{2+} . Calcium release from the endoplasmic reticulum activates the TRPM5 channel, which depolarizes the cell membrane. Increased intracellular Ca^{2+} also activates PDE that causes the reduction of intracellular cAMP levels. Increase in intracellular Ca^{2+} by activation of T2Rs has been shown to activate innate immune responses such as generation of nitrous oxide (NO) and antimicrobial peptides (AMPs). Salt taste sensation is mediated independent of Ca^{2+} signaling. The amiloride-sensitive epithelial sodium channel (ENaC) is suggested as the sensor for sodium taste. ENaC-mediated entry of Na^+ drives the voltage-dependent ATP release via the CALHM1/3-channel to elicit the sodium taste (Nomura et al., 2020). The fat taste sensation is modulated by GPCR 40/120 and cluster of differentiation 36 (CD36) receptors. Activation of these receptors has been shown to be mediated via increase in the intracellular Ca^{2+} levels as well as the mitogen activated protein kinase (MAPK) and the extracellular signal-regulated kinase (ERK) pathway (Subramaniam et al., 2016). The schematic was drawn using the Adobe Illustrator software. Reproduced with permission from Royal Society of Chemistry.

Activated $G\alpha$ -gustducin or released $G_{\beta\gamma}$ can activate phospholipase C- β_2 ($PLC\beta_2$) to generate inositol-1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG); IP_3 and DAG elicit Ca^{2+} release from internal stores, leading to depolarization of the TRC and neurotransmitter release (Bernhardt et al., 1996). These two pathways can coexist in same taste cells. Sweet-responsive taste cells from the circumvallate papillae of rat had an influx of Ca^{2+} in response to sucrose, whereas the artificial sweeteners saccharin and SC45647 elevated Ca^{2+} via release from internal stores (Bernhardt et al., 1996). Stimulation of umami receptor heterodimer T1R1 + T1R3 by stimuli activates $G_{\beta\gamma}$ that leads to activation of $PLC\beta_2$ (Wei He et al., 2004; Sue C Kinnamon, 2009). $G\alpha$ -gustducin is related to $G\alpha$ -transducin, which is also expressed in taste buds. Both α -gustducin and α -transducin activate PDEs, which results in a decrease in intracellular cAMP concentration. Furthermore, biochemical studies have shown that glutamate decreases cAMP concentration in taste buds, and the response is potentiated by 5'-nucleotides (Abaffy et al., 2003). Experiments performed on rat circumvallate taste buds suggest that cAMP modulates umami signaling (Abaffy et al., 2003). $G\alpha_i$ is abundantly expressed in taste buds, so it is hypothesized that $G\alpha_i$ may couple to T1R1/T1R3 in circumvallate and foliate taste buds (Kusakabe et al., 1998; Eduardo Sainz, Margaret M. Cavenagh, et al., 2007).

The human genome encodes 25 T2Rs localized as clusters on chromosomes 5p15, 7q31, and 12p13 (Conte et al., 2002; Shi et al., 2003). Our group has previously reported a diversity in the sequence and the number of T2Rs between different species (Nisha Singh et al., 2011). The canonical bitter signal transduction pathway starts with the binding of a bitter ligand to a T2R. This induces a conformational change in the T2R resulting in the activation of its cognate heterotrimeric G protein and signaling occurs as shown in **Figure 1.2**. In taste cells, the functional heterotrimeric G protein is composed predominantly of $G\alpha$ -gustducin ($G\alpha_{\text{gust}}$), $G\beta_3$ and $G\gamma_{13}$ subunits (Caicedo et al., 2003; Chandrashekar et al., 2000; Huang et al., 1999; Rössler

et al., 2000). Recent studies have shown that T2Rs can couple to multiple heterotrimeric G proteins in a tissue and cell specific manner (Donghwa Kim et al., 2017). The downstream effects of T2R activation depend largely on the tastant binding to the receptor. Structure-function studies on T2Rs have shown that while some T2Rs recognize a wide range of ligands, others like T2R38 are very specific and selective for ligands (Wolfgang Meyerhof et al., 2009).

It is now well-established through studies in our lab and others that, T2Rs are expressed in tissues not directly related to the detection of tastants, which strongly suggests their role in the monitoring of internal environments and in mitigating chronic diseases (Behrens et al., 2011; Foster et al., 2014; Jaggupilli et al., 2017; S. J. Lee et al., 2019; Shaik et al., 2016). Amongst others, T2Rs have been identified in ‘solitary’ chemosensory cells throughout the respiratory system in the epithelium, as well as in the airway smooth muscle cells, where they are shown to mediate airway reflexes and bronchodilation (Deepak A Deshpande et al., 2010; Finger et al., 2003; Tizzano et al., 2011).

1.3. T2R classification

T2Rs were previously classified under frizzled receptor family, however, recent classification by International Union of Basic and Clinical Pharmacology (IUPAC) has annotated T2Rs as a separate class that is denoted by “T” (Pándy-Szekeres et al., 2018). According to HUGO nomenclature the genes that code for T2Rs are denoted as TAS2R followed by the number of the receptor (Braschi et al., 2019). T2Rs are known to recognize diverse and a wide range of compounds. Similar to other GPCRs, the molecular structure of T2Rs consists of seven transmembrane helices, 3 extracellular loops, 3 intracellular loops, short N-terminus and a C-terminus. From an evolutionary standpoint, T2Rs have been hypothesized to have evolved as sentinels to detect noxious substances in food (Behrens et al., 2021; Shi et al., 2003).

1.3.1. T2R G protein selectivity and signaling

Several studies have used co-expression systems where both T2R and the G proteins are co-expressed to analyze receptor activation. The idea that bitter and sweet taste transduction occurs via the GTP-binding proteins was first conceptualized in 1992 (S. C. Kinnamon et al., 1992). Studies that followed have identified gustducin, as a unique G protein α -subunit expressed primarily in TRCs, that is required for bitter taste signaling in vivo (Wong et al., 1996). In a classic study, (Ueda et al., 2003) determined that the last 37 to 44 C-terminal amino acids of gustducin were critical for T2R coupling by creating a series of chimeric G proteins from G α 16 (which couples many GPCRs to calcium signalling) that incorporated different lengths of gustducin-specific C-terminal amino acid sequences. Resulting G16/gust44 and G16/gust37 chimeras effectively coupled T2Rs to calcium signaling, whereas chimeric G proteins with shorter terminal segments did not. Replacing the terminal 44 amino acids of G α 16 with the equivalent domains of G α t (transducin) and G α i also produced functional chimeras (Ueda et al., 2003). Besides gustducin and its close cousin transducin, several members of the Gi/o family have been demonstrated to functionally couple to T2Rs in vitro (Ozeck et al., 2004). Indeed, studies have shown selectivity of different G proteins to mT2R5, hT2R4, hT2R7, hT2R14, and hT2R43 and thus it is possible that binding of a ligand to a T2R can lead to biased downstream signalling in different tissues. All these T2Rs were able to couple with G α t, G α i, G α o but not with G α s, and G α q (E. Sainz et al., 2007). Finally, gustducin knockout mice have been shown to recognize bitter compounds in various physiological and behavioural assays (W. He et al., 2002). Therefore, the idea of T2R coupling to only G α gustducin is questionable.

Analyzing T2R activation also depends on the intracellular calcium detection methods employed (Freund et al., 2018; Jaggupilli et al., 2018). Two calcium-sensing dyes Fura-2 and

Fluo-4 are routinely used in T2R assays. Fura-2 is a ratiometric dye with an excitation ratio of 340/380 nm and emission around 500 nm. The Fluo-4 is a single wave calcium probe with excitation maxima of 490 nm and emission at 520 nm. The emission intensity in the Fluo-4 system depends on the levels of calcium bound to the dye (i.e. higher calcium released by cell leads to brighter signal intensity). Fura-2 is a high affinity calcium probe (K_d : 140 nM) whereas Fluo-4 is a low affinity calcium probe (K_d : 345 nM). The Fura-2 dye would be useful in accurately measuring resting calcium and minute calcium increases with a rapid saturation. In contrast, Fluo-4 dye can measure higher calcium spikes with lower saturation. The Fura-2 dye is used to measure global Ca^{2+} levels as well as live single cell imaging that uses a fluorescent microscope (Freund et al., 2018; I. Gopallawa et al., 2020). The Fura-2 dye coupled with microfluorimetry is useful in analyzing calcium changes in individual cells or a group of cells. On the other hand, Fluo-4 is mainly used in Fluorescent Imaging Plate Reader (FLIPR) based assays. This approach has been mainly used in determining the efficacy of bitter agonists, inverse agonists and antagonists or when screening novel bitter compounds (W. Meyerhof et al., 2010; Pydi et al., 2015; Pydi, Sobotkiewicz, et al., 2014). Apart from measuring the transient intracellular calcium mobilization several recent studies also measured the more stable and upstream secondary messengers such as inositol phosphate (IP)₁ and IP₃ along with calcium assays (Itoigawa et al., 2019; Pydi, Sobotkiewicz, et al., 2014). Interestingly, IP₃ assay has shown to be effective in measuring basal or constitutive activity of T2R4 mutants A90F and K270A, whereas the basal calcium levels in these mutant cells were comparable to that of wild type (WT) cells (Pydi, Sobotkiewicz, et al., 2014). GPCRs are known to activate a multitude of downstream signaling such as activation of GPCR kinases (GRKs) and β -arrestin recruitment (Wei et al., 2003). The activation of GRK and β -arrestin signaling pathways lead to receptor desensitization and downstream activation of mitogen-activated protein kinase (MAPK) pathways (DeWire et

al., 2007). T2R activation can also lead to the recruitment of arrestin (Woo et al., 2019) and the activation of MAPK (Ozeck et al., 2004).

1.4. CHALLENGES IN T2R MOLECULAR PHARMACOLOGY

Significant progress has been achieved over the past decade in characterizing the molecular pharmacology of T2Rs. However, there remain significant challenges, some of them are addressed in this section.

1.4.1. T2R ligand specificity

The experimental evidence from several studies reveals that T2Rs exhibit a substantial degree of agonist non-specificity. The bitter agonists identified thus far are known to activate multiple T2Rs and a single T2R is known to recognize several bitter molecules. For example, the bitter compound denatonium benzoate is known to activate 4 T2Rs (T2R4, T2R8, T2R10 and T2R13), caffeine is known to activate 5 T2Rs (T2R7, T2R10, T2R14, T2R43 and T2R46), chloroquine is known to activate 5 T2Rs (T2R3, T2R7, T2R10, T2R14, and T2R39) and the widely used bitter compound quinine is known to activate 9 T2Rs (T2R4, T2R7, T2R10, T2R14, T2R39, T2R40, T2R43, T2R44 and T2R46) (W. Meyerhof et al., 2010; Pydi, Bhullar, et al., 2012). Similar pattern of activation of multiple T2Rs are observed with QSMs and antibiotics. Recent studies using heterologous cells revealed activation of T2R4, T2R14 and T2R20 by AHLs and by broad-spectrum antibiotics such as tobramycin, levofloxacin and azithromycin (A. Jaggupilli et al., 2019). In airway epithelial cells, the AHLs and quinolones are shown to activate multiple T2Rs (T2R4, T2R10, T2R14, T2R16 and T2R38 (Freund et al., 2018; R. J. Lee et al., 2012b). Such broad spectrum T2R agonist activity poses significant challenges in delineating T2R specific physiological functions. In contrast, the lack of broad spectrum T2R blockers is a major challenge.

Over the past decade, T2R structure-function studies have identified bitter blockers, antagonists and inverse agonists. For example, α , α -bis (carboxymethyl)-L-Lysine (BCML), γ -aminobutyric acid (GABA) and peptides isolated from beef protein hydrolysates (ETSARHL and AGDDAPRAVF) were effective in blocking quinine and PQS mediated calcium responses with an IC_{50} concentration ranging from 50 nM to 150 μ M respectively (Freund et al., 2018; Pydi, Sobotkiewicz, et al., 2014; C. Zhang et al., 2018). The broadly expressed T2R14 receptor is blocked by plant flavonoids 4'-fluoro-6-methoxyflavonone, 6,3'-dimethoxyflavanone and 6-methoxyflavanone (Hariri et al., 2017; Roland et al., 2014). However, the blocking ability of these compounds has been characterized mostly in heterologous systems. In endogenous cell systems and in-vivo studies the expression of other T2Rs might affect the ability of these bitter blockers to effectively block the response generated by a single receptor.

Constitutive receptor activity and inverse agonism are very important concepts in pharmacology and are of immense value in understanding receptor structure-function and mechanisms, as well as guiding new drug discovery. In the T2R field only few studies have addressed this topic. Structure-function studies on T2R4 identified the first constitutively active mutant (CAM), S285A in TM7 that showed ≈ 3 fold agonist-independent activity over wild type receptor (Pydi, Bhullar, et al., 2012). This was followed by the discovery of many CAMs in T2Rs that has been extensively reviewed (Pydi, Bhullar, et al., 2014). The discovery of T2R4 CAMs led to the characterization of the first inverse agonist, BCML for T2R4 (Pydi, Sobotkiewicz, et al., 2014). An interesting study on the primate lemur identified the β -glucoside arbutin acted as an agonist in the ring-tailed lemur but as an inverse agonist in black and black-and-white ruffed lemurs (Itoigawa et al., 2019). This constitutive activity was attributed to a naturally occurring mutation in TM7 of T2R16. This intriguing study opens the possibility of naturally occurring and constitutively active T2Rs existing in humans. Though this would add another layer of

complexity in T2R pharmacology. There is an urgent need for broad spectrum T2R blockers. Until such blockers are available, the field must rely on blocking T2R downstream signalling partners $G\beta\gamma$, $PLC\beta$, IP_3R or using specific T2R knockdown approaches, all of which are tedious and cumbersome.

1.4.2. T2R expression and detection

Different methods have been employed to express T2Rs in heterologous systems. Earlier studies used epitope tagged T2R constructs for expression in HEK293 cells that yielded low plasma membrane expression (Adler et al., 2000; Chandrashekar et al., 2000). The chaperoning of sensory receptors was identified as important for proper folding and targeting to the plasma membrane (Baker et al., 1994; Dwyer et al., 1998). In fact, a recent study has demonstrated T2R14 to be chaperoned to the plasma membrane by β_2 -AR (D. Kim et al., 2016). Interestingly, a pharmacochaperone activity with the bitter agonist quinine was observed for T2R4, T2R7, T2R10, T2R39 and T2R46 (J. D. Upadhyaya et al., 2016). Studies have shown T2R chimeric protein sequences to be efficiently targeted to the plasma membrane. For example, rhodopsin-T2R chimera with the first 39 amino acids of bovine rhodopsin added to the N-terminus allowed mT2R5, hT2R4, and mT2R8 to be effectively targeted to the plasma membrane (Chandrashekar et al., 2000). Later it was concluded that the rhodopsin N-terminus 33 amino acids are sufficient to enhance expression of T2R4 by 2.5 fold (Pydi et al., 2013). Another common approach is to tag the N-terminus of T2Rs with the first 45 amino acids of the rat somatostatin receptor 3 (SSTR3) to enable efficient cell membrane localization of T2Rs (Lossow et al., 2016). Hence the use of T2R chimeras can be employed to achieve efficient cell surface expression without substantially compromising the structural integrity and functionality of the receptors.

The characterization of endogenous T2R expression is mostly done at mRNA levels using qPCR and RNA *in-situ* hybridization approaches (Chen et al., 2019; Taher et al., 2019). To date

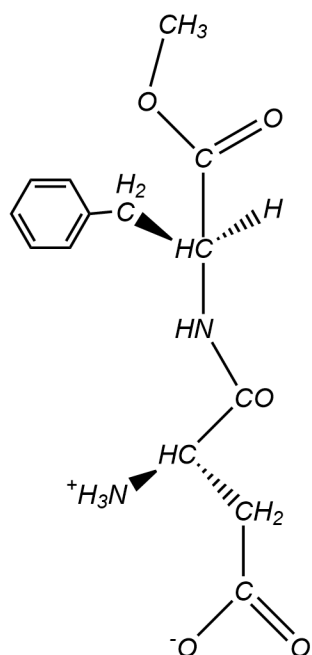
there are few studies that correlate mRNA and T2R protein expression in cell systems (I. Gopallawa et al., 2020; Shaik et al., 2019; Singh et al., 2020; Tran et al., 2018). A limiting factor is the availability of T2R antibodies that can target the extracellular epitopes. Such antibodies shall be useful in determining the cell surface expression of T2Rs without permeabilizing cells. Due to the lack of monoclonal and/or highly specific polyclonal antibodies most of the studies rely on T2R blockers (antagonists or inverse agonists) and siRNA or CRISPR-Cas9 approaches to confirm protein expression of T2Rs. Hence there is an urgent need to develop specific antibodies to detect T2R conformations (active or inactive) and cell surface expression.

1.5. Taste modulatory peptides

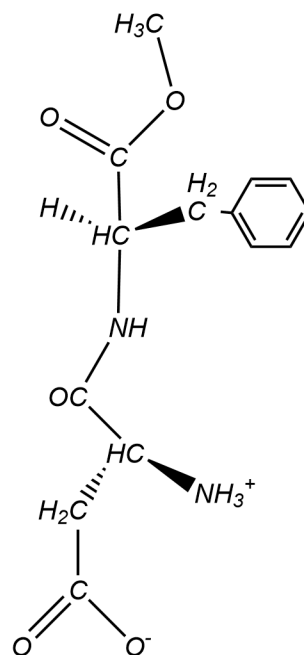
Digestive enzymes as well as proteolytic microbial enzymes breakdown proteins within the food into short oligopeptides often referred to as biopeptides or bioactive peptides. Bioactive peptides are defined as protein fragments that generally have a positive impact on body functions or conditions and may ultimately influence health (David et al., 2003). The physiological effect of these biopeptides depends in a large part upon their ability to successfully reach the target site, their amino acid composition, and their sequence. Upon binding to a suitable target these biopeptides may regulate several activities including nutrient utilization and exert systemic effects (Korhonen et al., 2003).

It has been long hypothesized that sweet, bitter, and umami tastes are the most important among the five recognized basic taste sensations, for food acceptance or rejection. Humans, like most animals, have a natural likeness for sweet-tasting molecules as an evolved mechanism for accepting sugars, the body's main source of energy. However, an innate aversion against bitter-tasting molecules is said to be an evolved protective mechanism from noxious compounds (Breslin, 2013; Wooding, 2005). Despite these contrasting responses, sweet and bitter molecules

belong to very diverse chemical families. In many cases, a seemingly minor modification of the ligand peptide can change a sweet tastant into a bitter one (Temussi, 2007; Verkade, 1968). The same observation is also made in the case of chiral isomerism of peptides (Kirimura et al., 1969; Mazur et al., 1969). A very classic example of this phenomenon is observed in the taste of Aspartame, a non-caloric sweetener. L-Aspartyl-L-phenylalanyl methyl ester is sweet, but L-Aspartyl-D-phenylalanyl methyl ester is not sweet, suggesting very precise steric requirements for taste perception (**Figure 1.3**). Further, all possible chiral isomers of aspartame, i.e. D,L; L,D and D,D, are actually bitter (Kirimura et al., 1969; Mazur et al., 1969).



Aspartame (Sweet L,L isomer)



Aspartame (Bitter D,L; L,D; D,D isomers)

Figure 1. 3: Molecular models of the two chiral isomers of Aspartame.

While the L, L isomer is sweet, the D, L isomer, elicits a bitter taste rather than a sweet taste.

Structures drawn using the ChemDraw software. Reproduced with permission from the Royal Society of Chemistry.

Peptides	Source	Functions (taste modulations)	Reference
Pro-glutamyl dipeptides	Cocoa beans	Umami taste enhancer	(Salger et al., 2019)
Doenjang water extract (DWE) acidic peptide Glu and Asp	Steamed soyabean	Mask bitter taste of hydrolysed animal protein and enhance Umami taste	(Rhyu et al., 2011)
SSRNEQSR and EGSEAPDGSSR	Peanut hydrolysate	Elicit umami taste	(Su et al., 2012)
Glutamyl-dipeptides (Glu-Glu) and (Glu-Asp)	Commercial umami peptides	Sweetness suppressor	(Shim et al., 2015)
GF and GL	Casein hydrolysates	Activate T2R1, 4, 14 and 16	(Kenji Maehashi et al., 2008)
GF, IF, FL and FFF	Commercial bitter peptides	Activate T2R1	(J. Upadhyaya et al., 2010)
FFPR	Commercial bitter peptide	Activate T2R8 and T2R39	(Ueno et al., 2011)
ETSARHL AGDDAPRAVF	Beef protein hydrolysate	T2R4 blocker	(Chunlei Zhang et al., 2018)
γ -glutamyl dipeptides EI, EL or EF	Dry-Cured hams	Bitterness suppressor	(Sforza et al., 2006)
ED, EE, ES, DES, EGS	Soybean	Bitterness suppressor	(M. J. Kim et al., 2015)
AGFAGDDAPR, AGGATAAQLE, ALGQNPTNAEINK, ASNPYS- TVT, FTQQIE, GDDAPR, ILGNPSKEE, ITTNPY, ITTNPYD, KALQEAHQ- QTLDD, KNLQEE, LEGSLE, LELNQ, PIGNIN, SMQSTLD, and TDISQIQS	Hen protein hydrolysate	Bitterness suppressor	(Q. Xu et al., 2019)
APPPPAEVHEV	Porcine troponin T derived peptide	Sour taste suppressor	(Okumura et al., 2004)

Table 1. 1: Diverse taste modulatory peptides and their potential functions. Reproduced with permission from the Royal Society of Chemistry.

Our understanding of taste perception has advanced significantly since these findings from the 1960s. Studies during the past decade have extensively explored both the agonists, the receptors, as well as their interaction and are extensively reviewed (Jaggupilli et al., 2016; Pydi, Bhullar, et al., 2014; Pydi, Upadhyaya, et al., 2012). Current interests for both the food and the pharmaceutical industry lie in including those peptides that can improve taste and suppress bitterness. In aqueous solution, proteins acquire a conformation with hydrophilic or polar groups on their outer surface, and the hydrophobic groups packed inside. Enzymatic digestion of proteins exposes the hydrophobic amino acid containing peptide moieties which give a sensation of bitterness. This is because hydrophobicity has been hypothesized as a contributing factor towards bitterness (Cho et al., 2004). Though not a sole factor, most bitter compounds demonstrate higher hydrophobic amino acids as compared to sweet compounds. Reducing hydrophobicity has in fact been suggested as a strategy for decreasing the bitter off-taste of peptide sweeteners (Di Pizio et al., 2019). Ney in 1971, first proposed the Q-rule as a correlation between the presence or absence of bitterness and the average hydrophobicity (Boudreau, 1979). The assessment of compounds on the Q-rule however, has yielded conflicting results (Toelstede et al., 2008). Studies on synthetic peptides have yielded important information on structural requirements of peptides for bitterness. Matoba et al. (Matoba et al., 1970) hydrolysed casein and identified three kinds of bitter peptides in a 0.1% solution. The primary structures of these peptides were proposed to be G-P-F-P-V-I, F-F-V-A-P-F-P-E-V-F-G-V-K, and F-A-L-P-Q-Y-L-K. Because F was common to these bitter peptides, it was predicted to be critical to the perception of bitter taste. Further, it was identified that though L-F has a bitter taste, its bitterness is increased significantly when acetyl and ethoxy groups block its amino and carboxyl groups, respectively. This suggests that modification of phenylalanine by carboxyl groups may contribute to the weakening of the bitterness of these peptides (Matoba et al., 1970).

Glycine containing peptides, such as G-L, V-G, and G-F-G, were bitter (Kenji Maehashi et al., 2008). However, other glycine peptides, such as G-G, G-A, and G-E, were not. The bitterness of amino acids intensified when both amino and carboxyl groups were blocked than when only one end was blocked (Matoba et al., 1972). Also, it was observed that the peptides whose amino and carboxyl groups were blocked by acetylation and esterification were 10 times more bitter than their corresponding unblocked peptides (Matoba et al., 1972). Ishibashi et al. found that the linear side chain of norvaline and norleucine were more intense for bitterness than the branched chain of their analogues (Ishibashi et al., 1988). A study that followed later examined the bitterness of the peptide R-G-P-P-F-I-V (Ishibashi et al., 1988). The study showed that the spatial structure of L-proline influenced bitterness significantly. The authors proposed that the hydrophobic imino ring of proline functioned as a binding unit for a putative T2R, and that the adjacent guanidino group in the arginine residue played a role as the stimulating unit. Also, bitter taste was apparent only when both the binding and the stimulating unit coexisted (Ishibashi et al., 1988). The perception of bitter taste was shown to depend on the presence and the distance between these two units. The size of the pocket in a putative T2R was proposed to be 15Å and a distance of 4.1Å between the binding and stimulating units was estimated to be ideal (Ishibashi et al., 1988). It was hypothesized that T2Rs detect the hydrophobicity of the peptide through the recognition zone in the wall of the pocket (Ishibashi et al., 1988).

Binding of a bitter ligand to the extracellular or transmembrane domains of a T2R mediates conformational change in the receptor to bring about the downstream signaling cascade. One of the earliest reports using heterologously expressed T2Rs showed that the human T2Rs are activated by the bitter tasting extracts of the milk protein, casein (Kenji Maehashi et al., 2008) . In that study, two synthetic bitter di-peptides, G-F and G-L, were shown to activate the bitter receptor T2R1. Following this report, a study in our lab identified several other bitter peptides as

well as characterized their interaction sites (J. Upadhyaya et al., 2010). One of the earlier studies suggested that the mouse T2R5 and rat T2R9 respond to cycloheximide (Chandrashekar et al., 2000). β -glucopyranosides (salicin and related compounds) can bind and activate T2R16 (Eduardo Sainz, Margaret M Cavenagh, et al., 2007). Thujone and picrotoxinin can activate T2R14 (Behrens et al., 2004). T2R43 and T2R47 are activated by 6-nitrosaccharin, aristolochic acid, and denatonium (Pronin et al., 2004). T2R43 and T2R44 also respond to the bitterness of saccharin (Kuhn et al., 2004). Bitter perception is complex, and further studies are needed to understand the functional details to target them in designing better masking agents.

1.5.1. Peptides as bitter blockers

Functional foods and peptides are an important category in the nutraceutical industry and are valued at approximately 75 billion annually. Bioactive peptides have been shown to exert various effects such as antihypertensive, antimicrobial, antithrombotic, immunomodulatory, opioid, antioxidant, and mineral binding functions (Chakrabarti et al., 2014; Sánchez et al., 2017; Soory, 2012). The effect exerted by these biopeptides depends largely on the sequence of amino acids (J. Wu et al., 2007). The structure and functional relationship of taste modulating bioactive peptides are not well established.

Peptides can elicit both bitter and sweet taste for example, Aspartame is intensely sweet but the aftertaste is bitter (Mazur et al., 1969). Several oligopeptides isolated from fish protein hydrolysate have a flavour resembling that of MSG (Noguchi et al., 1975). It is the bitter taste of peptides that is of most concern to the food industry. Bitter peptides are naturally produced during food processing and are an important concern for food palatability. Hydrolysates of soy (Cho et al., 2004) and cheese (Karametsi et al., 2014) proteins are perceived as bitter. Casein is known to produce the most bitter hydrolysates which is attributable to peptides formed as it matures during cheese formation. Casein hydrolysates have been shown to elicit aversive

responses in several animals and have been widely investigated (K. Maehashi et al., 2009). Using heterologous expression system it was shown that several casein peptides were able to activate T2R1, T2R4, T2R14, and T2R16 (Kenji Maehashi et al., 2008). Among these, T2R1 was most strongly activated by bitter di-peptides G-F and G-L, and none of the receptors were activated by the non-bitter dipeptide G-G (Kenji Maehashi et al., 2008). Studies on soy peptides identified that the bitter tasting peptides carried a leucine at the C-terminus, which when degraded by carboxypeptidases markedly reduced bitterness (Arai et al., 1970). There is increasing interest in identifying biologically derived peptides that can specifically mask the bitter taste.

Structure-function studies that followed observed that peptide bitterness was most apparent when the hydrophobic amino-acids are located at the C-terminal (H. O. Kim et al., 2006; Tamura et al., 1990; J. Wu et al., 2006). For tri-peptides, amino acids located in the middle are considered more important for bitterness than the N- and C-terminal amino acids (J. Wu et al., 2007). It was shown that both di- and tri- peptides were able to activate T2R1 (J. Upadhyaya et al., 2010). Nucleotides such as cytosine monophosphate and 2-deoxyadenosine triphosphate were demonstrated to cause a 40% and 60% reduction in bitterness of a 10 mM quinine solution, respectively (Ley, 2008). Yet, another study showed that the amino acid tryptophan could function as a T2R4 agonist (Kohl et al., 2013). Thus, the role of peptides in T2R mediated responses is still incomplete and needs further investigation.

Structure-function studies identified that seven amino acids located in the extracellular side of TM3, TM4, extracellular loop 2 (ECL2), and ECL3 were involved in the binding of T2R4 to its agonist quinine (Pydi, Sobotkiewicz, et al., 2014). This study identified that residues N173 and T174 in ECL2 are essential for quinine binding (Pydi, Sobotkiewicz, et al., 2014). γ -aminobutyric acid (GABA) and $N\alpha,N\alpha$ -bis(carboxymethyl)-L-lysine (BCML) were identified as competitive inhibitors of quinine-activated T2R4 with an IC_{50} of $3.2 \pm 0.3 \mu M$ and $59 \pm 18 nM$,

respectively (Pydi, Sobotkiewicz, et al., 2014). BCML is a derivative of the advanced glycation end product (AGE) called carboxymethyl-lysine (CML). CML inhibits quinine-activated T2R4 with an IC_{50} of $32.62 \pm 9.5 \mu\text{M}$ (C.-H. Wu et al., 2011). As discussed previously, umami tasting peptides have shown promise in masking the bitter taste. Umami peptides can suppress T2R16 by non-competitive inhibition (M. J. Kim et al., 2014). Deamidation of peptides by specific enzymes, has been shown to increase umami-taste and thus decrease bitterness (B. Y. Liu et al., 2017; C. Zhang et al., 2019).

Peptides isolated from beef protein hydrolysates led to reduced bitterness intensity of quinine (Chunlei Zhang et al., 2018). The most effective suppression of calcium mobilization was observed for the peptides AGDDAPRAVF and ETSARHL. In contrast to the observation with soy peptides, hydrophobic leucine-containing peptides had similar or even lower ability to release calcium than peptides that did not contain leucine (Chunlei Zhang et al., 2018). The results of this study suggested that short peptide lengths or the presence of multiple serine residues may not be effective in blocking T2Rs (Chunlei Zhang et al., 2018).

AGEs have been shown to elicit several beneficial health effects and are also recognized for generating a unique aroma and taste for processed foods. Glycation of beef protein alcalase hydrolysates (BPAH) and beef protein chymotrypsin hydrolysates (BPCH) led to up to 38% reduction in bitterness intensity of quinine (C. Zhang et al., 2019). Glycation is a non-enzymatic reaction between the carbonyl group of a reducing sugar and an amino group of a protein, also termed as the Maillard reaction. Treatment of HEK293T cells stably expressing the T2R4 receptor by glycated BPAH and glycated BPCH significantly attenuated the intracellular calcium as compared to non-glycated controls and quinine (C. Zhang et al., 2019). Casein peptide derived AGEs exhibited decreased bitterness intensity as compared to the unmodified casein peptide alone (Dong et al., 2011). Further, glycation of soy protein hydrolysates exhibited caramel-like

odor and a much weaker bitter taste (P. Liu et al., 2012). Recent studies have shown two AGEs, a glyoxal-derived lysine dimer (GOLD) and CML, activated T2R20 and inhibited T2R4 (Appalaraju Jaggupilli et al., 2019). The mechanism of action of AGEs is unclear, however, it is proposed that the higher charged state of the glycated peptides could reduce ligand-receptor interactions, hence the weak intracellular calcium response (Appalaraju Jaggupilli et al., 2019).

A recent study has shown peptides obtained from hen proteins hydrolysates were effective blockers for T2Rs (Q. Xu et al., 2019). The most effective responses were observed for peptides with the least bitterness quotient as measured using electronic tongue. These peptides exhibited lowest hydrophobic amino acids and effectively inhibited calcium mobilization induced by quinine or diphenhydramine in HEK293T cells expressing human T2R4, T2R7, or T2R14 receptors (Q. Xu et al., 2019). These recent findings are exciting, as these biologically derived peptides can inhibit several T2Rs and mask the bitter taste. Further studies are needed, especially to analyze the effect of these peptides on the remaining T2Rs. Such future studies combined with a better understanding of the structural features of these peptides, that can block several receptors simultaneously, will allow to devise better masking strategies.

1.6. Extraoral expression of T2Rs

Studies in recent years have highlighted the expression of T2Rs in several extraoral tissues including, gut, brain, cancer cells and endometrium, which are briefly reviewed in this section. For a more comprehensive discussion on extraoral roles of T2Rs, readers are referred to an earlier review (Shaik et al., 2016).

1.6.1. T2Rs in brain, gut, and cancers

The first study to suggest expression of TAS2Rs in brain cells was pursued in rats (N. Singh et al., 2011). This study showed by RT-PCR and immunohistochemistry analysis that TAS2R4, TAS2R107 and TAS2R38 were expressed in rat brain stem, cerebellum, cortex and nucleus accumbens (N. Singh et al., 2011). After this study, there were reports of TAS2R expression in brain tissues of both, animal models and humans, but the functional role of these T2Rs in these tissues remain to be understood and characterized. The human fundic primary cells were shown to express T2R10 and its activation was shown to influence ghrelin secretion (Wang et al., 2019), TAS2R5 and TAS2R38 was detected in human duodenal sections and enteroendocrine cells (EEC) responsible for secreting anorexigenic peptides such cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) (Latorre et al., 2016; Park et al., 2015). Studies have shown increased secretion of GLP-1 and PYY hormones in NCI-H716 cells upon treatment with T2R5 agonist 1,10-phenantroline (Park et al., 2015). The GLP-1 and PYY secretion was decreased upon down regulation of α -gustducin and multiple T2Rs (K. S. Kim et al., 2014). TAS2R3, TAS2R4 and TAS2R10 along with their downstream signal transducing partners are expressed in the human gastric smooth muscle cells (Avau et al., 2015). The presence of T2Rs in the brain and gut suggests their involvement in neuroendocrine systems; gut-brain feedback systems and the regulation of energy homeostasis. Recently, a study has shown that intra-gastric administration of quinine resulted in enhanced brain activity in the

hypothalamus and the hedonic regions (Iven et al., 2019). In contrast, decreased plasma levels of octanoylated ghrelin, total ghrelin and motilin hormones were observed in subjects treated with quinine versus placebo leading to decreased hedonic food intake (Iven et al., 2019). Several questions have been raised about the role of T2Rs in obesity and fat tissue differentiation. T2R38 was shown to be expressed in human adipocytes and was found to be highly expressed in obese subjects as compared to the lean subjects (Cancello et al., 2020). The same study also showed T2R38 to be responsible for adipocyte differentiation and delipidation (Cancello et al., 2020).

Recent studies suggest the expression of T2Rs in many cancers including breast, pancreatic, neuroblastoma, prostate and ovarian cancers (M. M. Gaida, C. Mayer, et al., 2016; Martin et al., 2019; Seo et al., 2017; Singh et al., 2020). However, the pathophysiological role of T2Rs in these cancers is varied and not fully understood. The tissue biopsies from pancreatic ductal adenocarcinoma patients and membrane preparations from Su8686 and HuH7 cell lines revealed co-localization of T2R38 with a lipid droplet marker perilin (M. M. Gaida, C. Mayer, et al., 2016). In another study, T2R8 and T2R10 were shown to suppress cancer stem cell markers such as DLK1, CD133, NOTCH1, and Sox2 and neuroblastoma cell migration (Seo et al., 2017). T2R4 and T2R14 are differentially expressed in breast cancer tissues and their activation induced anti-proliferative and anti-migratory responses in highly metastatic breast cancer cells (Singh et al., 2020). In ovarian and prostate cancer cell lines, T2R14 specific responses were shown to induce pro-apoptotic proteins Bcl-XL and caspase-3 leading to apoptosis as measured by increased annexin V on cell membranes (Martin et al., 2019). The expression of 9 TAS2Rs genes was observed in post-meiotic spermatids of mouse testis (J. Xu et al., 2013).

1.7. T2R HOST-MICROBE INTERACTIONS

The sections that follow will discuss the role of T2Rs in sensing various bacterial quorum systems and the associated molecular pharmacology

1.7.1. Bacterial QSMs as a new class of ligands for T2Rs

Bacteria secrete QSMs during colonization. Once colonized these bacteria are very difficult to eradicate and result in episodic exacerbations and eventual system failures. The QSMs as the name suggests, are important for the bacteria to sense their numbers (hence, quorum) and then signal each other to mediate relevant responses.

Both Gram-negative and Gram-positive bacteria employ quorum sensing (QS) for communication, but they use different molecules. Gram-negative bacteria such as *Pseudomonas aeruginosa* secrete three molecules: N-acyl homoserine lactone (AHL) molecules (autoinducer-1, AI-1) forming its main QS signal, followed by Autoinducer-2 (4,5-dihydroxy-2,3-pentanedione (DPD) and 2-heptyl-3-hydroxy-4-quinolone (PQS) (J. Lee et al., 2015; H. Li et al., 2015). In contrast, Gram-positive bacteria use modified oligopeptides (autoinducer peptides, AIP) (Taga et al., 2003). These peptides possess a large structural diversity and frequently undergo post-translational modifications (Sturme et al., 2002). Both Gram-positive and Gram-negative bacteria can produce and detect a third type of QSMs known as AI-2. These are boron-furan-derived signal molecules (Z. Li et al., 2012). Besides these 3 main groups, there is also a fourth group of miscellaneous QSMs (Barber et al., 1997; Flavier et al., 1997; Holden et al., 1999). In host-microbe interactions, these mechanisms become relevant when bacteria infect and colonize the host, thus understanding the effects the QSMs have is paramount.

It is well understood that eukaryotic host and colonizing prokaryotes have co-evolved (Lyte et al., 1993). For example, Enterohaemorrhagic *E. coli* (EHEC) is able to modify its

motility and virulence expression as per the host's adrenaline and noradrenaline concentration (Pacheco et al., 2009). The neurotransmitter γ -aminobutyric acid (GABA) produced by various Lactobacilli plays an important role in mammalian brains (Barrett et al., 2012). Thus, it is no longer surprising that their communication systems evolved as well (González et al., 2013; E. K. Shiner et al., 2005). In a major finding, it was reported that nasal solitary chemosensory cells (SCCs) expressed T2Rs and responded to AHLs produced by bacteria (Tizzano et al., 2010) establishing QSMs as a new class of ligands for T2Rs.

1.7.2. AHLs

These are the most common class of autoinducers and structurally, possess a homoserine lactone (HSL) moiety, acyl side chains with 4–18 carbons that determine the structure diversity and a substitution primarily belonging to one of the three: (a) simple acyl, (b) 3-hydroxyacyl, and (c) 3-oxoacyl groups. Though, there are several subtypes of AHLs, most of the available studies so far have focused on C-12 AHL. Indeed, it is a key and a very well-studied molecule in the biofilm formation and regulation of virulence in *P. aeruginosa*. AHLs are also now recognized to be important for inter-species communication between bacteria (Bhargava et al., 2012), which highlights their importance in the development of multi-species chronic infections. The first clue that AHLs could play a role as signaling molecules in inter-kingdom communication was N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL or C12-AHL) secreted by *P. aeruginosa* (Telford et al., 1998). Given the high hydrophobicity of C12-AHL it was hypothesized that it could diffuse freely through lipid membranes and mediate responses in the host (Barrett et al., 2012; Barth et al., 2012).

Studies in the recent past have also shown the effects of C12-AHL on respiratory epithelial cells, such as enhanced ciliary movement and nitric oxide production (R. J. Lee et al., 2012b), indicating that C12-AHL induces activation of defense-related functions in these cells.

Cystic fibrosis (CF) is a life-threatening genetic disease characterized by pulmonary exacerbations mainly due to infections with *P. aeruginosa*. The concentrations of C12-AHL in CF sputum are believed to be in the nanomolar range (E. Shiner et al., 2006) but reach 5 μ M in *P. aeruginosa* culture supernatants and may be greater than 100 μ M in regions adjacent to biofilms (Kravchenko et al., 2008; Kravchenko et al., 2006). An interesting study suggested that C12-AHL stimulates CFTR-dependent Cl^- and fluid secretion in an intact CFTR expressing, CaLu3 airway epithelial cells by activating the inositol trisphosphate receptor and store-operated cAMP production (Schwarzer et al., 2010). C12-AHL treatment of myeloid or non-myeloid cells resulted in the morphological alteration of mitochondria and especially endoplasmic reticulum (ER) (Kravchenko et al., 2006). Further the study also showed that the N- (3-oxo-acyl) homoserine lactone ring moiety in AHLs is required for the induction of phosphorylated mitogen-activated protein kinase p38, and that of eukaryotic translation initiation factor 2 α (eIF2 α) (Kravchenko et al., 2006). Canonical host recognition molecules such as MyD88, TRIF, TLR2, TLR4, Nod1, and Nod2 were also found to be dispensable for the C12-AHL mediated signaling and it was hypothesized that C12-AHL could in fact function as a pathogen-associated molecular pattern (Kravchenko et al., 2006).

1.7.3. AHLs interaction with T2Rs

Recent studies have shown expression of T2R38 on peripheral blood leukocytes, monocytes and neutrophils as well as monocytic cell lines (Matthias Martin Gaida et al., 2016; Maurer et al., 2015). T2R38 has been found to associate with IQGAP1, a scaffolding protein, which participates critically in the rearrangement of the cytoskeleton and in regulating cell morphogenesis (Matthias Martin Gaida et al., 2016). Among others, it is a key regulator of chemotaxis. C12-AHL has been found to induce chemotaxis (Karlsson et al., 2012) and thus, it is

hypothesized that C12-AHL may cause chemotaxis via the T2R38-IQGAP pathway. There is a correlation between T2R38 receptor allotypes and susceptibility to airway infections (R. J. Lee et al., 2012b; A. S. Shah et al., 2009; Tizzano et al., 2010). T2R38 has been shown to respond to at least two other AHLs produced by *P. aeruginosa*: N-butyryl-L-homoserine lactone and N-hexanoyl-L-homoserine lactone (R. J. Lee et al., 2012b).

Though the T2R-AHL relationship is being explored, the mechanism is poorly understood. For example, in heterologous systems T2R38 is not activated by AHLs (Lossow et al., 2016). However, co-immunoprecipitation, immunofluorescence and antibody blocking has shown C12-AHL interaction with T2R38 on myeloid cells (Matthias Martin Gaida et al., 2016; Maurer et al., 2015). In another study, it was suggested that the solvent (DMSO) for these AHLs caused activation of T2R38 (Verbeurgt et al., 2017). An extensive structure-function study was conducted to analyze the interaction between several T2Rs and AHLs (Jaggupilli et al., 2018). This study identified that C4-AHL could activate T2R14 while C8-AHL and C12-AHL activated T2R4, T2R14, and T2R20. Taken together, these results suggest that AHLs can bind to T2Rs and participate in local host defense.

1.7.4. Pharmacological characterization of AHL-T2R interactions

The binding sites of the QSMs in T2R4, T2R14 and T2R20 were recently characterized (Jaggupilli et al., 2018). In addition, the analysis of bitter sensory profile of C4-AHL, C8-AHL, 3-oxo-C12-AHL and T2R agonists quinine, caffeine, dextromethorphan (DXM), diphenhydramine (DPH), cromolyn using E-tongue (Alpha MOS) suggest that the bitterness score of the above AHLs (~10) was slightly higher compared to quinine (8.45). These E-tongue experiments provide the first sensory insights into the bitter intensity profiles of bacterial AHLs.

Future studies involving human taste panels are warranted to analyze the sensory properties of AHLs and to support the notion that humans can taste bacterial biofilms.

The QSM mediated T2R activation was functionally characterized using HEK293T cells stably expressing T2R1, T2R4, T2R14 and T2R20 (Jaggupilli et al., 2018; Jaggupilli et al., 2017). The $(Ca^{2+})_i$ assays in HEK293T cells showed no activation of T2R1 by any of the AHLs tested. However, T2R4 was activated by 50 μ M C8-AHL and 100 μ M C12-AHL, while T2R14 was activated by C4-AHL, C8-AHL and C12-AHL. Finally, T2R20 was activated by C8-AHL and C12-AHL. Moreover, C8-AHL and C12-AHL were able to activate T2R4 and T2R14 in a concentration dependent manner. Surprisingly, the AHLs were able to activate T2Rs in the lower micromolar ranges with higher potencies than conventional bitter taste receptor agonists. The maximum effect (E_{max}) caused by AHLs in terms of intracellular calcium mobilization is much lower than that of conventional bitter agonists (**Table 2.1**). Unfortunately, some of these AHLs could not be used at a concentration higher than 200 μ M to reduce any unwanted cross effect caused by the solvent DMSO. Hence direct comparison between AHLs and bitter agonists remains elusive. Nevertheless, these results establish AHLs as new class of agonists in the T2R family.

QSMs and bacterial metabolites (Conc)*	Heterologous system (HEK293T cells)	Primary or immortalized cells	References
C4-AHL (50 μ M)	T2R14 EC ₅₀ ND Emax ND		(Jaggupilli et al., 2018)
C8-AHL (3.15-200 μ M)	T2R4 EC ₅₀ 30.13 $\pm$ 12 μ M, Emax 37.81 $\pm$ 5.0 RFU T2R14 EC ₅₀ 19.65 $\pm$ 10 μ M, Emax 20.33 $\pm$ 3.8 RFU		(Jaggupilli et al., 2018)
C12-AHL (3.15-200 μ M)	T2R4 EC ₅₀ 40.9 $\pm$ 13 μ M, Emax 26.89 $\pm$ 3.2 RFU T2R14 EC ₅₀ 69.54 $\pm$ 40 μ M, Emax 29.59 $\pm$ 5.1 RFU T2R20 EC ₅₀ 58.31 $\pm$ 12 μ M, Emax 24.31 $\pm$ 4.1 RFU		(Jaggupilli et al., 2018)
PQS (100 μ M)	T2R4, T2R16, T2R38, T2R39 EC ₅₀ ND Emax ND	Airway epithelial cells and macrophages	(Freund et al., 2018; I. Gopallawa et al., 2020)
HHQ (100 μ M)	T2R14 EC ₅₀ ND Emax ND	Airway epithelial cells and macrophages	(Freund et al., 2018; I. Gopallawa et al., 2020)
Acetone * (3.5-185 mM)	T2R38 EC ₅₀ 60 mM		(Verbeurgt et al., 2017)
2-Butanone (0.3-80 mM)	T2R38 EC ₅₀ 11 mM		(Verbeurgt et al., 2017)
2-Pentanone (0.1-20 mM)	T2R38 EC ₅₀ 4 mM		(Verbeurgt et al., 2017)
2-Methylpropanal (0.39-25 mM)	T2R38 EC ₅₀ 11 mM		(Verbeurgt et al., 2017)
γ -Butyrolactone (1-100 mM)	T2R38 EC ₅₀ 12 mM		(Verbeurgt et al., 2017)
Dimethylsulfoxide* (0.25-3 mM)	T2R38 EC ₅₀ 3 mM		(Verbeurgt et al., 2017)
Methylmercaptan* (0.36-10 mM)	T2R38 EC ₅₀ 8 mM		(Verbeurgt et al., 2017)

* Solvents used to solubilize the QSMs.

ND-not determined

Table 2. 1: Pharmacological characterization of QSMs and bacterial metabolites activating T2Rs in heterologous and endogenous cell systems. Reproduced with permission from the Handbook of Experimental Pharmacology; License number- 5015650539319

1.7.5. T2R amino acid residues interacting with QSMs

Since there is no crystal structure available for any of the T2Rs, thus far structure-function studies have utilized site directed mutagenesis and computational based approaches (Jaggupilli et al., 2016). To understand the nature of interaction between AHLs and T2Rs, the binding sites of C8-AHL and C12-AHL in several T2Rs was characterized (Jaggupilli et al., 2018). The validated homology models of T2R4, T2R14 and T2R20 were docked with C8-AHL and C12-AHL using Libdock algorithm. The analysis of different ligand binding poses revealed several amino acids on extracellular side in T2Rs to be within 4Å distance of the AHLs (**Figure 1.4**). The amino acids identified to be interacting with homoserine lactone group in AHLs include N165, T166, K262 in T2R4; R160, K163, T182 in T2R14; and H65, W88, W164, Q265 in T2R20. The homoserine lactone group in AHLs were shown to interact predominantly with extracellular residues in T2Rs (**Figure 1.4**). Both C8-AHL and C12-AHL activated the three T2Rs (T2R4, T2R14 and T2R20) with different intrinsic efficacies. This is due to the orientation of AHL molecules within the ligand-binding site. Within the AHLs, it was suggested that the homoserine lactone group is essential in binding to the extracellular orthosteric site in T2Rs and the acyl chain is shown to interact with hydrophobic amino acid residues located in the TM regions of T2Rs. In T2R4, the acyl chain of C12-AHL is extended towards TM3-TM7, while in T2R14 and T2R20, the acyl chains were located deep within the TM core. The orientation of acyl chains of C8-AHL is observed to be opposite in the TM core of T2R4 and T2R14. These observations suggested that hydrophobic amino acids in the TM regions of T2Rs might play an important role in stabilizing AHL binding to receptor and/or facilitate T2R activation. In the absence of an actual ligand binding assay that measures K_d (ligand affinity) it is currently not possible to differentiate between T2R amino acids important for ligand binding and/or T2R activation.

However, the above study reinforced the significance of amino acids in extracellular loop 2 in T2R ligand binding and activation.

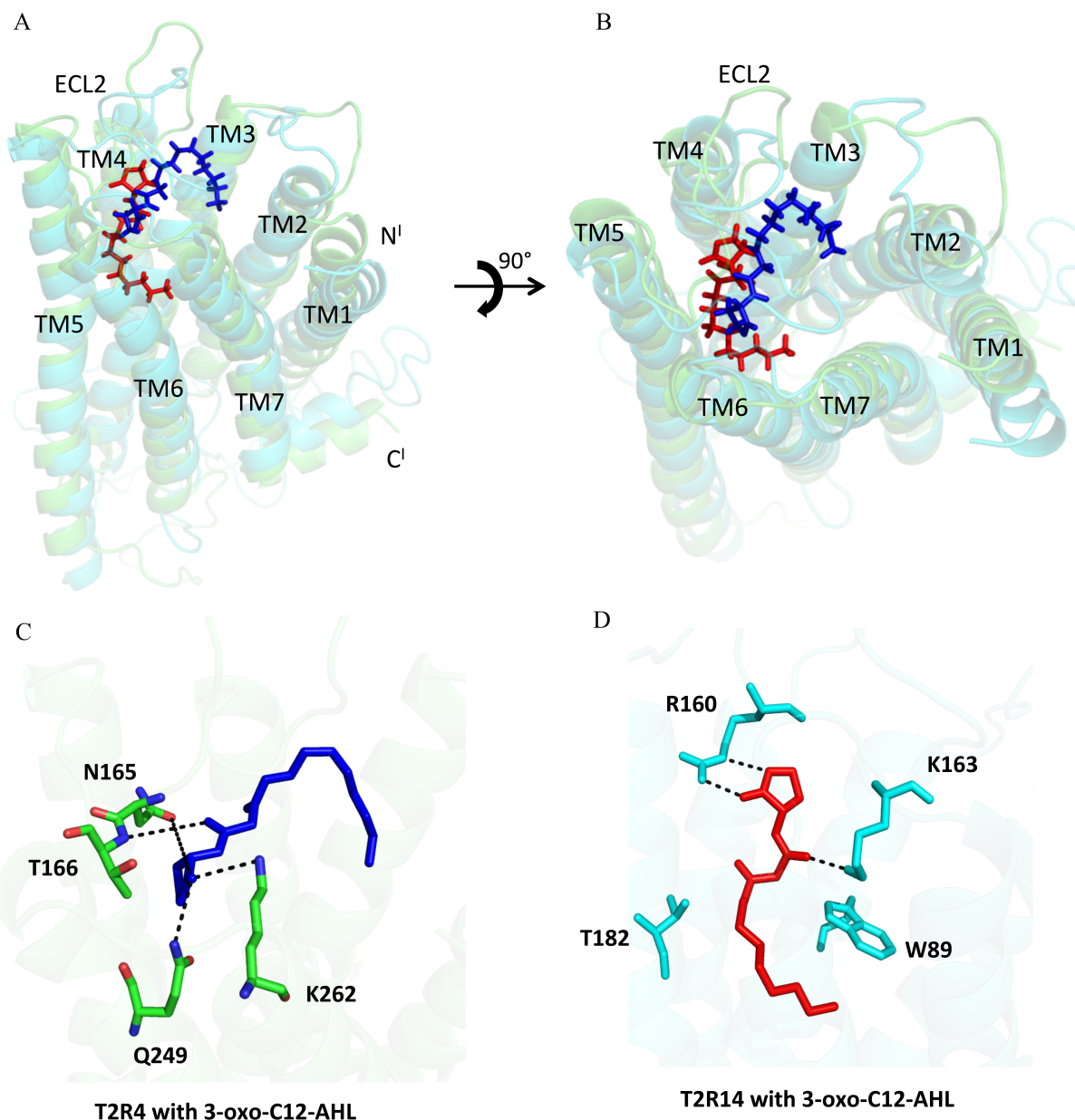


Figure 1. 4: The binding site of 3-oxo-C12-AHL in T2R4 and T2R14 based on site-directed mutagenesis and molecular modeling analysis (Jaggupilli et al., 2018).

(A) Superimposed molecular models of T2R4 (Green) and T2R14 (Cyan) docked with the 3-oxo-C12-AHL (blue in T2R4 and red in T2R14). (B) Top view of the ligand binding pocket showing the arrangement of the cylindrical seven transmembrane (TM 1-7) helices, extracellular loop 2

(ECL2) and the 3-oxo-C12-AHL (blue in T2R4 and red in T2R14). (C-D) Amino acids interacting with 3-oxo-C12-AHL in T2R4 and T2R14 respectively (Medapati, Bhagirath, et al., 2021). Reproduced with permission from the Handbook of Experimental Pharmacology; License number- 5015650539319.

1.7.6. Quinolones interaction with T2Rs

The second class of molecules for QS in *P. aeruginosa* is 4-hydroxy-2-alkylquinolines (HAQs). HAQs include derivatives of 4-hydroxy-2-heptylquinoline (HHQ) and the dihydroxylated derivatives, such as 2-heptyl-3, 4-dihydroxyquinoline (PQS, pseudomonas quinolone signal) and 2-nonyl-4(1H)-quinolone (NHQ) (Heeb et al., 2011). HHQ is synthesized from anthranilic acid and PQS is synthesized by hydroxylation of HHQ. Both HHQ and PQS act as co-inducing ligands for the transcriptional regulator controlling genes encoding for HAQs (Bredenbruch et al., 2005; Diggle et al., 2006; Lepine et al., 2004; Wade et al., 2005). The sensory profiles of these QSs are yet to be characterized by human taste panels. Though, bitter sensory profiles analyzed by emerging technologies such as the E-tongue suggest HHQ and NHQ with higher bitterness scores (>10) than AHLs, quinine or caffeine (Jaggupilli et al., 2018).

In chronic infections such as in CF, the environment over time becomes hypoxic, or oxygen deficient (Montgomery et al., 2017). Under anaerobic conditions, PQS is not produced due to the absence of oxygen. HHQ is the predominant signaling molecule under chronic hypoxic environments and PQS during early infections (Schertzer et al., 2010). Studies on both HHQ and PQS have shown that they can strongly suppress innate immune responses *in vitro* and *ex vivo*, mediated by the NF- κ B signaling pathway (K. Kim et al., 2010). A recent study also showed that PQS stimulates neutrophil chemotaxis via activation of the p38 MAP kinase possibly employing the same pathway as C-12 AHL (Hansch et al., 2014) (**Figure 1.5 and 1.6**). A recent study has shown that PQS activated T2R4, T2R16, and T2R38, whereas HHQ activated only T2R14

(Freund et al., 2018). PQS and HHQ also increased calcium responses but decreased both baseline and stimulated cAMP levels in cultured and primary airway cells (Freund et al., 2018). Further activation of T2Rs also increased nitric oxide generation, a robust innate immune response against *P. aeruginosa*, suggesting a role in innate immune responses (Freund et al., 2018).

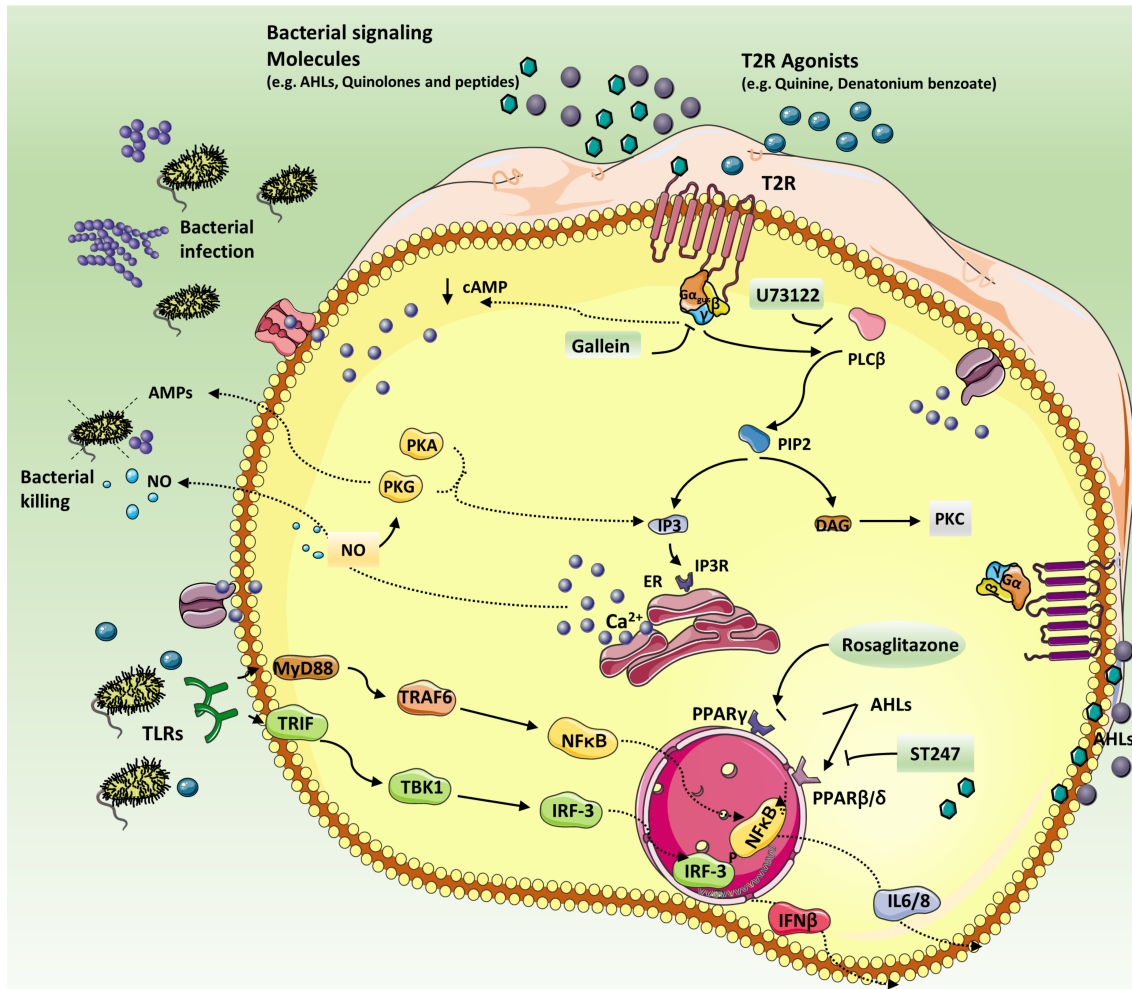
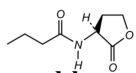


Figure 1. 5: Schematic of bacterial QSM mediated activation of canonical T2R signaling pathways.

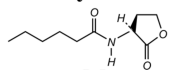
P. aeruginosa secreted QSMs (AHLs, quinolones, and AI-2), *S. aureus* Gram-positive bacterial peptides (AIPs) and bitter agonists activate Gβγ-PLCβ-IP3 pathway leading to intracellular calcium mobilization. The intracellular calcium mobilization leads to regulation of downstream signaling elements including activation of PKA, PKG pathways and secretion of antimicrobial

peptides (AMP) and NO, which facilitate bacterial killing. The AHLs are also shown to enter the cells and activate PPAR ($\gamma\beta\delta$) receptors expressed on nuclear membrane. The PPAR receptors then activate NF- κ B signaling pathway and several transcriptional factors leading to secretion of pro-inflammatory cytokines (Medapati, Bhagirath, et al., 2021). Reproduced with permission from the Handbook of Experimental Pharmacology; License number- 5015650539319.

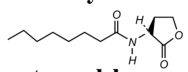
AHLs



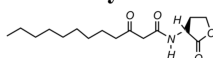
N-butanoyl-homoserine lactone (C₄-HSL)



N-hexanoyl-homoserine lactone (C₆-HSL)

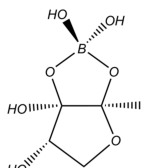


N-octanoyl-homoserine lactone (C₈-HSL)



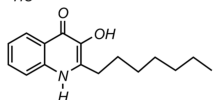
N-3-oxododecanoyl-homoserine lactone (3OC₁₂-HSL)

Autoinducer-2

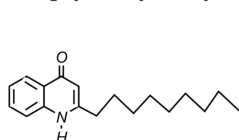


**Furanosyl borate diester
AI-2
Streptococcus oralis**

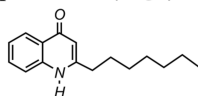
Quinolones



2-heptyl-3-hydroxy-4(1H)-quinolone (PQS)



2-nonyl-4(1H)-quinolone (NHQ)



4-hydroxy-2-heptylquinoline (HHQ)

Autoinducer Peptides

SIFTLVA-iAM373, *Enterococcus faecalis*
 EMRKSNNFFHFLRRI-CSP, *Streptococcus mitis*
 GVNACSSLF-AIP2, *Staphylococcus aureus*
 NNWNN-EDF, *Escherichia coli*
 VNYGNGVSCSKTKCSVNWGQ-CbnB2-*Carnobacterium piscicola*
 GIFWEQ-ComX, *Bacillus subtilis*

Figure 1. 6: Chemical structures of AHLs, AI-2, Quinolones and AIPs.

The structures are drawn using ChemDraw software (Medapati, Bhagirath, et al., 2021).

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1.7.7. Autoinducer peptides (AIP) interaction with T2Rs

The AIPs are oligopeptide QSMs used mainly by Gram-positive bacteria. These peptides are typically 2-20 amino acids long often with side chain modifications (Lazazzera et al., 1998). Classically, these peptides are synthesized as precursor peptides by the ribosomes and undergo posttranslational modification to form an active (leader) peptide. The secretion of the active peptide is usually facilitated by a membrane associated ATP-binding cassette (ABC) transporter (Kleerebezem et al., 1997). When the concentration of the AIP in the external environment reaches a threshold, the peptide binds to a membrane sensor. The activated sensor kinase phosphorylates the intracellular response regulator. The response regulator influences the transcription of downstream target genes to mediate a feedback loop (Verbeke et al., 2017). In contrast to the previous two categories (AHLs and Quinolones) AIPs cannot pass through the membrane easily and require dedicated transporters or receptors (Kleerebezem et al., 1997). AIPs are known to be involved in playing two important roles: one, to induce the production of class II antimicrobial peptides (bacteriocins) as seen in *Lactobacillus platarum* (Miloni et al., 2015) and second, providing genetic competence as seen in *Streptococcus pneumoniae* (Salvadori et al., 2019). These are mostly linear peptides and are characterized by the double-glycine leader peptide. Autoinducing peptides from *Escherichia coli* and *Staphylococcus aureus* contain a cyclic thiolactone structure (Thoendel et al., 2011). In *Bacillus subtilis*, the 10 amino acid AIP is actually processed from a 55 amino acid precursor peptide and then modified at a tryptophan

residue possibly by an isoprenoid group (Schneider et al., 2002). Crosstalk by AIPs is an important factor in shaping the microbial ecosystems (Miller et al., 2018).

The 17 amino acid AIP is referred to as the competence stimulating peptide (CSP) in *S. pneumoniae* (Håvarstein et al., 1995). A structure-activity analysis identified that the N-terminus region of the CSP and the hydrophobic residues at the central region of the peptides were most important for its recognition and activity (Yang et al., 2017). CSPs are now identified in various other streptococcal species including *S. gordonii*, *S. intermedius*, and *S. mutans* (Cvitkovitch, 2001). *S. mutans* is a key organism in causing dental caries. It secretes a 21 amino acid competence stimulating peptide (CSP-1) to mediate biofilm formation and bacteriocin production (Hossain et al., 2012a). Interestingly, AIP derived from *S. aureus* (Ard D1 thiolactone) and *S. pneumoniae* CSP-1 (EMRLSKFFRDFILQRKK) did not activate T2R38 (Verbeurgt et al., 2017). The ability of CSPs from different streptococci to activate T2Rs remains to be investigated. Apart from AIPs, D-amino acids secreted by several gram negative and positive bacteria (Brandenburg et al., 2013; Lam et al., 2009; Sarkar et al., 2015) are known to activate T1Rs in upper airways (Robert J. Lee et al., 2017). However, their interaction with T2Rs is not characterized so far.

1.8. T2Rs IN INNATE IMMUNITY

The expression of T2Rs in extraoral tissues and interaction with bacterial QSMs suggests that T2Rs are involved in mediating some aspects of innate immunity, which are discussed below.

1.8.1. Role of T2R38 genetics in innate immunity

T2R38 expressed in primary human sinonasal epithelial cells is probably activated by QSMs, mainly C4-AHL and C12-AHL (R. J. Lee et al., 2012b). These QSMs were able to mobilize intracellular Ca^{2+} and nitric oxide (NO) secretion leading to mucociliary clearance (R. J. Lee et al., 2012b). Moreover, the AHL mediated Ca^{2+} and NO was suggested to be TAS2R38 genotype dependent. The cells containing PAV/PAV (super-taster) genotype were able to induce better innate immune responses compared to PAV/AVI (intermediate-taster) and AVI/AVI (non-taster) genotypes. Therefore, the polymorphisms in T2Rs probably play an important role in ligand recognition and downstream signalling functions. In another recent study on 217 subjects from different ethnic backgrounds it was shown people expressing T2R38^{PAV/PAV} genotype have less sinonasal symptoms as compared to both the intermediate-taster and non-taster genotypes (Farquhar et al., 2015). A study with a small sample size of 25 subjects with chronic rhinosinusitis (CRS) suggested increased bacterial populations isolated from sinuses in AVI/AVI and PAV/AVI compared to PAV/PAV genotype (Rom et al., 2017). It was suggested that TAS2R38^{PAV/PAV} genotype, as opposed to non-functional AVI/AVI genotype, is associated with the absence of *P. aeruginosa* sinus infection (R. J. Lee et al., 2012b). In contrast, the studies that followed could not establish this association (Gallo et al., 2016; Turnbull et al., 2018).

1.8.2. T2R mediated innate immune responses in chemosensory and immune cells

The solitary chemosensory cells expressed in sinonasal and gingival tissues express T2Rs and taste signaling components such as Gnat3 (gustducin), TrpM5 and PLC β 2 (Barham et al., 2013; Saunders et al., 2014; Zheng et al., 2019). T2R agonist denatonium was able to induce Ca²⁺ signalling leading to robust antimicrobial peptide (AMP) β -defensin1 and 2 secretions in these SCCs (Carey, Workman, Hatten, et al., 2017; R. J. Lee et al., 2014). The denatonium treated SCC supernatants were able to decrease growth of *P. aeruginosa* as compared to control cultures (R. J. Lee et al., 2014). A recent study has shown activation of T2R38 by several organic bacterial metabolites but not AHLs in heterologous cell systems (**Table 1**) (Verbeurgt et al., 2017). Surprisingly, T2Rs expressed in sinonasal epithelial cells were also activated by Gram-positive bacteria such as *Bacillus cereus* leading to increased intracellular calcium mobilization and NO production, however, these effects were observed to be independent of T2R38 leading to speculation that other T2Rs are also involved in recognizing bacteria and their molecules (Carey, Workman, Yan, et al., 2017).

The well-known T2R agonist quinine induced T2R signalling dependent NO generation and CBF (Ciliary beat frequency) in sinonasal cells (Workman et al., 2018). Quinolones, a class of bacterial QSMs that activate multiple T2Rs (**Table 2.1**), lead to intracellular calcium mobilization and decreased cAMP signalling in heterologous systems as well as in primary human lung epithelial cells (Freund et al., 2018). A recent study demonstrated the expression of T2R4, T2R14, T2R38 and T2R46 in monocyte-derived macrophages (I. Gopallawa et al., 2020). The T2R14 in unprimed macrophages was activated by *P. aeruginosa* derived QSMs along with other known bitter agonists such as quinine, denatonium benzoate, and Flufenamic acid (FFA) (I. Gopallawa et al., 2020). The T2R dependent calcium signalling in macrophages resulted in increased cGMP and NO dependent phagocytosis of bacteria. This recognition of bacteria and

their metabolites by T2Rs is consistent with observations in other species as well. A recent study also demonstrated increased expression of T2Rs in rainbow trout fish (*Onchorhynchus mykiss*) treated with bacteria *Flavobacterium columnare*, infectious hematopoietic necrosis virus (IHNV) and *Ichthyophthirius multifiliis* (X. Liu et al., 2020). Collectively these studies suggest T2Rs as one of the innate immune targets during bacterial infections.

1.9. Microvesicles and exosomes

The cells in our body secrete membrane bound nano-carriers that are termed as extracellular vesicles (EVs). EVs are released both under physiological and pathological conditions and are means of intracellular communication (Mittelbrunn et al., 2012). EVs are classified into three categories namely exosomes, microvesicles (MVs) and apoptotic bodies. Exosomes are smaller vesicles that are surrounded by a phospholipid bilayer with a size range of 30 -100 nm and are formed via exocytosis of multivesicular bodies containing intraluminal vesicles (Trams et al., 1981). Contrary to exosomes, MVs are larger and surrounded by phospholipid bilayer with a size of 0.1 to 1.0 μm . These MVs are released by shedding of plasma membrane evaginations (György et al., 2011). Among the EVs, the apoptotic bodies are larger in size (1 to 5 μm) and are released in late apoptotic stages of the cells (György et al., 2011).

The mechanism of release of exosomes and MVs are entirely different. Exosomes are released either constantly or upon activation and are mainly involved in transfer of mRNA, miRNA, antigen presentation, immunostimulatory and inhibitory activities (Hurley et al., 2010; Raposo et al., 1996; Valadi et al., 2007). Exosomes are characterized by the presence of phosphatidylserine on the membrane and other unique markers such as CD9, CD63, CD81, LAMP1 and TSG101 (Chaput et al., 2011; Dignat-George et al., 2011; Mathivanan et al., 2010). The release of MVs is not constitutive and is dependent on activation of cell surface receptors

and increase in intracellular Ca^{2+} levels. The increased Ca^{2+} levels activate calpain that modulates actin remodelling thereby removing membrane bound proteins (**Figure 1.7**). The remodelling of actin protein network leads to formation of membrane blebs and their eventual release as MVs (Hartwig et al., 1989; Pasquet et al., 1996). Platelets, RBCs and endothelial cells are known to predominantly form MVs (Baroni et al., 2007; Kahner et al., 2008).

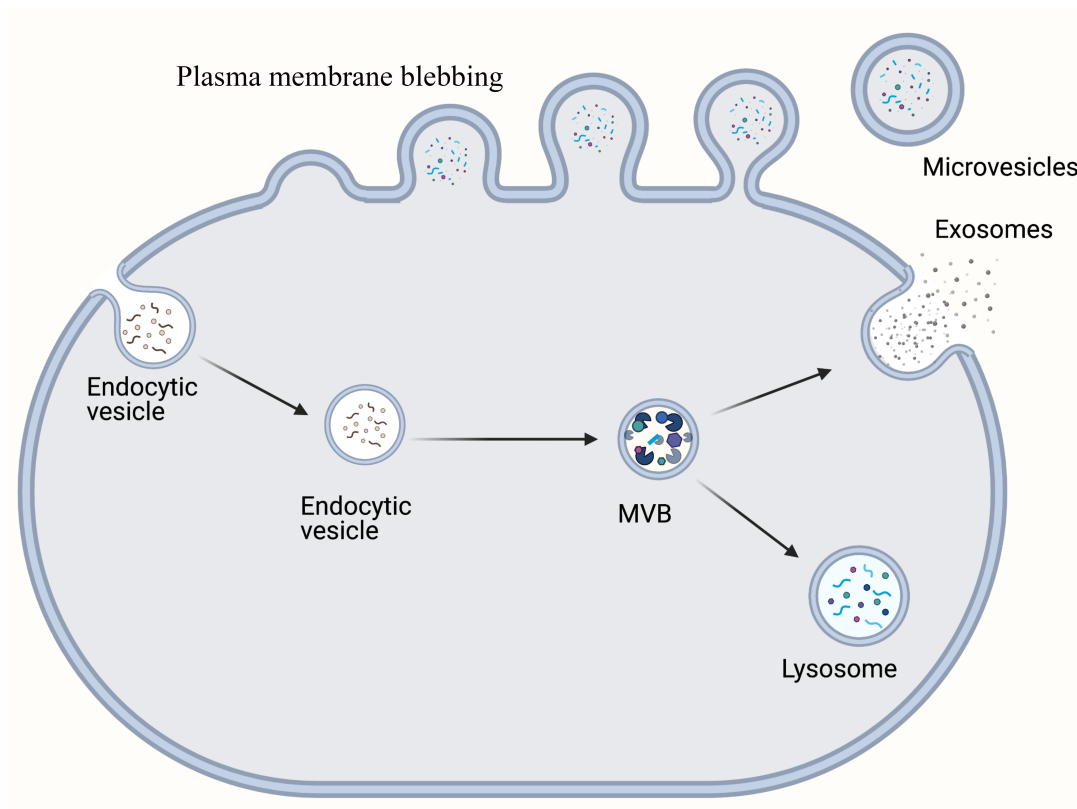


Figure 1. 7: Schematic representation of EV biogenesis.

The exosomes are formed by plasma membrane invagination followed by formation of endocytic vesicles consisting of intraluminal vesicles. These intraluminal vesicles progress to form multi vesicular bodies (MVB), which are then sorted for lysosomal degradation or progress to fuse with plasma membrane leading to exocytosis as exosomes. The microvesicles or (MVs) were formed as a result of blebbing of plasma membrane due to activation of cell surface receptors. The

plasma membrane blebbing undergo periodical shedding to form MVs. The figure was drawn using Biorender.

Recent studies point out the role of GPCRs in EV life cycle and also the role played by EVs in GPCR signal transduction pathways. Recently, The sorting of GPCRs into EVs has gained significant focus, recently. For example, the ciliary GPCR, NPY2R with dysregulated internalization mechanism is sorted into EVs (Nager et al., 2017), as well as angiotensin II type 1 receptor (AT1R) in rat cardiac pressure model (Pironti et al., 2015). Other GPCRs such as GPRC5B and GPR177 (orphan Wnt receptor) (Gross et al., 2012; Kwon et al., 2014) are sorted from golgi complex to intraluminal vesicles which are then sorted for extracellular release. The above few studies suggest a link between GPCR sorting into EVs and their extracellular release. Hence it is important to study the EV localization of GPCRs, especially chemosensory GPCRs in physiological and pathological conditions.

Chapter 2: Hypothesis and Objectives

2.1 Study Rationale

The epithelial cells in the oral cavity are the first responders to oral pathogens. They recognize several bacterial molecules with the help of Toll-like receptors (TLRs). TLRs are known to work in tandem with other cell surface receptors to mediate innate immune responses. In humans there are 25 T2Rs that are known to interact with several bacterial QSMs and modulate innate immunity. Several recent studies have shown T2Rs interacting with QSMs from *P aeruginosa* such as AHLs (3-Oxo-C12-AHL, C8-AHL), HHQ and NHQ. These interactions activate innate immune responses such as nitric oxide (NO) release, and increased ciliary beat frequency (CBF) in upper airway epithelial cells. Also, in immune cells such as macrophages, bitter agonists mediated activation of T2Rs induces NO secretion and phagocytosis. Hence there is well-documented evidence of T2R-QSM interactions, however most of these studies are limited to Gram-negative bacterial QSMs in the diseases CF and CRS. The interaction of T2Rs with Gram-positive bacterial QSMs and their physiological effects are not documented in oral epithelial cells.

Significant number of agonist activated GPCRs are internalized and recycled back to the plasma membrane through endosomes. Emerging evidence suggests that some of these GPCRs are also exported outside the cells by EVs. Recent studies have shown the localization of GPCRs such as somatostatin receptor 2 (SSTR-2), chemokine receptors 4 and 7 (CXCR4, CCR7) and angiotensin II type 1 receptor (AT1R) in EVs. However, the internalization mechanism of T2Rs, their sorting into endosomes, and the presence of T2Rs in EVs has not been characterized thus far.

2.2 Hypothesis

T2Rs are expressed in oral epithelial cells and interact with *S. mutans* QSMs, modulating innate immune responses and bacterial survival. The EVs from saliva express GPCRs including T2Rs.

2.3 Objectives

To test the above hypothesis, I pursued the three objectives described below using a combination of pharmacological, biochemical, microbial and physiological approaches.

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

The first focus of this objective is to analyze the expression of T2Rs in GECs. The second focus is to study the interaction of the highly expressed T2R in GECs with CSPs secreted by *S. mutans*, and the resulting innate immune responses.

2.3.2 Bitter taste receptor T2R14 modulates Gram-positive bacterial internalization and survival in gingival epithelial cells.

The primary focus of this objective is to elucidate the role of T2R14 in internalization of Gram-positive bacteria and to characterize the T2R14 mediated bactericidal effect on *S. aureus* and *S. mutans*.

2.3.3 Characterization of GPCRs in Extracellular Vesicles (EV).

The focus of this objective is to characterize the presence of T2Rs in EVs isolated from a heterologous cell system and from saliva.

Chapter 3 General method

3.1 Materials

3.1.1 Reagents used in the study

The following reagents were commonly used in the study: Diphenhydramine (DPH), dimethyl sulphoxide (DMSO), fetal bovine serum (FBS), trypsin-EDTA, phospho lipase C inhibitor (U73122), phosphatase inhibitor cocktail 3 (Cat. #P0044) were acquired from Sigma Aldrich (Oakville, ON, Canada). Apigenin (API) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Fluo-4 NW calcium assay kit, Dulbecco's modified Eagle medium (DMEM)/Ham's F12 (F12), DMEM (High glucose), penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml), hygromycin B (Cat. #10687010), lipofectamine 2000, opti-MEM, western blot stripping buffer, Gβγ inhibitor (Gallein) (Cat. #3090) was purchased from Tocris. Protease inhibitor cocktail (Cat. #539131) was obtained from Millipore. QIAprep plasmid extraction kit and RNeasy Mini Kit (Cat. #74104) were acquired from Qiagen (Toronto, ON, Canada).

3.1.2 Media for bacterial culture

Luria-Bertani (LB) media: 10 g of tryptone, 10 g of sodium chloride, 5 g of yeast extract were dissolved in 1 L of deionized water and autoclaved for 45 mins.

LB agar: To the LB media prepared above, 15 g of agar was added and the medium was autoclaved for 45 mins. After autoclaving approximately 15 ml of medium was added to petri dishes and allowed to solidify at room temperature. For plates with antibiotic, 100 µg/ml ampicillin was added to 1 L of LB agar. The plates were stored at 4°C until use.

Brain heart infusion (BHI) broth: 40 g of BHI is dissolved in 1 L of deionized water and was autoclaved for 45 mins.

BHI agar: 40 g of BHI and 10 g of agar were dissolved in 1 L of deionized water and were autoclaved for 45 mins. After autoclaving, approximately 15 ml of media was added to each petri plates. The media was allowed to solidify and the plates were stored at 4°C until use.

3.1.3 Media for mammalian cell culture

For culture of GEC cell lines (OKF6 and TIGK) the Keratinocyte growth medium -2 (KGM-2) is supplemented with bovine pituitary extract (0.004 ml/ml), recombinant epidermal growth factor (EGF) (0.125 ng/ml) recombinant insulin (5 µg/ml), hydrocortisone (0.33 µg/ml), epinephrine (0.39 µg/ml), recombinant human transferrin (10 µg/ml) and CaCl₂ (0.06 mM).

For culture of promyeloblast (HL-60) cells, the Iscove's modified dulbecco's medium (IMDM) was supplemented with 20% FBS.

For culture of HEK293T cells the Dulbecco's modified Eagle medium (DMEM)/Ham's F12 (F12) was supplemented with 10% heat inactivated FBS, and penicillin (10,000 U/ml)-streptomycin (10,000 µg/ml).

3.1.4 Buffers

PBS: 137 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl (pH 7.6)

SDS-PAGE running buffer: 25 mM Tris base, 192 mM Glycine, 0.1% SDS (pH 8.3)

TAE buffer: 40 mM Tris base, 20 mM Acetic acid, 10 mM EDTA (pH 8.0)

TBS: 20 mM Tris base, 150 mM NaCl (pH 7.6)

TBST: TBS + 0.05% Tween20

Turbo blot transfer buffer: 20 ml of 5x Turbo blot transfer buffer was diluted with 20 ml of methanol and 60 ml of deionized water.

Cell lysis buffer (Immunoblot analysis): 50 mM Tris base, 150 mM NaCl, 10% Glycerol, 1% DM with 1X protease inhibitor and 1X phosphatase inhibitor cocktails.

Sample loading buffer (2X Laemmli buffer): 125 mM Tris base, 20% 2-Mercaptoethanol, 20% Glycerol, 4% SDS, 0.004% Bromophenol blue (pH 6.8).

Agarose loading buffer: 10 mM Tris-base (pH 7.6), 60 mM EDTA, 60% Glycerol, 0.03% Bromophenol blue.

FACS buffer: 1X PBS with 0.5% BSA.

Chapter 4: Bitter taste receptor T2R14 detects quorum sensing molecules from cariogenic *Streptococcus mutans* and mediates innate immune responses in gingival epithelial cells.

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Author contributions to chapter 4

Manoj Reddy Medapati designed the study, performed majority of the experiments (calcium mobilization assays, generation of T2R14 KO cells, western blots and migration assays) and wrote the first-draft of the manuscript. Dr. Nisha Singh performed Meso Scale cytokine ELISA. Dr. Anjali Bhagirath performed genomic DNA extractions and PCR. Dr. Barbara Triggs-Raine designed the CRISPR/Cas9 probes. Dr. Kangmin Duan and Dr. Eraldo L Batista Jr gave guidance with different experiments. Dr. Prashen Chelikani designed the study, obtained funding, overall guidance and edited the manuscript.

4.1 Abstract

Host-pathogen interactions play an important role in defining the outcome of a disease. Recent studies have shown that the bacterial QSM can interact with host cell membrane proteins, mainly 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 CSPs secreted by cariogenic bacterium *S. 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 show 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.

4.2 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) (Kirschning et al., 2002; Lu et al., 2008). 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 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 (Alvarez-Curto et al., 2016; Bufe et al., 2015; Pundir et al., 2019; Rasoamanana et al., 2012). Recent studies have shown that the 25 T2Rs in humans which form the third largest GPCR group, act as important sensors for the bacterial QSMs (Alfonso-Prieto et al., 2019; Freund et al., 2018; Munk et al., 2016; A. S. Shah et al., 2009).

T2Rs are predominantly involved in taste chemosensing though studies have identified their extra-oral localization (Adler et al., 2000; Jaggupilli et al., 2017; Lindemann, 1996; Shaik et al., 2016). Emerging evidence shows T2R interaction with bacterial QSMs leads to innate immune responses in diseases such as CF and Chronic Rhinosinusitis (CRS) (Adappa et al., 2016; R. J. Lee et al., 2012a). In CF, the *P. aeruginosa* derived QSMs, mainly AHLs, interact with T2Rs in airway epithelial cells (Freund et al., 2018; A. S. Shah et al., 2009). In our previous study, we characterized the binding sites on T2R4, T2R14 and T2R20 for the QSMs 3-Oxo-C12-AHL and C8-AHL (Jaggupilli et al., 2018). 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 (Freund et al., 2018). A recent study suggested T2Rs are immune sentinels in the gingival solitary

chemosensory cell (GSC) of a mouse periodontitis infection model (Zheng et al., 2019). 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 (Y. H. Li et al., 2001; Senadheera et al., 2008; Tanzer et al., 2001). 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 (Pundir et al., 2019). 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.

4.3 Material and Methods

4.3.1 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), IkBa (#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).

4.3.2 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 (Dickson et al., 2000; McMahon et al., 2011). All the GECs were cultured in serum free KGM-2 supplemented with 0.6 mM CaCl₂. HEK293T and HEK293T cells overexpressing T2R14 and T2R39 receptors were generated as previously described (Shaik et al., 2019). 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 (Millius et al., 2010). Briefly, 2×10^5 cells/ml were seeded in 25 ml medium 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.

4.4 Methods

4.4.1 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/well. 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 µg 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 $\leq .05$. The signal intensity values were used to generate heat maps for transcripts in the RStudio using ggplot2 package (Version 3.2.1) (H, 2016).

4.4.2 Generation of T2R14 knock down (KD) OKF6 cells using CRISPR-Cas9 system

According to the HUGO nomenclature the T2R coding genes are represented as TAS2R (Braschi et al., 2019). A total of 100 000 OKF6 cells/well were seeded in a 6-well plate. After 24 hours, 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 CRISPRcrRNA (#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 (Zuris et al., 2015). The efficiency of transfection was analyzed using CytoFLEX flow cytometer on the basis that only positive cells contain ATTO550 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/10 s, 35 cycles of 95°C/30 s, 62°C/30s, 72°C/30 s, and a final extension at 72°C/1 min. 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 as 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.

4.4.3 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 (Jaggupilli et al., 2018; Shaik et al., 2019). 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 minutes followed by another incubation at room temperature for 30 minutes. 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 (Jaggupilli et al., 2017). ATP (1 μM) a well-known activator of PLC signaling induced by purinergic receptors was used as a control (Takenouchi et al., 2005). 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).

4.4.4 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 (Shultz et al., 2008). 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).

4.4.5 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 (Singh et al., 2020). 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-IkBa^{Ser 32}, IKK α , IKK β , IkBa (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).

4.4.6 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) (Qiao et al., 2009), NF-kB signaling inhibitor BAY 11-7082 (10 μ M) (L. Zhang et al., 2011), peptide inhibitor for TLR adaptor protein MyD88 Pepinh-MyD (40 μ M) (L. Zhang et al., 2011), 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) (Chandler et al., 2015). 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.

4.4.7 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 fluorescence intensity

was measured using FACS canto analyzer and quadrant gating was used to identify the percentage of cells expressing the markers.

4.4.8 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 (Singh et al., 2020).

4.4.9 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).

4.5 Results

4.5.1 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 (Allen et al., 2017; Kaur et al., 2019; Wiley et al., 2018). 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* (Braschi et al., 2019). Our analysis identified 26 GPCR transcripts that are medium to highly expressed in both GECs and *TAS2RI4* is present among these (**Figure 4.1A**). The normalized signal intensity values were compared using one-way analysis of variance (Anova) to examine the differential gene expression. The expression of *TAS2RI4* 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 ($p<0.9$) (**Figure 4.1B**). To verify abundant expression of the T2R14 protein in these GECs, we used western blot analysis (**Figure 4.1C**). The detected T2R14 was much higher than that of another T2R, T2R39 (**Figure 4.1D**). These findings were consistent with the mRNA expression profiles shown in Figure. 1B where *TAS2RI4* mRNA levels were significantly higher than those of *TAS2R39*.

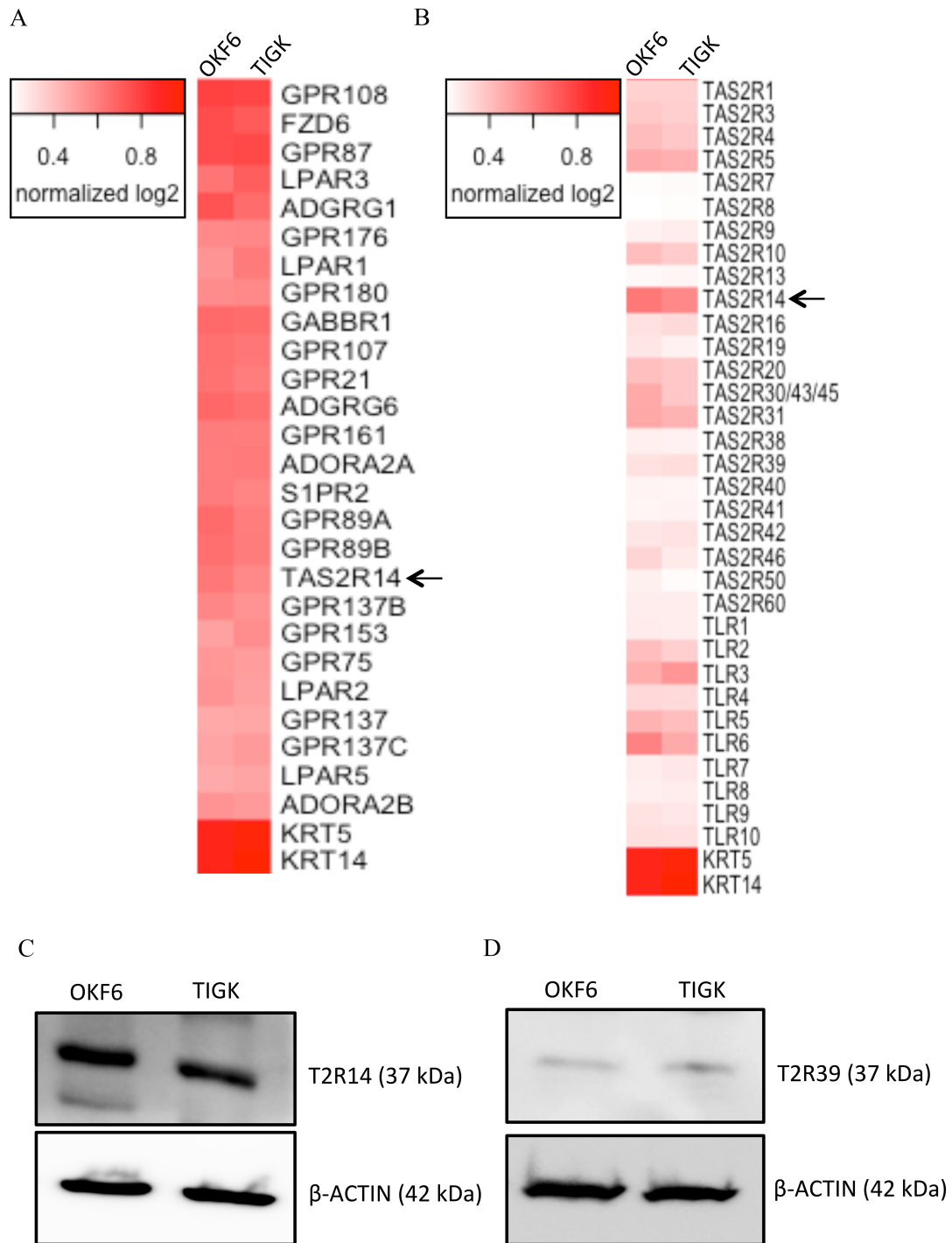


Figure 4. 1: Expression analysis of GPCRs, TAS2Rs and TLRs in GECs.

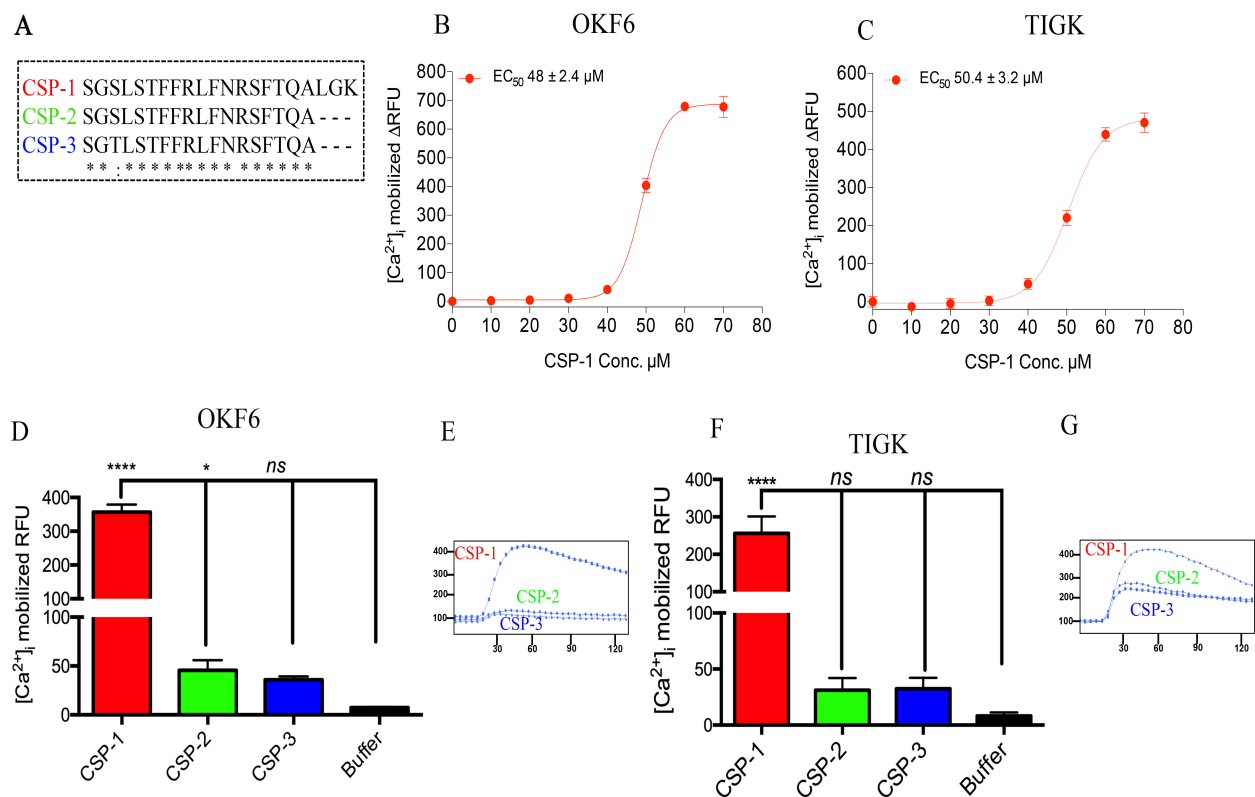
A: Heat map of highly expressed GPCRs in OKF6 and TIGK cells. **B:** Heat map of TAS2Rs and TLRs. The signal intensities were normalized using *RPS23* transcript. Epithelial cell markers (*KRT5* and *KRT14*) were used as a reference in the analysis. The arrow represents *TAS2R14*.

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 (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

4.5.2 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 (Y. H. Li et al., 2001). CSP-1 is cleaved at the C-terminus by membrane protease sepM into an 18 amino acid CSP-2 (Hossain et al., 2012b). 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 (Hillman et al., 1987). The *S. mutans* strains secreting CSP-1 were shown to have higher transformation efficiency compared to strains containing c-terminal truncated CSP (Y. H. Li et al., 2001). To further study CSP-1, CSP-2 and CSP-3, we had these peptides synthesized commercially with >98% purity (**Figure 4.2A**). 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 EC_{50} of ≈ 50 μ M (**Figure 4.2B, C**). Next, we treated the GECs with CSP-2 and CSP-3 using the EC_{50} 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 (**Figure 4.2D-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) (Figure 2H). CSP-1 (Δ K21) showed an EC_{50} 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 (**Figure 4.2I-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 (**Figure 4.2M,N**).



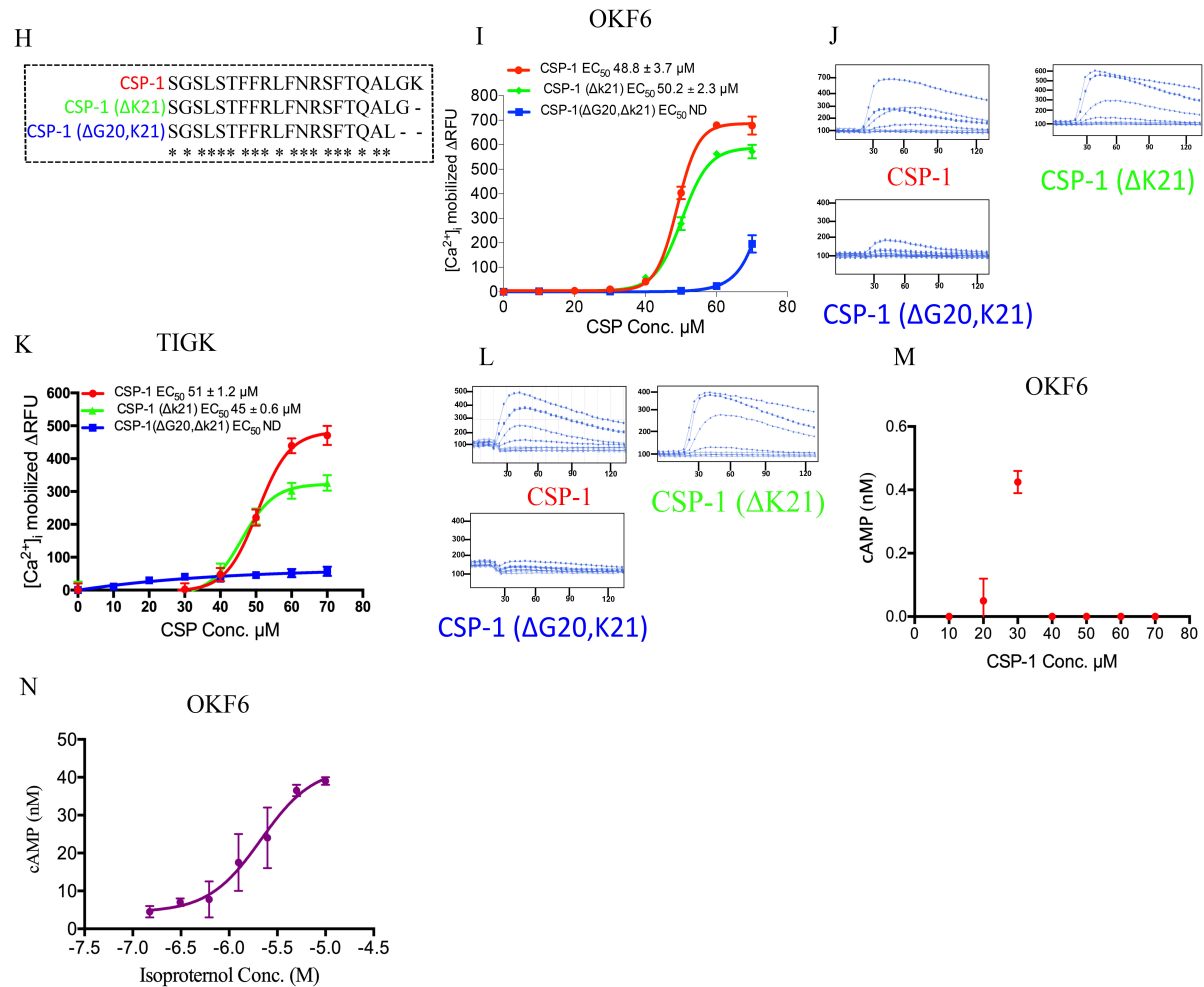


Figure 4. 2: Intracellular calcium mobilization in GECs in response to *S. mutans* CSPs.

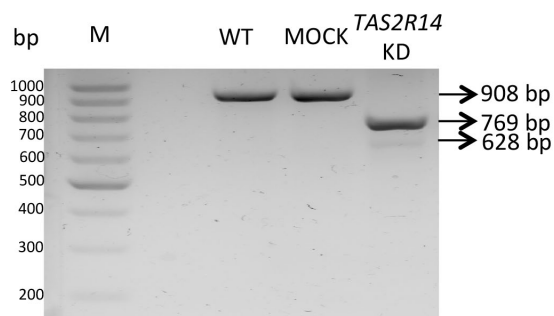
A: Multiple sequence alignment of *S. mutans* CSPs used in this study. **B and C:** OKF6 and TIGK cells are treated with increasing concentrations of synthetic CSP-1 (10 μ M-70 μ M) and the intracellular calcium mobilization was measured using Fluo-4NW fluorescent dye. The EC_{50} values were obtained 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 μ M) responses of CSP-1 (red curve), C- terminal amino acid deletion peptides CSP-1 (Δ K21, green curve) and CSP-1 (Δ G20, Δ K21 blue curve) treated OKF6 and TIGK cells. The intracellular calcium mobilized was measured and EC₅₀ 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 μ M) 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 (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

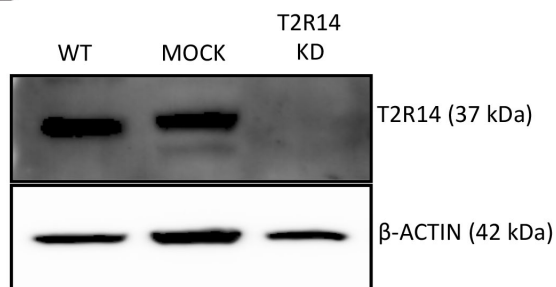
4.5.3 CSP-1 induced calcium response in GECs occurs through T2R14.

To test whether CSP-1 induced calcium responses are dependent on T2R14. T2R14 KD was performed 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 (**Figure 4.3A**). 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 (**Figure 4.3B**). 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 ($***p<0.001$) (**Figure 4.3C-F**). We also assayed CSP-1 induced calcium mobilization in the presence of the T2R14 antagonist 6-MF (Hariri et al., 2017). 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 (**Figure 4.3G and H**). Previous studies have shown 6-MF as an antagonist for T2R39 (Roland et al., 2014). However, our western blot analysis revealed T2R39 expression was minimal in GECs (Figure 1D) and CSP-1 did not induce intracellular calcium mobilization in HEK293T cell overexpressing T2R39 (**Figure 4.4A and B**). In contrast, HEK293T cells overexpressing T2R14 (**Figure 4.3I**) showed a concentration dependent increase in calcium mobilization upon treatment with CSP-1 and DPH (**Figure 4.3J-M**). These data indicate CSP-1 induced calcium responses are predominantly mediated through T2R14 in GECs.

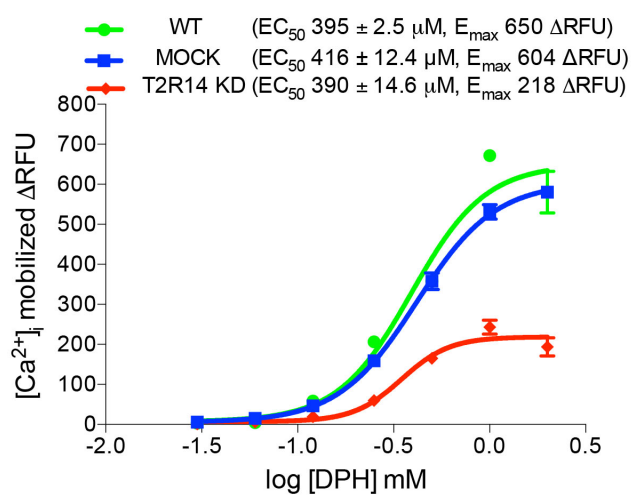
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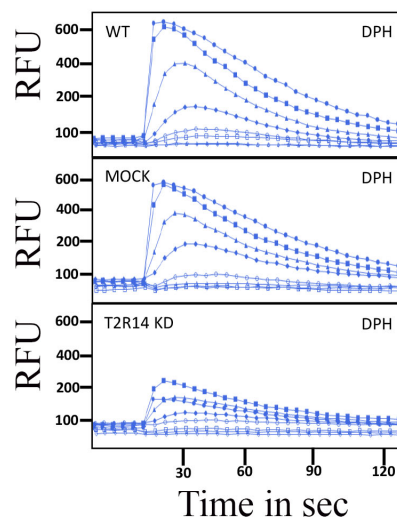
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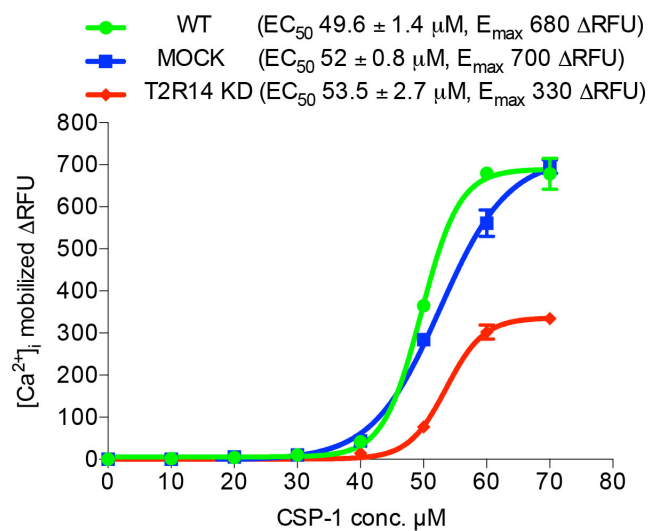
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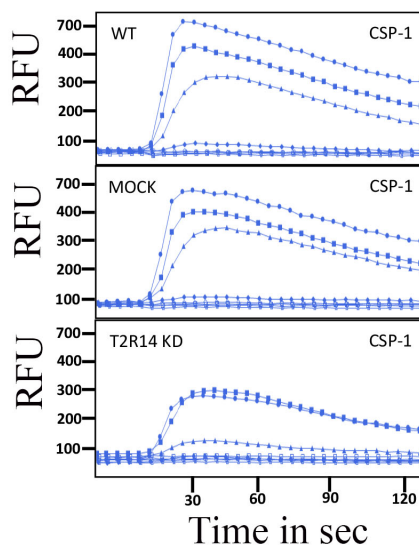
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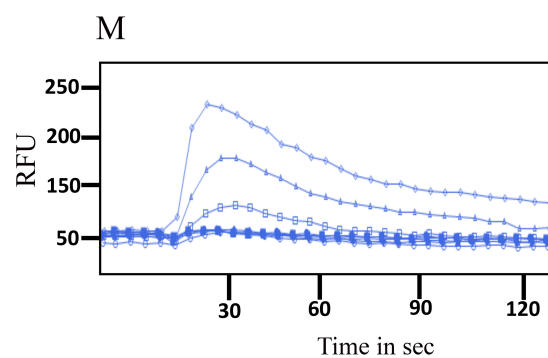
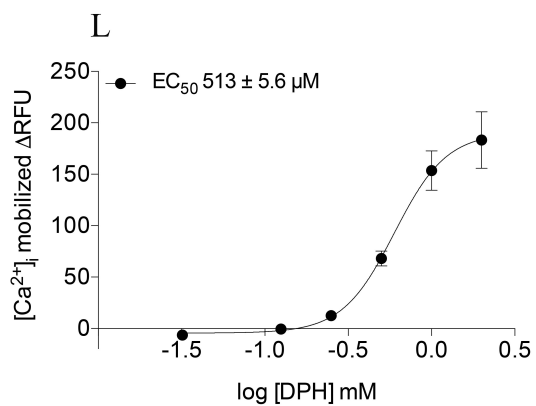
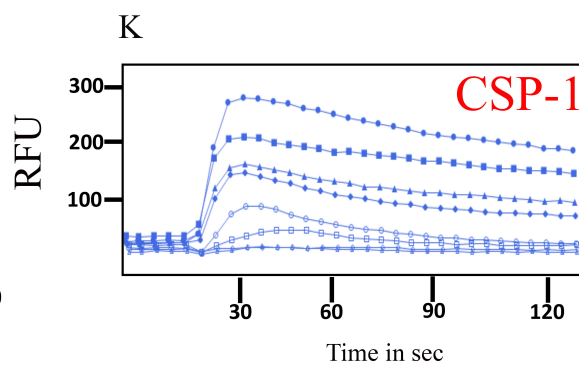
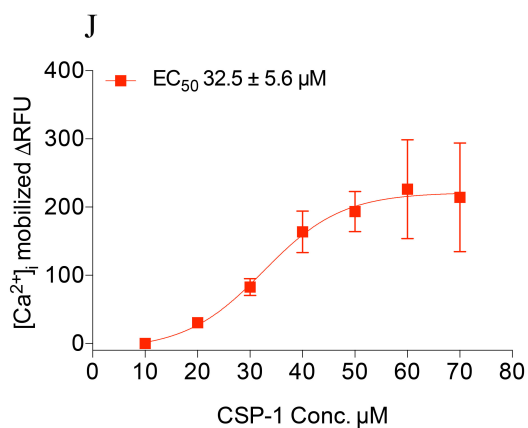
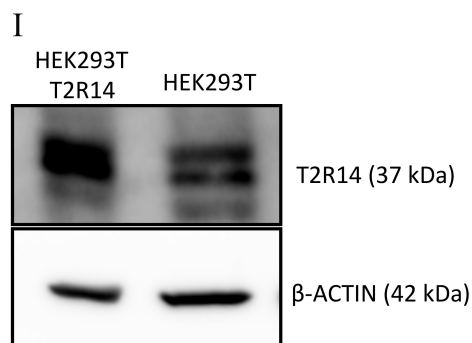
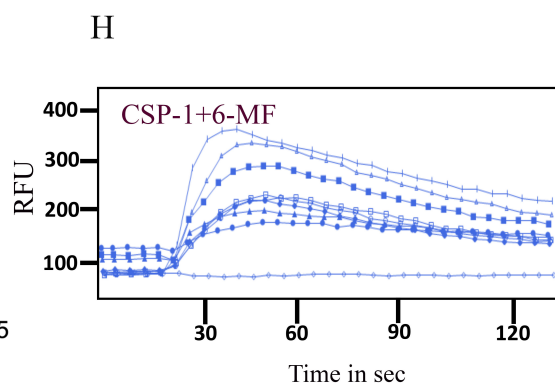
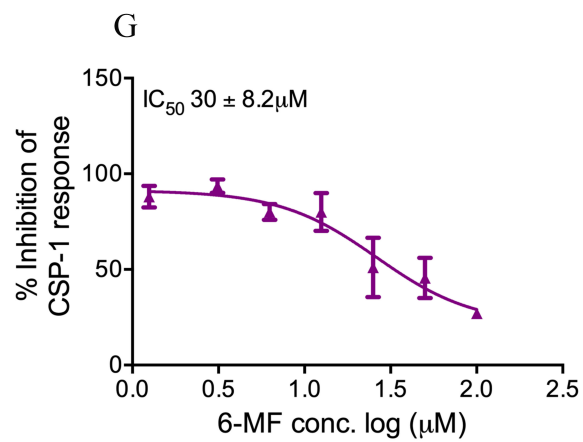


Figure 4. 3: Characterization of CSP-1 and T2R14 dependent calcium signaling in OKF6 and in heterologous cells overexpressing T2R14.

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 µM) 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 µM) and T2R14 antagonist 6-MF (1.25 – 100 µM). 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 µM) 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 (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

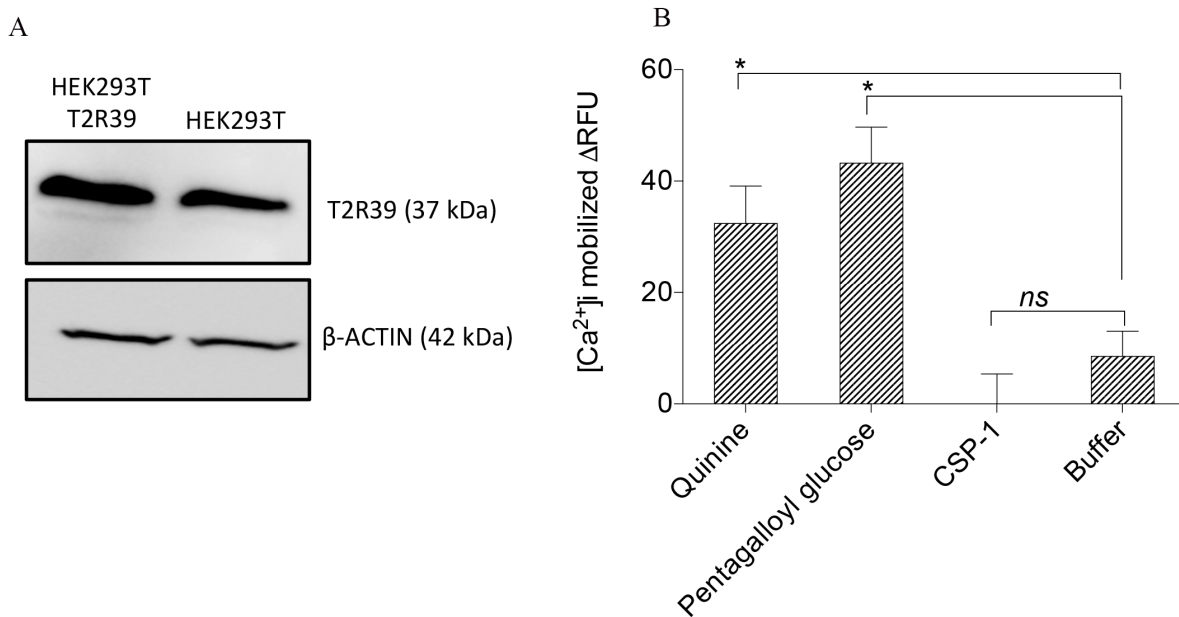


Figure 4. 4: Characterization of T2R39 activity using heterologous cells.

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 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 (** $p < 0.02$) (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

4.5.4 CSP-1 induces calcium response in GECs through the canonical T2R14- $G_{\alpha\beta\gamma}$ -PLC $_{\beta}$ 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 $_{\beta}$ 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) (**Figure 4.5A-D**).

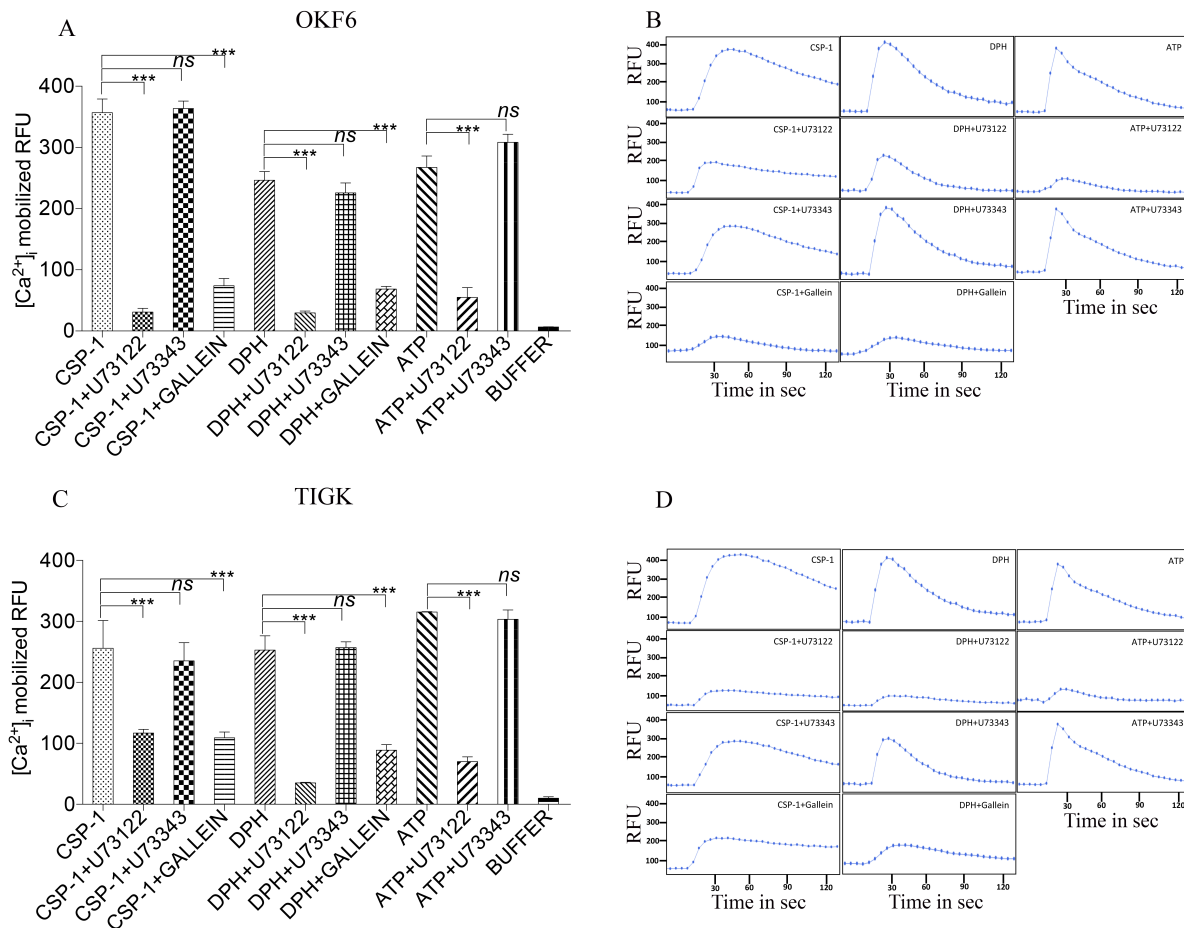


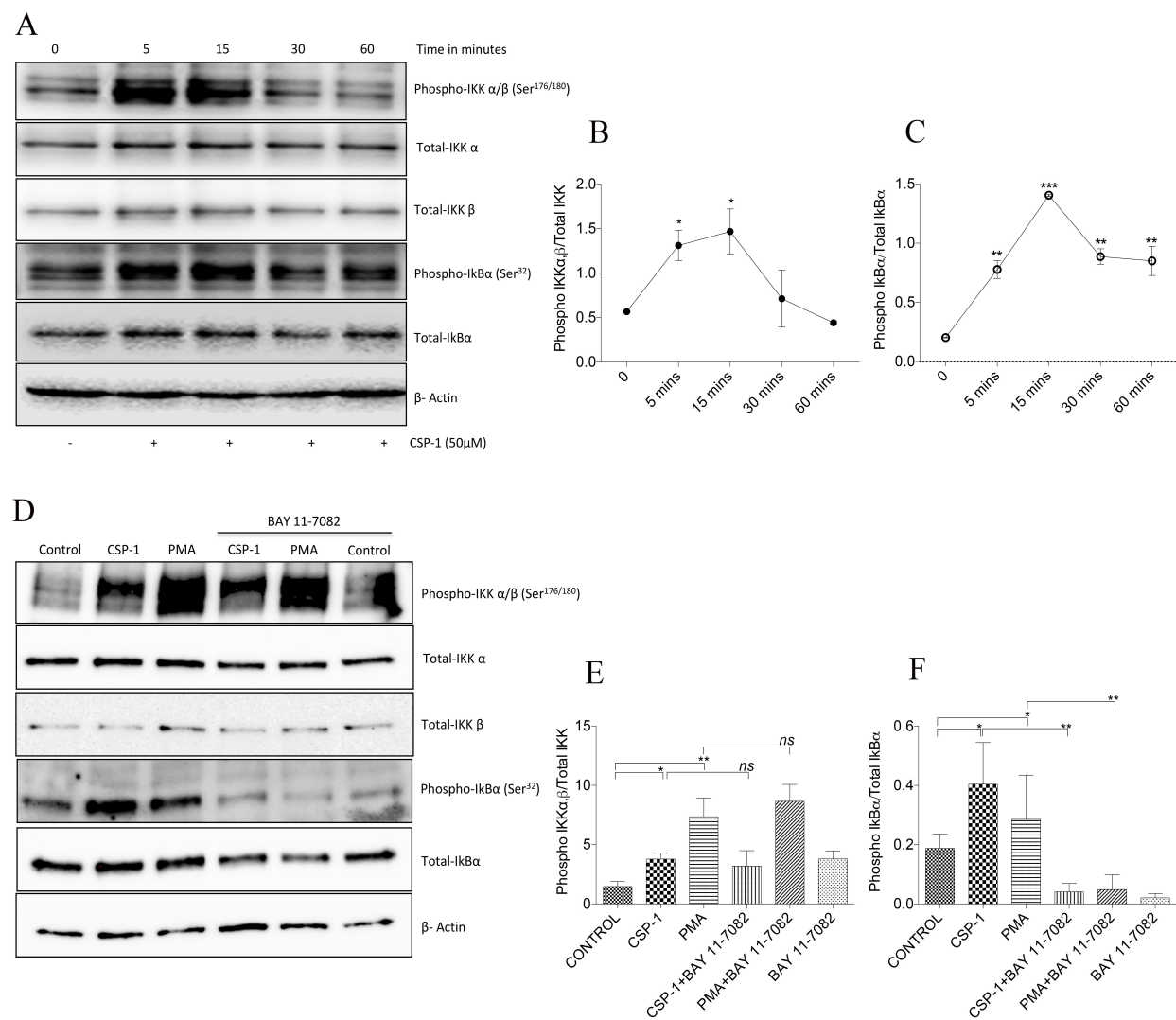
Figure 4. 5: Characterization of effect of PLC_β and G_{βγ} inhibitors on calcium mobilization in CSP-1 and T2R14 agonist DPH treated GECs.

A and C: OKF6 and TIGK cells are treated with CSP-1 (50 μM), DPH (500 μM) and ATP (1 μM) alone or in combination with 5 μM of U73122 (PLC_β inhibitor) and its analogue U73343 and 10 μM gallein (G_{βγ} 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 triplicate (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

4.5.5 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 (Dickson et al., 2000). The PLC signaling is shown to induce PKC isoforms (Gukovsky et al., 2004) which can activate the NF-kB signaling pathway (Suh et al., 2008). 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 (**Figure 4.6A-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 (**Figure 4.6B, C**). The phosphorylation of IKK α/β reached basal levels after 30 mins (**Figure 4.6B**), while phosphorylation of I κ B α did not reach basal levels (**Figure 4.6C**). 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 (**Figure 4.6D 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 (**Figure 4.6G-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 (**Figure 4.6J-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-κB signaling intermediates in OKF6 cells (**Figure 4.7A and B**).



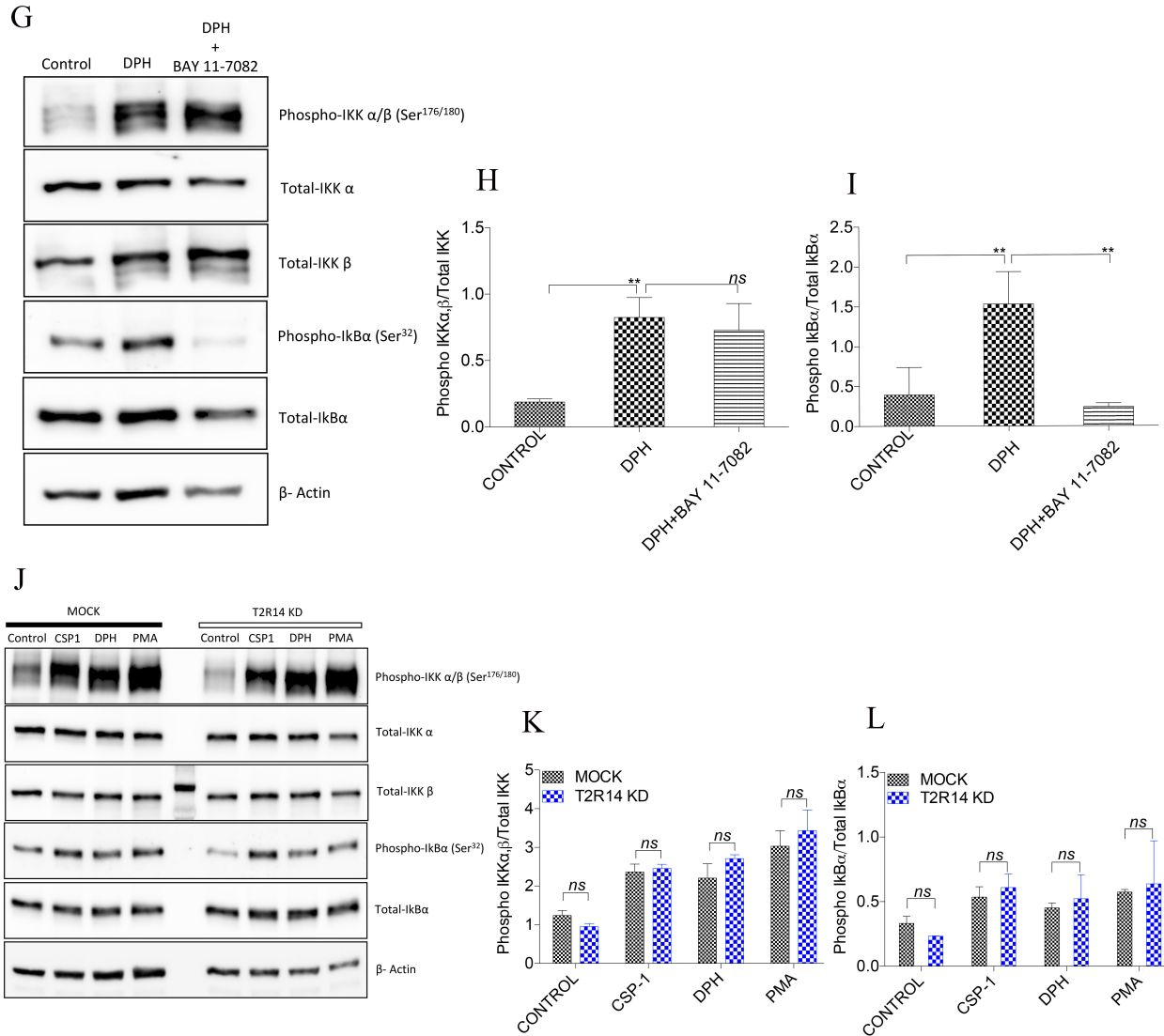


Figure 4. 6: *S. mutans* CSP-1 induced activation of NF-κB signaling pathway in WT, MOCK and T2R14 KD OKF6 cells.

Western blot analysis of total protein extracts collected and separated on 10% SDS-PAGE. The blots were probed with phospho and total antibodies for IKKα/β, IκBα (1:1000) and HRP- Goat anti-rabbit or mouse (1:3000). **A:** OKF6 cells treated with CSP-1 (50 μM) for different time points (0-60 mins). **B and C:** Densitometry analysis of phosphorylation of IKKα/β^{Ser176/180} and IκBα^{Ser32} are represented by quantifying the band intensity using imageJ analysis software. **D and G:** Cells treated with CSP-1 (50 μM), DPH (500 μM), PMA (100 nM) alone or in combination

with IKB α phosphorylation inhibitor BAY 11-7082 (10 μ M). **E, F, H and I:** Densitometry analysis of phosphorylation of IKK α/β ^{Ser176/180}, IKB 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 IKB 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 (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

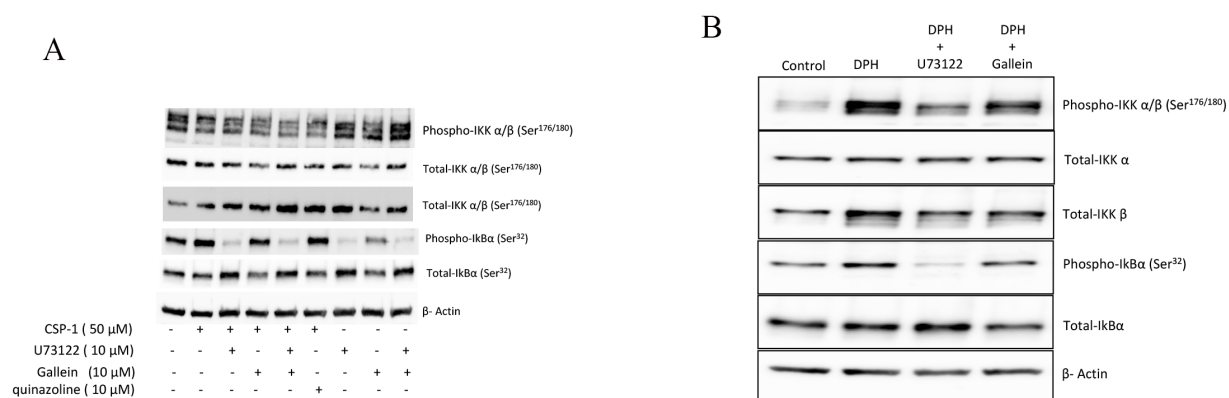


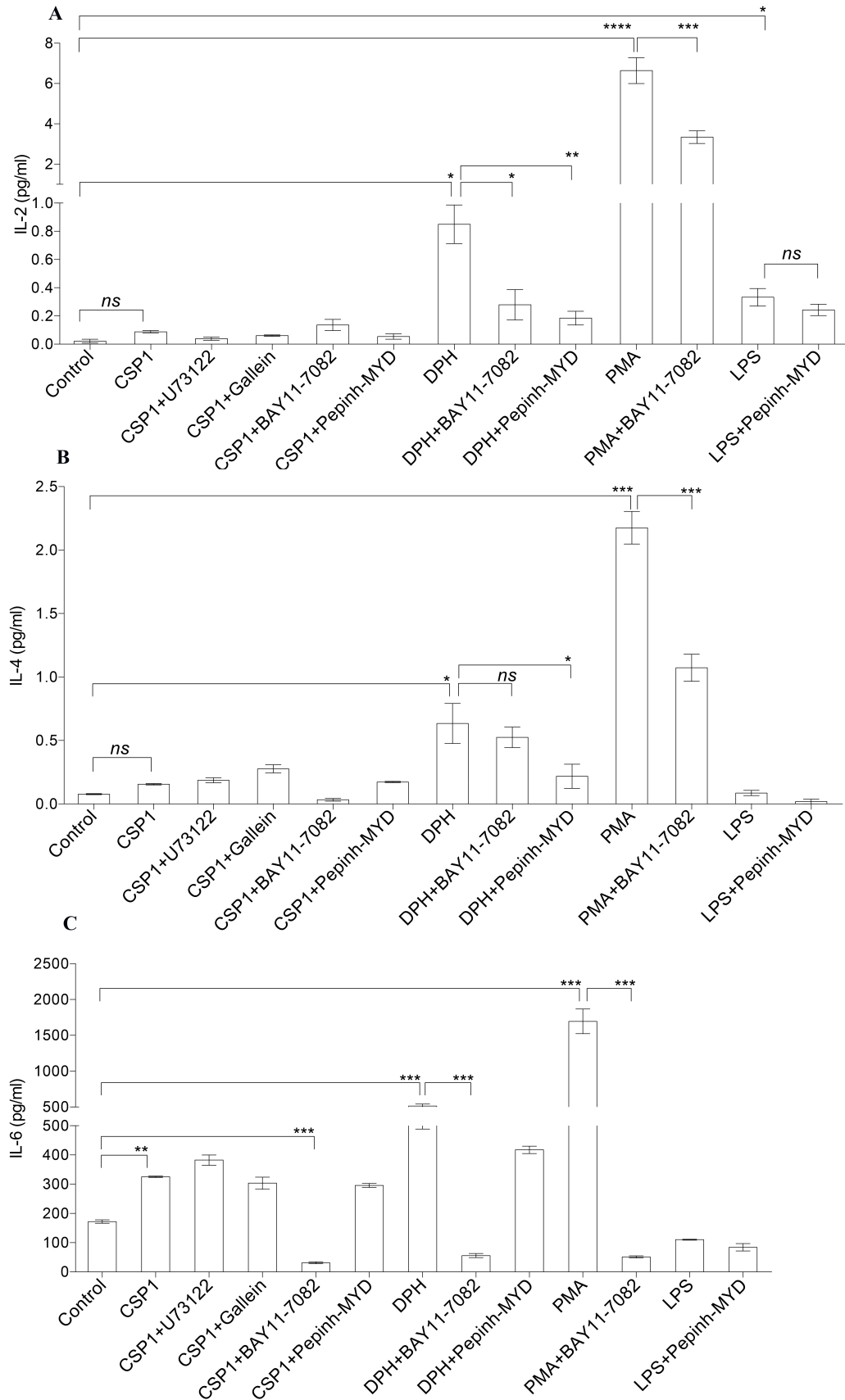
Figure 4. 7: Characterization of NF-kB signaling pathway upon inhibition of T2R downstream signaling.

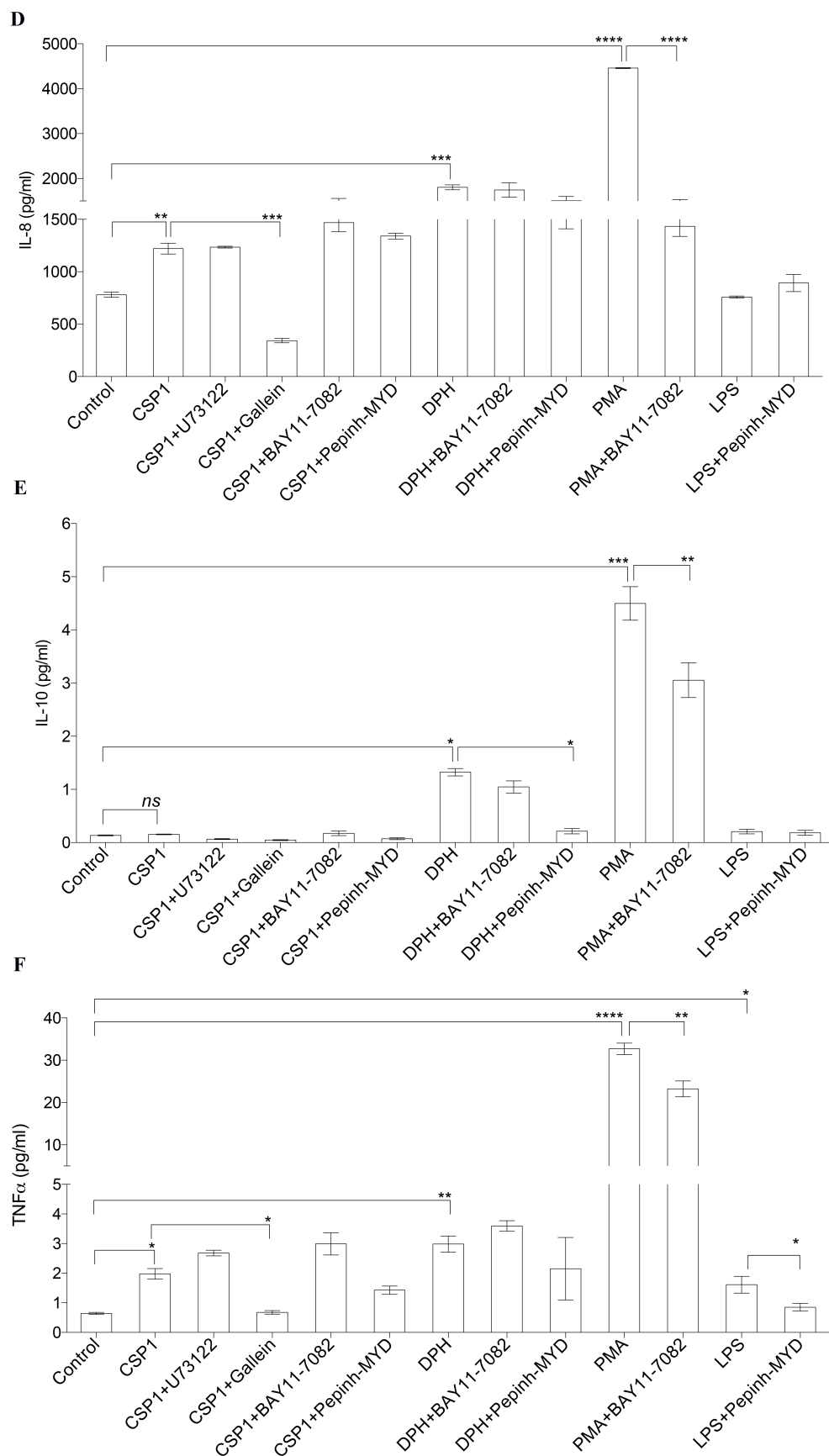
Western blot analysis of total protein extracts separated on 10% SDS-PAGE. The blots were probed with phospho and total antibodies for IKK α/β , IKB α (1:1000) and HRP- Goat anti-rabbit or mouse (1:3000) to quantify IKK α/β ^{Ser176/180} and IKB α ^{Ser32} protein phosphorylation. **A and B:** OKF6 cells 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) (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

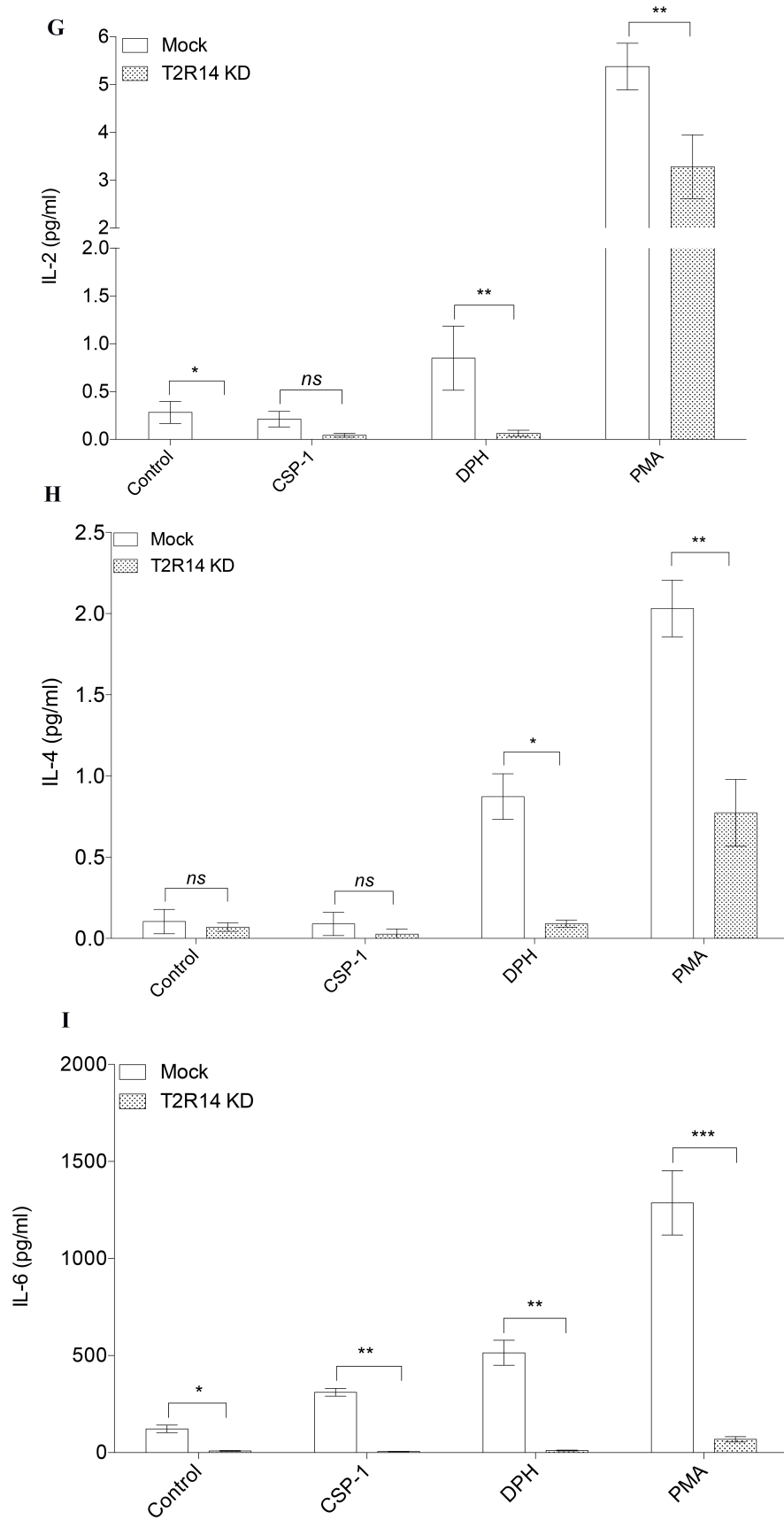
4.5.6 CSP-1 induces cytokine secretion in GECs.

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 (Pundir et al., 2019; Zhu et al., 2008). 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 and hence this was not pursued in further analysis. We observed increased levels of IL-6 (** p <0.0034) (**Figure 4.8C**), IL-8 (** p <0.0014) (**Figure 4.8D**) and TNF- α (* p <0.0177) (**Figure 4.8F**) in CSP-1 treated cells. However, no significant change in levels of IL-2 (**Figure 4.8A**), IL-4 (**Figure 4.8B**) and IL-10 (**Figure 4.8E**) 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) (**Figure 4.8A**), IL-4 (* p <0.05, *** p <0.0008) (**Figure 4.8B**), IL-6 (** p <0.002, *** p <0.0009) (**Figure 4.8C**), IL-8 (** p <0.002, **** p <0.0001) (**Figure 4.8D**), IL-10 (* p <0.05, *** p <0.0008) (**Figure 4.8E**) and TNF- α (** p <0.002, **** p <0.0001) (**Figure 4.8F**). 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) (**Figure 4.8F**). 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 ($***p<0.001$) (**Figure 4.8D**) and TNF- α ($*p<0.05$) (**Figure 4.8F**) 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 (**Figure 4.8G-L**). A significant decrease in basal IL-2, IL-6, IL-8, TNF- α levels were observed ($*p<0.05$) upon T2R14 KD (**Figure 4.8G-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 $**p<0.01$) (**Figure 4.8G-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 $*p<0.05$) (**Figure 4.8G-L**).







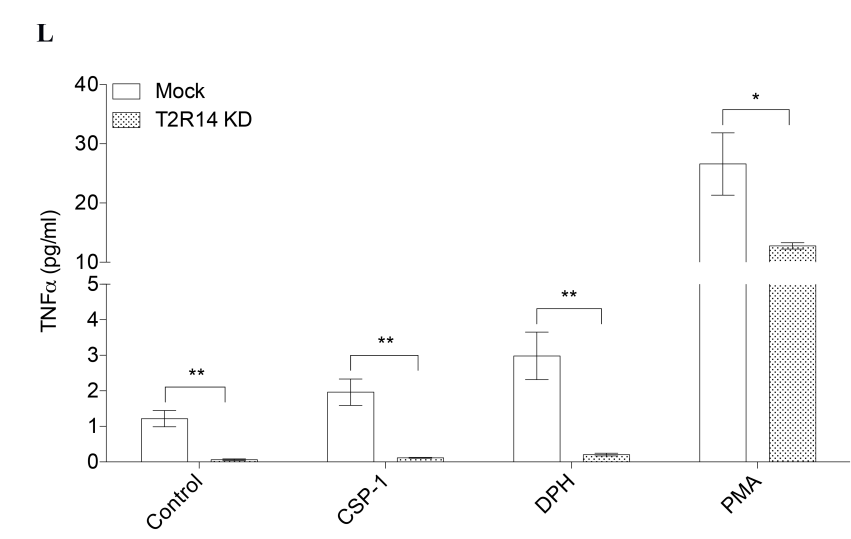
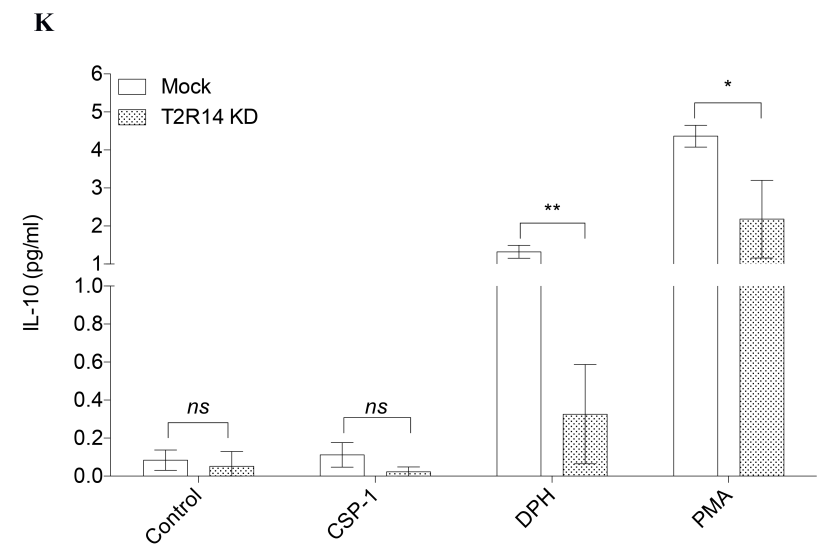
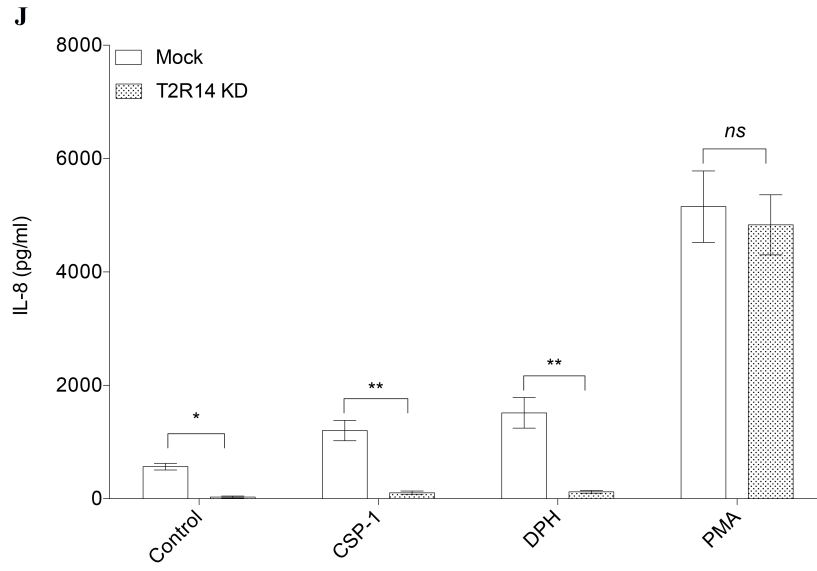


Figure 4. 8: Quantification of cytokines secreted in OKF6 WT, MOCK and T2R14 KD OKF6 cells by Meso Scale multiplexing ELISA.

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 (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

4.5.7 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 dHL-60 as a model (**Figure 4.9A**) (Millius et al., 2010). FACS analysis showed an increased expression of CD11b and CD14 cell surface markers in dHL-60 cells as compared to the undifferentiated cells (**Figure 4.9A**). 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 (*** $p < 0.002$) (**Figure 4.10B, 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 (**** $p < 0.0001$, ** $p < 0.01$, *** $p < 0.001$) (**Figure 4.10B, 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 (**Figure 4.10D, E**). However, the migratory effect was the same when PMA treated CM from MOCK and T2R14 KD cells was used (**Figure 4.10D, E**). These results confirm that CSP-1 activation of T2R14 in OKF6 cells creates a microenvironment that can act as an attractant for neutrophils.

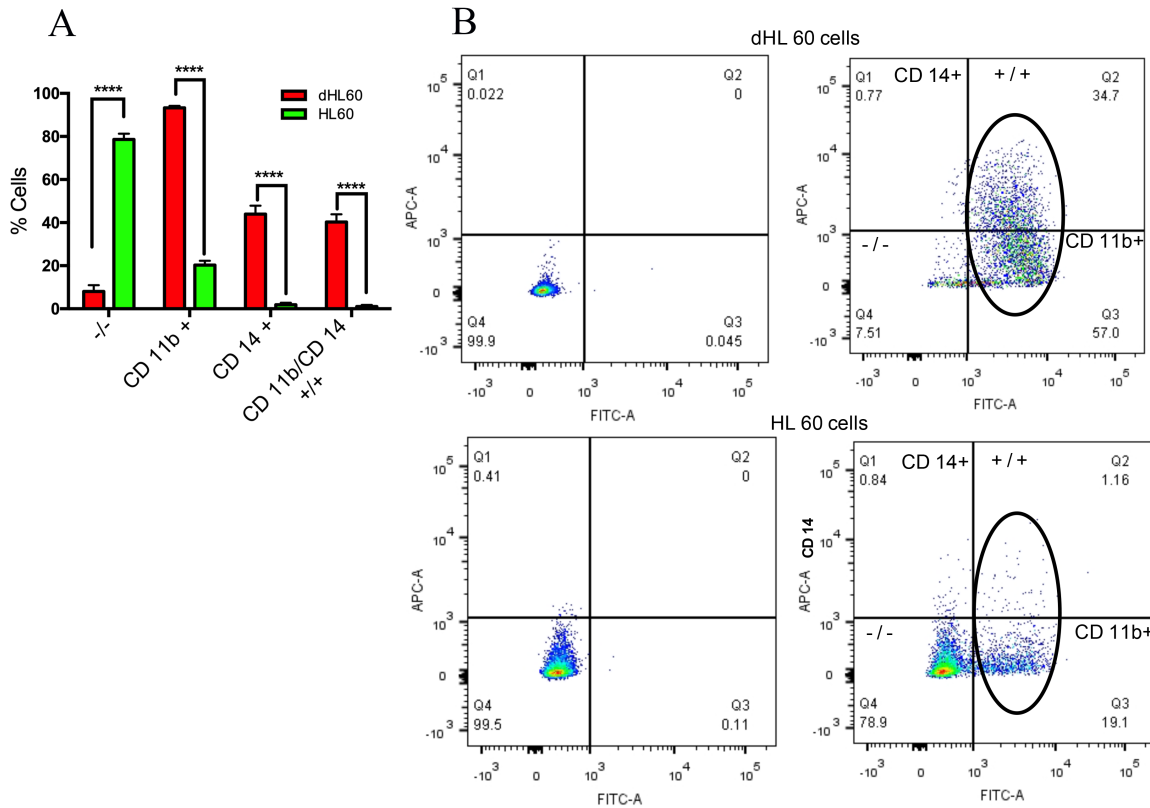
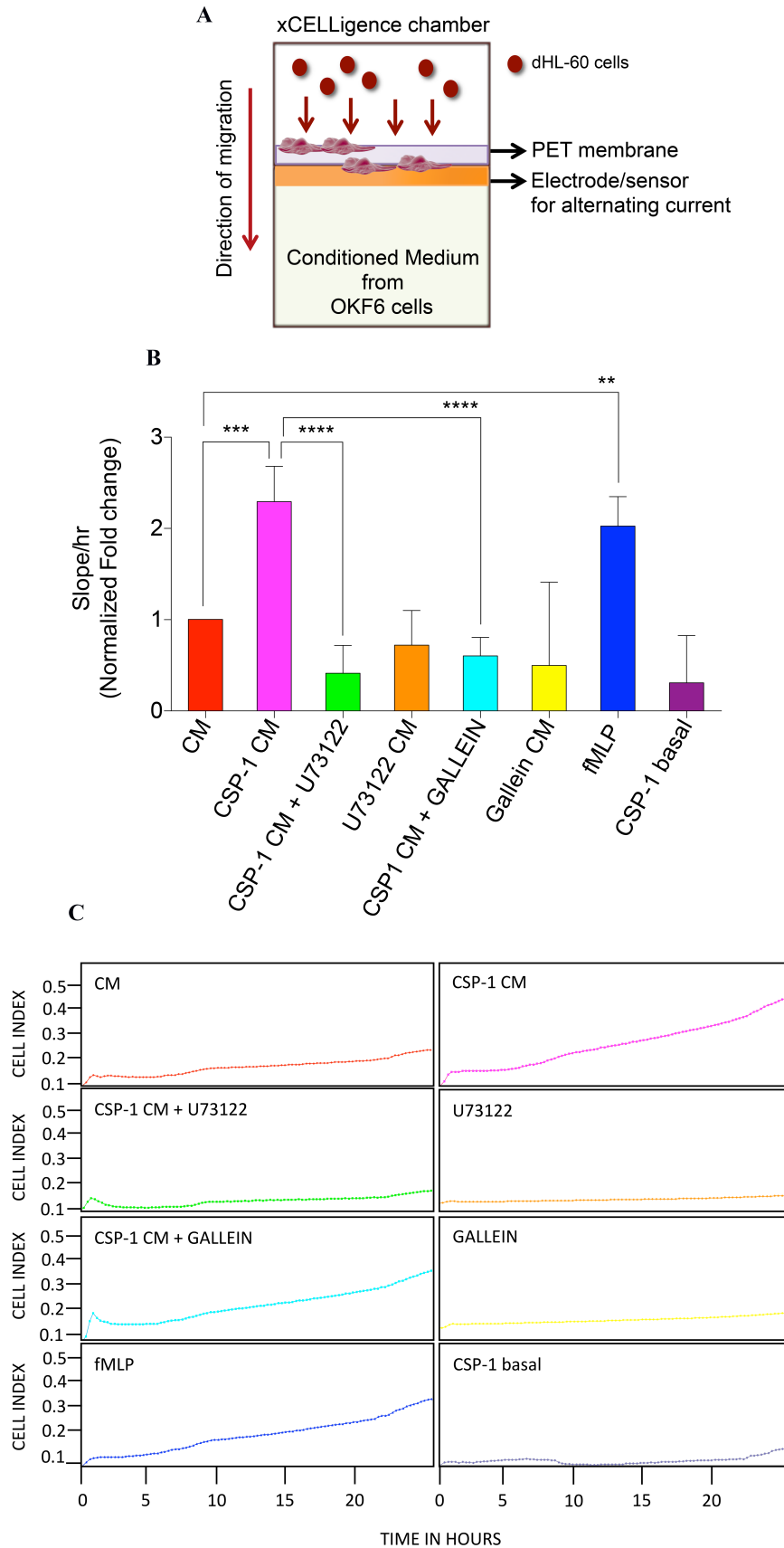


Figure 4. 9: Cell surface expression of CD14 and CD11b in differentiated and undifferentiated HL-60 cells.

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 (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.



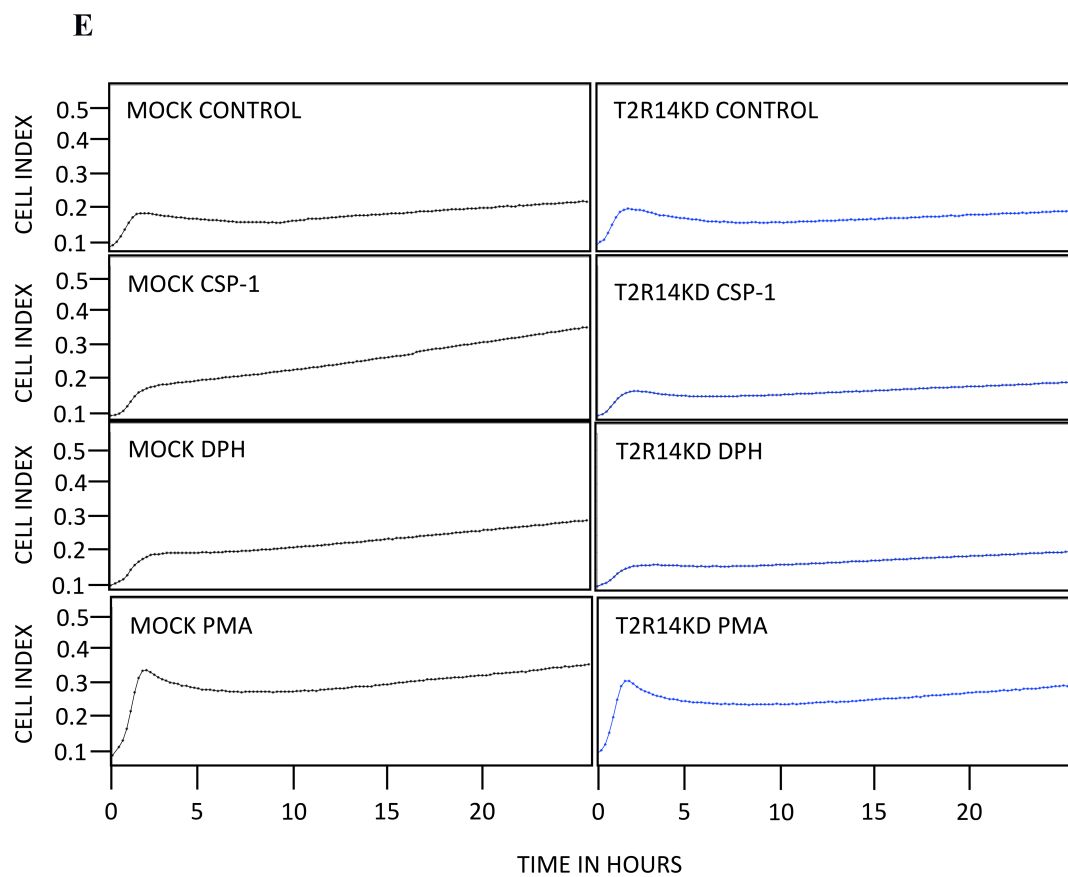
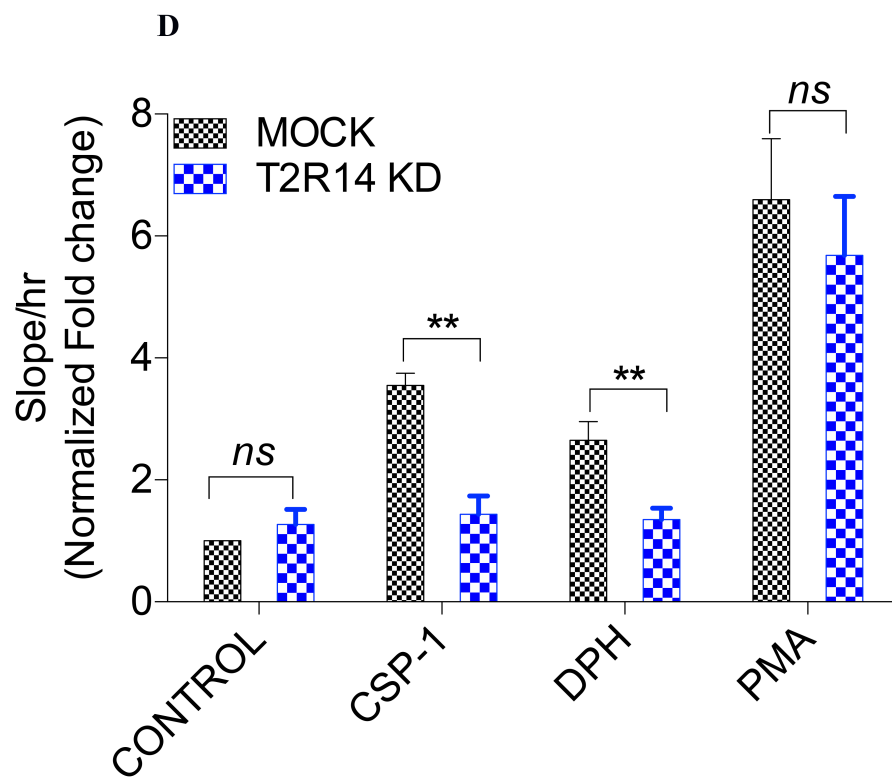


Figure 4. 10: Effect of migration of differentiated HL-60 (dHL-60) cells treated with conditioned medium from OKF6 cells.

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 (Medapati, Singh, et al., 2021). Reproduced with permission from the FASEB journal; License number-5015461264517.

4.6 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 (Munk et al., 2016) 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 (Freund et al., 2018; I. Gopallawa et al., 2020). 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 (**Figure 4.1A 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 (**Figure 4.1B**). 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 (Freund et al., 2018; Gil et al., 2015; Jaggupilli et al., 2018; Jaggupilli et al., 2017). 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 (**Figure 4.2B and C**). The cariogenic *S. mutans* is shown to secrete CSP-1 that is cleaved by membrane bound protease sepM to produce CSP-2 (Hossain et al., 2012b; Y. H. Li et al., 2001). 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 (Y. H. Li et al., 2001) and NCTC10449 strain secreting 23-mer CSP containing LGKIR at the C-terminus (Petersen et al., 2006). This variability of C-terminal amino acids is known to effect genetic transformation efficiency in these strains (Y. H. Li et al., 2001). 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 (**Figure 4.2I 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 (Pundir et al., 2019). 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 (Syvitski et al., 2007). 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 (Bikash et al., 2018). 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 (**Figure 4.3C 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 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 (Freund et al., 2018; Yadav et al., 2018). 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-kB pathway by CSP-1. We observed time-dependent increase in the phosphorylation of NF-kB signaling intermediates IKK $\alpha\beta$ and IKB α (**Figure 4.5A-C**). Interestingly the NF-kB signaling induced by either CSP-1 or DPH is not inhibited upon T2R14 KD (**Figure 4.5J-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 4.6A and B**). Several studies have shown PKC dependent activation of NF-kB pathway and PKC is a known downstream target for PLC β (Gukovsky et al., 2004; Satoh et al., 2006; Suh et al., 2008). Studies have also reported Gq-PLC-PI3K mediated activation of NF-kB signaling (Rahman et al., 2001; Shahrestanifar et al., 1999) and a recent study involving rat pulmonary nociceptors showed T2R-PLC-PKC signaling mediated sensitization of transient receptor potential vanilloid receptor 1 (TRPV1) (Gu et al., 2017). Hence, we believe CSP-1 mediated NF-kB 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 (**Figure 4.6C, 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 (Horst et al., 2011; Ra et al., 2017). TNF- α is highly expressed in taste buds and its signaling in these cells preferentially modulates bitter taste responses (Feng et al.,

2015). Interestingly, IL-8 and TNF- α secretion are shown to be dependent on G $\beta\gamma$ subunit activation but not PLC β , NF-kB 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-kB, CREB, AP-1, NF-IL6 in human bronchial epithelial cells treated with *P. aeruginosa* (Bezzetti et al., 2011). Currently there exists no direct evidence of T2R mediated transcriptional regulation of AP-1, CREB or NF-kB; 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 (Shandilya et al., 2016). 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-kB 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 (**Figure 4.6I**). 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-kB 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 KD on transcription factor NF-IL6, which along with NF-kB, is a known transcriptional regulator for IL-6 (Matsusaka et al., 1993). 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 (Stephens et al., 1994) and Rho family GTPase (Siripurapu et al., 2017). 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 (Meyle et al., 2017). 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 (**Figure 4.10B**). This effect was abolished when GECs were treated with G $\beta\gamma$ or PLC β inhibitors and the T2R14 antagonist 6-MF (**Figure 4.10B**). 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 (**Figure 4.6J**). Previously, IL-8 is shown to be a potent chemoattractant for neutrophils (Boneschansker et al., 2014). 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) (Pundir et al., 2019). 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.

BRIDGE TO CHAPTER 5

In chapter 4, using transcriptomics and western blot analysis I show T2R14 is highly expressed in GECs. CRISPR-Cas9 knock down of T2R14 in GECs followed by a series of signaling assays, I show that T2R14 is involved in interaction with CSPs secreted by *S. mutans*. Further, I characterized the innate immune responses induced by CSP-1/T2R14 activation in GECs. In the following chapter 5, I test the hypothesis whether the innate immune responses activated by T2R14 in GECs are effective against many Gram-positive bacteria or are selective for some.

Chapter 5: Bitter taste receptor T2R14 modulates Gram-positive bacterial internalization and survival in gingival epithelial cells.

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Manuscript currently under review.

Author contributions for chapter 5

Manoj Reddy Medapati designed the study, performed majority of the experiments including bacterial internalization experiments, Rac1 activity assays and wrote the first-draft of the manuscript. Dr. Anjali Bhagirath cultured bacteria and performed internalization assays. Dr. Nisha Singh performed nitric oxide, hBD-2 and IL-8 ELISA. Dr. Rajinder Bhullar provided expert guidance on Rac-1 activation assay. Dr. Robert J Scroth and Dr. Kangmin Duan gave insightful guidance and edited the manuscript. Dr. Prashen Chelikani designed the study, obtained funding, overall guidance and edited the manuscript.

5.1 ABSTRACT

Bitter taste receptors (T2Rs) have emerged as key players in host-pathogen interactions and important modulators of oral innate immunity. Previously, we reported that T2R14 is highly expressed in gingival epithelial cells (GECs) and interacts with competence stimulating peptides (CSPs) secreted by the cariogenic *S. mutans*. The underlying mechanisms of the innate immune responses and physiological effects of T2R14 on Gram-positive bacteria are not well characterized. In this study, we examined the role of T2R14 in internalization and growth inhibitory effects on Gram-positive bacteria, *Staphylococcus aureus* and *S. mutans*. We utilized CRISPR-Cas9 T2R14 KD GECs as the study model to address these key physiological mechanisms. Our data reveals that the internalization of *S. aureus* is significantly decreased, while the internalization of *S. mutans* remains unaffected upon knock down of T2R14 in GECs. Surprisingly, GECs primed with *S. mutans* CSP-1 resulted in an inhibition of growth for *S. aureus*, but not for *S. mutans*. The GECs infected with *S. aureus* induced T2R14 dependent human β -defensin-2 (hBD-2) secretion, however *S. mutans* infected GECs did not induce hBD-2 secretion, but induced T2R14 dependent IL-8 secretion. Interestingly, our results suggest that T2R14 KD might affect the cytoskeletal reorganization in GECs thereby inhibiting *S. aureus* internalization. Our study highlights the distinct mechanisms and a direct role of T2R14 in influencing physiologic responses to Gram-positive bacteria in the oral cavity.

5.2 INTRODUCTION

Oral epithelial cells are in constant communication with several diverse microbes under both normal and pathological conditions. However, the signaling mechanisms for pathogen recognition and innate immune responses are not yet fully understood. Host cells express toll-like receptors (TLRs), which can recognize pathogen-associated molecular patterns (PAMPs). PAMPs are bacterial cell-wall components such as lipoteichoic acid in Gram-positive organisms, lipopolysaccharide in Gram-negative organisms, and sometimes other bacterial products (Ooi et al., 2008). Apart from these above-mentioned molecules, bacteria secrete QSMs that are recognized by T2Rs (Freund et al., 2018; Jaggupilli et al., 2018; Medapati, Singh, et al., 2021).

T2Rs belong to GPCR superfamily of proteins and were first identified in the oral tissues (Adler et al., 2000; Lindemann, 1996). However, several studies have shown both, extra-oral expression as well as extra-gustatory functions for T2Rs in physiological as well as pathophysiological conditions (Jaggupilli et al., 2017; Shaik et al., 2016). T2Rs have now been identified as important modulators of innate immune responses in several cell types including sinonasal epithelial cells, upper airway epithelial cells, immune cells such as macrophages and in GECs (Carey, Workman, Yan, et al., 2017; I. Gopallawa et al., 2021; R. J. Lee et al., 2012a; Medapati, Singh, et al., 2021). The stimulation of bronchial airway epithelial cells by bitter compounds resulted in an increase in intracellular Ca^{2+} resulting in an increase in ciliary beat frequency (Alok S Shah et al., 2009), nitric oxide (NO) production and increased bactericidal effects in sinonasal epithelial cells (R. J. Lee et al., 2012a) as well as regulation of type-2 immunity against gut pathogens (Howitt et al., 2016; von Moltke et al., 2016).

Apart from the epithelial cells, T2Rs are also expressed in immune cells such as monocytes, Natural killer (NK) cells, B cells, T cells, and polymorphonuclear leucocytes (PMNs)

(Malki et al., 2015). Human neutrophils and phagocytes have been shown to express T2R38, bind to bacterial QSM AHL-12, and cause neutrophil migration (M. M. Gaida, U. Dapunt, et al., 2016). In contrast, T2Rs expressed on human mast cells were not activated upon stimulation with agonists (Ekoff et al., 2014). These studies suggest a tissue-specific role for T2Rs. A recent study showed that stimulation of human monocyte derived macrophages by agonists such as denatonium benzoate, quinine as well as some bacterial signaling molecules activated several T2Rs (Indiwari Gopallawa et al., 2019). Activation of T2Rs resulted in increased NO production and enhanced phagocytosis of *Escherichia coli* and *S. aureus* (Indiwari Gopallawa et al., 2019). All these studies enumerate the role of T2Rs in innate immunity. However, the physiological effect of T2R14 on Gram-positive bacteria is not well established.

We hypothesized that T2R14 activation in GECs results in an innate immune response that is effective against selective Gram-positive bacteria. To test this hypothesis, we followed up on our recent studies where we analyzed the effects of the CSPs secreted by the cariogenic *S. mutans* in GECs (Medapati, Singh, et al., 2021). *S. mutans* CSP-1 is shown to activate T2R14 leading to an innate immune response in GECs. However, the underlying mechanisms of these innate immune responses and physiological effects of T2R14 on Gram-positive bacteria are not characterized. Therefore, one of the major questions we addressed in this study is whether this innate immune response is deleterious for *S. mutans* or is *S. mutans* using the host immune system to inhibit other Gram-positive bacteria. We show that the CSP-1 and bitter agonist mediated T2R14 activation in GECs inhibits the growth of *S. aureus* but not *S. mutans*. Our results suggest that T2R14 influences cytoskeletal rearrangement and therefore internalization of Gram-positive bacteria in GECs.

5.3 MATERIALS AND METHODS

5.3.1 Reagents used in the study

Methylpiperidino pyrazole (MPP)(#13863), apigenin (#10010275) and nitrate/nitrite fluorometric assay kit (#780001) were purchased from Cayman chemical company. Cytochalasin D (# C8273), TRITC-phalloidin (#P1951) and diphenhydramine hydrochloride (#PHR1015) were purchased from Sigma Aldrich (Oakville, ON). 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) (# B1170) was purchased from Thermo Fisher Scientific. Human β -defensin 2 (hBD-2) ELISA kit (# 900-K172) was purchased from PeptoTech and IL-8/CXCL8 ELISA kit (# D8000C) was purchased from R&D systems. Keratinocyte growth medium-2 (KGM-2) for OKF6 cell culture was purchased from Promo Cell (Heidelberg, Germany). The following antibodies were purchased: mouse monoclonal anti- β -actin (#A5441) from Sigma Aldrich (Oakville, ON), rabbit polyclonal anti-T2R14 (#OSR00161W), 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), mouse monoclonal anti-Rac1 (#05-389) from Millipore (Oakville, ON).

5.3.2 Cell line used in the study

The oral keratinocyte cell line OKF6 was an in-kind gift from Dr. Gill Diamond, University of Florida (Dickson et al., 2000; McMahon et al., 2011). The T2R14 KD and a non-targeting (MOCK) Alt®-R-control CRISPR-crRNA or MOCK OKF6 cells were generated using CRISPR-Cas9 (Zuris et al., 2015), and published in our previous study (Medapati, Singh, et al., 2021). The expression of T2R14 in WT, MOCK and T2R14 KD cells is confirmed by western-blot analysis (**Figure S1**). The protocol for western blot detection of T2R14 is previously described (Medapati, Singh, et al., 2021).

5.3.3 Bacterial strains used in the study

The *S. aureus* (ATCC strain 6538) and *S. mutans* strain UA159 were purchased from ATCC. The *S. aureus* strain is propagated in Luria-Bertani (LB) broth at 37°C under constant agitation. The *S. mutans* strain is propagated in Brain Heart Infusion (BHI) broth at 37°C and 5% CO₂ under constant agitation.

5.3.4 Bacterial internalization assay

To assay internalization, *S. aureus* and *S. mutans* were grown overnight at 37°C under constant agitation in the corresponding liquid medium. The early log phase cultures for both *S. aureus* and *S. mutans* were used for infections. For infection with *S. aureus* an MOI of 50:1 (50 bacteria per 1 epithelial cell) and for *S. mutans* an MOI of 100:1 (100 bacteria per 1 epithelial cell) were re-suspended in GEC medium (Al Kindi et al., 2019; Maisonneuve et al., 2020). The OKF6 WT, MOCK and T2R14 KD cells were infected with *S. aureus* for 1 hour and with *S. mutans* for 2 hours at 37°C (Al Kindi et al., 2019; Maisonneuve et al., 2020). These conditions were optimized prior to the experiments and the best MOI and infection times were chosen based

on cell viability assays. After infection, the cells were washed four times with sterile PBS. The extracellular bacteria were killed by treating the cells with gentamycin (100 µg/ml) for 1 hour. After antibiotic treatment the cells were washed four times using sterile PBS and were incubated with 1% saponin for 15 mins (Wunsch et al., 2015). Then, 100 µl of lysate was spread on LB agar and BHI plates and incubated for 24 hours at 37°C. After 24 hours the plates were removed, colonies were counted manually and represented in colony forming units (CFUs).

5.3.5 Immunofluorescence microscopy of internalized bacteria in host cells

The log phase cultures of *S. aureus* and *S. mutans* were labeled for 45 mins with BCECF-AM (10 µM) (Wunsch et al., 2015). BCECF-AM is a non-fluorescent membrane permeable dye that is converted to fluorescein (BCECF) by intracellular esterases. The labeled bacteria were washed using sterile PBS and re-suspended in GEC medium. The OKF6 WT, MOCK and T2R14 KD cells were infected using BCECF-AM labeled *S. aureus* (50 MOI for 1 hour) and *S. mutans* (100 MOI for 2 hours). After infection, the GECs were washed three times using PBS and were fixed using 4% paraformaldehyde. After fixation, GECs were washed three times using PBS and the actin filaments were labeled using TRITC-phalloidin (50µM) for 45 mins followed by washing thrice with PBS. The nucleus was stained with DAPI (1:10.000 dilution) for 5 mins. The cover slips were then mounted on to a glass slide using fluoromount G mounting medium (Southern Biotech). The cells were imaged using a Nikon Eclipse Ti microscope using narrow standard band pass filters for DAPI, FITC for BCECF-AM and Texas Red for TRITC-phalloidin. The images were acquired at different focal points using a Z-stack program. In total, 30 stacks were obtained and with each stack having a size of 0.3µm. The 30-stack image is then processed to correct for out-of-focus light by deconvolution using a wide-field point spread function algorithm in NIS-elements software.

5.3.6 GTP-Rac1 pull-down assay

The OKF6 WT, MOCK and T2R14 KD cells were lysed and the total protein was extracted according to the manufacturer's protocol (#BK030, cytoskeleton). The protein concentration was quantified and 500 µg of total protein was incubated with 50 µg of PAK1 beads for 1 hour at 4°C. After 1-hour incubation the beads were centrifuged at 5000xg at 4°C for 1 min. The supernatant was carefully removed and the beads were washed using wash buffer and centrifuged at 5000xg at 4°C for 3 min. The wash buffer was carefully discarded and 2x Laemmli buffer was added to the beads. The sample was then heated at 100°C for 2 min and western blot analysis was performed and the membranes were probed with anti-Rac1 and anti-actin antibodies.

5.3.7 Bacterial survival assay

The early log phase cultures of *S. aureus* and *S. mutans* were used for the survival assay. The bacteria were centrifuged at 5000xg for 5 mins and the pellet was re-suspended with conditioned medium (CM) from OKF6 WT, MOCK and T2R14 KD cells treated using DPH, CSP-1 and apigenin. For real-time analysis the bacterial pellet was re-suspended in 100µl of CM and was plated in a clear bottom 96-well plate layered with sterile white mineral oil to prevent evaporation of the media. The OD₆₀₀ was measured every 10 minutes for a period of 24 hours using a Synergy H4 Multimode Microplate Reader (BioTek).

5.3.8 Human β-defensin 2 (hBD-2) ELISA

Briefly, OKF6 WT, MOCK and T2R14 KD cells were seeded in a 12-well plate at a density of 1.5×10^5 cells/well. These cells were infected with *S. aureus* and *S. mutans* for 18

hours at an MOI of 50 and 100 respectively. After the treatments, supernatant was collected and filtered using 0.2 μ m nylon filter. The filtered supernatant was used to determine the secreted levels of hBD-2 using ELISA (Peprotech). The detection range of the assay is (1500 – 23 pg/ml). The absorbance A_{450} nm was measured using a Flexstation3 plate reader (Molecular devices, CA).

5.3.9 Measurement of Nitrate/Nitrite secretion

The supernatant from OKF6 WT, MOCK and T2R14 KD cells was collected and 50 μ l was used to measure the secreted levels of nitrate/nitrite using a fluorescent assay kit (Cayman Chemical). The reductase enzyme cofactor and reductase enzyme were mixed with supernatant and incubated for 30 mins at RT followed by addition of 2,3-diaminophthalene (DAN) and NaOH. The detection limit of the assay is (60 nM – 3.85 μ M). The fluorescence is measured at an excitation wavelength of 365 nm and an emission wavelength of 430 nm using a Flexstation3 plate reader (Molecular devices, CA).

5.3.10 Measurement of IL-8/CXCL8 secretion

Briefly 1.5×10^5 cells were infected with *S. aureus* (50 MOI) or *S. mutans* (100 MOI) for 18 hours. After infection the supernatant was collected and filtered using a 0.2 μ m nylon filter. The supernatant was diluted (1:10 dilution) and IL-8/CXCL8 levels in the diluted supernatant were measured by commercially available quantikine ELISA as per manufacturer instructions (#D8000C, R&D systems). The detection limit of the assay is (2000 – 31 pg/ml). The absorbance is measured using a Flexstation3 plate reader at the wavelength of 450 nm (Molecular devices, CA).

5.4 RESULTS

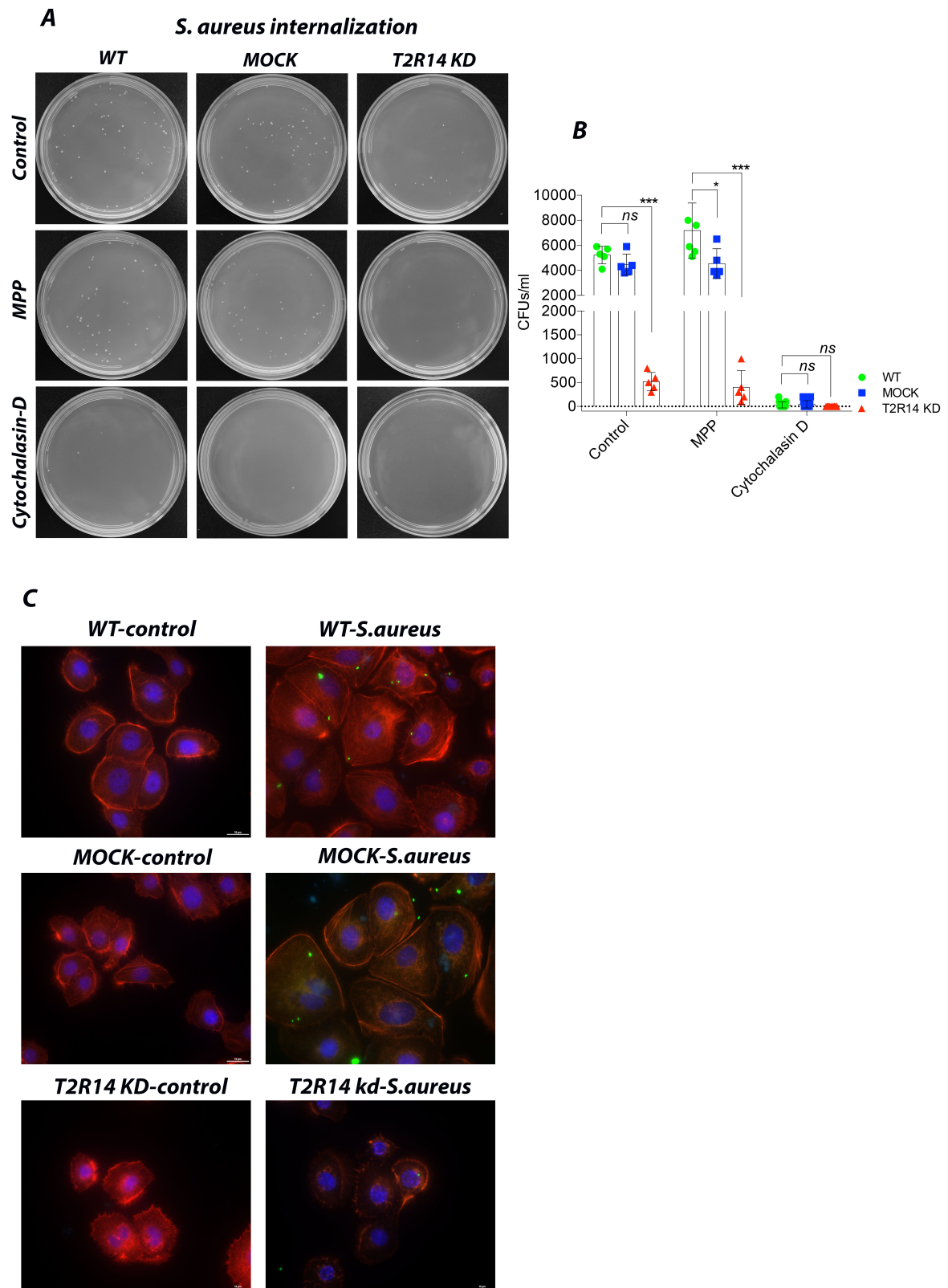
5.4.1 T2R14 mediates internalization of *S. aureus* and *S. mutans* in GECs

A recent study on macrophages suggests T2Rs as one of the important receptors in the phagocytic machinery (I. Gopallawa et al., 2020). Although immune cells are the primary mediators of bacterial phagocytosis, epithelial cells in the oral cavity and the upper respiratory tract are the first line of defense against microbes. These epithelial cells are also known to internalize several species of bacteria and also express several T2Rs. However, the role of T2Rs in internalization of bacteria, especially Gram-positive pathogens such as the cariogenic *S. mutans* and the methicillin-resistant *S. aureus* is not studied in GECs. To characterize the role of T2R14 in bacterial internalization, the OKF6 WT, MOCK and T2R14 KD cells were infected using *S. aureus* and *S. mutans*.

In OKF6 T2R14KD cells the internalization of *S. aureus* was significantly decreased as compared to the WT and MOCK cells, as indicated by the decreased colony forming units (CFUs) (**Figure 5.1A and B**) and confirmed by immunofluorescence (**Figure 5.1C**). When compared to the control GECs pretreated with TLR signaling inhibitor MPP, no changes were observed in the pattern of internalization between WT, MOCK and T2R14 KD cells. However, the internalization levels decreased significantly in WT and MOCK cells treated with cytochalasin D (**Figure 5.1A and B**).

Next, we characterized the internalization of cariogenic bacteria *S. mutans* in WT, MOCK and T2R14 KD cells. In the control group there was no significant change in internalization between WT, MOCK and T2R14 KD cells. In the MPP treated group the internalization of *S. mutans* has significantly increased in WT and MOCK cells but not in T2R14 KD cells. In the

cytochalasin D treated group the internalization of *S. mutans* is similar between WT, MOCK and T2R14 KD cells (**Figure 5.1D-F**).



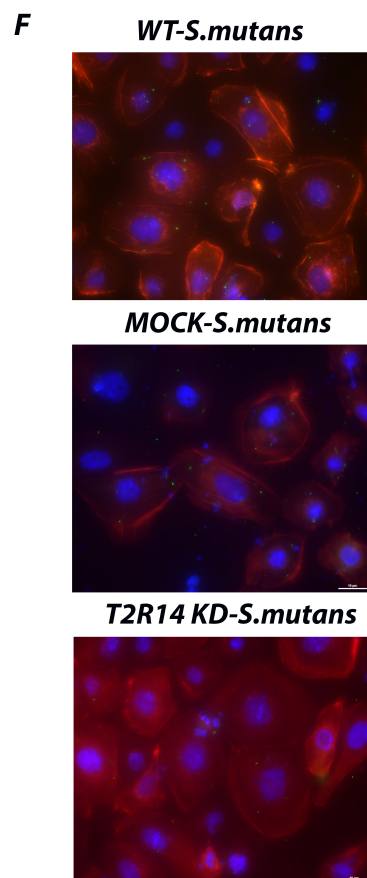
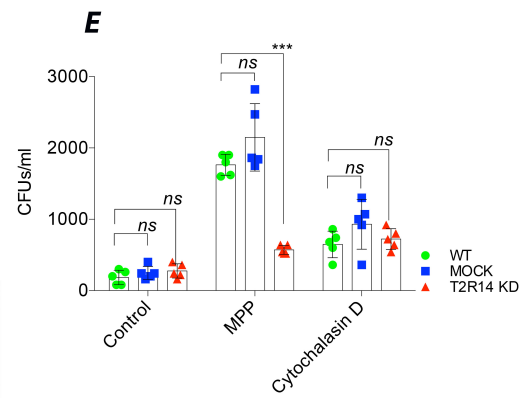
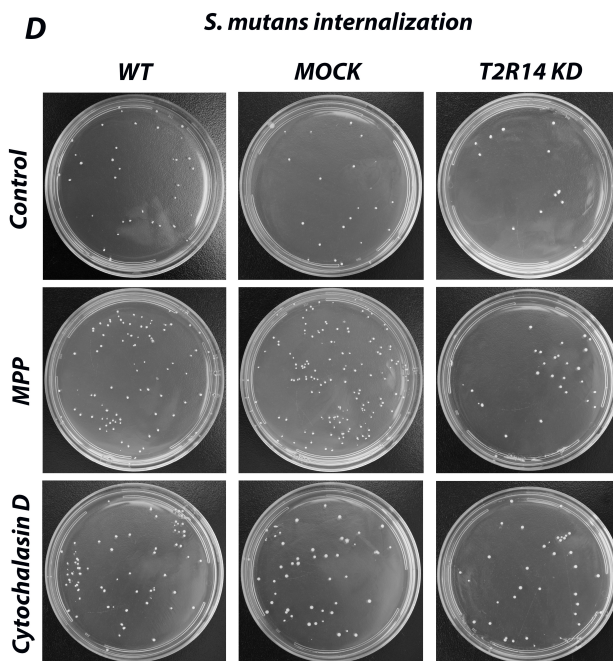


Figure 5. 1: Internalization of *S. aureus* and *S. mutans* in OKF6 WT, MOCK and T2R14 KD cells.

A, The OKF6 WT, MOCK and T2R14 KD cells pre-treated with MPP (5 μ M) and cytochalasin D (1 μ g/ml) were infected with *S. aureus* (50 MOI) for 1 hour and the internalized *S. aureus* bacteria were released from OKF6 cells using 1% saponin and the lysate was diluted in 1:10 dilution. 100 μ l of the diluent containing bacteria were grown on LB agar plates for 24 hours to form CFUs. B, The corresponding CFUs in the representative LB agar plate were calculated and were represented in a grouped bar graph. C, OKF6 cells infected with BCECF-AM (10 μ M) labelled *S. aureus* for 1 hour. Cells were fixed with paraformaldehyde and labelled with TRITC-phalloidin (50 μ M) and DAPI (1:10,000 dilution). The immunofluorescence image shows nucleus stained with DAPI (blue), F-actin stained with TRITC-phalloidin (red) and *S. aureus* stained with BCECF-AM (green). D, OKF6 cells pre-treated with MPP (5 μ M) and cytochalasin D (1 μ g/ml) were infected with *S. mutans* (100 MOI) for 2 hours and the internalized *S. mutans* were released using 1% saponin and the lysate containing bacteria were grown on BHO agar plate to form CFUs. E, The corresponding *S. mutans* CFUs from the representative BHI agar plate were calculated and were represented in a grouped bar graph. F, The immunofluorescence image shows OKF6 cells infected with BCECF-AM (green) labeled *S. mutans*, and the OKF6 cells nucleus stained with DAPI (blue) and TRITC-phalloidin (red). The images were captured using a Nikon Ti microscope using 60X oil immersion objective. The data represented in the graphs are SEM of ≥ 5 independent experiments. Two-way ANOVA analysis using Tukey's multiple comparison analysis was performed and the observed *P* value is ****P*=0.002.

5.4.2 Knock down of T2R14 decreases PAK1 associated actin but not GTP-Rac1 in GECs

One of several bacterial internalization mechanisms involves activation of Src pathway leading to cortactin recruitment followed by the polymerization of actin (Agerer et al., 2005; Fowler et al., 2003; Selbach et al., 2005). The actin polymerization and cytoskeletal rearrangements are controlled by small GTPases, especially Rac1. To characterize the role of these GTPases and their relationship to T2R14 activation, pull-down assays were performed in MOCK and T2R14 KD cells. The MOCK and T2R14 KD cells exhibited similar levels of GTP-Rac1 (First panel, **Figure 5.2**). However, in T2R14 KD cells there is a decreased pull-down of PAK1 associated actin (Third panel, **Figure 5.2**).

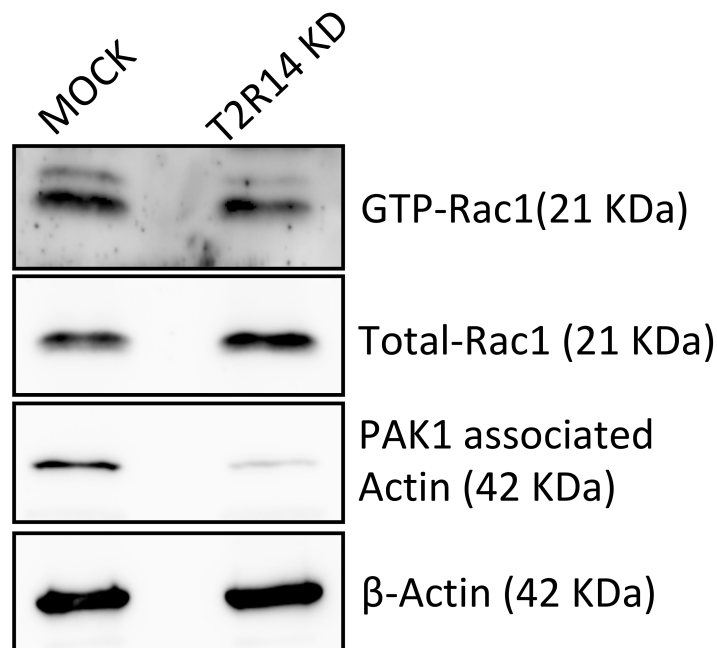


Figure 5. 2: Characterizing the GTP-Rac1 in MOCK and T2R14 KD OKF6 cells.

The MOCK and T2R14 KD OKF6 cells lysates (500 μ g) were incubated with PAK1 agarose beads (20 μ g) for 1 hour at 4°C. After incubation the beads were washed and re-suspended in 2x Laemmli buffer and were separated by SDS-PAGE using a 12% TGX gel and blotted onto a PVDF membrane. Western blot analysis of PAK1 pull-down and total protein lysates of MOCK

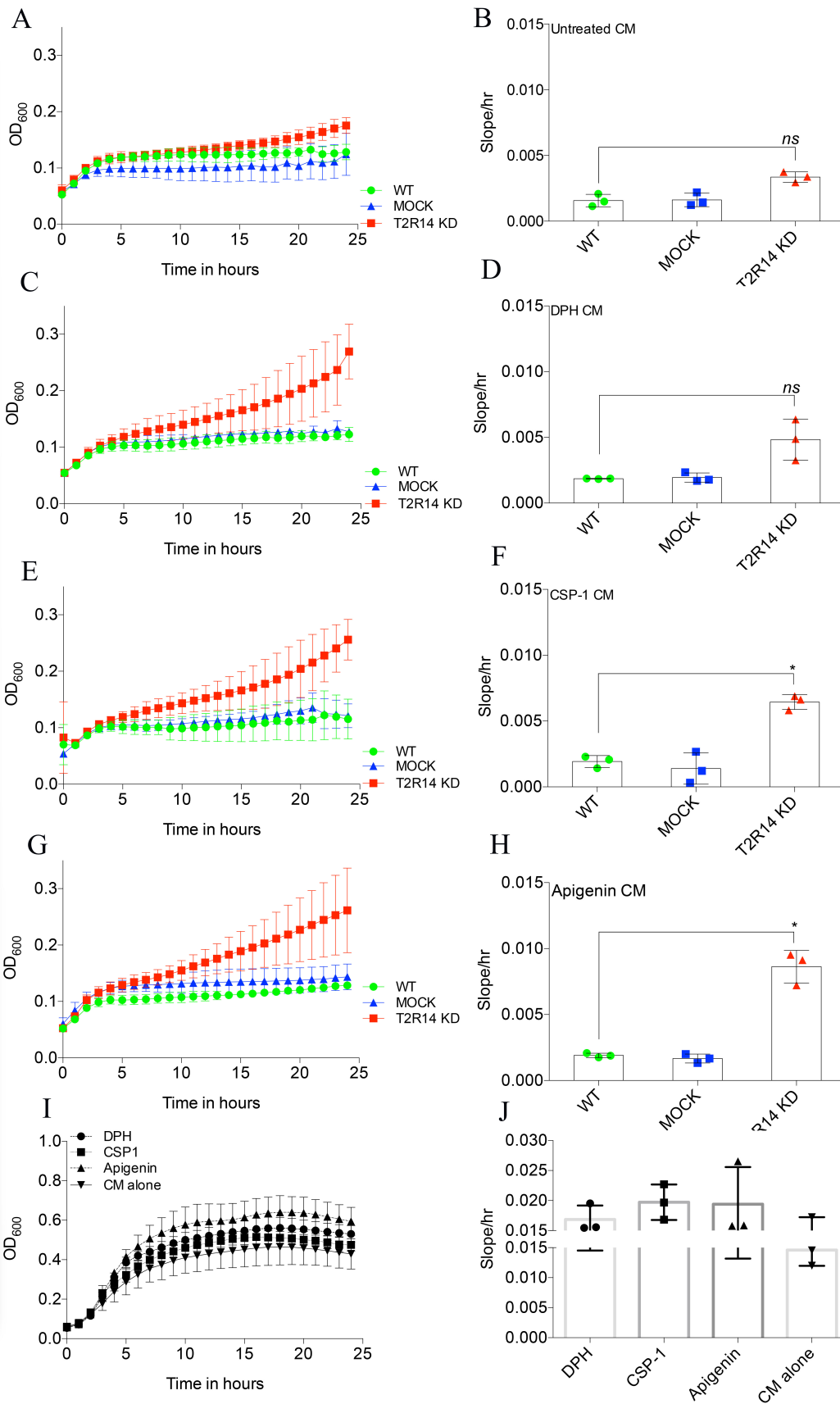
and T2R14 KD cells probed using primary mouse monoclonal anti-Rac1 antibody (1:1000), mouse monoclonal anti β -Actin antibody (1:25000) and secondary anti-mouse HRP antibody (1:5000 and 1:25000) respectively. The blots were developed using chemiluminescence detection system and were imaged using a ChemiDoc MP imaging system.

5.4.3 Activation of T2R14 in GECs by CSP-1 and agonist has a bactericidal effect on *S. aureus* but not *S. mutans*.

To characterize the bactericidal effect of T2R14 the OKF6 WT, MOCK and T2R14 KD cells were treated with the recently identified T2R14 agonist CSP-1 secreted by *S. mutans* (Medapati, Singh, et al., 2021), and bitter agonists DPH, apigenin. The CM from the above treated GECs was used to test the bactericidal effect on *S. aureus* and *S. mutans*. The results show that CM from untreated WT, MOCK and T2R14 KD inhibited the growth of *S. aureus* (**Figure 5.3A and B**). The CM from DPH treated T2R14 KD cells appears to have rescued *S. aureus* growth as observed in the real-time growth curve (**Figure 5.3C**). However, the slope analysis did not show any significance between WT, MOCK and T2R14 KD cells (**Figure 5.3D**). The CM from CSP-1 and apigenin treated T2R14 KD cells rescued *S. aureus* growth as evident in real-time growth curves and slope when compared to WT and MOCK treated cells (**Figure 5.3E-H**). The T2R14 agonists alone did not inhibit *S. aureus* growth (**Figure 5.3I and J**).

Next, we characterized the bactericidal effect of T2R14 activation on cariogenic bacterium *S. mutans*. The growth of *S. mutans* is similar upon treatment with CM from untreated WT, MOCK and T2R14 KD cells (**Figure 5.3K and L**). However, none of the T2R14 agonists including CSP-1 had any noticeable effect on *S. mutans* growth (**Figure 5.3M-R**). The growth of bacteria in untreated and those treated with agonists remained same. The T2R14 agonists alone did not inhibit *S. mutans* growth (**Figure 5.3S and T**). These data suggest that T2R14 mediated bactericidal effect inhibits *S. aureus* growth, but not *S. mutans*.

S. aureus



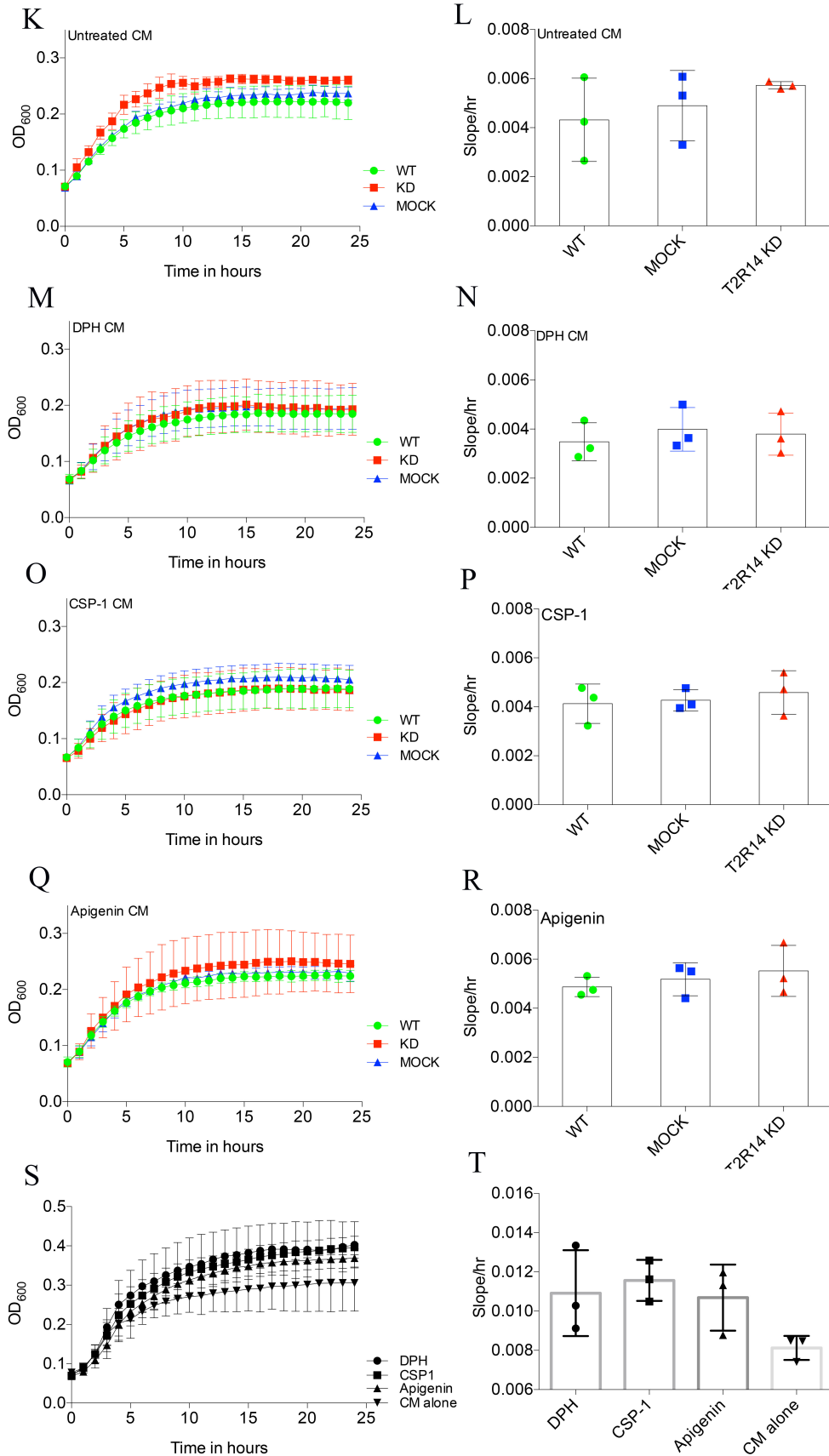


Figure 5. 3: Characterizing the bactericidal affect of T2R14 on *S. aureus* and *S. mutans*.

Analysis of growth of *S. aureus* and *S. mutans* over a period of 24 hours measured at OD600.

A,C,E,G and I, Real-time growth curves of *S. aureus* in response to WT, MOCK and T2R14 KD CM alone or with T2R14 agonists DPH (250 μ M), CSP-1 (50 μ M), apigenin (50 μ M) and compound alone treatment. B,D,F,H and J: The graphs depict the growth rate of *S. aureus* as slope/hr. K,M,O,Q and S, Real-time growth curves of *S. mutans* in response to WT, MOCK and T2R14 KD CM alone or treated with the above mentioned T2R14 agonists and compound alone treatment. L,N,P,R and S, The graphs depict the growth rate of *S. mutans* as slope/hr. The data represented in the graphs are SEM of ≥ 3 independent experiments. Two-way ANOVA analysis using Tukey's multiple comparison analysis was performed and the observed *P* value is $*P<0.05$.

5.4.4 *S. aureus*-induced hBD-2 secretion is T2R14 dependent

Defensins are small cationic peptides that are known to exert antimicrobial responses towards both Gram-negative and Gram-positive bacteria by destroying their cell-wall (Lehrer, 2004). To characterize the role of T2R14 in hBD-2 secretion, the OKF6 WT, MOCK and T2R14 KD cells were infected with *S. aureus* and *S. mutans* for 18 hours. Post-infection, the CM from WT and MOCK cells infected with *S. aureus* showed a significant increase in hBD-2 levels as compared to the T2R14 KD cells. Interestingly, WT, MOCK and T2R14 KD cells infected with *S. mutans* did not show any significant change in the secretion of hBD-2. The basal levels of hBD-2 between WT, MOCK and T2R14 KD cells remain unaffected (**Figure 5.4A**). This data suggests that different bacteria elicit differential innate immune responses in the host cells.

5.4.5 Gram-positive bacteria do not induce Nitrite/Nitrate secretion in GECs

Epithelial cells produce NO as a part of the innate immune response against bacteria. Previous studies have shown a T2R mediated increase in NO upon treatment with Gram -positive bacteria such as *Staphylococcus epidermidis* and *Bacillus cereus* (Carey et al., 2016; Carey, Workman, Yan, et al., 2017). However, the T2R mediated NO secretion in oral epithelial cells has not been characterized thus far. To characterize the T2R14 mediated NO secretion, OKF6 WT, MOCK and T2R14 KD cells were infected with *S. aureus* and *S. mutans* respectively. After infection, the supernatants were collected and the end products of NO (Nitrite/Nitrate) were measured by ELISA using a reference standard. The results from these assays show that OKF6 cells secrete basal levels of Nitrite/Nitrate and these levels remain unchanged upon infection with Gram-positive bacteria *S. aureus* and *S. mutans* (**Figure 5.4B**).

5.4.6 IL-8/CXCL-8 secretion in GECs is T2R14 dependent

Oral epithelial cells are known to secrete IL-8 chemokine, a potent neutrophil attractant upon bacterial insult. Previously, we have shown an increased IL-8 secretion in GECs upon treatment with T2R14 agonists, including *S. mutans* CSP-1, as well as a decreased secretion of IL-8 upon knock down of T2R14 (Medapati, Singh, et al., 2021). However, the T2R14 dependent IL-8 secretion profile upon bacterial insult has not been investigated.

To analyze the secretion of IL-8 upon bacterial insult, OKF6 WT, MOCK and T2R14 KD cells were infected with *S. aureus* and *S. mutans* for 18 hours. The analysis of conditioned medium from these cells showed a significant decrease in basal IL-8 secretion in T2R14 KD cells compared to WT and MOCK cells. Interestingly, the levels of IL-8 did not change upon infection with *S. aureus* while the infection of WT and MOCK cells with *S. mutans* significantly increased IL-8 secretion. In T2R14 KD cells *S. mutans* treatment did not increase IL-8 secretion (**Figure 5.4C**).

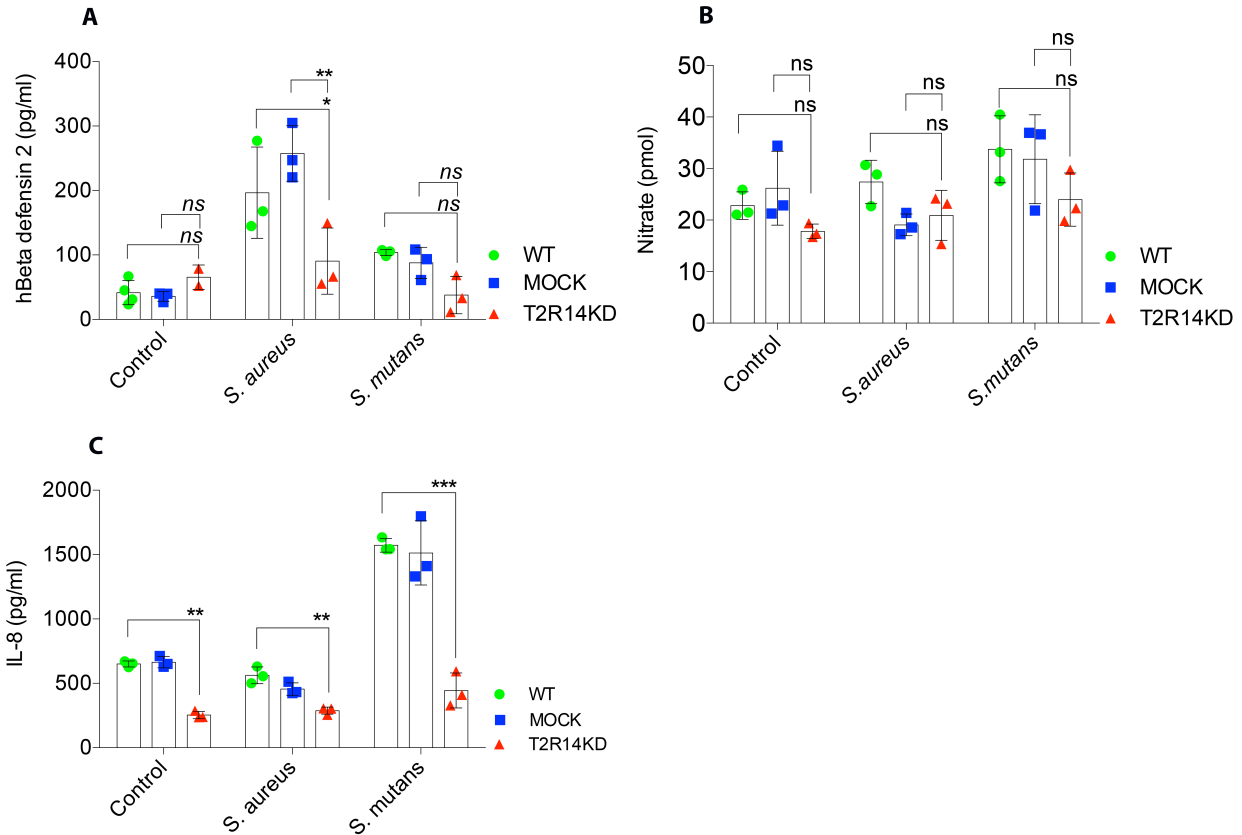


Figure 5. 4: Characterizing the secretion of Antimicrobial peptide hBD-2, Nitrite/Nitrate and IL-8/CXCL8 by ELISA.

The OKF6 WT, MOCK and T2R14 KD cells were infected with *S. aureus* (50 MOI) and *S. mutans* (100 MOI) for 18 hours. After infection the supernatants were collected and filtered using 0.25µm nylon filter to remove cellular debris and bacteria. The supernatant was then used to determine secreted innate immune markers such as A, hBD-2, B, Nitrite/Nitrate and C, IL-8/CXCL8 by standard ELISA. The representative grouped bar graphs were generated using graph pad prism 7.0. The data represented in the graphs are SEM of ≥ 3 independent experiments. Two-way ANOVA analysis using Tukey's multiple comparison analysis was performed and the observed P values are $*P=0.01$, $**P=0.004$, $***P=0.002$.

5.5 DISCUSSION

T2Rs have recently emerged as important receptors in innate immunity. Studies suggest different bacterial metabolites and QSMs to be interacting and signaling through several T2Rs (Freund et al., 2018; Jaggupilli et al., 2018; Medapati, Singh, et al., 2021). The T2R-QSM interactions are shown to induce several innate immune responses such as NO secretion mainly in the sinonasal cavity, upper airway epithelial cells and in macrophages (Carey, Workman, Yan, et al., 2017; I. Gopallawa et al., 2020; Hariri et al., 2017). Although these findings provide insightful knowledge into T2R mediated innate immune mechanisms there exists a gap in knowledge mainly in deciphering the physiological effects of T2Rs on bacteria. In this context, our study addresses the physiological effects of T2R14 on Gram-positive pathogenic bacteria *S. aureus* and *S. mutans*.

Our results show that *S. aureus*, but not *S. mutans* internalization is significantly decreased upon knock down of T2R14 (**Figure 5.1B and E**). To identify the mechanisms of internalization we have utilized TLR inhibitor MPP and actin polymerization inhibitor cytochalasin D. Our results show that upon inhibition of actin polymerization the internalization of *S. aureus* is significantly decreased in WT, MOCK and in T2R14 KD cells. Interestingly, the TLR signaling inhibitor MPP did not effect on the internalization of *S. aureus*. Previous studies have shown that *S. aureus* is known to internalize via F-actin mediated host-directed endocytosis (Agerer et al., 2005). Therefore, the GECs were pretreated with cytochalasin D a well-known inhibitor of F-actin polymerization. The *S. aureus* internalization was completely abolished in WT, MOCK and T2R14 KD upon treatment with cytochalasin D (**Figure 5.1A and B**) suggesting F-actin mediated internalization of *S. aureus* in GECs. The internalization of *S. aureus* is regulated mainly by receptor mediated endocytosis in non-phagocytotic cells in a fibronectin-binding

protein (FnBP) dependent manner (Patti et al., 1994). In several cell types such as endothelial cells (Massey et al., 2001), osteoblasts (Ahmed et al., 2001), HEK293T cells (Schaefer et al., 2001) and human keratinocyte cell line, HaCat (Mempel et al., 2002), *S. aureus* FnBP is known to bind with integrin $\alpha 5 \beta 1$ on the host cells for internalization (Grundmeier et al., 2004). The internalization mechanism of *S. aureus* involves several molecular events such as recruitment of focal adhesion kinase (FAK) followed by activation of the Src pathway leading to cortactin recruitment and polymerization of actin (Agerer et al., 2005; Fowler et al., 2003; Selbach et al., 2005). Recent study using macrophages has shown T2R mediated phagocytosis of bacteria via Ca^{2+} , NO and cGMP signaling (I. Gopallawa et al., 2020). In our GECs, although the internalization of *S. aureus* is T2R14 dependent, it might not be dependent on NO mediated cGMP signaling cascade as the treatment of GECs with *S. aureus* and *S. mutans* did not affect NO secretion (**Figure 5.4B**). Hence, the internalization mechanism involving T2R14 might differ based on the cell type being infected. It is possible that knock down of T2R14 can lead to changes in the protein expression of *S. aureus* binding proteins on the host cell membranes such as $\alpha 5 \beta 1$ integrins, cortactins and FAK leading to a decrease in internalization. Further studies into the analysis of expression profiles of the above-mentioned proteins in T2R14 KD cells may shed light on this mechanism.

Previously, we have shown that QSMs mainly CSP from cariogenic bacterium *S. mutans* are recognized by T2R14 in GECs (Medapati, Singh, et al., 2021) and mediate an innate immune response. However, the role played by T2R14 on *S. mutans* infection is not well characterized. The results from our internalization experiments show that T2R14 KD does not directly affect the internalization of *S. mutans* as compared to *S. aureus* (**Figure 5.1B and E**). Interestingly, the internalization of *S. mutans* is significantly increased in T2R14 expressing GECs treated with

TLR signaling inhibitor MPP (**Figure 5.1E**). However, the internalization of *S. mutans* in control and cytochalasin D groups is not significantly different between WT, MOCK and T2R14 KD cells (**Figure 5.1E**). These results indicate that *S. mutans* internalization in GECs might not be dependent on F-actin. Our results are similar to an earlier study in which cytochalasin D did not affect internalization of *S. gordonii* strain DL1 in HEp-2 cells and the internalization of *S. gordonii* is mediated by host $\beta 1$ integrin (Nobbs et al., 2007). However, the *S. mutans* internalization mechanisms need to be characterized.

To better understand the molecular mechanism behind T2R14 regulated bacterial internalization we performed small GTPase activation assays for Rac1, as it is primarily involved in actin cytoskeleton reorganization that is known to facilitate bacterial internalization and endocytosis. Our results indicate decreased PAK1 associated actin pull-down in T2R14 KD cells compared to MOCK cells. Previous studies on platelet cells have shown PAK1 (GTP-Rac1 binding partner) to interact with cortical actin binding protein (cortactin) and N-WASP, which interact with actin (Grassart et al., 2010; Vidal et al., 2002). However, in GECs the expression of cortactin and N-WASP and their interaction with PAK1 remains to be determined. In our study the internalization of *S. aureus* is significantly inhibited by cytochalasin D. Taken together, these results suggest that T2R14 KD in GECs might affect the cytoskeletal reorganization thereby inhibiting *S. aureus* internalization (**Figure 5.5**). In line with this assumption our previous study has shown that bitter agonist quinine mediated activation of T2R4 inhibits Rac1 activation (Sidhu et al., 2017). Hence there exists a link between T2Rs and small GTPases and their possible role in cytoskeletal reorganization. The internalization data from our study suggest that *S. aureus* and *S. mutans* invoke different mechanisms of internalization both of which are regulated by T2R14. However, the internalization of T2R14 into endocytic vesicles is not yet characterized (**Figure 5.5**). Previously, we reported that some T2Rs such as T2R4 and T2R38 are localized in

extracellular vesicles (Medapati et al., 2017). Therefore, studies focused on understanding the internalization mechanisms of T2Rs in epithelial and immune cells infected with bacteria are warranted.

T2Rs have emerged as important receptors in modulating innate immune responses and their activation is known to induce protective responses mainly in upper airway epithelial cells. In SCCs the bitter agonist denatonium is shown to induce antimicrobial peptide secretion and invoke bactericidal effect of Gram-negative CF bacterium *P. aeruginosa* (Carey, Workman, Hatten, et al., 2017). We performed bacterial survival assays to test T2R14 mediated bactericidal effect on *S. aureus* and *S. mutans*. Our data show that DPH, CSP-1 and apigenin CM from T2R14 KD cells increased *S. aureus* growth compared to that of WT and MOCK. Intriguingly, the CM from untreated cells showed growth inhibitory effects on *S. aureus* and addition of bitter agonist blunted the inhibitory response only in T2R14 KD cells. However, we did not observe similar effects on cariogenic *S. mutans*. Our data is in agreement with earlier findings in which bactericidal effects on Gram-negative bacteria such as *P. aeruginosa* were mediated by T2Rs agonists such as denatonium (Carey, Workman, Hatten, et al., 2017; R. J. Lee et al., 2014). In our cell model the bactericidal effect was minimal compared to effect induced by denatonium in sinonasal ciliary cells (Carey, Workman, Hatten, et al., 2017; R. J. Lee et al., 2014). However, the effect we observed in GECs is comparable to that in sinonasal ciliary cells in which lower concentrations of bitter agonist (0.1mM denatonium) had a minimal bactericidal effect compared to higher concentrations of agonist (10 mM denatonium). In our study, lower concentrations of bitter agonists and QSMs were chosen to better reflect the physiological concentrations. While the differential bactericidal effects on *S. aureus* and *S. mutans* are interesting, one possible explanation could be the high biofilm formation by strain used in this study, *S. mutans* UA159, thus evading host response mechanisms (Ahn et al., 2008). Another interesting observation is the

inhibition of *S. aureus* upon treatment of GECs with CSPs from *S. mutans*. This result is in agreement with a recent study, which isolated bioactive molecules from biofilms of *S. mutans* and observed a bactericidal effect on other commensal bacteria (Tang et al., 2020). Our finding provides hints into a host-directed mechanism by which commensal bacteria such as *S. mutans* overcome competition by other commensals such as *S. aureus* in dysbiotic states (**Figure 5.5**).

To understand the mechanisms behind the T2R mediated bactericidal effects, the AMP and NO secretions were analyzed upon infection with *S. aureus* and *S. mutans*. Interestingly, only WT and MOCK cells treated with *S. aureus* secreted higher levels of hBD-2 but not T2R14 KD cells. Recent study of gingival tissues of gustducin knock-out mice showed decreased levels of beta-defensins and LL-37 (Zheng et al., 2019). Intriguingly, in our study *S. mutans* treatment did not elicit significant hBD-2 response in the presence or absence of T2R14 (**Figure 5.4A**). A recent study utilizing primary GECs also reported minimal secretion of hBD-2 upon stimulation with *S. mutans* (Gil et al., 2015). It is not surprising to observe that only certain bacterial species induce AMP secretions, as *S. mutans* is the most abundant inhabitant of the healthy oral cavity. While *S. aureus* is also a low-abundance commensal, infection with *S. aureus* is often associated with mucositis, angular cheilitis and parotitis. An earlier study has shown differential hBD-2 regulation in oral keratinocytes in response to pathogenic and commensal bacterial species (Chung et al., 2004). Hence in depth analysis of other AMPs in presence of different bacterial strains is warranted.

The gingival epithelial barrier is the first line of defense against several pathogenic bacteria. In response to increasing numbers of bacteria it is known to secrete several cytokines and chemokines such as IL-8/CXCR8 to attract neutrophils and monocytes to the site of infection. In our previous study we have shown that T2R14 agonist and CSP-1 from *S. mutans* induce the secretion of IL-8 and this is abolished upon a knock-down of T2R14 leading to

decreased neutrophil chemotaxis (Medapati, Singh, et al., 2021). In the same study we have observed IL-8 secretion regulated by expression of T2R14 in GECs. However, the mechanisms for T2R14 mediated IL-8 secretion are yet to be investigated. In the present study we observed a similar effect in which T2R14 expressing GECs were able to secrete significantly higher amounts of IL-8 upon infection with *S. mutans* as compared to the T2R14 KD cells (**Figure 5.4C**).

Although the LPS from Gram-negative bacteria and *S. mutans* are known to signal via TLRs the regulation of IL-8 by T2R14 KD can affect the TLR signaling responses. Hence our findings point to a possible cross-talk between T2R and TLR signaling pathways (**Figure 5.5**). This interesting finding highlights the importance of T2R14 in regulating innate immune responses in oral epithelial cells.

In summary, our findings identify previously unexplored mechanisms of T2R14 mediated Gram-positive bacterial internalization and bactericidal affects. Future studies should be focused on examining the global cellular effects of T2R14KD. Further, generating T2R14 CRISPR knock-down animal models might help to better understand the role of microbial colonization in the oral cavity and in deciphering the mechanisms involved in TLR/T2R cross talk.

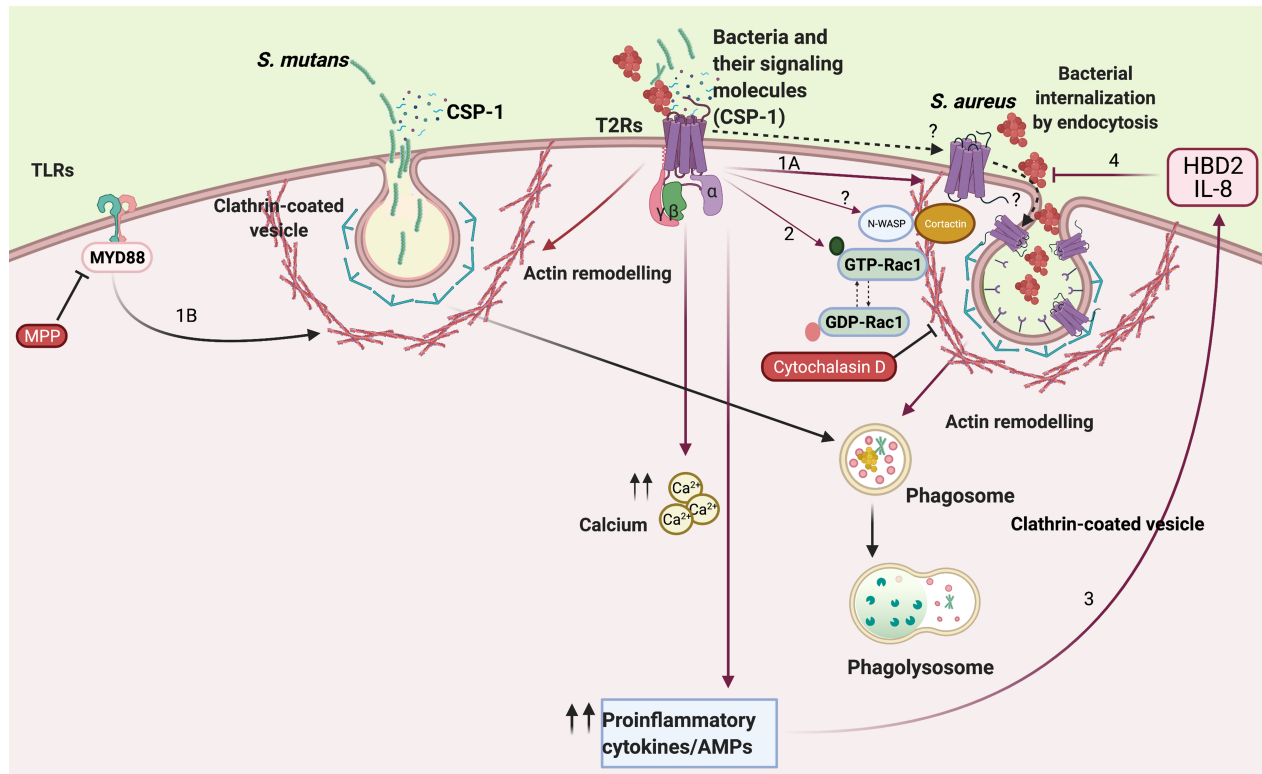


Figure 5. 5: Schematic showing the mechanism of T2R mediated Gram-positive bacterial internalization and survival in GECs.

T2R14 mediated internalization of *S. aureus* might involve actin remodeling, while *S. mutans* internalize via T2R/TLR dependent mechanisms in GECs (pathway **1A, B**). T2R14 in GEC influences actin remodeling (based on cytochalasin D treatment and PAK1 associated actin pull-down) thus modulating bacterial internalization (pathway **2**). The activation to T2R14 signaling by bacteria or their signaling molecules leads to an increased secretion of innate immune markers such as IL-8/CXCL8 and antimicrobial peptide hBD-2 in GECs (pathway **3**). The T2R14 dependent AMP secretion by GECs inhibits the growth of *S. aureus* (pathway **4**). It is currently unknown whether T2R14 internalizes into clathrin-coated vesicles and signals (dotted arrows on the membrane). Figure drawn using BioRender.

BRIDGE TO CHAPTER 6

In chapter 4, I reported that T2R14 is highly expressed in GECs and interacts with CSPs secreted by *S. mutans*. In chapter 5, I report an intriguing finding that the *S. mutans* CSP-1 mediated activation of T2R14 leading to innate immune responses in GECs is not deleterious for *S. mutans*, in contrast, *S. mutans* might be using the host immune system to inhibit other Gram-positive bacteria. These results suggest that CSP-1 and bitter agonist mediated T2R14 activation in GECs inhibits growth of *S. aureus* but not for *S. mutans*. Further, the result suggests that T2R14 influences cytoskeleton rearrangement in GECs and modulates internalization of Gram-positive bacteria. The two chapters (4 and 5), show a major role for T2R14 in host-microbe interaction and oral innate immunity. In chapter 6, I test the hypothesis whether extracellular vesicles (EVs) isolated from saliva express GPCRs including T2Rs. To keep this hypothesis feasible, I focus on the well-studied, Angiotensin II receptor type 1 (AT1R), T2R38 and T2R4. In chapter 6, I characterized the localization of the three GPCRs in EVs isolated from cell lines and saliva using two different isolation techniques, ultracentrifugation and PEG mediated precipitation.

Chapter 6: Characterization of G protein-coupled receptors (GPCRs) in Extracellular Vesicles (EVs).

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Author contributions for chapter 6

Manoj Reddy Medapati designed the study, performed majority of the experiments (exosome isolation by ultracentrifugation and immunoTEM) and wrote the first draft of the manuscript. Anula Singh and Ranjit R Korupally assisted with salivary exosome isolation. Dr. Sasidhar V Manda was responsible for acquiring normal salivary samples and provided insightful guidance for the experiments. Dana Henderson performed electron microscope on EVs. Dr. Thomas Klonisch analyzed the EM data and gave insightful guidance. Dr. Prashen Chelikani designed the study, obtained funding, and edited the manuscript.

6.1 Abstract

Extracellular vesicle (EV) are tiny membranous vesicles usually <500 nm in size that recently emerged as a new paradigm in human intercellular signaling. EVs have shown a promising role in development of diagnostic markers in many pathophysiological disorders. The presence of chemosensory and therapeutically relevant GPCRs on EV membranes is poorly characterized. Here we compare different methods including ultracentrifugation and polymer-charge based separation to isolate EVs from cell culture media and human saliva. The presence of bitter taste GPCRs (T2R4 and T2R38) and a class A GPCR Angiotensin II type 1 receptor (AT1R) on these EVs was characterized by ELISA and immuno-transmission electron microscopy (TEM).

6.2 Introduction

Extracellular vesicles (EVs) are tiny membranous vesicles of varying sizes (50 -500 nm) and origin (plasma membrane vs. endosome) released by human cells. They are produced by endocytosis of membrane components, formation, and exocytosis of multivesicular bodies (MVBs) to the plasma membrane of a cell (Trams et al., 1981). Several studies have identified EVs as intercellular messengers in several biological processes such as inflammation, cell proliferation, and neuronal function (Raposo et al., 1996; Simhadri et al., 2008). EVs were also identified to be involved in various pathophysiological conditions such as atherosclerosis, thrombosis, liver disease, neurodegenerative disease and cancer (Azevedo et al., 2007; Masyuk et al., 2013; Rak et al., 2012; Vella et al., 2008). Both *in vitro* and *in vivo* studies have shown that normal cells (non-malignant) and tumors cells can engulf EVs and facilitate the release of EV contents within the cells (Kourembanas, 2015; Tian et al., 2010). Previously, EVs are believed to carry waste products of the cells, but studies suggest that they can carry functional nucleic acids (DNA, RNA) and proteins, as well as other biochemical products (Stoorvogel et al., 2002). They have been shown to carry proteins that are ubiquitous (GAPDH), tetraspanins (CD9, CD63, CD81), heat shock proteins (Hsp70 and Hsp90) (Record et al., 2011), and proteins involved in EV synthesis (ALIX, TSG101) (Nabhan et al., 2012; Raiborg et al., 2009). EVs also carry unique biological molecules from the parent cells which releases them. Some of these molecules are CD3 in T cells (Blanchard et al., 2002), transferrin receptor (TFR) in reticulocytes (Blanchard et al., 2002), GPCRs like somatostatin receptor 2 (SSTR-2), chemokine receptors 4 and 7 (CXCR4, CCR7) and AT1R (Delcayre et al., 2005; Estelles et al., 2007; Pironti et al., 2015).

In humans, the majority of the cell surface proteins or membrane proteins belong to the GPCR family. The GPCR family is categorized into different classes, with Class A containing

the highest number of well characterized receptors. A recent nomenclature places the 25 human T2Rs in Class T (Munk et al., 2016). T2Rs are initially discovered in the oral cavity and are involved in the perception of bitter compounds (Adler et al., 2000; Chandrashekar et al., 2000). Several recent studies, including a recent Nanostring sequencing analysis, suggest expression of T2Rs in many extra-oral tissues (D. A. Deshpande et al., 2010; Foster et al., 2014; Jaggupilli et al., 2017). The roles of T2Rs in these tissues are being investigated, with some T2Rs implicated in pathophysiological disorders (Shaik et al., 2016; Singh et al., 2014).

In this study, we describe a detailed methodology to isolate EVs from cell culture media of GPCR expressing stable cell lines (T2R4-FLAG tag, T2R38-Avi tag and AT1R-Avi tag) and from human saliva. Different methods to isolate EVs are compared and the immunolocalization of these GPCRs on the isolated EVs by immuno-TEM and ELISA is described.

6.3. Materials

All solutions were prepared using ultrapure double deionized water and are filtered through 0.2 μ m nylon filter to remove a particulate matter and contaminants (unless otherwise indicated).

6.3.1 Preparation of 10ml PEG solution:

Items required

1. PEG of molecular weight 6000-8000 (Sigma Aldrich).
2. Sodium Chloride (*NaCl*).
3. Filtered 1x PBS.
4. Vortex.
5. 50 ml and 15 ml conical tubes.

Procedure

- A. 2x (24% w/v) PEG stock solution (*see Note i*): Add 10ml (*see Note ii*) of filtered sterile 1x PBS to a 15 ml conical tube.
- B. Weigh 2.5 grams of PEG and 0.584 grams of *NaCl* to obtain a final concentration of 1M.
- C. The above contents are mixed gently using a vortex at low speed settings (*see Note ii*) for 5 min with an intermittent break of 5 min. This should be carried out for about 30 – 40 min during which the contents are completely dissolved.

Note

- i. Stock solution of PEG is prepared fresh one hour before the experiment at room temperature.*
- ii. Care should be taken while dissolving PEG. Higher vortex speed will cause foam, which will be hard to remove.*

6.3.2 EV depleted cell culture media

Cell culture medium is the same as previously described for growth of HEK293 cells (Chakraborty et al., 2015). However, this medium supplemented with FBS contains EVs that may interfere with the isolation procedures. Hence EVs were depleted from cell culture media containing FBS as follows.

Items required

1. Thick walled polycarbonate ultra-centrifuge tubes (Thermo Fisher, #75000610).
2. Micro ultracentrifuge (Sorvall™ MTX 150).
3. DMEM-F12 cell culture media with 1% (or 1x) penicillin, streptomycin and glutamine (Chakraborty et al., 2015).
4. Fetal Bovine Serum (FBS) heat inactivated (Chakraborty et al., 2015).

Procedure

A. 20 ml of medium (DMEM-F12) containing 20% FBS (*see Note iii*) was taken in polycarbonate thick walled tube in a total of six tubes (*120 ml in total*).

B. Centrifuge the medium overnight at 120,000xg at 4°C for 16 hours.

C. Collect the supernatant carefully without disturbing the pellet.

D. Filter the supernatant through 0.2µm filter connected to a vacuum pump.

120 ml of DMEM-F 12 medium is added to 120ml of EV depleted medium to obtain a final concentration of 10% FBS in DMEM-F12.

E. Store the depleted medium up to 2 weeks at 4°C until further use.

6.3.3 Cell culture

Items required

1. HEK 293T cells, and GPCR-T2R4, T2R38 and AT1R (with either a FLAG at the N-terminus and/or Avi tag at the C-terminus) overexpressing cells prepared as described elsewhere (Chakraborty et al., 2015).

2. 100 mm and 150 mm cell culture petri dishes.

3. EV depleted medium from *section 2.2*.

4. 50 ml and 15 ml conical tubes.

6.3.4 Cell seeding for isolation of EVs by Ultra Centrifugation (UC)

A. Cells were seeded in two 150 mm petri dishes at a density of 1×10^6 cells/dish in 30 ml of DMEM-F12 containing 10% FBS.

B. After 24 hours, the medium was removed and the cells were washed with 5ml of calcium and magnesium free 1x PBS *and* 30 ml of EV depleted cell culture media was added to each petri dish.

C. The cells were allowed to grow for six days and EV depleted cell culture media was replenished on the 3rd day. The medium collected on the 3rd day is centrifuged at 500xg to remove cell debris and stored at -80°C for EV isolation along with medium collected on 6th in *step D*.

D. After six days the medium collected will used for EVs isolation immediately or can be stored at -80°C for further use.

Note

iii. Cells used in all the experiment were between passages 1 to 5. It is important to use cells of lower passage number. Cells with higher passage numbers are subjected to stress. This leads to shedding of membrane fragments and other particles that show up during EVs isolation.

6.3.5 Cell seeding for isolation of EVs by PEG precipitation

A. Cells were seeded in two 100mm petri dishes at a density of 5×10^5 cells/dish in 10 ml of DMEM-F12 containing 10% FBS.

B. After 24 hours, the medium was removed and the cells were washed with 2ml of calcium and magnesium free 1x PBS and 10ml of EV depleted cell culture media was added to each petri dish.

C. The cells were allowed to grow for three days and EV depleted cell culture media was collected on the third day. The medium collected was used for EVs isolation immediately or can be stored at -80°C until further use.

6.4 Methods

6.4.1 Isolation of EVs by UC

Items required

1. Conditioned medium from step D of section 2.3.1.
2. 0.45µm nylon filter (fisher scientific).
3. Polycarbonate ultracentrifuge tubes.
4. Micro ultracentrifuge (Sorvall™ MTX 150) with fixed angle rotor of maximum 209,438xg (Thermo Fisher, Sorvall, #S50-A).
5. 1x PBS ice cold with 1µM protease inhibitor.

Procedure

- A. Medium harvested from cells in step D of section 2.3.1 is centrifuged at 500xg for 5 min to remove cells.
- B. The supernatant is spun down at 3000xg for 30 min to remove cell debris.
- C. The supernatant is filtered through a 0.4µm nylon filter to remove apoptotic bodies and large vesicles.
- D. The filtrate is then centrifuged for 2 hours at 120,000xg at 4°C and the supernatant is discarded without disturbing the pellet.
- E. The pellet is carefully washed with 20 ml of 1x PBS and centrifuged for 2 hours at 120,000xg at 4°C.

F. The supernatant is carefully discarded without disturbing the pellet and the pellet is dissolved in 200µl of 1xPBS containing 1µM protease inhibitor.

G. EVs can be used immediately for downstream applications or can be stored at -80°C in aliquots for future use.

6.4.2 Isolation of EVs by PEG precipitation

Items required

1. Conditioned medium from step C of section 2.3.2.
2. 0.45µm nylon filter (fisher scientific).
3. 15 ml conical tubes.
4. Centrifuge with swing bucket M4 rotor (Thermo Fisher, #11175338) at 10,000xg (Thermo Fisher, Sorvall, RT3).
5. 1x PBS ice cold with 1µM protease inhibitor.

Procedure

- A. The processing of harvested medium is same as in *steps A, B and C* of section 3.1.
- B. The filtrate is mixed with PEG in ratio of 1:1 (10 ml medium and 10 ml of 24% PEG).
- C. The mixture is incubated at 4°C for at least 14 hours.
- D. After overnight incubation the mixture was centrifuged for 30 min at 3000xg at 4°C
- E. The supernatant is discarded and the tubes are centrifuged twice for 5 min to remove excess medium.
- F. The obtained pellet is re-suspended in washed with 5 ml of 1xPBS to remove FBS contamination.
- G. The above suspension is incubated with 5 ml of 24% PEG for 2 hours at 4°C.
- H. After incubation for 2 hours, the mixture is centrifuged for 30 min at 3000xg at 4°C.

- I. The pellet obtained is suspended in 100 µl of 1x PBS containing 1µM protease inhibitor.
- J. EVs can be used immediately for downstream applications or can be stored at -80°C in aliquots for future use.

6.4.3 Immuno Transmission Electron Microscopy (TEM) of EVs:

Items required

1. EVs isolated from step G and J of section 3.1 and 3.2.
2. **4% (w/v) Paraformaldehyde (PFA):** Dissolve 2g of PFA in 40 ml of 1x PBS and heat the solution to 65°C while stirring. Add drops of 1N NaOH until the solution turns clear. Add 1x PBS to make it up to 50ml and filter the solution through a 0.2µm nylon filter. Make 1ml aliquots and store them at -20°C, thaw them for future use and do not refreeze them.
3. Formvar carbon coated EM grids (Cedarlane, #01800-F).
4. Clean forceps.
5. Transmission Electron Microscope (TEM) (Philips CM10).
6. **Antibodies used:** Anti CD63 mouse monoclonal (Abcam, #ab59479), Anti Flag-M2 monoclonal (Sigma, #F1804), Anti Avi mouse monoclonal (Genscript, #A01738), Normal mouse IgG (Upstate cell signaling solutions, #), Goat anti mouse secondary (10nm gold) conjugate (Abcam, #ab39619).
7. **0.5 % BSA blocking solution:** Dissolve 0.25g of BSA in 50 ml of 1x PBS and filter the solution through a 0.2µm nylon filter.
8. **Permeabilization solution:** Dissolve 10 µl of Tween-20 in 50 ml of 1x PBS and store the solution at 4°C for future use.

9. **1% glutaraldehyde:** Dissolve 40 μ l of 25% glutaraldehyde (Electron Microscopy Sciences, #16210) in 1 ml of 1x PBS and use the solution immediately. Do not store the solution for future use.

10. **2% (w/v) Uranyl Acetate (UA):** Dissolve 4g of UA crystals in 100 ml of distilled water by stirring. Once the crystals are completely dissolved filter the solution through 0.2 μ m nylon filter. Store the solution in a glass bottle protected from light at 4°C.

Procedure

Fixing EVs on to EM grids

A. 10 μ l of EVs are diluted in 10 μ l of 4% PFA.

B. 10 μ l of resuspended EVs are deposited on Formvar carbon coated grids. The grids are covered and EVs are adsorbed for 30 min at room temperature.

Wash and block EVs

C. Grids are transferred to 100 μ l of 1x PBS drops (EVs coated side facing the drop) and washed twice for 3 min each.

D. Grids are transferred to 100 μ l of 50mM glycine drops and washed three times for 3 min each.

E. EVs were permeabilized in 1x PBS containing 0.02% Tween-20 for 30 min at room temperature (*See Note v*).

Note v: The permeabilization step is only done for Flag and Avi tag antibodies. These antibodies were unable to bind to the respective GPCRs when the EVs were not permeabilized.

F. Grids are blocked with 0.5% BSA in 1x PBS for 10 min at room temperature.

Antibody incubation

G. Grids are incubated with 100 μ l of corresponding primary antibodies at a concentration of 20 μ g/ml for 2 hours at room temperature.

EVs incubated with non-immunized IgG serve as a negative control.

H. Grids are washed in 100µl of 1x PBS three times for 3 min each.

I. Grids are then incubated with corresponding gold conjugated secondary antibodies at 1:10 dilution for 2 hours at room temperature (*See Note vi*).

Note vi: *EVs incubated only with secondary antibody serve as negative control.*

J. Grids are washed in 100µl of 1x PBS three times for 3 min each.

Post fixation of EVs

K. Grids are fixed in 100µl of 1% glutaraldehyde for 5 min.

L. Grids are washed in 100µl of filtered distilled water twice for 2 min each.

Contrast staining of EVs

H. Grids are counter stained using 50µl of 2% UA for 3 min and air-dried.

I. Grids are stored in EM boxes and imaged using TEM.

6.4.4 Preparation of Saliva for EV isolation:

Unstimulated saliva is collected in a collection tube supplemented with Sigma FASTTM protease inhibitor. All the samples are incubated on ice until further processing.

Items required

1. Collection tubes (Tarsons, #510030).
2. Micro centrifuge (Eppendorf, #5424).
3. Sigma FASTTM protease inhibitor, (Catalogue #S8820, Sigma Aldrich).

Procedure

A. 500µl of Sigma FASTTM (*See Note vii*) is added to each labeled collection sterile tube before collecting the saliva sample.

Note vii: One tablet of Sigma FAST™ is dissolved in 100ml of nuclease free water and 500µl of the inhibitor solution is added to each tube used in the saliva collection.

Whole unstimulated saliva (1 ml) is collected from ten healthy individuals in sterile collection tubes.

B. The saliva samples are diluted 1:1 with 1x PBS and centrifuged at 1500xg for 10 minutes at room temperature.

C. The clarified saliva mixture is filtered through a 0.2µM sterile filter and processed for EV isolation.

6.4.5 Isolation of salivary EVs

All solutions are prepared using ultrapure double deionized water and are filtered through 0.2µm nylon filter to remove particulate matter and contaminants.

Items required

1. PEG of molecular weight ~35,000 (Sigma Aldrich, #81310).
2. Protamine chloride (Sigma Aldrich, #P3880).
3. BCA Protein Assay Kit II (Biovision, SKU K812)
4. Filtered 1x PBS.
5. Micro centrifuge (Eppendorf, #5424)
6. 2ml conical Eppendorf tubes
7. Weigh balance

Procedure

Polymer-based precipitation with enhancement of a charge-based isolation leads to increased salivary EV yields.

A. EV precipitation solution contains 0.2gm of PEG 35,000 and 0.1 mg protamine chloride in 1ml of distilled water (*See Note viii*):

B. 1 volume of precipitation solution is added to 4 volumes of diluted saliva derived from Section 3.5.

C. The above contents are mixed on a rotary shaker for 15 min at room temperature and then incubated at 4°C overnight.

D. After overnight incubation, samples are centrifuged at 1500g for 30min at room temperature.

E. Supernatant is discarded and the pellet containing salivary EV is resuspended in 200µl PBS.

F. Isolated salivary EV are stored at -20°C for downstream experiments.

Note viii: The maximal yield of salivary EV is found at 0.1 mg/ml protamine chloride along with PEG 35,000 polymer; higher concentration of protamine chloride ranging from 0.25 to 1mg/ml may be used.

G. Protein content of the isolated salivary EVs was determined using BCA protein assay (item #3) and was 276 ± 122 µg/ml (SD).

6.4.6 ELISA on salivary EVs:

T2R38 expression levels in salivary EVs were quantified using an ELISA established in the laboratory. All antibodies and peptides used in this ELISA are from Santa Cruz, unless otherwise mentioned.

Items required

1. T2R38 peptide (#Sc-34294-P)
2. T2R38 primary antibody (T-16, #Sc-34294, goat polyclonal IgG)

3. Secondary Antibody (rabbit anti-goat IgG), horseradish peroxidase conjugate (#R21459 from Invitrogen Life Technologies)
4. TMB substrate (HiMedia, #ML-168)
5. *ELISA wash buffer* (0.05% Tween-20 in PBS)
6. ELISA diluent buffer (0.5% BSA, 0.01% Tween-20 in PBS)
7. ELISA blocking buffer (0.5% BSA, 0.01% Tween-20 in PBS)
8. 96 well – Maxisorb plate (ThermoFisher, #44-2404-21)
9. Plate reader - SynergyH1 Hybrid multimode plate reader (Biotek)

Procedure

- A. A standard curve is generated using T2R38-peptide in concentration range of 30 – 1200 pg/ml. Peptide standards along with 30µg of EV protein in PBS are dispensed into respective wells and incubated at 4°C overnight.
- B. After overnight incubation, wells are washed thrice with ELISA wash buffer (item #5) followed by blocking for 1 hour at 37°C using ELISA blocking buffer (item #7).
- C. 50µl of primary antibody is diluted 1:250 in ELISA diluent buffer (item # 6) and incubated for 90 min at room temperature followed by 4-5 washes with ELISA wash buffer (item # 5).
- D. 50µl of secondary antibody diluted 1:5000 in ELISA diluent buffer was added to all wells and plate was incubated for 120min at room temperature.
- E. 100µl of TMB substrate is added per well and incubated for 30 min at 37°C in the dark.
- F. Equal volume of 1N H₂SO₄ is added to stop the reaction.
- G. Color development is quantified with a plate reader at A450nm. T2R38 protein level on the surface of EVs is derived from T2R38-Peptide standard curve.

6.5 Results

6.5.1 Bitter taste receptors are localized in EVs isolated by ultracentrifugation.

To characterize the localization of T2Rs in EVs we have utilized HEK 293T cells overexpressing FLAG tagged T2R4 and Avi tagged T2R38 and AT1R a well-known GPCR to be localized in EVs. The cell culture supernatants from the above mentioned cell lines were ultracentrifuged and the pellet obtained was re-suspended in PBS. The immuno-TEM was performed on EVs as described earlier (**Section 6.4.3**). The immune-TEM images show localization of Flag-T2R4 (**Figure 6.1A**) and Avi-T2R38 (**Figure 6.1B**) in EVs. We have also shown AT1R to be present in EVs isolated from HEK 293T-AT1R cells (**Figure 6.1C**). The EVs isolated from the T2R expressing cells were also positive for EV marker CD81 (**Figure 6.1D**). The EVs isolated from HEK 293T and HEK 293S parental cells not expressing Flag or Avi constructs did not stain for Flag and Avi antibodies (**Figure 6.1E-G**). The Flag-T2R4, Avi-T2R38 expressing cells stained using mouse IgG or secondary antibodies alone did not show any staining (**Figure 6.1H and I**).

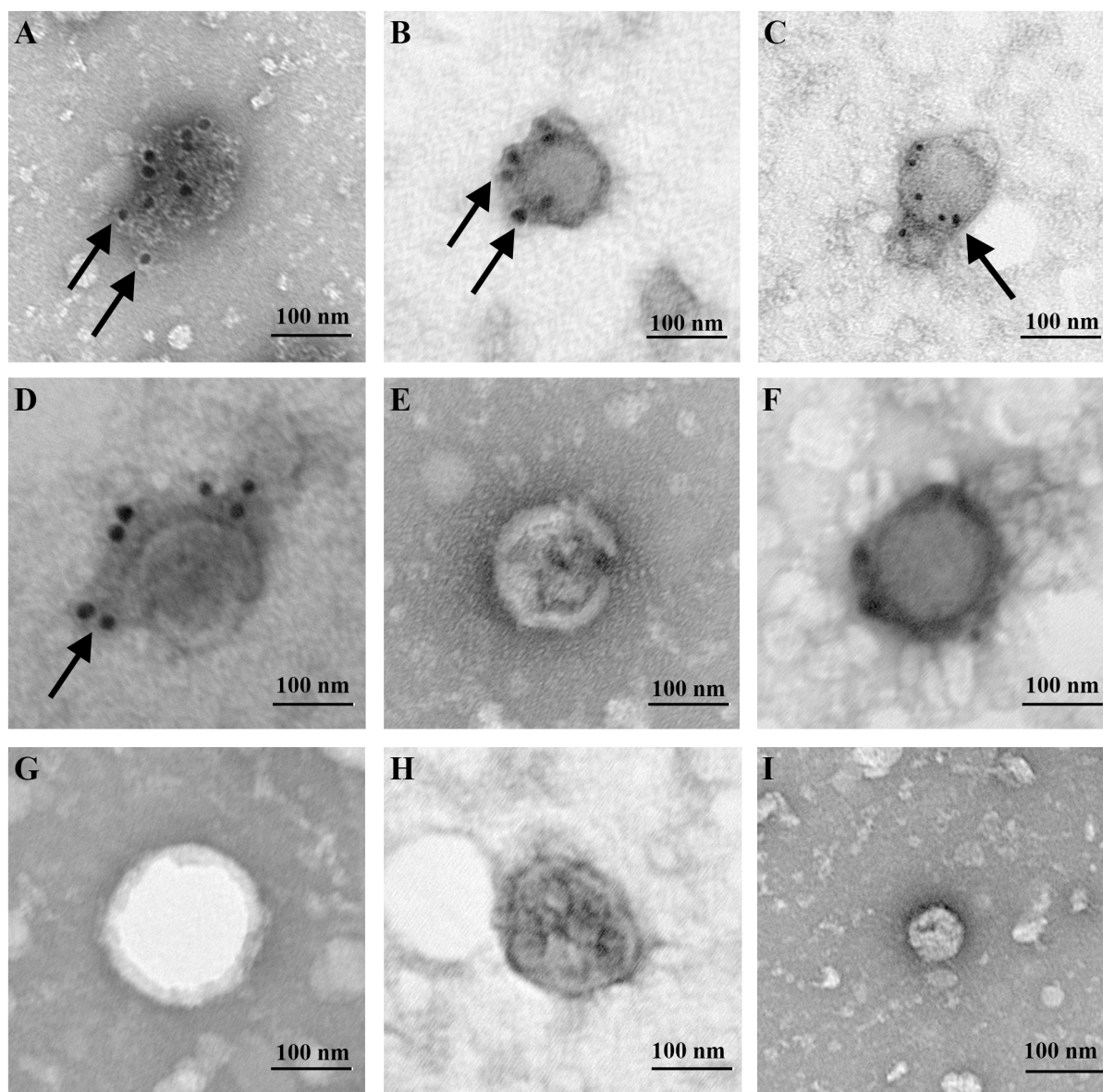


Figure 6. 1: Immuno TEM on EVs isolated from HEK293 parental and GPCR overexpressing HEK293 cells.

(A–C) Immuno TEM of EVs isolated from T2R4 (A), T2R38 (B), and AT1R (C) overexpressing HEK293 cells using specific antibodies against FLAG or Avi tags. T2R4 has a FLAG tag at its N-terminus, while T2R38 and AT1R have an Avi tag at the C-terminus. (D) EVs isolated from the same cell lines were positive for CD81, a marker of EVs. (E–G) EVs isolated from HEK293T and HEK293S parental cells did not stain or showed minimal staining with either FLAG or

Avi antibodies. **(H-I)** EVs incubated with mouse IgG or with secondary gold conjugated antibody alone served as negative controls and did not show any staining. The *arrows* represent positive staining for the proteins on EVs.

6.5.2 EVs isolated from saliva express T2R38.

EVs are known to be present in several biological fluids such as serum, urine and saliva. However the expression of T2Rs in these salivary EVs has not been identified thus far. To characterize T2R38 localization the salivary EVs were isolated using PEG based precipitation as described earlier (**section 6.4.5**). The T2R38 expression in salivary EVs is characterized using ELISA (**Figure 6.2A and B**). Our results show that levels of T2R38 in salivary EVs ranged from 80-1000 ng/mg of EV protein. Also, the expression of T2R38 in EVs varied between different subjects (**Figure 6.2B**).

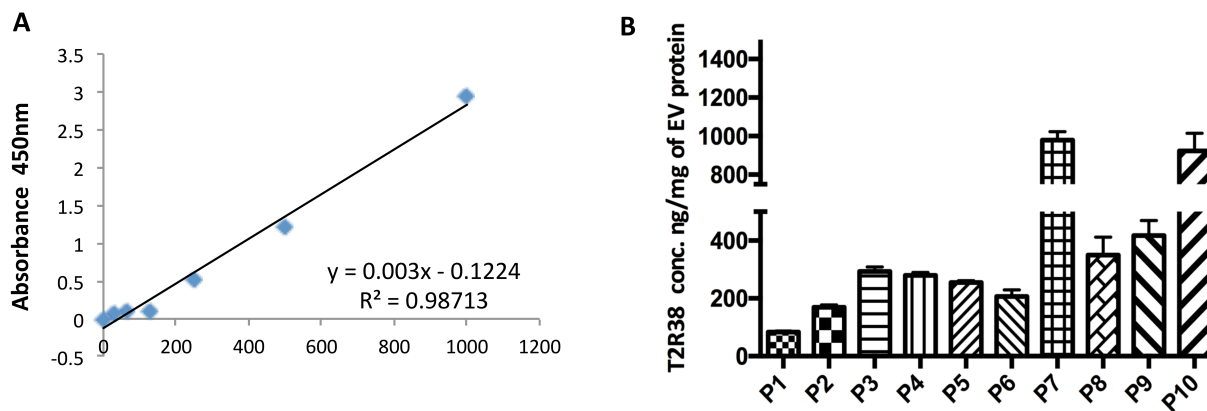


Figure 6. 2: T2R38 ELISA on EVs isolated from saliva of healthy individuals.

(A) ELISA standard curve. Duplicate readings were averaged for each standard, and blank values were subtracted. Standard curve was plotted from the mean absorbance and concentration for each standard and best-fit curve was plotted by regression analysis. **(B)** T2R38 ELISA. Salivary EVs were isolated from $n = 10$ (healthy individuals, P1–P10). T2R38-specific ELISA was done

on the salivary EVs according to the procedure mentioned in the text. Absorbance at 450 nm was measured, and T2R38 concentrations were deduced from the standard curve. All experiments were run in triplicate.

6.6 Discussion

The EVs are nanocarriers released by many human cells. EVs are membranous vesicles ranging between 50 to 500 nm in size and are secreted by endocytosis of membranes ultimately leading to exocytosis of MVBs or by shedding of plasma membrane of blebs (Trams et al., 1981). EVs play an important role in intercellular communication and are involved in regulating several biological processes such as inflammation, cell proliferation, and neuronal function (Raposo et al., 1996; Simhadri et al., 2008).

EVs carry unique biological molecules from the parent cells they are released such as CD3 in T-cells, transferrin receptor (TFR) in reticulocytes. However, recent studies have also shown the localization of GPCRs like SSTR-2, CXCR4, CCR7 and AT1R (Blanchard et al., 2002; Delcayre et al., 2005; Estelles et al., 2007; Pironti et al., 2015). In this study we have shown the localization of T2Rs in EVs isolated from HEK293T cells and in saliva using immuno TEM and ELISA assays. It was the first report to suggest expression of T2R4 and T2R38 in EVs isolated from saliva of healthy individuals. However, the specific role of T2Rs in EV life cycle and conversely, the contributions of EVs in T2R signaling remain to be determined.

The intertwined mechanism of intercellular communications between GPCRs and EVs was recently reviewed (Bebelman et al., 2020). Although the role of T2Rs in EVs is not yet known, the notion of extracellular localization of GPCRs is well established in the case of AT1R (Pironti et al., 2015), GPCR5B (Kwon et al., 2014) and kinin1B receptors (Kahn et al., 2017). The process of GPCR internalization and sorting into endosomal compartments is similar to that of MVB formation in EV life cycle. Hence there exists a mechanistic link between GPCR internalization via endosomal sorting leading to their EV secretion. Conventional knowledge suggests endosomal GPCR MVBs fuse with lysosomes leading to degradation or recycling to the

plasma membrane. However, these GPCR MVBs can also fuse with plasma membrane leading to their extracellular release.

The localization of T2R4 and T2R38 in EVs might offer renewed focus onto T2R recycling in cells via EV secretion pathways. Currently, few studies were pursued on T2R internalization or recycling upon activation by cognate ligands (J. D. Upadhyaya et al., 2016; Woo et al., 2019). Studies on T2R4 suggest that it does not internalize upon agonist treatment and the agonist quinine has a pharmacochaperone activity increasing the cell surface expression of T2R4 (J. D. Upadhyaya et al., 2016). In contrast, recent studies on T2R14 suggest all agonists except diphenhydramine cause T2R14 internalization (Woo et al., 2019). These studies suggest that internalization might be T2R and agonist specific.

Chapter 7: Conclusion and future directions

7.1 Conclusion

T2Rs are primarily expressed in gustatory tissues and recognize bitter compounds. However, over the past few years several studies including ours have identified extra-oral expression and extra-gustatory roles for T2Rs. T2Rs along with TLRs have emerged as the sentinels of the innate immune system in several tissues and cell types. The innate immune responses modulated by T2Rs have been studied mainly in pathophysiological conditions such as CRS and CF. Most of these earlier studies have focused on QSMs secreted by Gram-negative bacteria.

In this study I have characterized the interaction of T2R14 and QSM-CSPs secreted by Gram-positive cariogenic bacterium *S. mutans*. The transcriptome analysis showed that T2R14 was expressed at high levels compared to the remaining 24 T2Rs and 10 TLRs in two GEC lines. CSP-1, a major QSM secreted by *S. mutans*, was shown to increase intracellular calcium levels but did not affect cAMP levels in GECs. Other CSP derivatives such as CSP-2 and CSP-3 lacking the C-terminal amino acids (LGK) did not induce significant intracellular calcium mobilization compared to CSP-1. The effect of different artificially synthesized CSPs having varying lengths of amino acids at the C-terminus on T2R14 signaling was examined. These experiments showed that the two C-terminal amino acids (GK) in CSP-1 are important in mediating intracellular calcium mobilization in GECs.

To characterize the interaction between CSP-1 and T2R14 in GECs, a CRISPR/Cas9 approach to knock down T2R14 was pursued. The mutation in the TAS2R14 gene created by a CRISPR-mediated approach yielded a complete gene cleavage as determined by T7EI assay. This resulted in complete knock down of T2R14 protein expression in OKF6 cells as determined by western blot analysis. Next, calcium mobilization assays in T2R14 KD cells using CSP-1 and the

T2R14 agonist DPH were performed; this resulted in decreased intracellular calcium mobilization. Interestingly, the EC₅₀ of CSP-1 and DPH did not change in T2R14 KD cells although the maximal calcium responses were significantly reduced. In a complementary approach, competition assays using T2R14 antagonist 6-MF and T2R signaling inhibitors show inhibition of CSP-1 mediated intracellular calcium mobilization in GECs. In T2R14 overexpressing HEK293T cells, intracellular calcium mobilization was increased upon treatment with CSP-1. Taken together, the above data suggested that *S. mutans* CSP-1 primarily signals via T2R14 leading to increased intracellular calcium mobilization in GECs.

Next, I characterized the downstream signaling pathways activated by CSP-1 in GECs. Since activation of PLC is shown to induce NF- κ B signaling via PKC, I quantified phosphorylation of the NF- κ B signaling intermediates IKK α / β and I κ B α . CSP-1 was found to induce phosphorylation of IKK α / β and I κ B α that was inhibited by PLC inhibitor but not with G β γ inhibitor or by knock down of T2R14. This showed that CSP-1 can also act independently of T2R14.

The main conclusions from the work in chapter 4 are as follows:

- 1) Among the CSP's secreted by *S. mutans*, CSP-1 caused significant activation of T2R14 in GECs.
- 2) The CSP-1 mediated activation of T2R14 induced innate immune responses such as cytokine/chemokine secretion that resulted in immune cell recruitment to the site of inflammation. T2R14 activation led to increased secretion of cytokines IL-6, TNF- α and chemokine IL-8/CXCL8 upon treatment with CSP-1. The CSP-1 CM from GECs increased migration of dHL-60 cells compared to untreated cells and this migration was decreased in T2R14 KD cells treated with CSP-1 or agonist DPH.

In the oral epithelium, GECs are in constant contact with several microbes. The bacteria and their products are known to interact and signal via host cell surface proteins. LPS from bacteria is recognized by TLRs and several QSMs are recognized by T2Rs. However, the physiological significance of these interactions involving T2Rs remain unclear. In chapter 5, I examined the role of T2R14 in mediating bacterial internalization into GECs and bactericidal effects of T2R14 activation on Gram-positive bacteria. Internalization of *S. aureus* was significantly decreased in T2R14 KD cells and the internalization of *S. mutans* remained unaffected. To further understand the mechanism of T2R14 mediated internalization of Gram-positive bacteria the GECs were treated with an actin polymerization inhibitor (cytochalasin-D) and TLR signaling inhibitor (MPP). The internalization of *S. aureus* was significantly decreased by cytochalasin-D but not with MPP. Interestingly, *S. mutans* internalization has increased only in WT cells treated with MPP. These results suggest that *S. aureus* internalization is dependent on T2R14 mediated actin polymerization and *S. mutans* internalization is TLR/T2R signaling mediated. Since actin cytoskeleton is a major player in internalization I performed pull-downs of GTP-Rac-1 a well-known cytoskeletal modulator. I observed a decreased pull-down of PAK-1 associated actin but not Rac-1 in T2R14 KD cells. These results show that T2R14 KD alters cytoskeletal dynamics in GECs thereby affecting internalization of Gram-positive bacteria.

Earlier studies have shown bactericidal affects of T2R activation in CRS. In this work, I have analyzed the effect of T2R14 activation in GECs on *S. aureus* and *S. mutans*. The *S. aureus* growth is decreased upon treatment with DPH, apigenin and CSP-1 in the presence of T2R14. On the contrary, *S. mutans* growth was unaffected. Interestingly, *S. mutans* CSP-1 is shown to inhibit another commensal bacteria *S. aureus*. These findings provide insight into a host-directed mechanism by which commensal bacteria inhibit other bacterial species. To better understand the mechanisms behind the observed bactericidal effects I have analyzed secretion of nitrates and

anti-microbial peptides such as hBD-2. The nitrate levels were not affected by Gram-positive bacterial infection of GECs. However, cells infected with *S. aureus* have shown T2R14 mediated hBD-2 secretion. I have also quantified secretion of chemokine IL-8/CXCL8 in response to bacterial infection and found that only *S. mutans* treated GECs increased T2R14-dependent IL-8 secretion. My findings provide a new and yet unexplored mechanism of T2R mediated innate immune responses in oral epithelial cells, and suggest possible T2R/TLR cross-talk.

The main conclusions from the work in chapter 5 are as follows:

- 1) The *S. aureus* internalization is dependent on T2R14 mediated actin polymerization and *S. mutans* internalization is TLR/T2R signaling mediated.
- 2) T2R14 mediates bactericidal effect on *S. aureus* but not *S. mutans*.

Results from the final chapter of this thesis provided the first evidence that T2Rs are present in EVs isolated from human saliva and cell cultures. This discovery might be of diagnostic significance as T2Rs might be useful as biomarkers for certain diseases. More importantly, the presence of T2R in EVs might be interesting from a mechanistic perspective in understanding T2R internalization and recycling. The results from chapters 5 and 6 suggest that T2Rs mediate bacterial internalization and T2Rs themselves can be internalized into endosomes leading to their EV release. However, the mechanisms of T2R internalization into EVs need to be deciphered. It is possible that T2R localization into MVBs upon bacterial exposure and their extracellular release might act as a secondary communication between cells. Hence, future studies should focus on understanding the mechanisms involved in T2R internalization into EVs upon bitter agonist treatment or QSMs from bacteria.

The main conclusions from the work in chapter 6 are as follows:

- 1) T2R4 and T2R38 are present in EVs isolated from cell lines and saliva.
- 2) The mechanisms of T2R internalization into EVs should be characterized.

7.2 Future directions

In recent years the extra-gustatory roles of T2Rs have been tremendously expanded with a major focus on mediating innate immune responses. These findings place T2Rs as important receptors in the field of host-pathogens interactions. The knowledge available till date about QSM-T2R interactions and signaling is mostly based on heterologous cell system over-expressing T2Rs or endogenous cell systems using T2R signaling blockers. Although these provide a preliminary understanding about the QSM-T2R interactions there is an urgent need to develop T2R knock out models to study these interactions. The work presented in this thesis is among the few that utilizes a T2R knock out cell model to study host-pathogen interactions in a cell-based system. Future studies should be focused on generating T2R knock out mouse models to study host-pathogen interactions in oral and upper respiratory tract. The T2R mediated innate immune responses identified thus far include release of Nitric oxide, AMPs and several cytokines. However, many of these are also known to be regulated by TLRs. So future studies are warranted to understand the mechanism of cross-talk between TLR and T2R signaling pathways. As T2Rs are known to affect secretion of AMPs and other innate immune markers it would be useful to identify oral and gut microbiome profile using T2R knock out mouse models. It would be useful in answering very important questions, including whether T2R expression is required for maintaining normal microbial flora, and knock out of T2Rs cause dysbiosis of oral and gut microbiota.

Most of the knowledge of T2R activation by bitter agonists and QSMs is based on intracellular calcium mobilization regulated via $G\alpha\beta\gamma$ -PLC pathways. However, recent studies have shown biased signaling where T2Rs can signal via G_i leading to inhibition of cAMP responses. Therefore, understanding T2R- G_i mediated signaling becomes of paramount

importance in several pathological conditions such as caries and CF. This study has also identified the role of T2Rs in Gram-positive bacterial internalization, and possible role of T2Rs in cytoskeletal rearrangement.

Non-phagocytic cells internalize Gram-positive bacteria by zipper and trigger mechanisms that are regulated by several signaling events leading to cytoskeletal rearrangements in the host cells. For example, Gram-positive food born bacteria *Listeria monocytogenes* is internalized by zipper mechanism by binding to host cell receptors E-cadherin and hepatocyte growth factor receptor (MET) (Mengaud et al., 1996; Shen et al., 2000). The binding to host cell receptors activates two signaling cascades. The first being, internalization of the bacteria into clathrin-coated vesicles that are later cleaved by dynamin family proteins. The other pathway activates downstream signaling cascades leading to phosphorylation of Src kinases and PI3 kinase to generate PIP3 followed by the recruitment of actin remodeling proteins N-WASP and Arp2/3. These induce actin polymerization thus facilitating host cell membrane reorganization and bacterial intake. Other Gram-positive bacteria internalize by fibronectin mediated host cell $\alpha 5\beta 1$ integrin receptor clustering (Nitsche-Schmitz et al., 2007). Thus, facilitating activation of focal adhesion kinases, Src-dependent phosphorylation of cortactin, recruitment of Arp2/3 complex responsible for actin polymerization and promoting internalization by membrane ruffling (Ozeri et al., 2001).

In this study I have shown T2R14 mediated internalization of *S. aureus* in GECs. However the molecular mechanisms behind T2R14 mediated internalization of bacteria is not characterized. Future studies should be focused on characterizing these internalization mechanisms. First, T2R mediated cytoskeletal remodeling such as F-actin formation and its effect on bacterial internalization should be characterized. This entails investigating the expression, phosphorylation, recruitment of actin remodeling proteins (cortactin, N-WASP and Arp2/3) and

$\alpha 5\beta 1$ integrin receptor clustering using T2R signaling inhibitors and knock out cell models.

In this study we have utilized cultured monolayer GECs for internalization. However, the epithelial cells in oral tissues are multilayered and are supported by fibroblasts. Future studies on T2R internalization can use organoid or 3D cultures to replicate the physiological conditions of the oral tissues. The use of 3D cultures will also be useful in characterizing T2R mediated effect on epithelial barrier integrity and transepithelial electrical resistance (TEER) upon oral bacterial infections.

My study has shown T2R dependent Gram-positive bacterial internalization in GECs, However T2Rs are also expressed in ciliated nasal epithelial cells and upper airway epithelial cells (Jaggupilli et al., 2017; Workman et al., 2018). In these cells, T2Rs are known to recognize QSMs from Gram-negative bacteria and induce innate immune responses. It would be interesting to study the role of T2Rs in the internalization of Gram-negative bacterial species mainly *P. aeruginosa* known to be predominant in CF. This would involve characterizing T2R mediated cytoskeletal rearrangements leading to airway epithelial cell remodeling and its effect on ciliary beat frequency and mucus secretion upon exposure to pathogens. To study the impact of T2Rs in microbial colonization of CF airways it would be useful to generate specific T2R knock out animal (mouse or pig) model containing CFTR mutation ($\Delta F508$). Further, these animal models can be used to study the microbiome during different stages of CF lung function decline and understand T2R mediated airway remodeling.

Finally my work has shown that T2Rs are present in EVs isolated from cell culture supernatants and saliva. However, the function of T2Rs in EVs is not characterized in this study. As discussed earlier GPCR internalization into endosomes and MVB formation leading to EV release are physiologically similar processes. Most of the agonist activated GPCRs are inhibited from binding to G protein binding by phosphorylation of the receptor by G protein regulated

kinases (GRK) and β -arrestin binding leading to receptor internalization into endosomes (Jean-Charles et al., 2017). These receptors present in the endosomes were then recycled back to the plasma membrane. However there exists a possibility by which these receptors are secreted into EVs without fusing to the plasma membrane (Bebelman et al., 2020). The internalization or desensitization mechanism of T2Rs is not characterized. However, the presence of T2Rs in EVs offers renewed focus on receptor trafficking. One interesting approach is to link T2R internalization upon bacterial infection and on exposure to bacterial QSMs. This study would further lead into understanding T2R sorting into EVs either by β -arrestin dependent or independent mechanisms. This could be more relevant in the case of immune cells such as macrophages and neutrophils, where functional T2Rs are expressed and their activation by QSMs or bitter agonists could lead to their release into EVs. These future studies can cement the role of T2Rs as sentinels of innate immune system and in governing host-pathogen interactions.

Chapter 8: REFERENCES

1. Abaffy, T., Trubey, K. R., & Chaudhari, N. (2003). Adenylyl cyclase expression and modulation of cAMP in rat taste cells. *American Journal of Physiology-Cell Physiology*, 284(6), C1420-C1428. doi:10.1152/ajpcell.00556.2002
2. Adappa, N. D., Workman, A. D., Hadjiliadis, D., Dorgan, D. J., Frame, D., Brooks, S., . . . Cohen, N. A. (2016). T2R38 genotype is correlated with sinonasal quality of life in homozygous DeltaF508 cystic fibrosis patients. *Int Forum Allergy Rhinol*, 6(4), 356-361. doi:10.1002/alr.21675
3. Adler, E., Hoon, M. A., Mueller, K. L., Chandrashekar, J., Ryba, N. J., & Zuker, C. S. (2000). A novel family of mammalian taste receptors. *Cell*, 100(6), 693-702. doi:10.1016/s0092-8674(00)80705-9
4. Agerer, F., Lux, S., Michel, A., Rohde, M., Ohlsen, K., & Hauck, C. R. (2005). Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. *J Cell Sci*, 118(Pt 10), 2189-2200. doi:10.1242/jcs.02328
5. Ahmed, S., Meghji, S., Williams, R. J., Henderson, B., Brock, J. H., & Nair, S. P. (2001). *Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. *Infect Immun*, 69(5), 2872-2877. doi:10.1128/iai.69.5.2872-2877.2001
6. Ahn, S. J., Ahn, S. J., Wen, Z. T., Brady, L. J., & Burne, R. A. (2008). Characteristics of biofilm formation by *Streptococcus mutans* in the presence of saliva. *Infect Immun*, 76(9), 4259-4268. doi:10.1128/iai.00422-08
7. Al Kindi, A., Alkahtani, A. M., Nalubega, M., El-Chami, C., O'Neill, C., Arkwright, P. D., & Pennock, J. L. (2019). *Staphylococcus aureus* Internalized by Skin Keratinocytes Evade Antibiotic Killing. *Front Microbiol*, 10, 2242. doi:10.3389/fmicb.2019.02242
8. Alfonso-Prieto, M., Navarini, L., & Carloni, P. (2019). Understanding Ligand Binding to G-Protein Coupled Receptors Using Multiscale Simulations. *Front Mol Biosci*, 6, 29. doi:10.3389/fmolb.2019.00029

9. Allen, J. A., Raval, S. R., Yang, R., & Xi, S. (2017). Genome-wide tissue transcriptome profiling and ligand screening identify seven striatum-specific human orphan GPCRs. *The FASEB Journal*, 31(1_supplement), lb576-lb576.
doi:10.1096/fasebj.31.1_supplement.lb576
10. Alvarez-Curto, E., & Milligan, G. (2016). Metabolism meets immunity: The role of free fatty acid receptors in the immune system. *Biochem Pharmacol*, 114, 3-13.
doi:10.1016/j.bcp.2016.03.017
11. Arai, S., Noguchi, M., Kurosawa, S., Kato, H., & Fujimaki, M. (1970). Applying proteolytic enzymes on soyabean. 6. Deodorization effect of aspergillopeptidase A and debittering effect of aspergillus acid carboxypeptidase. *Journal of Food Science*, 35(4), 392-395. doi:10.1111/j.1365-2621.1970.tb00940.x
12. Assadi-Porter, F. M., Tonelli, M., Maillet, E. L., Markley, J. L., & Max, M. (2010). Interactions between the human sweet-sensing T1R2-T1R3 receptor and sweeteners detected by saturation transfer difference NMR spectroscopy. *Biochim Biophys Acta*, 1798(2), 82-86. doi:10.1016/j.bbamem.2009.07.021
13. Avau, B., Rotondo, A., Thijs, T., Andrews, C. N., Janssen, P., Tack, J., & Depoortere, I. (2015). Targeting extra-oral bitter taste receptors modulates gastrointestinal motility with effects on satiation. *Sci Rep*, 5, 15985. doi:10.1038/srep15985
14. Azevedo, L. C., Janiszewski, M., Pontieri, V., Pedro Mde, A., Bassi, E., Tucci, P. J., & Laurindo, F. R. (2007). Platelet-derived exosomes from septic shock patients induce myocardial dysfunction. *Critical Care*, 11(6), R120. doi:10.1186/cc6176
15. Baker, E. K., Colley, N. J., & Zuker, C. S. (1994). The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. *Embo j*, 13(20), 4886-4895.
16. Barber, C., Tang, J., Feng, J., Pan, M., Wilson, T., Slater, H., . . . Daniels, M. (1997). A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol*, 24(3), 555-566.
17. Barham, H. P., Cooper, S. E., Anderson, C. B., Tizzano, M., Kingdom, T. T., Finger, T. E., . . . Ramakrishnan, V. R. (2013). Solitary chemosensory cells and bitter taste receptor signaling in human sinonasal mucosa. *Int Forum Allergy Rhinol*, 3(6), 450-457.
doi:10.1002/alr.21149

18. Baroni, M., Pizzirani, C., Pinotti, M., Ferrari, D., Adinolfi, E., Calzavarini, S., . . . Di Virgilio, F. (2007). Stimulation of P2 (P2X7) receptors in human dendritic cells induces the release of tissue factor-bearing microparticles. *The FASEB Journal*, 21(8), 1926-1933. doi:10.1096/fj.06-7238com
19. Barrett, E., Ross, R., O'toole, P., Fitzgerald, G., & Stanton, C. (2012). γ - Aminobutyric acid production by culturable bacteria from the human intestine. *Journal of Applied Microbiology*, 113(2), 411-417.
20. Barth, C., Jakubczyk, D., Kubas, A., Anastassacos, F., Brenner-Weiss, G., Fink, K., . . . Koelsch, P. (2012). Interkingdom signaling: integration, conformation, and orientation of N-acyl-L-homoserine lactones in supported lipid bilayers. *Langmuir*, 28(22), 8456-8462.
21. Bartoshuk, L. M. (2000). Comparing sensory experiences across individuals: recent psychophysical advances illuminate genetic variation in taste perception. *Chem Senses*, 25(4), 447-460.
22. Baumer-Harrison, C., Raymond, M. A., Myers, T. A., 2nd, Sussman, K. M., Rynberg, S. T., Ugartechea, A. P., . . . Breza, J. M. (2020). Optogenetic Stimulation of Type I GAD65(+) Cells in Taste Buds Activates Gustatory Neurons and Drives Appetitive Licking Behavior in Sodium-Depleted Mice. *The Journal of Neuroscience*. doi:10.1523/JNEUROSCI.0597-20.2020
23. Bebelman, M. P., Crudden, C., Pegtel, D. M., & Smit, M. J. (2020). The Convergence of Extracellular Vesicle and GPCR Biology. *Trends Pharmacol Sci*, 41(9), 627-640. doi:10.1016/j.tips.2020.07.001
24. Behrens, M. (2021). Pharmacology of TAS1R2/TAS1R3 Receptors and Sweet Taste. *Handb Exp Pharmacol*. doi:10.1007/164_2021_438
25. Behrens, M., Brockhoff, A., Kuhn, C., Bufo, B., Winnig, M., & Meyerhof, W. (2004). The human taste receptor hTAS2R14 responds to a variety of different bitter compounds. *Biochemical and biophysical research communications*, 319(2), 479-485. doi:10.1016/j.bbrc.2004.05.019
26. Behrens, M., Di Pizio, A., Redel, U., Meyerhof, W., & Korsching, S. I. (2021). At the Root of T2R Gene Evolution: Recognition Profiles of Coelacanth and Zebrafish Bitter Receptors. *Genome Biol Evol*, 13(1). doi:10.1093/gbe/evaa264

27. Behrens, M., & Meyerhof, W. (2011). Gustatory and extragustatory functions of mammalian taste receptors. *Physiology & Behaviour*, 105(1), 4-13.
doi:10.1016/j.physbeh.2011.02.010
28. Bernhardt, S. J., Naim, M., Zehavi, U., & Lindemann, B. (1996). Changes in IP3 and cytosolic Ca²⁺ in response to sugars and non-sugar sweeteners in transduction of sweet taste in the rat. *The Journal of Physiology*, 490(2), 325-336.
doi:10.1113/jphysiol.1996.sp021147
29. Bezzerri, V., Borgatti, M., Finotti, A., Tamanini, A., Gambari, R., & Cabrini, G. (2011). Mapping the transcriptional machinery of the IL-8 gene in human bronchial epithelial cells. *Journal of Immunology*, 187(11), 6069-6081. doi:10.4049/jimmunol.1100821
30. Bhargava, N., Sharma, P., & Capalash, N. (2012). N-acyl homoserine lactone mediated interspecies interactions between *A. baumannii* and *P. aeruginosa*. *Biofouling*, 28(8), 813-822. doi:10.1080/08927014.2012.714372
31. Bikash, C. R., Hamry, S. R., & Tal-Gan, Y. (2018). Structure-Activity Relationships of the Competence Stimulating Peptide in *Streptococcus mutans* Reveal Motifs Critical for Membrane Protease SepM Recognition and ComD Receptor Activation. *ACS Infectious Diseases*, 4(9), 1385-1394. doi:10.1021/acsinfecdis.8b00115
32. Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G., & Hivroz, C. (2002). TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *Journal of Immunology*, 168(7), 3235-3241.
33. Boneschansker, L., Yan, J., Wong, E., Briscoe, D. M., & Irimia, D. (2014). Microfluidic platform for the quantitative analysis of leukocyte migration signatures. *Nat Commun*, 5, 4787. doi:10.1038/ncomms5787
34. Boudreau, J. C. (1979). *Food taste chemistry: based on a symposium sponsored by the Division of Agricultural and Food Chemistry at the ACS/CSJ Chemical Congress, Honolulu, Hawaii, April 2-6, 1979*. Paper presented at the ACS symposium series (USA).
35. Brandenburg, K. S., Rodriguez, K. J., McAnulty, J. F., Murphy, C. J., Abbott, N. L., Schurr, M. J., & Czuprynski, C. J. (2013). Tryptophan inhibits biofilm formation by *Pseudomonas aeruginosa*. *Antimicrobial agents and chemotherapy*, 57(4), 1921-1925.

36. Braschi, B., Denny, P., Gray, K., Jones, T., Seal, R., Tweedie, S., . . . Bruford, E. (2019). Genenames.org: the HGNC and VGNC resources in 2019. *Nucleic Acids Research*, 47(D1), D786-D792. doi:10.1093/nar/gky930
37. Bredenbruch, F., Nimtz, M., Wray, V., Morr, M., Muller, R., & Haussler, S. (2005). Biosynthetic pathway of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines. *J Bacteriol*, 187(11), 3630-3635. doi:10.1128/jb.187.11.3630-3635.2005
38. Breslin, Paul A. S. (2013). An Evolutionary Perspective on Food and Human Taste. *Current Biology*, 23(9), R409-R418. doi:<https://doi.org/10.1016/j.cub.2013.04.010>
39. Bufe, B., Schumann, T., Kappl, R., Bogeski, I., Kummerow, C., Podgorska, M., . . . Zufall, F. (2015). Recognition of bacterial signal peptides by mammalian formyl peptide receptors: a new mechanism for sensing pathogens. *Journal of Biological Chemistry*, 290(12), 7369-7387. doi:10.1074/jbc.M114.626747
40. Caicedo, A., Pereira, E., Margolskee, R. F., & Roper, S. D. (2003). Role of the G-protein subunit α -gustducin in taste cell responses to bitter stimuli. *Journal of Neuroscience*, 23(30), 9947-9952.
41. Canello, R., Micheletto, G., Meta, D., Lavagno, R., Bevilacqua, E., Panizzo, V., & Invitti, C. (2020). Expanding the role of bitter taste receptor in extra oral tissues: TAS2R38 is expressed in human adipocytes. *Adipocyte*, 9(1), 7-15. doi:10.1080/21623945.2019.1709253
42. Carey, R. M., Chen, B., Adappa, N. D., Palmer, J. N., Kennedy, D. W., Lee, R. J., & Cohen, N. A. (2016). Human upper airway epithelium produces nitric oxide in response to *Staphylococcus epidermidis*. *Int Forum Allergy Rhinol*, 6(12), 1238-1244. doi:10.1002/alr.21837
43. Carey, R. M., Workman, A. D., Hatten, K. M., Siebert, A. P., Brooks, S. G., Chen, B., . . . Cohen, N. A. (2017). Denatonium-induced sinonasal bacterial killing may play a role in chronic rhinosinusitis outcomes. *Int Forum Allergy Rhinol*, 7(7), 699-704. doi:10.1002/alr.21949
44. Carey, R. M., Workman, A. D., Yan, C. H., Chen, B., Adappa, N. D., Palmer, J. N., . . . Cohen, N. A. (2017). Sinonasal T2R-mediated nitric oxide production in response to *Bacillus cereus*. *American Journal of Rhinology & Allergy*, 31(4), 211-215. doi:10.2500/ajra.2017.31.4453

45. Chakrabarti, S., Jahandideh, F., & Wu, J. (2014). Food-derived bioactive peptides on inflammation and oxidative stress. *BioMed research international*, 2014.
46. Chakraborty, R., Xu, B., Bhullar, R. P., & Chelikani, P. (2015). Expression of g protein-coupled receptors in Mammalian cells. *Methods in Enzymology*, 556, 267-281. doi:10.1016/bs.mie.2014.12.013
47. Chandler, J. D., Min, E., Huang, J., McElroy, C. S., Dickerhof, N., Mocatta, T., . . . Day, B. J. (2015). Antiinflammatory and Antimicrobial Effects of Thiocyanate in a Cystic Fibrosis Mouse Model. *American journal of respiratory cell and molecular biology*, 53(2), 193-205. doi:10.1165/rcmb.2014-0208OC
48. Chandrashekar, J., Mueller, K. L., Hoon, M. A., Adler, E., Feng, L., Guo, W., . . . Ryba, N. J. (2000). T2Rs function as bitter taste receptors. *Cell*, 100(6), 703-711. doi:10.1016/s0092-8674(00)80706-0
49. Chaput, N., & Théry, C. (2011). Exosomes: immune properties and potential clinical implementations. *Semin Immunopathol*, 33(5), 419-440. doi:10.1007/s00281-010-0233-9
50. Chen, J., Larson, E. D., Anderson, C. B., Agarwal, P., Frank, D. N., Kinnamon, S. C., & Ramakrishnan, V. R. (2019). Expression of Bitter Taste Receptors and Solitary Chemosensory Cell Markers in the Human Sinonasal Cavity. *Chem Senses*, 44(7), 483-495. doi:10.1093/chemse/bjz042
51. Cho, M. J., Unklesbay, N., Hsieh, F. H., & Clarke, A. D. (2004). Hydrophobicity of bitter peptides from soy protein hydrolysates. *Journal of Agricultural and Food Chemistry*, 52(19), 5895-5901. doi:10.1021/jf0495035
52. Chung, W. O., & Dale, B. A. (2004). Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species. *Infect Immun*, 72(1), 352-358. doi:10.1128/iai.72.1.352-358.2004
53. Conte, C., Ebeling, M., Marcuz, A., Nef, P., & Andres-Barquin, P. J. (2002). Identification and characterization of human taste receptor genes belonging to the TAS2R family. *Cytogenet Genome Res*, 98(1), 45-53. doi:10.1159/000068546
54. Cvitkovitch, D. (2001). Genetic competence and transformation in oral streptococci. *Critical Reviews in Oral Biology & Medicine*, 12(3), 217-243.

55. David, D. K., & Katie, W. (2003). Bioactive Proteins and Peptides from Food Sources. Applications of Bioprocesses used in Isolation and Recovery. *Current Pharmaceutical Design*, 9(16), 1309-1323. doi:<http://dx.doi.org/10.2174/1381612033454883>
56. Delcayre, A., Estelles, A., Sperinde, J., Roulon, T., Paz, P., Aguilar, B., . . . Le Pecq, J. B. (2005). Exosome Display technology: applications to the development of new diagnostics and therapeutics. *Blood Cells, Molecules Diseases*, 35(2), 158-168. doi:10.1016/j.bcmed.2005.07.003
57. Deshpande, D. A., Wang, W. C., McIlmoyle, E. L., Robinett, K. S., Schillinger, R. M., An, S. S., . . . Liggett, S. B. (2010). Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nature medicine*, 16(11), 1299.
58. Deshpande, D. A., Wang, W. C., McIlmoyle, E. L., Robinett, K. S., Schillinger, R. M., An, S. S., . . . Liggett, S. B. (2010). Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction. *Nat Med*, 16(11), 1299-1304.
59. DeWire, S. M., Ahn, S., Lefkowitz, R. J., & Shenoy, S. K. (2007). Beta-arrestins and cell signaling. *Annu Rev Physiol*, 69, 483-510. doi:10.1146/annurev.physiol.69.022405.154749
60. Di Pizio, A., Behrens, M., & Krautwurst, D. (2019). Beyond the Flavour: The Potential Druggability of Chemosensory G Protein-Coupled Receptors. *International journal of molecular sciences*, 20(6), 1402. doi:10.3390/ijms20061402
61. Dickson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., . . . Rheinwald, J. G. (2000). Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol*, 20(4), 1436-1447. doi:10.1128/mcb.20.4.1436-1447.2000
62. Diggle, S. P., Cornelis, P., Williams, P., & Camara, M. (2006). 4-quinolone signalling in *Pseudomonas aeruginosa*: old molecules, new perspectives. *International Journal of Medical Microbiology*, 296(2-3), 83-91.

63. Dignat-George, F., & Boulanger, C. M. (2011). The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol*, 31(1), 27-33.
doi:10.1161/atvbaha.110.218123
64. DiPatrizio, N. V. (2014). Is fat taste ready for primetime? *Physiology & Behaviour*, 136, 145-154. doi:10.1016/j.physbeh.2014.03.002
65. Dong, S., Wei, B., Chen, B., McClements, D. J., & Decker, E. A. (2011). Chemical and antioxidant properties of casein peptide and its glucose Maillard reaction products in fish oil-in-water emulsions. *Journal of Agricultural and Food Chemistry*, 59(24), 13311-13317.
66. Dutta Banik, D., & Medler, K. F. (2021). Taste Receptor Signaling. *Handb Exp Pharmacol*. doi:10.1007/164_2021_442
67. Dwyer, N. D., Troemel, E. R., Sengupta, P., & Bargmann, C. I. (1998). Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. *Cell*, 93(3), 455-466. doi:10.1016/s0092-8674(00)81173-3
68. Ekoff, M., Choi, J.-H., James, A., Dahlén, B., Nilsson, G., & Dahlén, S.-E. (2014). Bitter taste receptor (TAS2R) agonists inhibit IgE-dependent mast cell activation. *Journal of allergy and clinical immunology*, 134(2), 475-478. doi:10.1016/j.jaci.2014.02.029
69. Estelles, A., Sperinde, J., Roulon, T., Aguilar, B., Bonner, C., LePecq, J. B., & Delcayre, A. (2007). Exosome nanovesicles displaying G protein-coupled receptors for drug discovery. *International Journal of Nanomedicine*, 2(4), 751-760.
70. Farquhar, D. R., Kovatch, K. J., Palmer, J. N., Shofer, F. S., Adappa, N. D., & Cohen, N. A. (2015). Phenylthiocarbamide taste sensitivity is associated with sinonasal symptoms in healthy adults. *Int Forum Allergy Rhinol*, 5(2), 111-118. doi:10.1002/alr.21437
71. Feng, P., Jyotaki, M., Kim, A., Chai, J., Simon, N., Zhou, M., . . . Wang, H. (2015). Regulation of bitter taste responses by tumor necrosis factor. *Brain, Behaviour, and Immunity*, 49, 32-42. doi:10.1016/j.bbi.2015.04.001
72. Finger, T. E., Böttger, B., Hansen, A., Anderson, K. T., Alimohammadi, H., & Silver, W. L. (2003). Solitary chemoreceptor cells in the nasal cavity serve as sentinels of respiration. *Proc Natl Acad Sci U S A*, 100(15), 8981-8986.

73. Flavier, A. B., Clough, S. J., Schell, M. A., & Denny, T. P. (1997). Identification of 3 - hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. *Mol Microbiol*, 26(2), 251-259.
74. Foster, S. R., Roura, E., & Thomas, W. G. (2014). Extrasensory perception: odorant and taste receptors beyond the nose and mouth. *Pharmacol Ther*, 142(1), 41-61. doi:10.1016/j.pharmthera.2013.11.004
75. Fowler, T., Johansson, S., Wary, K. K., & Höök, M. (2003). Src kinase has a central role in in vitro cellular internalization of *Staphylococcus aureus*. *Cellular microbiology*, 5(6), 417-426. doi:10.1046/j.1462-5822.2003.00290.x
76. Freund, J. R., Mansfield, C. J., Doghramji, L. J., Adappa, N. D., Palmer, J. N., Kennedy, D. W., . . . Lee, R. J. (2018). Activation of airway epithelial bitter taste receptors by *Pseudomonas aeruginosa* quinolones modulates calcium, cyclic-AMP, and nitric oxide signaling. *Journal of Biological Chemistry*, 293(25), 9824-9840. doi:10.1074/jbc.RA117.001005
77. Gaida, M. M., Dapunt, U., & Hänsch, G. M. (2016). Sensing developing biofilms: the bitter receptor T2R38 on myeloid cells. *Pathogens and Disease*, 74(3). doi:10.1093/femspd/ftw004
78. Gaida, M. M., Dapunt, U., & Hänsch, G. M. (2016). Sensing developing biofilms: the bitter receptor T2R38 on myeloid cells. *Pathog Dis*, 74(3). doi:10.1093/femspd/ftw004
79. Gaida, M. M., Mayer, C., Dapunt, U., Stegmaier, S., Schirmacher, P., Wabnitz, G. H., & Hansch, G. M. (2016). Expression of the bitter receptor T2R38 in pancreatic cancer: localization in lipid droplets and activation by a bacteria-derived quorum-sensing molecule. *Oncotarget*, 7(11), 12623-12632. doi:10.18632/oncotarget.7206
80. Gallo, S., Grossi, S., Montrasio, G., Binelli, G., Cinquetti, R., Simmen, D., . . . Campomenosi, P. (2016). TAS2R38 taste receptor gene and chronic rhinosinusitis: new data from an Italian population. *BMC medical genetics*, 17(1), 54.
81. Gil, S., Coldwell, S., Drury, J. L., Arroyo, F., Phi, T., Saadat, S., . . . Chung, W. O. (2015). Genotype-specific regulation of oral innate immunity by T2R38 taste receptor. *Mol Immunol*, 68(2 Pt C), 663-670. doi:10.1016/j.molimm.2015.10.012

82. Gilbertson, T. A., Avenet, P., Kinnamon, S. C., & Roper, S. D. (1992). Proton currents through amiloride-sensitive Na channels in hamster taste cells. Role in acid transduction. *J Gen Physiol*, 100(5), 803-824. doi:10.1085/jgp.100.5.803
83. González, J. F., & Venturi, V. (2013). A novel widespread interkingdom signaling circuit. *Trends in plant science*, 18(3), 167-174.
84. Gopallawa, I., Freund, J. R., & Lee, R. J. (2019). Bitter taste receptors stimulate phagocytosis in human macrophages through calcium, nitric oxide, and cyclic-GMP signaling. *bioRxiv*, 776344. doi:10.1101/776344
85. Gopallawa, I., Freund, J. R., & Lee, R. J. (2020). Bitter taste receptors stimulate phagocytosis in human macrophages through calcium, nitric oxide, and cyclic-GMP signaling. *Cellular and Molecular Life Sciences*. doi:10.1007/s00018-020-03494-y
86. Gopallawa, I., Freund, J. R., & Lee, R. J. (2021). Bitter taste receptors stimulate phagocytosis in human macrophages through calcium, nitric oxide, and cyclic-GMP signaling. *Cellular and Molecular Life Sciences*, 78(1), 271-286. doi:10.1007/s00018-020-03494-y
87. Grassart, A., Meas-Yedid, V., Dufour, A., Olivo-Marin, J. C., Dautry-Varsat, A., & Sauvonnnet, N. (2010). Pak1 phosphorylation enhances cortactin-N-WASP interaction in clathrin-caveolin-independent endocytosis. *Traffic*, 11(8), 1079-1091. doi:10.1111/j.1600-0854.2010.01075.x
88. Gross, J. C., Chaudhary, V., Bartscherer, K., & Boutros, M. (2012). Active Wnt proteins are secreted on exosomes. *Nat Cell Biol*, 14(10), 1036-1045. doi:10.1038/ncb2574
89. Grundmeier, M., Hussain, M., Becker, P., Heilmann, C., Peters, G., & Sinha, B. (2004). Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. *Infect Immun*, 72(12), 7155-7163. doi:10.1128/iai.72.12.7155-7163.2004
90. Gu, Q. D., Joe, D. S., & Gilbert, C. A. (2017). Activation of bitter taste receptors in pulmonary nociceptors sensitizes TRPV1 channels through the PLC and PKC signaling pathway. *American Journal of Physiology-Lung Cellular Molecular Physiology*, 312(3), L326-L333. doi:10.1152/ajplung.00468.2016
91. Gukovsky, I., Cheng, J. H., Nam, K. J., Lee, O. T., Lugea, A., Fischer, L., . . . Gukovskaya, A. S. (2004). Phosphatidylinositol 3-kinase gamma regulates key

- pathologic responses to cholecystokinin in pancreatic acinar cells. *Gastroenterology*, 126(2), 554-566. doi:10.1053/j.gastro.2003.11.017
92. György, B., Szabó, T. G., Pásztói, M., Pál, Z., Misják, P., Aradi, B., . . . Buzás, E. I. (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cellular and Molecular Life Sciences*, 68(16), 2667-2688. doi:10.1007/s00018-011-0689-3
 93. H, W. (2016). *Ggplot2 elegant graphics for data analysis* (1 ed.): Springer-Verlag New York.
 94. Hansch, G. M., Prior, B., Brenner-Weiss, G., Obst, U., & Overhage, J. (2014). The Pseudomonas quinolone signal (PQS) stimulates chemotaxis of polymorphonuclear neutrophils. *J Appl Biomater Funct Mater*, 12(1), 21-26. doi:10.5301/jabfm.5000204
 95. Hariri, B. M., McMahon, D. B., Chen, B., Freund, J. R., Mansfield, C. J., Doghramji, L. J., . . . Lee, R. J. (2017). Flavones modulate respiratory epithelial innate immunity: Anti-inflammatory effects and activation of the T2R14 receptor. *Journal of Biological Chemistry*, 292(20), 8484-8497. doi:10.1074/jbc.M116.771949
 96. Hartwig, J. H., Chambers, K. A., & Stossel, T. P. (1989). Association of gelsolin with actin filaments and cell membranes of macrophages and platelets. *Journal of Cell Biology*, 108(2), 467-479. doi:10.1083/jcb.108.2.467
 97. Håvarstein, L. S., Coomaraswamy, G., & Morrison, D. A. (1995). An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proceedings of the National Academy of Science of the United States of America*, 92(24), 11140-11144. doi:10.1073/pnas.92.24.11140
 98. He, W., Danilova, V., Zou, S., Hellekant, G., Max, M., Margolskee, R. F., & Damak, S. (2002). Partial rescue of taste responses of alpha-gustducin null mice by transgenic expression of alpha-transducin. *Chem Senses*, 27(8), 719-727. doi:10.1093/chemse/27.8.719
 99. He, W., Yasumatsu, K., Varadarajan, V., Yamada, A., Lem, J., Ninomiya, Y., . . . Damak, S. (2004). Umami taste responses are mediated by alpha-transducin and alpha-gustducin. *The Journal of Neuroscience*, 24(35), 7674-7680. doi:10.1523/JNEUROSCI.2441-04.2004

100. Heck, G. L., Mierson, S., & DeSimone, J. A. (1984). Salt taste transduction occurs through an amiloride-sensitive sodium transport pathway. *Science*, 223(4634), 403-405.
101. Heeb, S., Fletcher, M. P., Chhabra, S. R., Diggle, S. P., Williams, P., & Camara, M. (2011). Quinolones: from antibiotics to autoinducers. *FEMS microbiology reviews*, 35(2), 247-274. doi:10.1111/j.1574-6976.2010.00247.x
102. Hichami, A., Khan, A. S., & Khan, N. A. (2021). Cellular and Molecular Mechanisms of Fat Taste Perception. *Handb Exp Pharmacol*. doi:10.1007/164_2021_437
103. Hillman, J. D., Dzuback, A. L., & Andrews, S. W. (1987). Colonization of the human oral cavity by a *Streptococcus mutans* mutant producing increased bacteriocin. *J Dent Res*, 66(6), 1092-1094. doi:10.1177/00220345870660060101
104. Holden, M. T., Ram Chhabra, S., De Nys, R., Stead, P., Bainton, N. J., Hill, P. J., . . . England, D. (1999). Quorum - sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram - negative bacteria. *Mol Microbiol*, 33(6), 1254-1266.
105. Horst, O. V., Horst, J. A., Samudrala, R., & Dale, B. A. (2011). Caries induced cytokine network in the odontoblast layer of human teeth. *BMC Immunol*, 12, 9. doi:10.1186/1471-2172-12-9
106. Hossain, M. S., & Biswas, I. (2012a). An extracellular protease, SepM, generates functional competence-stimulating peptide in *Streptococcus mutans* UA159. *Journal of Bacteriology*, 194(21), 5886-5896. doi:10.1128/jb.01381-12
107. Hossain, M. S., & Biswas, I. (2012b). An extracellular protease, SepM, generates functional competence-stimulating peptide in *Streptococcus mutans* UA159. *J Bacteriol*, 194(21), 5886-5896. doi:10.1128/jb.01381-12
108. Houpt, T. A., Frankmann, S. P., & Berlin, R. (1996). Cyclic AMP tastes aversive, not sweet, to rats. *Physiology & Behaviour*, 59(3), 495-498. doi:[https://doi.org/10.1016/0031-9384\(95\)02090-X](https://doi.org/10.1016/0031-9384(95)02090-X)
109. Howitt, M. R., Lavoie, S., Michaud, M., Blum, A. M., Tran, S. V., Weinstock, J. V., . . . Osborne, L. C. (2016). Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. *Science*, 351(6279), 1329-1333.
110. Huang, L., Shanker, Y. G., Dubauskaite, J., Zheng, J. Z., Yan, W., Rosenzweig, S., . . . Margolskee, R. F. (1999). Ggamma13 colocalizes with gustducin in taste receptor

- cells and mediates IP3 responses to bitter denatonium. *Nat Neurosci*, 2(12), 1055-1062. doi:10.1038/15981
111. Hurley, J. H., Boura, E., Carlson, L. A., & Różycki, B. (2010). Membrane budding. *Cell*, 143(6), 875-887. doi:10.1016/j.cell.2010.11.030
 112. Isberg, V., Mordalski, S., Munk, C., Rataj, K., Harpsoe, K., Hauser, A. S., . . . Gloriam, D. E. (2017). GPCRdb: an information system for G protein-coupled receptors. *Nucleic Acids Research*, 45(5), 2936. doi:10.1093/nar/gkw1218
 113. Ishibashi, N., Kubo, T., Chino, M., Fukui, H., Shinoda, I., Kikuchi, E., . . . Fukui, S. (1988). Taste of Proline-containing Peptides. *Agricultural and Biological Chemistry*, 52(1), 95-98. doi:10.1080/00021369.1988.10868632
 114. Itoigawa, A., Hayakawa, T., Suzuki-Hashido, N., & Imai, H. (2019). A natural point mutation in the bitter taste receptor TAS2R16 causes inverse agonism of arbutin in lemur gustation. *Proceedings of the Royal Society of Biological Sciences*, 286(1904), 20190884. doi:10.1098/rspb.2019.0884
 115. Iven, J., Biesiekierski, J. R., Zhao, D., Deloof, E., O'Daly, O. G., Depoortere, I., . . . Van Oudenhove, L. (2019). Intragastric quinine administration decreases hedonic eating in healthy women through peptide-mediated gut-brain signaling mechanisms. *Nutrition Neuroscience*, 22(12), 850-862. doi:10.1080/1028415x.2018.1457841
 116. Jaggupilli, A., Howard, R., Aluko, R. E., & Chelikani, P. (2019). Advanced glycation end-products can activate or block bitter taste receptors. *Nutrients*, 11(6), 1317.
 117. Jaggupilli, A., Howard, R., Upadhyaya, J. D., Bhullar, R. P., & Chelikani, P. (2016). Bitter taste receptors: Novel insights into the biochemistry and pharmacology. *International Journal of Biochemistry & Cell Biology*, 77(Pt B), 184-196. doi:10.1016/j.biocel.2016.03.005
 118. Jaggupilli, A., Singh, N., De Jesus, V. C., Gounni, M. S., Dhanaraj, P., & Chelikani, P. (2019). Chemosensory bitter taste receptors (T2Rs) are activated by multiple antibiotics. *The FASEB Journal*, 33(1), 501-517. doi:10.1096/fj.201800521RR
 119. Jaggupilli, A., Singh, N., Jesus, V. C., Duan, K., & Chelikani, P. (2018). Characterization of the Binding Sites for Bacterial Acyl Homoserine Lactones (AHLs) on Human Bitter Taste Receptors (T2Rs). *ACS Infectious Diseases*, 4(7), 1146-1156. doi:10.1021/acsinfecdis.8b00094

120. Jaggupilli, A., Singh, N., Upadhyaya, J., Sikarwar, A. S., Arakawa, M., Dakshinamurti, S., . . . Chelikani, P. (2017). Analysis of the expression of human bitter taste receptors in extraoral tissues. *Molecular Cellular Biochemistry*, 426(1-2), 137-147. doi:10.1007/s11010-016-2902-z
121. Jean-Charles, P. Y., Kaur, S., & Shenoy, S. K. (2017). G Protein-Coupled Receptor Signaling Through β -Arrestin-Dependent Mechanisms. *J Cardiovasc Pharmacol*, 70(3), 142-158. doi:10.1097/fjc.0000000000000482
122. Kahn, R., Mossberg, M., Ståhl, A. L., Johansson, K., Lopatko Lindman, I., Heijl, C., . . . Karpman, D. (2017). Microvesicle transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis. *Kidney Int*, 91(1), 96-105. doi:10.1016/j.kint.2016.09.023
123. Kahner, B. N., Dorsam, R. T., & Kunapuli, S. P. (2008). Role of P2Y receptor subtypes in platelet-derived microparticle generation. *Frontiers in Bioscience*, 13, 433-439. doi:10.2741/2690
124. Karametsi, K., Kokkinidou, S., Ronningen, I., & Peterson, D. G. (2014). Identification of bitter peptides in aged cheddar cheese. *Journal of Agriculture and Food Chemistry*, 62(32), 8034-8041. doi:10.1021/jf5020654
125. Karlsson, T., Turkina, M. V., Yakymenko, O., Magnusson, K.-E., & Vikström, E. (2012). The *Pseudomonas aeruginosa* N-acylhomoserine lactone quorum sensing molecules target IQGAP1 and modulate epithelial cell migration. *PLOS Pathogens*, 8(10), e1002953. doi:10.1371/journal.ppat.1002953
126. Kaur, H., Carvalho, J., Looso, M., Singh, P., Chennupati, R., Preussner, J., . . . Wettschureck, N. (2019). Author Correction: Single-cell profiling reveals heterogeneity and functional patterning of GPCR expression in the vascular system. *Nat Commun*, 10(1), 1448. doi:10.1038/s41467-019-09334-3
127. Kim, D., Pauer, S. H., Yong, H. M., An, S. S., & Liggett, S. B. (2016). beta2-Adrenergic Receptors Chaperone Trapped Bitter Taste Receptor 14 to the Cell Surface as a Heterodimer and Exert Unidirectional Desensitization of Taste Receptor Function. *J Biol Chem*, 291(34), 17616-17628. doi:10.1074/jbc.M116.722736

128. Kim, D., Woo, J. A., Geffken, E., An, S. S., & Liggett, S. B. (2017). Coupling of airway smooth muscle bitter taste receptors to intracellular signaling and relaxation is via Gai1, 2, 3. *American journal of respiratory cell and molecular biology*, 56(6), 762-771.
129. Kim, H. O., & Li-Chan, E. C. (2006). Quantitative structure-activity relationship study of bitter peptides. *J Agric Food Chem*, 54(26), 10102-10111. doi:10.1021/jf062422j
130. Kim, K., Kim, Y. U., Koh, B. H., Hwang, S. S., Kim, S.-H., Lépine, F., . . . Lee, G. R. (2010). HHQ and PQS, two *Pseudomonas aeruginosa* quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway. *Immunology*, 129(4), 578-588. doi:10.1111/j.1365-2567.2009.03160.x
131. Kim, K. S., Egan, J. M., & Jang, H. J. (2014). Denatonium induces secretion of glucagon-like peptide-1 through activation of bitter taste receptor pathways. *Diabetologia*, 57(10), 2117-2125. doi:10.1007/s00125-014-3326-5
132. Kim, M. J., Son, H., Kim, Y., Misaka, T., & Rhyu, M. (2014). Umami–bitter interactions: The suppression of bitterness by umami peptides via human bitter taste receptor. *Biochemical and biophysical research communications*, 456. doi:10.1016/j.bbrc.2014.11.114
133. Kim, M. J., Son, H. J., Kim, Y., Misaka, T., & Rhyu, M. R. (2015). Umami-bitter interactions: the suppression of bitterness by umami peptides via human bitter taste receptor. *Biochem Biophys Res Commun*, 456(2), 586-590. doi:10.1016/j.bbrc.2014.11.114
134. Kinnamon, S. C. (2009). Umami taste transduction mechanisms. *The American Journal of Clinical Nutrition*, 90(3), 753S-755S. doi:10.3945/ajcn.2009.27462K
135. Kinnamon, S. C., & Cummings, T. A. (1992). Chemosensory transduction mechanisms in taste. *Annu Rev Physiol*, 54, 715-731. doi:10.1146/annurev.ph.54.030192.003435
136. Kirimura, J., Shimizu, A., Kimizuka, A., Ninomiya, T., & Katsuya, N. (1969). Contribution of peptides and amino acids to the taste of foods. *Journal of Agricultural and Food Chemistry*, 17(4), 689-695. doi:10.1021/jf60164a031
137. Kirschning, C. J., & Schumann, R. R. (2002). TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr Top Microbiol Immunol*, 270, 121-144. doi:10.1007/978-3-642-59430-4_8

138. Kleerebezem, M., Quadri, L. E., Kuipers, O. P., & de Vos, W. M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in gram-positive bacteria. *Mol Microbiol*, 24(5), 895-904. doi:10.1046/j.1365-2958.1997.4251782.x
139. Kohl, S., Behrens, M., Dunkel, A., Hofmann, T., & Meyerhof, W. (2013). Amino acids and peptides activate at least five members of the human bitter taste receptor family. *Journal of Agricultural and Food Chemistry*, 61(1), 53-60.
140. Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides--opportunities for designing future foods. *Curr Pharm Des*, 9(16), 1297-1308. doi:10.2174/1381612033454892
141. Kourembanas, S. (2015). Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy. *Annu Rev Physiol*, 77, 13-27. doi:10.1146/annurev-physiol-021014-071641
142. Kravchenko, V. V., Kaufmann, G. F., Mathison, J. C., Scott, D. A., Katz, A. Z., Grauer, D. C., . . . Ulevitch, R. J. (2008). Modulation of gene expression via disruption of NF- κ b signaling by a bacterial small molecule. *Science*, 321(5886), 259-263. doi:10.1126/science.1156499
143. Kravchenko, V. V., Kaufmann, G. F., Mathison, J. C., Scott, D. A., Katz, A. Z., Wood, M. R., . . . Ulevitch, R. J. (2006). N-(3-Oxo-acyl)homoserine Lactones Signal Cell Activation through a Mechanism distinct from the Canonical Pathogen-associated Molecular Pattern Recognition Receptor Pathways. *Journal of Biological Chemistry*, 281(39), 28822-28830. doi:10.1074/jbc.M606613200
144. Kuhn, C., Bufo, B., Winnig, M., Hofmann, T., Frank, O., Behrens, M., . . . Meyerhof, W. (2004). Bitter Taste Receptors for Saccharin and Acesulfame K. *The Journal of Neuroscience*, 24(45), 10260-10265. doi:10.1523/jneurosci.1225-04.2004
145. Kusakabe, Y., Yamaguchi, E., Tanemura, K., Kameyama, K., Chiba, N., Arai, S., . . . Abe, K. (1998). Identification of two α -subunit species of GTP-binding proteins, G α 15 and G α q, expressed in rat taste buds. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1403(3), 265-272. doi:[https://doi.org/10.1016/S0167-4889\(98\)00062-7](https://doi.org/10.1016/S0167-4889(98)00062-7)

146. Kwon, S. H., Liu, K. D., & Mostov, K. E. (2014). Intercellular transfer of GPRC5B via exosomes drives HGF-mediated outward growth. *Curr Biol*, 24(2), 199-204. doi:10.1016/j.cub.2013.12.010
147. Lam, H., Oh, D.-C., Cava, F., Takacs, C. N., Clardy, J., de Pedro, M. A., & Waldor, M. K. (2009). D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science*, 325(5947), 1552-1555.
148. Latorre, R., Huynh, J., Mazzoni, M., Gupta, A., Bonora, E., Clavenzani, P., . . . Sternini, C. (2016). Expression of the bitter taste receptor, T2R38, in enteroendocrine cells of the colonic mucosa of overweight/obese vs. lean subjects. *PLoS One*, 11(2), e0147468. doi:10.1371/journal.pone.0147468
149. Laugerette, F., Passilly-Degrace, P., Patris, B., Niot, I., Febbraio, M., Montmayeur, J. P., & Besnard, P. (2005). CD36 involvement in orosensory detection of dietary lipids, spontaneous fat preference, and digestive secretions. *J Clin Invest*, 115(11), 3177-3184. doi:10.1172/jci25299
150. Lazazzera, B. A., & Grossman, A. D. (1998). The ins and outs of peptide signaling. *Trends in microbiology*, 6(7), 288-294.
151. Lee, J., & Zhang, L. (2015). The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein & cell*, 6(1), 26-41. doi:10.1007/s13238-014-0100-x
152. Lee, R. J., Hariri, B. M., McMahon, D. B., Chen, B., Doghramji, L., Adappa, N. D., . . . Cohen, N. A. (2017). Bacterial class d-amino acids suppress sinonasal innate immunity through sweet taste receptors in solitary chemosensory cells. *Science Signaling*, 10(495), eaam7703. doi:10.1126/scisignal.aam7703
153. Lee, R. J., Kofonow, J. M., Rosen, P. L., Siebert, A. P., Chen, B., Doghramji, L., . . . Cohen, N. A. (2014). Bitter and sweet taste receptors regulate human upper respiratory innate immunity. *J Clin Invest*, 124(3), 1393-1405. doi:10.1172/jci72094
154. Lee, R. J., Xiong, G., Kofonow, J. M., Chen, B., Lysenko, A., Jiang, P., . . . Cohen, N. A. (2012a). T2R38 taste receptor polymorphisms underlie susceptibility to upper respiratory infection. *J Clin Invest*, 122(11), 4145-4159. doi:10.1172/jci64240
155. Lee, R. J., Xiong, G., Kofonow, J. M., Chen, B., Lysenko, A., Jiang, P., . . . Cohen, N. A. (2012b). T2R38 taste receptor polymorphisms underlie susceptibility to

- upper respiratory infection. *Journal of Clinical Investigation*, 122(11), 4145-4159.
doi:10.1172/jci64240
156. Lee, S. J., Depoortere, I., & Hatt, H. (2019). Therapeutic potential of ectopic olfactory and taste receptors. *Nat Rev Drug Discov*, 18(2), 116-138. doi:10.1038/s41573-018-0002-3
 157. Lehrer, R. I. (2004). Primate defensins. *Nat Rev Microbiol*, 2(9), 727-738.
doi:10.1038/nrmicro976
 158. Lepine, F., Milot, S., Deziel, E., He, J., & Rahme, L. G. (2004). Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by *Pseudomonas aeruginosa*. *J Am Soc Mass Spectrom*, 15(6), 862-869.
doi:10.1016/j.jasms.2004.02.012
 159. Lewandowski, B. C., Sukumaran, S. K., Margolskee, R. F., & Bachmanov, A. A. (2016). Amiloride-Insensitive Salt Taste Is Mediated by Two Populations of Type III Taste Cells with Distinct Transduction Mechanisms. *J Neurosci*, 36(6), 1942-1953.
doi:10.1523/jneurosci.2947-15.2016
 160. Ley, J. P. (2008). Masking bitter taste by molecules. *Chemosensory Perception*, 1(1), 58-77.
 161. Li, H., Li, X., Wang, Z., Fu, Y., Ai, Q., Dong, Y., & Yu, J. (2015). Autoinducer-2 regulates *Pseudomonas aeruginosa* PAO1 biofilm formation and virulence production in a dose-dependent manner. *BMC microbiology*, 15, 192-192. doi:10.1186/s12866-015-0529-y
 162. Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M., & Adler, E. (2002). Human receptors for sweet and umami taste. *Proc Natl Acad Sci U S A*, 99(7), 4692-4696.
doi:10.1073/pnas.072090199
 163. Li, Y. H., Lau, P. C., Lee, J. H., Ellen, R. P., & Cvitkovitch, D. G. (2001). Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J Bacteriol*, 183(3), 897-908. doi:10.1128/jb.183.3.897-908.2001
 164. Li, Z., & Nair, S. K. (2012). Quorum sensing: how bacteria can coordinate activity and synchronize their response to external signals? *Protein Science*, 21(10), 1403-1417.
 165. Lindemann, B. (1996). Taste reception. *Physiol Rev*, 76(3), 719-766.
doi:10.1152/physrev.1996.76.3.719

166. Liu, B. Y., Zhu, K. X., Guo, X. N., Peng, W., & Zhou, H. M. (2017). Effect of deamidation - induced modification on umami and bitter taste of wheat gluten hydrolysates. *Journal of the Science of Food and Agriculture*, 97(10), 3181-3188.
167. Liu, P., Huang, M., Song, S., Hayat, K., Zhang, X., Xia, S., & Jia, C. (2012). Sensory characteristics and antioxidant activities of Maillard reaction products from soy protein hydrolysates with different molecular weight distribution. *Food and Bioprocess Technology*, 5(5), 1775-1789.
168. Liu, X., Yu, Y., Qin, D., Song, Z., Huang, Z., Meng, K., . . . Xu, Z. (2020). Expression analysis of taste receptor genes (T1R1, T1R3, and T2R4) in response to bacterial, viral and parasitic infection in rainbow trout, *Oncorhynchus mykiss*. *Fish Shellfish Immunol*, 101, 176-185. doi:10.1016/j.fsi.2020.03.055
169. Lossow, K., Hubner, S., Roudnitzky, N., Slack, J. P., Pollastro, F., Behrens, M., & Meyerhof, W. (2016). Comprehensive Analysis of Mouse Bitter Taste Receptors Reveals Different Molecular Receptive Ranges for Orthologous Receptors in Mice and Humans. *J Biol Chem*, 291(29), 15358-15377. doi:10.1074/jbc.M116.718544
170. Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, 42(2), 145-151. doi:10.1016/j.cyto.2008.01.006
171. Lyte, M., & Ernst, S. (1993). Alpha and beta adrenergic receptor involvement in catecholamine-induced growth of gram-negative bacteria. *Biochemical and biophysical research communications*, 190(2), 447-452.
172. Maehashi, K., & Huang, L. (2009). Bitter peptides and bitter taste receptors. *Cellular and Molecular Life Sciences*, 66(10), 1661-1671. doi:10.1007/s00018-009-8755-9
173. Maehashi, K., Matano, M., Wang, H., Vo, L. A., Yamamoto, Y., & Huang, L. (2008). Bitter peptides activate hTAS2Rs, the human bitter receptors. *Biochemical and biophysical research communications*, 365(4), 851-855.
174. Maisonneuve, E., Chevrier, J., Dubus, M., Varin, J., Sergheraert, J., Gangloff, S. C., . . . Kerdjoudj, H. (2020). Infection of Human Dental Pulp Stromal Cells by *Streptococcus mutans*: Shedding Light on Bacteria Pathogenicity and Pulp Inflammation. *Front Cell Dev Biol*, 8, 785. doi:10.3389/fcell.2020.00785

175. Malki, A., Fiedler, J., Fricke, K., Ballweg, I., Pfaffl, M. W., & Krautwurst, D. (2015). Class I odorant receptors, TAS1R and TAS2R taste receptors, are markers for subpopulations of circulating leukocytes. *Journal of leukocyte biology*, 97(3), 533-545.
176. Martin, L. T. P., Nachtigal, M. W., Selman, T., Nguyen, E., Salsman, J., Dellaire, G., & Dupre, D. J. (2019). Bitter taste receptors are expressed in human epithelial ovarian and prostate cancers cells and nospapine stimulation impacts cell survival. *Mol Cell Biochem*, 454(1-2), 203-214. doi:10.1007/s11010-018-3464-z
177. Massey, R. C., Kantzanou, M. N., Fowler, T., Day, N. P., Schofield, K., Wann, E. R., . . . Peacock, S. J. (2001). Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells. *Cell Microbiol*, 3(12), 839-851. doi:10.1046/j.1462-5822.2001.00157.x
178. Masyuk, A. I., Masyuk, T. V., & Larusso, N. F. (2013). Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases. *J Hepatol*, 59(3), 621-625. doi:10.1016/j.jhep.2013.03.028
179. Mathivanan, S., Ji, H., & Simpson, R. J. (2010). Exosomes: extracellular organelles important in intercellular communication. *J Proteomics*, 73(10), 1907-1920. doi:10.1016/j.jprot.2010.06.006
180. Matoba, T., & Hata, T. (1972). Relationship between bitterness of peptides and their chemical structures. *Agricultural and Biological Chemistry*, 36(8), 1423-1431. doi:10.1080/00021369.1972.10860410
181. Matoba, T., Hayashi, R., & Hata, T. (1970). Isolation of Bitter Peptides from Tryptic Hydrolysate of Casein and their Chemical Structure. *Agricultural and Biological Chemistry*, 34(8), 1235-1243. doi:10.1080/00021369.1970.10859753
182. Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T., & Akira, S. (1993). Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci U S A*, 90(21), 10193-10197. doi:10.1073/pnas.90.21.10193
183. Maurer, S., Wabnitz, G. H., Kahle, N. A., Stegmaier, S., Prior, B., Giese, T., . . . Hänsch, G. M. (2015). Tasting *Pseudomonas aeruginosa* biofilms: human neutrophils

- express the bitter receptor T2R38 as sensor for the quorum sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Frontiers in immunology*, 6, 369.
184. Mazur, R. H., Schlatter, J. M., & Goldkamp, A. H. (1969). Structure-taste relationships of some dipeptides. *Journal of the American Chemical Society*, 91(10), 2684-2691. doi:10.1021/ja01038a046
 185. McMahon, L., Schwartz, K., Yilmaz, O., Brown, E., Ryan, L. K., & Diamond, G. (2011). Vitamin D-mediated induction of innate immunity in gingival epithelial cells. *Infect Immun*, 79(6), 2250-2256. doi:10.1128/iai.00099-11
 186. Medapati, M. R., Bhagirath, A. Y., Singh, N., & Chelikani, P. (2021). Pharmacology of T2R Mediated Host-Microbe Interactions. *Handb Exp Pharmacol*. doi:10.1007/164_2021_435
 187. Medapati, M. R., Singh, A., Korupally, R. R., Henderson, D., Klonisch, T., Manda, S. V., & Chelikani, P. (2017). Characterization of GPCRs in extracellular vesicle (EV). *Methods Cell Biol*, 142, 119-132. doi:10.1016/bs.mcb.2017.07.004
 188. Medapati, M. R., Singh, N., Bhagirath, A. Y., Duan, K., Triggs-Raine, B., Batista, E. L., Jr., & Chelikani, P. (2021). Bitter taste receptor T2R14 detects quorum sensing molecules from cariogenic *Streptococcus mutans* and mediates innate immune responses in gingival epithelial cells. *FASEB J*, 35(3), e21375. doi:10.1096/fj.202000208R
 189. Mempel, M., Schnopp, C., Hojka, M., Fesq, H., Weidinger, S., Schaller, M., . . . Abeck, D. (2002). Invasion of human keratinocytes by *Staphylococcus aureus* and intracellular bacterial persistence represent haemolysin-independent virulence mechanisms that are followed by features of necrotic and apoptotic keratinocyte cell death. *Br J Dermatol*, 146(6), 943-951. doi:10.1046/j.1365-2133.2002.04752.x
 190. Mengaud, J., Ohayon, H., Gounon, P., Mege, R. M., & Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell*, 84(6), 923-932. doi:10.1016/s0092-8674(00)81070-3
 191. Meyerhof, W., Batram, C., Kuhn, C., Brockhoff, A., Chudoba, E., Buße, B., . . . Behrens, M. (2009). The Molecular Receptive Ranges of Human TAS2R Bitter Taste Receptors. *Chem Senses*, 35(2), 157-170. doi:10.1093/chemse/bjp092

192. Meyerhof, W., Batram, C., Kuhn, C., Brockhoff, A., Chudoba, E., Bufe, B., . . . Behrens, M. (2010). The molecular receptive ranges of human TAS2R bitter taste receptors. *Chem Senses*, 35(2), 157-170.
193. Meyle, J., Dommisch, H., Groeger, S., Giacaman, R., Costalonga, M., & Herzberg, M. (2017). The innate host response in caries and periodontitis. *Journal of Clinical Periodontology*, 44. doi:10.1111/jcpe.12781
194. Milioni, C., Martínez, B., Degl'Innocenti, S., Turchi, B., Fratini, F., Cerri, D., & Fischetti, R. (2015). A novel bacteriocin produced by *Lactobacillus plantarum* LpU4 as a valuable candidate for biopreservation in artisanal raw milk cheese. *Dairy Science & Technology*, 95(4), 479-494. doi:10.1007/s13594-015-0230-9
195. Miller, E. L., Kjos, M., Abrudan, M. I., Roberts, I. S., Veening, J.-W., & Rozen, D. E. (2018). Eavesdropping and crosstalk between secreted quorum sensing peptide signals that regulate bacteriocin production in *Streptococcus pneumoniae*. *The ISME Journal*, 12(10), 2363-2375. doi:10.1038/s41396-018-0178-x
196. Millius, A., & Weiner, O. D. (2010). Manipulation of neutrophil-like HL-60 cells for the study of directed cell migration. *Methods in molecular biology (Clifton, N.J.)*, 591, 147-158. doi:10.1007/978-1-60761-404-3_9
197. Mittelbrunn, M., & Sánchez-Madrid, F. (2012). Intercellular communication: diverse structures for exchange of genetic information. *Nat Rev Mol Cell Biol*, 13(5), 328-335. doi:10.1038/nrm3335
198. Montgomery, S. T., Mall, M. A., Kicic, A., & Stick, S. M. (2017). Hypoxia and sterile inflammation in cystic fibrosis airways: mechanisms and potential therapies. *European Respiratory Journal*, 49(1), 1600903. doi:10.1183/13993003.00903-2016
199. Munk, C., Isberg, V., Mordalski, S., Harpsoe, K., Rataj, K., Hauser, A. S., . . . Gloriam, D. E. (2016). GPCRdb: the G protein-coupled receptor database - an introduction. *Br J Pharmacol*, 173(14), 2195-2207. doi:10.1111/bph.13509
200. Nabhan, J. F., Hu, R., Oh, R. S., Cohen, S. N., & Lu, Q. (2012). Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proc Natl Acad Sci U S A*, 109(11), 4146-4151. doi:10.1073/pnas.1200448109

201. Nager, A. R., Goldstein, J. S., Herranz-Pérez, V., Portran, D., Ye, F., Garcia-Verdugo, J. M., & Nachury, M. V. (2017). An Actin Network Dispatches Ciliary GPCRs into Extracellular Vesicles to Modulate Signaling. *Cell*, 168(1-2), 252-263.e214. doi:10.1016/j.cell.2016.11.036
202. Nelson, G., Chandrashekar, J., Hoon, M. A., Feng, L., Zhao, G., Ryba, N. J., & Zuker, C. S. (2002). An amino-acid taste receptor. *Nature*, 416(6877), 199-202. doi:10.1038/nature726
203. Nitsche-Schmitz, D. P., Rohde, M., & Chhatwal, G. S. (2007). Invasion mechanisms of Gram-positive pathogenic cocci. *Thromb Haemost*, 98(3), 488-496.
204. Nobbs, A. H., Shearer, B. H., Drobni, M., Jepson, M. A., & Jenkinson, H. F. (2007). Adherence and internalization of *Streptococcus gordonii* by epithelial cells involves beta1 integrin recognition by SspA and SspB (antigen I/II family) polypeptides. *Cell Microbiol*, 9(1), 65-83. doi:10.1111/j.1462-5822.2006.00768.x
205. Noguchi, M., Arai, S., Yamashita, M., Kato, H., & Fujimaki, M. (1975). Isolation and identification of acidic oligopeptides occurring in a flavor potentiating fraction from a fish protein hydrolysate. *Journal of Agricultural and Food Chemistry*, 23(1), 49-53. doi:10.1021/jf60197a003
206. Nomura, K., Nakanishi, M., Ishidate, F., Iwata, K., & Taruno, A. (2020). All-Electrical Ca(2+)-Independent Signal Transduction Mediates Attractive Sodium Taste in Taste Buds. *Neuron*. doi:10.1016/j.neuron.2020.03.006
207. Okumura, T., Yamada, R., & Nishimura, T. (2004). Sourness-suppressing peptides in cooked pork loins. *Biosci Biotechnol Biochem*, 68(8), 1657-1662. doi:10.1271/bbb.68.1657
208. Ooi, E. H., Wormald, P.-J., & Tan, L. W. (2008). Innate immunity in the paranasal sinuses: a review of nasal host defenses. *American journal of rhinology*, 22(1), 13-19.
209. Ozeck, M., Brust, P., Xu, H., & Servant, G. (2004). Receptors for bitter, sweet and umami taste couple to inhibitory G protein signaling pathways. *Eur J Pharmacol*, 489(3), 139-149. doi:10.1016/j.ejphar.2004.03.004
210. Ozeri, V., Rosenshine, I., Ben-Ze'Ev, A., Bokoch, G. M., Jou, T. S., & Hanski, E. (2001). De novo formation of focal complex-like structures in host cells by invading *Streptococci*. *Mol Microbiol*, 41(3), 561-573. doi:10.1046/j.1365-2958.2001.02535.x

211. Pacheco, A. R., & Sperandio, V. (2009). Inter-kingdom signaling: chemical language between bacteria and host. *Current opinion in microbiology*, 12(2), 192-198.
212. Pándy-Szekeres, G., Munk, C., Tsonkov, T. M., Mordalski, S., Harpsøe, K., Hauser, A. S., . . . Gloriam, D. E. (2018). GPCRdb in 2018: adding GPCR structure models and ligands. *Nucleic Acids Res*, 46(D1), D440-d446. doi:10.1093/nar/gkx1109
213. Park, J., Kim, K. S., Kim, K. H., Lee, I. S., Jeong, H. S., Kim, Y., & Jang, H. J. (2015). GLP-1 secretion is stimulated by 1,10-phenanthroline via colocalized T2R5 signal transduction in human enteroendocrine L cell. *Biochemical and biophysical research communications*, 468(1-2), 306-311. doi:10.1016/j.bbrc.2015.10.107
214. Pasquet, J. M., Dachary-Prigent, J., & Nurden, A. T. (1996). Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. *Eur J Biochem*, 239(3), 647-654. doi:10.1111/j.1432-1033.1996.0647u.x
215. Patti, J. M., Allen, B. L., McGavin, M. J., & Höök, M. (1994). MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu Rev Microbiol*, 48, 585-617. doi:10.1146/annurev.mi.48.100194.003101
216. Petersen, F. C., Fimland, G., & Scheie, A. A. (2006). Purification and functional studies of a potent modified quorum-sensing peptide and a two-peptide bacteriocin in *Streptococcus mutans*. *Mol Microbiol*, 61(5), 1322-1334. doi:10.1111/j.1365-2958.2006.05312.x
217. Pironti, G., Strachan, R. T., Abraham, D., Mon-Wei Yu, S., Chen, M., Chen, W., . . . Rockman, H. A. (2015). Circulating Exosomes Induced by Cardiac Pressure Overload Contain Functional Angiotensin II Type 1 Receptors. *Circulation*, 131(24), 2120-2130. doi:10.1161/CIRCULATIONAHA.115.015687
218. Pronin, A. N., Tang, H., Connor, J., & Keung, W. (2004). Identification of ligands for two human bitter T2R receptors. *Chem Senses*, 29(7), 583-593. doi:10.1093/chemse/bjh064
219. Pundir, P., Liu, R., Vasavda, C., Serhan, N., Limjunyawong, N., Yee, R., . . . Dong, X. (2019). A Connective Tissue Mast-Cell-Specific Receptor Detects Bacterial Quorum-Sensing Molecules and Mediates Antibacterial Immunity. *Cell Host Microbe*, 26(1), 114-122.e118. doi:10.1016/j.chom.2019.06.003

220. Pydi, S. P., Bhullar, R. P., & Chelikani, P. (2012). Constitutively active mutant gives novel insights into the mechanism of bitter taste receptor activation. *Journal of Neurochemistry*, 122(3), 537-544. doi:10.1111/j.1471-4159.2012.07808.x
221. Pydi, S. P., Bhullar, R. P., & Chelikani, P. (2014). Constitutive activity of bitter taste receptors (T2Rs). *Adv Pharmacol*, 70, 303-326. doi:10.1016/b978-0-12-417197-8.00010-9
222. Pydi, S. P., Chakraborty, R., Bhullar, R. P., & Chelikani, P. (2013). Role of rhodopsin N-terminus in structure and function of rhodopsin-bitter taste receptor chimeras. *Biochem Biophys Res Commun*, 430(1), 179-182.
223. Pydi, S. P., Jaggupilli, A., Nelson, K. M., Abrams, S. R., Bhullar, R. P., Loewen, M. C., & Chelikani, P. (2015). Absciscic Acid Acts as a Blocker of the Bitter Taste G Protein-Coupled Receptor T2R4. *Biochemistry*, 54(16), 2622-2631. doi:10.1021/acs.biochem.5b00265
224. Pydi, S. P., Sobotkiewicz, T., Billakanti, R., Bhullar, R. P., Loewen, M. C., & Chelikani, P. (2014). Amino acid derivatives as bitter taste receptor (T2R) blockers. *J Biol Chem*, 289(36), 25054-25066. doi:10.1074/jbc.M114.576975
225. Pydi, S. P., Upadhyaya, J., Singh, N., Pal Bhullar, R., & Chelikani, P. (2012). Recent advances in structure and function studies on human bitter taste receptors. *Curr Protein Pept Sci*, 13(6), 501-508. doi:10.2174/138920312803582942
226. Qiao, H., & May, J. M. (2009). Macrophage differentiation increases expression of the ascorbate transporter (SVCT2). *Free Radic Biol Med*, 46(8), 1221-1232. doi:10.1016/j.freeradbiomed.2009.02.004
227. Ra, C., Kl, L., Salako, N., & Streckfus, C. (2017). Relationship between Salivary Cytokines, and Caries Experience in Children with Different Body Mass Indices. *Journal of Dental and Oral Health*, 3, 1-5.
228. Rahman, A., Anwar, K. N., Uddin, S., Xu, N., Ye, R. D., Platanias, L. C., & Malik, A. B. (2001). Protein kinase C-delta regulates thrombin-induced ICAM-1 gene expression in endothelial cells via activation of p38 mitogen-activated protein kinase. *Mol Cell Biol*, 21(16), 5554-5565. doi:10.1128/mcb.21.16.5554-5565.2001

229. Raiborg, C., & Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature*, 458(7237), 445-452. doi:10.1038/nature07961
230. Rak, J., & Guha, A. (2012). Extracellular vesicles--vehicles that spread cancer genes. *Bioessays*, 34(6), 489-497. doi:10.1002/bies.201100169
231. Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., & Geuze, H. J. (1996). B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*, 183(3), 1161-1172.
232. Rasoamanana, R., Darcel, N., Fromentin, G., & Tome, D. (2012). Nutrient sensing and signalling by the gut. *Proc Nutr Soc*, 71(4), 446-455. doi:10.1017/s0029665112000110
233. Record, M., Subra, C., Silvente-Poirot, S., & Poirot, M. (2011). Exosomes as intercellular signalosomes and pharmacological effectors. *Biochem Pharmacol*, 81(10), 1171-1182. doi:10.1016/j.bcp.2011.02.011
234. Rhyu, M. R., & Kim, E. Y. (2011). Umami taste characteristics of water extract of Doenjang, a Korean soybean paste: Low-molecular acidic peptides may be a possible clue to the taste. *Food Chem*, 127(3), 1210-1215. doi:10.1016/j.foodchem.2011.01.128
235. Roland, W. S., Gouka, R. J., Gruppen, H., Driesse, M., van Buren, L., Smit, G., & Vincken, J. P. (2014). 6-Methoxyflavanones as Bitter Taste Receptor Blockers for hTAS2R39. *PLoS One*, 9(4), e94451. doi:10.1371/journal.pone.0094451
236. PONE-D-13-51328 [pii]
237. Rom, D. I., Christensen, J. M., Alvarado, R., Sacks, R., & Harvey, R. J. (2017). The impact of bitter taste receptor genetics on culturable bacteria in chronic rhinosinusitis. *Rhinology*, 55(1), 90-94. doi:10.4193/Rhin16.181
238. Rössler, P., Boekhoff, I., Tareilus, E., Beck, S., Breer, H., & Freitag, J. (2000). G protein $\beta\gamma$ complexes in circumvallate taste cells involved in bitter transduction. *Chem Senses*, 25(4), 413-421.
239. Sainz, E., Cavenagh, M. M., Gutierrez, J., Battey, J. F., Northup, J. K., & Sullivan, S. L. (2007). Functional characterization of human bitter taste receptors. *Biochem J*, 403(3), 537-543.

240. Sainz, E., Cavenagh, M. M., Gutierrez, J., Battey, J. F., Northup, J. K., & Sullivan, S. L. (2007). Functional characterization of human bitter taste receptors. *Biochemical Journal*, 403(3), 537-543.
241. Sainz, E., Cavenagh, M. M., LopezJimenez, N. D., Gutierrez, J. C., Battey, J. F., Northup, J. K., & Sullivan, S. L. (2007). The G-protein coupling properties of the human sweet and amino acid taste receptors. *Developmental Neurobiology*, 67(7), 948-959. doi:10.1002/dneu.20403
242. Salger, M., Stark, T. D., & Hofmann, T. (2019). Taste Modulating Peptides from Overfermented Cocoa Beans. *J Agric Food Chem*, 67(15), 4311-4320. doi:10.1021/acs.jafc.9b00905
243. Salvadori, G., Junges, R., Morrison, D. A., & Petersen, F. C. (2019). Competence in *Streptococcus pneumoniae* and close commensal relatives: mechanisms and implications. *Frontiers in Cellular and Infection Microbiology*, 9(94). doi:10.3389/fcimb.2019.00094
244. Sánchez, A., & Vázquez, A. (2017). Bioactive peptides: A review. *Food Quality and Safety*, 1(1), 29-46.
245. Sarkar, S., & Pires, M. M. (2015). d-Amino acids do not inhibit biofilm formation in *Staphylococcus aureus*. *PLoS One*, 10(2), e0117613. doi:10.1371/journal.pone.0117613
246. Satoh, A., Gukovskaya, A. S., Reeve, J. R., Jr., Shimosegawa, T., & Pandol, S. J. (2006). Ethanol sensitizes NF-kappaB activation in pancreatic acinar cells through effects on protein kinase C-epsilon. *Am J Physiol Gastrointest Liver Physiol*, 291(3), G432-438. doi:10.1152/ajpgi.00579.2005
247. Saunders, C. J., Christensen, M., Finger, T. E., & Tizzano, M. (2014). Cholinergic neurotransmission links solitary chemosensory cells to nasal inflammation. *Proc Natl Acad Sci U S A*, 111(16), 6075-6080. doi:10.1073/pnas.1402251111
248. Schaefer, F., Bruttin, O., Zografos, L., & Guex-Crosier, Y. (2001). Bacterial keratitis: a prospective clinical and microbiological study. *Br J Ophthalmol*, 85(7), 842-847. doi:10.1136/bjo.85.7.842

249. Schertzer, J. W., Brown, S. A., & Whiteley, M. (2010). Oxygen levels rapidly modulate *Pseudomonas aeruginosa* social behaviours via substrate limitation of PqsH. *Mol Microbiol*, 77(6), 1527-1538. doi:10.1111/j.1365-2958.2010.07303.x
250. Schneider, K. B., Palmer, T. M., & Grossman, A. D. (2002). Characterization of *comQ* and *comX*, Two Genes Required for Production of ComX Pheromone in *Bacillus subtilis*. *J Bacteriol*, 184(2), 410-419. doi:10.1128/jb.184.2.410-419.2002
251. Schwarzer, C., Wong, S., Shi, J., Matthes, E., Illek, B., Ianowski, J. P., . . . Machen, T. E. (2010). *Pseudomonas aeruginosa* homoserine lactone activates store-operated cAMP and cystic fibrosis transmembrane regulator-dependent Cl⁻ secretion by human airway epithelia. *Journal of Biological Chemistry*, 285(45), 34850-34863. doi:10.1074/jbc.M110.167668
252. Selbach, M., & Backert, S. (2005). Cortactin: an Achilles' heel of the actin cytoskeleton targeted by pathogens. *Trends Microbiol*, 13(4), 181-189. doi:10.1016/j.tim.2005.02.007
253. Senadheera, D., & Cvitkovitch, D. G. (2008). Quorum sensing and biofilm formation by *Streptococcus mutans*. *Adv Exp Med Biol*, 631, 178-188. doi:10.1007/978-0-387-78885-2_12
254. Seo, Y., Kim, Y. S., Lee, K. E., Park, T. H., & Kim, Y. (2017). Anti-cancer stemness and anti-invasive activity of bitter taste receptors, TAS2R8 and TAS2R10, in human neuroblastoma cells. *PLoS One*, 12(5), e0176851. doi:10.1371/journal.pone.0176851
255. Servant, G., & Frerot, E. (2021). Pharmacology of the Umami Taste Receptor. *Handb Exp Pharmacol*. doi:10.1007/164_2021_439
256. Sforza, S., Galaverna, G., Schivazappa, C., Marchelli, R., Dossena, A., & Virgili, R. (2006). Effect of extended aging of parma dry-cured ham on the content of oligopeptides and free amino acids. *J Agric Food Chem*, 54(25), 9422-9429. doi:10.1021/jf061312+
257. Shah, A. S., Ben-Shahar, Y., Moninger, T. O., Kline, J. N., & Welsh, M. J. (2009). Motile cilia of human airway epithelia are chemosensory. *Science*, 325(5944), 1131-1134.

258. Shah, A. S., Ben-Shahar, Y., Moninger, T. O., Kline, J. N., & Welsh, M. J. (2009). Motile cilia of human airway epithelia are chemosensory. *Science*, 325(5944), 1131-1134. doi:10.1126/science.1173869
259. Shahrestanifar, M., Fan, X., & Manning, D. R. (1999). Lysophosphatidic acid activates NF-kappaB in fibroblasts. A requirement for multiple inputs. *J Biol Chem*, 274(6), 3828-3833. doi:10.1074/jbc.274.6.3828
260. Shaik, F. A., Medapati, M. R., & Chelikani, P. (2019). Cholesterol modulates the signaling of chemosensory bitter taste receptor T2R14 in human airway cells. *Am J Physiol Lung Cell Mol Physiol*, 316(1), L45-L57. doi:10.1152/ajplung.00169.2018
261. Shaik, F. A., Singh, N., Arakawa, M., Duan, K., Bhullar, R. P., & Chelikani, P. (2016). Bitter taste receptors: Extraoral roles in pathophysiology. *Int J Biochem Cell Biol*, 77(Pt B), 197-204. doi:10.1016/j.biocel.2016.03.011
262. Shandilya, J., Gao, Y., Nayak, T. K., Roberts, S. G., & Medler, K. F. (2016). AP1 transcription factors are required to maintain the peripheral taste system. *Cell Death Dis*, 7(10), e2433. doi:10.1038/cddis.2016.343
263. Sharma, P., Conaway, S., Jr., & Deshpande, D. (2021). Bitter Taste Receptors in the Airway Cells Functions. *Handb Exp Pharmacol*. doi:10.1007/164_2021_436
264. Shen, Y., Naujokas, M., Park, M., & Ireton, K. (2000). InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell*, 103(3), 501-510. doi:10.1016/s0092-8674(00)00141-0
265. Shi, P., Zhang, J., Yang, H., & Zhang, Y. P. (2003). Adaptive diversification of bitter taste receptor genes in Mammalian evolution. *Mol Biol Evol*, 20(5), 805-814. doi:10.1093/molbev/msg083
266. Shim, J., Son, H. J., Kim, Y., Kim, K. H., Kim, J. T., Moon, H., . . . Rhyu, M. R. (2015). Modulation of sweet taste by umami compounds via sweet taste receptor subunit hT1R2. *PLoS One*, 10(4), e0124030. doi:10.1371/journal.pone.0124030
267. Shiner, E., Terentyev, D., Bryan, A., Sennoune, S., Martinez - Zaguilan, R., Li, G., . . . Rumbaugh, K. (2006). *Pseudomonas aeruginosa* autoinducer modulates host cell responses through calcium signalling. *Cellular microbiology*, 8(10), 1601-1610.

268. Shiner, E. K., Rumbaugh, K. P., & Williams, S. C. (2005). Interkingdom signaling: deciphering the language of acyl homoserine lactones. *FEMS microbiology reviews*, 29(5), 935-947.
269. Shultz, S., Worzella, T., Gallagher, A., Shieh, J., Goueli, S., Hsiao, K., & Vidugiriene, J. (2008). Miniaturized GPCR signaling studies in 1536-well format. *J Biomol Tech*, 19(4), 267-274.
270. Sidhu, C., Jaggupilli, A., Chelikani, P., & Bhullar, R. P. (2017). Regulation of Rac1 GTPase activity by quinine through G-protein and bitter taste receptor T2R4. *Mol Cell Biochem*, 426(1-2), 129-136. doi:10.1007/s11010-016-2886-8
271. Simhadri, V. R., Reiners, K. S., Hansen, H. P., Topolar, D., Simhadri, V. L., Nohroudi, K., . . . Pogge von Strandmann, E. (2008). Dendritic cells release HLA-B-associated transcript-3 positive exosomes to regulate natural killer function. *PLoS One*, 3(10), e3377. doi:10.1371/journal.pone.0003377
272. Singh, N., Chakraborty, R., Bhullar, R. P., & Chelikani, P. (2014). Differential expression of bitter taste receptors in non-cancerous breast epithelial and breast cancer cells. *Biochem Biophys Res Commun*, 446(2), 499-503. doi:10.1016/j.bbrc.2014.02.140
273. Singh, N., Pydi, S. P., Upadhyaya, J., & Chelikani, P. (2011). Structural basis of activation of bitter taste receptor T2R1 and comparison with class A G-protein-coupled receptors (GPCRs). *Journal of Biological Chemistry*, 286(41), 36032-36041. doi:10.1074/jbc.M111.246983
274. Singh, N., Shaik, F. A., Myal, Y., & Chelikani, P. (2020). Chemosensory bitter taste receptors T2R4 and T2R14 activation attenuates proliferation and migration of breast cancer cells. *Mol Cell Biochem*, 465(1-2), 199-214. doi:10.1007/s11010-019-03679-5
275. Singh, N., Vrontakis, M., Parkinson, F., & Chelikani, P. (2011). Functional bitter taste receptors are expressed in brain cells. *Biochem Biophys Res Commun*, 406(1), 146-151.
276. Siripurapu, P., Kankanamge, D., Ratnayake, K., Senarath, K., & Karunarathne, A. (2017). Two independent but synchronized Gbetagamma subunit-controlled pathways are essential for trailing-edge retraction during macrophage migration. *J Biol Chem*, 292(42), 17482-17495. doi:10.1074/jbc.M117.787838

277. Soory, M. (2012). Nutritional antioxidants and their applications in cardiometabolic diseases. *Infectious Disorders-Drug Targets*, 12(5), 388-401.
278. Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., & Hawkins, P. T. (1994). A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. *Cell*, 77(1), 83-93. doi:10.1016/0092-8674(94)90237-2
279. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., & Raposo, G. (2002). The biogenesis and functions of exosomes. *Traffic*, 3(5), 321-330.
280. Striem, B. J., Naim, M., & Lindemann, B. (1991). Generation of Cyclic AMP in Taste Buds of the Rat Circumvallate Papilla in Response to Sucrose. *Cellular Physiology and Biochemistry*, 1(1), 46-54. doi:10.1159/000154592
281. Sturme, M. H., Kleerebezem, M., Nakayama, J., Akkermans, A. D., Vaughan, E. E., & De Vos, W. M. (2002). Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie Van Leeuwenhoek*, 81(1-4), 233-243.
282. Su, G., Cui, C., Zheng, L., Yang, B., Ren, J., & Zhao, M. (2012). Isolation and identification of two novel umami and umami-enhancing peptides from peanut hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS. *Food Chem*, 135(2), 479-485. doi:10.1016/j.foodchem.2012.04.130
283. Subramaniam, S., Ozdener, M. H., Abdoul-Azize, S., Saito, K., Malik, B., Maquart, G., . . . Khan, N. A. (2016). ERK1/2 activation in human taste bud cells regulates fatty acid signaling and gustatory perception of fat in mice and humans. *FASEB journal*, 30(10), 3489-3500. doi:10.1096/fj.201600422R
284. Suh, P. G., Park, J. I., Manzoli, L., Cocco, L., Peak, J. C., Katan, M., . . . Ryu, S. H. (2008). Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep*, 41(6), 415-434. doi:10.5483/bmbrep.2008.41.6.415
285. Syvitski, R. T., Tian, X. L., Sampara, K., Salman, A., Lee, S. F., Jakeman, D. L., & Li, Y. H. (2007). Structure-activity analysis of quorum-sensing signaling peptides from *Streptococcus mutans*. *J Bacteriol*, 189(4), 1441-1450. doi:10.1128/jb.00832-06
286. Taga, M. E., & Bassler, B. L. (2003). Chemical communication among bacteria. *Proceedings of the National Academy of Sciences*, 100(suppl 2), 14549-14554.

287. Taher, S., Borja, Y., Cabanela, L., Costers, V. J., Carson-Marino, M., Bailes, J. C., . . . Conrad, K. P. (2019). Cholecystokinin, gastrin, cholecystokinin/gastrin receptors, and bitter taste receptor TAS2R14: trophoblast expression and signaling. *Am J Physiol Regul Integr Comp Physiol*, 316(5), R628-r639. doi:10.1152/ajpregu.00153.2018
288. Takenouchi, T., Ogihara, K., Sato, M., & Kitani, H. (2005). Inhibitory effects of U73122 and U73343 on Ca²⁺ influx and pore formation induced by the activation of P2X7 nucleotide receptors in mouse microglial cell line. *Biochim Biophys Acta*, 1726(2), 177-186. doi:10.1016/j.bbagen.2005.08.001
289. Tamura, M., Miyoshi, T., Mori, N., Kinomura, K., Kawaguchi, M., Ishibashi, N., & Okai, H. (1990). Mechanism for the bitter tasting potency of peptides using O-aminoacyl sugars as model compounds. *Agric Biol Chem*, 54(6), 1401-1409.
290. Tang, X., Kudo, Y., Baker, J. L., LaBonte, S., Jordan, P. A., McKinnie, S. M. K., . . . Edlund, A. (2020). Cariogenic Streptococcus mutans Produces Tetramic Acid Strain-Specific Antibiotics That Impair Commensal Colonization. *ACS Infect Dis*, 6(4), 563-571. doi:10.1021/acsinfecdis.9b00365
291. Tanzer, J. M., Livingston, J., & Thompson, A. M. (2001). The microbiology of primary dental caries in humans. *J Dent Educ*, 65(10), 1028-1037.
292. Telford, G., Wheeler, D., Williams, P., Tomkins, P., Appleby, P., Sewell, H., . . . Pritchard, D. I. (1998). The Pseudomonas aeruginosa quorum-sensing signal molecule (3-oxododecanoyl)-L-homoserine lactone has immunomodulatory activity. *Infect Immun*, 66(1), 36-42.
293. Temussi, P. (2007). The sweet taste receptor: a single receptor with multiple sites and modes of interaction. *Adv Food Nutr Res*, 53, 199-239. doi:10.1016/s1043-4526(07)53006-8
294. Thoendel, M., Kavanaugh, J. S., Flack, C. E., & Horswill, A. R. (2011). Peptide signaling in the staphylococci. *Chemical Reviews*, 111(1), 117-151. doi:10.1021/cr100370n
295. Tian, T., Wang, Y., Wang, H., Zhu, Z., & Xiao, Z. (2010). Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy. *J Cell Biochem*, 111(2), 488-496. doi:10.1002/jcb.22733

296. Tizzano, M., Cristofolletti, M., Sbarbati, A., & Finger, T. E. (2011). Expression of taste receptors in solitary chemosensory cells of rodent airways. *BMC pulmonary medicine*, 11(1), 3.
297. Tizzano, M., Gulbransen, B. D., Vandenbeuch, A., Clapp, T. R., Herman, J. P., Sibhatu, H. M., . . . Finger, T. E. (2010). Nasal chemosensory cells use bitter taste signaling to detect irritants and bacterial signals. *Proceedings of the National Academy of Sciences*, 107(7), 3210-3215.
298. Toelstede, S., & Hofmann, T. (2008). Sensomics mapping and identification of the key bitter metabolites in Gouda cheese. *J Agric Food Chem*, 56(8), 2795-2804. doi:10.1021/jf7036533
299. Trams, E. G., Lauter, C. J., Salem, N., Jr., & Heine, U. (1981). Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta*, 645(1), 63-70.
300. Tran, H. T. T., Herz, C., Ruf, P., Stetter, R., & Lamy, E. (2018). Human T2R38 Bitter Taste Receptor Expression in Resting and Activated Lymphocytes. *Front Immunol*, 9, 2949. doi:10.3389/fimmu.2018.02949
301. Tu, Y. H., Cooper, A. J., Teng, B., Chang, R. B., Artiga, D. J., Turner, H. N., . . . Liman, E. R. (2018). An evolutionarily conserved gene family encodes proton-selective ion channels. *Science*, 359(6379), 1047-1050. doi:10.1126/science.aao3264
302. Turnbull, A. R., Murphy, R., Behrends, V., Lund-Palau, H., Simbo, A., Mariveles, M., . . . Davies, J. C. (2018). Impact of T2R38 receptor polymorphisms on *Pseudomonas aeruginosa* infection in cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 197(12), 1635-1638.
303. Ueda, T., Ugawa, S., Yamamura, H., Imaizumi, Y., & Shimada, S. (2003). Functional interaction between T2R taste receptors and G-protein alpha subunits expressed in taste receptor cells. *J Neurosci*, 23(19), 7376-7380.
304. Ueno, Y., Sakurai, T., Okada, S., Abe, K., & Misaka, T. (2011). Human bitter taste receptors hTAS2R8 and hTAS2R39 with differential functions to recognize bitter peptides. *Biosci Biotechnol Biochem*, 75(6), 1188-1190. doi:10.1271/bbb.100893
305. Upadhyaya, J., Pydi, S. P., Singh, N., Aluko, R. E., & Chelikani, P. (2010). Bitter taste receptor T2R1 is activated by dipeptides and tripeptides. *Biochemical and*

- biophysical research communications*, 398(2), 331-335.
doi:<https://doi.org/10.1016/j.bbrc.2010.06.097>
306. Upadhyaya, J. D., Chakraborty, R., Shaik, F. A., Jaggupilli, A., Bhullar, R. P., & Chelikani, P. (2016). The Pharmacochaperone Activity of Quinine on Bitter Taste Receptors. *PLoS One*, 11(5), e0156347. doi:10.1371/journal.pone.0156347
 307. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., & Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*, 9(6), 654-659. doi:10.1038/ncb1596
 308. Vella, L. J., Sharples, R. A., Nisbet, R. M., Cappai, R., & Hill, A. F. (2008). The role of exosomes in the processing of proteins associated with neurodegenerative diseases. *Eur Biophys J*, 37(3), 323-332. doi:10.1007/s00249-007-0246-z
 309. Verbeke, F., De Craemer, S., Debunne, N., Janssens, Y., Wynendaele, E., Van de Wiele, C., & De Spiegeleer, B. (2017). Peptides as quorum sensing molecules: measurement techniques and obtained levels in vitro and in vivo. *Frontiers in Neuroscience*, 11(183). doi:10.3389/fnins.2017.00183
 310. Verbeurgt, C., Veithen, A., Carlot, S., Tarabichi, M., Dumont, J. E., Hassid, S., & Chatelain, P. (2017). The human bitter taste receptor T2R38 is broadly tuned for bacterial compounds. *PLoS One*, 12(9), e0181302. doi:10.1371/journal.pone.0181302
 311. Verkade, P. E. (1968). On organic compounds with a sweet and-or a bitter taste. *Farmaco Sci*, 23(3), 248-291.
 312. Vidal, C., Geny, B., Melle, J., Jandrot-Perrus, M., & Fontenay-Roupie, M. (2002). Cdc42/Rac1-dependent activation of the p21-activated kinase (PAK) regulates human platelet lamellipodia spreading: implication of the cortical-actin binding protein cortactin. *Blood*, 100(13), 4462-4469. doi:10.1182/blood.V100.13.4462
 313. von Moltke, J., Ji, M., Liang, H.-E., & Locksley, R. M. (2016). Tuft-cell-derived IL-25 regulates an intestinal ILC2–epithelial response circuit. *Nature*, 529(7585), 221-225.
 314. Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P., & Pesci, E. C. (2005). Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa. *J Bacteriol*, 187(13), 4372-4380. doi:10.1128/jb.187.13.4372-4380.2005

315. Wang, Q., Liszt, K. I., Deloose, E., Canovai, E., Thijs, T., Farre, R., . . . Depoortere, I. (2019). Obesity alters adrenergic and chemosensory signaling pathways that regulate ghrelin secretion in the human gut. *FASEB J*, 33(4), 4907-4920. doi:10.1096/fj.201801661RR
316. Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M., & Lefkowitz, R. J. (2003). Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A*, 100(19), 10782-10787. doi:10.1073/pnas.1834556100
317. Wiley, S. Z., Sriram, K., Liang, W., Chang, S. E., French, R., McCann, T., . . . Insel, P. A. (2018). GPR68, a proton-sensing GPCR, mediates interaction of cancer-associated fibroblasts and cancer cells. *FASEB J*, 32(3), 1170-1183. doi:10.1096/fj.201700834R
318. Wong, G. T., Gannon, K. S., & Margolskee, R. F. (1996). Transduction of bitter and sweet taste by gustducin. *Nature*, 381(6585), 796-800.
319. Woo, J. A., Castano, M., Goss, A., Kim, D., Lewandowski, E. M., Chen, Y., & Liggett, S. B. (2019). Differential long-term regulation of TAS2R14 by structurally distinct agonists. *FASEB J*, 33(11), 12213-12225. doi:10.1096/fj.201802627RR
320. Wooding, S. (2005). Evolution: a study in bad taste? *Curr Biol*, 15(19), R805-807. doi:10.1016/j.cub.2005.09.021
321. Workman, A. D., Maina, I. W., Brooks, S. G., Kohanski, M. A., Cowart, B. J., Mansfield, C., . . . Cohen, N. A. (2018). The Role of Quinine-Responsive Taste Receptor Family 2 in Airway Immune Defense and Chronic Rhinosinusitis. *Front Immunol*, 9, 624. doi:10.3389/fimmu.2018.00624
322. Wu, C.-H., Huang, S.-M., Lin, J.-A., & Yen, G.-C. (2011). Inhibition of advanced glycation endproduct formation by foodstuffs. *Food & function*, 2(5), 224-234.
323. Wu, J., & Aluko, R. E. (2007). Quantitative structure - activity relationship study of bitter di - and tri - peptides including relationship with angiotensin I - converting enzyme inhibitory activity. *Journal of peptide science*, 13(1), 63-69.
324. Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure- activity relationship study of di-and tripeptides. *Journal of Agricultural and Food Chemistry*, 54(3), 732-738.

325. Wunsch, C. M., & Lewis, J. P. (2015). Porphyromonas gingivalis as a Model Organism for Assessing Interaction of Anaerobic Bacteria with Host Cells. *J Vis Exp*(106), e53408. doi:10.3791/53408
326. Xu, J., Cao, J., Iguchi, N., Riethmacher, D., & Huang, L. (2013). Functional characterization of bitter-taste receptors expressed in mammalian testis. *Mol Hum Reprod*, 19(1), 17-28. doi:10.1093/molehr/gas040
327. Xu, Q., Singh, N., Hong, H., Yan, X., Yu, W., Jiang, X., . . . Wu, J. (2019). Hen protein-derived peptides as the blockers of human bitter taste receptors T2R4, T2R7 and T2R14. *Food Chem*, 283, 621-627. doi:10.1016/j.foodchem.2019.01.059
328. Yadav, V. K., Singh, P. K., Kalia, M., Sharma, D., Singh, S. K., & Agarwal, V. (2018). Pseudomonas aeruginosa quorum sensing molecule N-3-oxo-dodecanoyl-L-homoserine lactone activates human platelets through intracellular calcium-mediated ROS generation. *Int J Med Microbiol*, 308(7), 858-864. doi:10.1016/j.ijmm.2018.07.009
329. Yang, Y., Koirala, B., Sanchez, L. A., Phillips, N. R., Hamry, S. R., & Tal-Gan, Y. (2017). Structure-activity relationships of the competence stimulating peptides (CSPs) in *Streptococcus pneumoniae* reveal motifs critical for intra-group and cross-group ComD receptor activation. *ACS chemical biology*, 12(4), 1141-1151. doi:10.1021/acscchembio.7b00007
330. Zhang, C., Alashi, A. M., Singh, N., Chelikani, P., & Aluko, R. E. (2019). Glycated beef protein hydrolysates as sources of bitter taste modifiers. *Nutrients*, 11(9). doi:10.3390/nu11092166
331. Zhang, C., Alashi, A. M., Singh, N., Liu, K., Chelikani, P., & Aluko, R. E. (2018). Beef Protein-Derived Peptides as Bitter Taste Receptor T2R4 Blockers. *J Agric Food Chem*, 66(19), 4902-4912. doi:10.1021/acs.jafc.8b00830
332. Zhang, C., Alashi, A. M., Singh, N., Liu, K., Chelikani, P., & Aluko, R. E. (2018). Beef protein-derived peptides as bitter taste receptor T2R4 blockers. *Journal of Agricultural and Food Chemistry*, 66(19), 4902-4912. doi:10.1021/acs.jafc.8b00830
333. Zhang, L., Lu, R., Zhao, G., Pflugfelder, S. C., & Li, D. Q. (2011). TLR-mediated induction of pro-allergic cytokine IL-33 in ocular mucosal epithelium. *Int J Biochem Cell Biol*, 43(9), 1383-1391. doi:10.1016/j.biocel.2011.06.003

334. Zheng, X., Tizzano, M., Redding, K., He, J., Peng, X., Jiang, P., . . . Margolskee, R. F. (2019). Gingival solitary chemosensory cells are immune sentinels for periodontitis. *Nat Commun*, 10(1), 4496. doi:10.1038/s41467-019-12505-x
335. Zhu, H., Conibear, T. C., Thuruthyil, S. J., & Willcox, M. D. (2008). *Pseudomonas aeruginosa* quorum-sensing signal molecules induce IL-8 production by human corneal epithelial cells. *Eye Contact Lens*, 34(3), 179-181. doi:10.1097/ICL.0b013e318159afde
336. Zuris, J. A., Thompson, D. B., Shu, Y., Guilinger, J. P., Bessen, J. L., Hu, J. H., . . . Liu, D. R. (2015). Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat Biotechnol*, 33(1), 73-80. doi:10.1038/nbt.3081