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Investigating the Effect of Taste Signaling Protein Abolishment on Gut Inflammation in an Inflammatory Bowel Disease Model

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TITLE:

Effects of Taste Signaling Protein Abolishment on Gut Inflammation in an Inflammatory Bowel Disease Mouse Model

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KEYWORDS:

Inflammatory bowel disease, intestinal illness, taste receptors, G protein-coupled receptors, α-gustducin, signaling pathway, dextran sulfate sodium, immune, inflammation

SUMMARY:

Here we present a protocol to investigate the effect of the nullification of gustation-related genes on immune responses in a dextran sulfate sodium (DSS)-induced inflammatory bowel disease (IBD) mouse model.

ABSTRACT:

Inflammatory bowel disease (IBD) is one of the immune-related gastrointestinal disorders, including ulcerative colitis and Crohn's disease, that affects the life quality of millions of people worldwide. IBD symptoms include abdominal pain, diarrhea, and rectal bleeding,

which may result from the interactions among gut microbiota, food components, intestinal epithelial cells, and immune cells. It is of particular importance to assess how each key gene expressed in intestinal epithelial and immune cells affects inflammation in the colon. G protein-coupled taste receptors, including G protein subunit α-gustducin and other signaling proteins, have been found in the intestines. Here, we use α-gustducin as a representative and describe a dextran sulfate sodium (DSS)-induced IBD model to evaluate the effect of gustatory gene mutations on gut mucosal immunity and inflammation. This method combines gene knockout technology with the chemically induced IBD model, and thus can be applied to assess the outcome of gustatory gene nullification as well as other genes that may exuberate or dampen the DSS-induced immune response in the colon. Mutant mice are administered with DSS for a certain period during which their body weight, stool, and rectal bleeding are monitored and recorded. At different timepoints during administration, some mice are euthanized, then the sizes and weights of their spleens and colons are measured and gut tissues are collected and processed for histological and gene expression analyses. The data show that the α-gustducin knockout results in excessive weight loss, diarrhea, intestinal bleeding, tissue damage, and inflammation *vs.* wild-type mice. Since the severity of induced inflammation is affected by mouse strains, housing environment, and diet, optimization of DSS concentration and administration duration in a pilot experiment is particularly important. By adjusting these factors, this method can be applied to assess both anti- and pro-inflammatory effects.

INTRODUCTION:

The two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC) are characterized by chronic remittent or progressive inflammatory conditions of the intestine with multifactorial etiology[1,2]. The development of IBD depends on genetic as well as certain environmental factors such as diet, antibiotic use, and importantly, pathogenic infections. However, the etiology and regulatory molecular mechanisms underlying IBD are still unclear. Hence, numerous chemically induced IBD animal models have been constructed and applied to delineate the pathogenesis and regulatory mechanisms and evaluate the effectiveness of human therapeutics[3].

Taste receptors are G protein-coupled receptors (GPCRs) and are classified as two major types: type I (T1Rs), and type II (T2Rs) that detect sweet, umami, and bitter compounds. Taste signaling cascades are initiated by tastant binding to T1Rs or T2Rs, activating the heterotrimeric G proteins consisting of α-gustducin and a Gβγ dimer and leading to release of the Gβγ subunits. The Gβγ moiety in turn stimulates the downstream effector enzyme phospholipase C-β2 (PLC-β2). Activated PLC-β2 then hydrolyzes phosphatidylinositol-4,5-bisphosphate into two intracellular secondary messengers [inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol], and IP_3 binds to and open its channel-receptor IP_3R3, releasing calcium ions from the endoplasmic reticulum. This eventually leads to the opening of transient receptor potential ion channel Trpm5 and release of the neurotransmitter ATP onto the gustatory nerves[4-7]. Yet, the signaling pathways of salty and sour tastes are different and

independent from sweet, umami, and bitter tastes[8]. In addition, the components of taste GPCRs and downstream proteins exist in various extra-oral tissues. Recent studies indicated that α-gustducin, the principal component of taste signaling, is found to be expressed in the intestinal mucosa. Further studies are needed to understand the functions of these taste signaling components in extra-oral tissues[9,10].

The method described here is used to characterize functions of the gustatory signaling proteins expressed in extra-oral tissues. We combine a transgenic mouse line developed for delineating signaling cascades in taste buds with the chemically induced colitis model. Largely due to its procedural simplicity and pathological similarities to human ulcerative colitis, the dextran sulfate sodium (DSS)-induced IBD model has been most widely used among the various chemically induced colitis models[11]. In this study, we used α-gustducin-deficient mice as a representative mouse line to reveal novel functions of α-gustducin in gut mucosal immunity and inflammation by 1) analyzing morphological changes in the tissue and 2) assaying differences in the expression of cytokines related to inflammation in the colon. This method can be used to quantitatively and qualitatively determine the contributions of gustatory signaling proteins (and other proteins expressed in the gut) to tissue damage and intestinal inflammation, when genetically modified mouse lines for the genes of interest are available. Advantages of this method are enabling users to obtain integrated data resulting from actions of both the chemical DSS and deficiency of the gene of interest. This method can be further improved to increase its sensitivity and reveal subtle intestinal changes at the cellular and molecular levels.

PROTOCOL:

All experiments involving mice were reviewed and approved by the Institutional Animal Care and Use Committees of Zhejiang University. It is advised to wear appropriate personal protective equipment before performing this protocol.

1. Preparation of Mice and DSS

1.1. Keep the knockout (α-gustducin$^{-/-}$) mice and age-, gender-, and body weight-matched wild-type control (α-gustducin$^{+/+}$) C57BL/6 mice individually in clean cages.

Note: The knockout mice have been backcrossed with C57BL/6 mice for over 20 generations and have a nearly 100% C57BL/6 genetic background.

1.2. Dissolve 30 g of dextran sulfate sodium (DSS) powder in 1 L of autoclaved water. Mix until the solution becomes clear to ensure that the final working concentration is 3% (w/v).

Note: The DSS solution can be stored at room temperature for up to 1 week or at 4 °C until use.

2. Induction and Evaluation of DSS Colitis in Mice

2.1. Weigh and record each mouse's initial body weight. Place the mice individually into standard plastic cages and label the cages.

2.2. Replace regular drinking water with 3% DSS solution for a total of 7 days to which both groups of mice have access *ad libitum*.

2.3. Measure the mouse's body weight, record DSS solution consumption, and collect and examine the stool of each mouse daily during the DSS administration. Observe the severity of diarrhea and rectal bleeding and convert this to the DSS-induced disease index[12].

2.4. During the experiment, the percentage of weight loss compared to initial weight and the disease index are calculated to evaluate the symptoms of colitis.

Note: The disease index is scored by combining observations of diarrhea and rectal bleeding and are defined as follows: 0 (normal stool, no blood), 1 (soft stool, no blood), 2 (soft stool, little blood), 3 (very soft stool, modest bleeding), and 4 (watery stool, significant bleeding)[12]. The disease index is analyzed daily for each mouse.

2.5. By the end of 7-day DSS treatment, sacrifice the mice by cervical dislocation and proceed with the remaining experiments.

3. Preparation of Tissue Samples

3.1. Place the mouse in the supine position and clean the skin of the abdomen with 70% ethanol. Make a 3 cm-long midline incision in the abdomen with a pair of small scissors to expose the abdominal cavity.

3.2. Use a pair of forceps to carefully separate the spleen from other tissues, then remove the spleen and measure its size.

3.3. Identify and lift the colon with forceps and separate it from the surrounding mesentery. Pull out the whole colon until the cecum and rectum are visible.

3.4. Isolate the colon by transecting it at the colonocecal margin and deep in the pelvis to free the proximal and distal colon, respectively. Then, measure and record the length of the isolated colon. Be careful not to damage the colonic tissue during the dissecting procedure.

3.5. Flush the colon with 10 mL of ice-cold phosphate-buffered saline (PBS) with a 10 mL syringe equipped with a gavage needle to remove the feces and blood until the eluate is completely clear.

3.6. For histological identification, divide the tissue samples equally into three parts: proximal, middle, and distal. Then, fix the tissue with 4% paraformaldehyde (PFA) overnight at 4 °C.

3.7. For the analysis of cytokine expression, freeze the entire colon rapidly with liquid nitrogen and store it at -80 °C until use.

4. Histological Assessment of the Severity of DSS-Induced Colitis

4.1. Hematoxylin and eosin (H&E) staining

4.1.1. After fixation, submerge the tissue in a solution of 30% sucrose in 1x PBS overnight in a 15 mL tube to cryoprotect the samples.

4.1.2. Embed the tissue in optimal cutting temperature (OCT) compound and place it at -20 °C until the OCT hardens.

4.1.3. Transfer the OCT block containing the tissue to a cryostat, set the thickness dial in the cryostat to 12 µm, and slice and collect 12 µm-thick frozen sections.

4.1.4. Heat the collected tissue sections from a cryostat at 65 °C for 20 min on a hot plate.

4.1.5. Wash the sections briefly in distilled water. Stain them with hematoxylin staining solution for 5 min and subsequently rinse with running tap water for 5 min.

4.1.6. Differentiate the sections with 0.5% hydrochloric acid-ethanol for 30 s and rinse them in running tap water for 1 min. Then, perform the wash in 1x PBS for 1 min and subsequently rinse with running tap water for 5 min.

4.1.7. Perform washing of the tissue sections in 70%, 75%, 80%, and 95% alcohol, each for 10 s. Counterstain in eosin staining solution for 2 min.

4.1.8. Perform dehydration through 95% alcohol and 2 changes of absolute alcohol for 5 min each time. Clear in 2 changes of xylene for 5 min.

4.1.9. Score tissue damage of the proximal, middle, and distal colon of each mouse for both the gene knockout and wild-type groups based on results of the above H&E staining.

Note: The disease index is a combined score of epithelial damage and inflammation in the mucosa, submucosa, muscularis, and serosa regions, which is defined as follows: 0 (no tissue damage and inflammation), 1 (focal tissue damage and inflammation), 2 (patchy tissue damage and inflammation), and 3 (diffuse tissue damage and inflammation)[12,13]. Three scores

per mouse for the proximal, middle, and distal parts of the colon are then summed to obtain a total score for each animal. The average scores for each group are then calculated.

4.2. Immunohistochemistry[14]

4.2.1. Heat the collected tissue sections from a cryostat at 65 °C for 20 min on a hot plate. Wash the sections in 1x PBS 3 times, for 10 min each. Incubate the tissue sections in 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase. Wash the sections 3 more times. Block the tissues with blocking buffer (3% BSA, 0.3% non-ionic detergent, 2% goat serum, 0.1% sodium azide in 1x PBS) at room temperature for 1 h or more.

4.2.2. Replace the blocking buffer with a solution containing the following immune cell type-specific primary antibodies: CD45 for leukocytes, CD3 for T cells, B220 for B cells, CD11b for macrophages, and Ly6G for neutrophils. Incubate at 4 °C overnight.

4.2.3. Remove the primary antibodies from tissue sections by aspiration. Wash the sections with 1x PBS 3 times, for 10 min each. Incubate the sections with biotinylated secondary antibody followed by incubation with the streptavidin-HRP complex at room temperature.

4.2.4. Incubate the sections with 3,3'-diaminobenziding (DAB) solution to develop a light- or dark-brown color and visualize the immunoreactive cells.

4.2.5. Counterstain the sections with hematoxylin and 0.3% (v/v) diluted ammonia. Take bright-field images at 10X magnification under a microscope.

4.2.6. Use an image processing program to identify and quantify the population of the marked immune cells in both the epithelium and the lamina propria (underneath the epithelium) by setting 2 masks: use the first mask in the color-cube-based feature to set a color detection threshold and measure the colored DAB-reactive areas; use the second mask to determine total areas of the epithelium and laminar propria in the examined section. Express the immunoreactive cell population as a ratio of the staining area of the infiltrated cells to the total area of the examined tissue.

5. Gene Expression Assessment of DSS-Induced Colitis

5.1. Retrieve the colon tissues from the DSS-treated gene knockout and wild-type mice from a -80 °C freezer and add 0.6 mL of lysis buffer to 25 mg of tissue, then homogenize in a homogenizer.

5.2. Follow the RNA extraction kit's protocol to extract the total RNA, and DNase I is used to eliminate any contaminating genomic DNA.

5.3. Run an agarose gel to check the quality of the extracted RNA. If its 28S RNA band is

brighter than 18S band, it is usable. Take 1 µL of the RNA sample to determine the RNA concentration on a microspectrophotometer.

5.4. Mix 1 µg of the total RNA with 2.5 µL of 20 mM oligo $(dT)^{12-18}$ primers and 1 µL of Moloney murine leukemia virus (MMLV) reverse transcriptase. Incubate at 42 °C for 60 min to prepare the cDNA.

5.5. Set up real-time PCR reactions by mixing 1µL of the cDNA with 0.5 µL of forward and reverse qPCR primers for TNF, IFN-γ, IL-5, IL-13, IL-10, TGF-β1, and β-Actin as a control in addition to 2x fluorescent green dye.

5.6. Run the qPCR with the following parameters: 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 50 °C for 25 s, and 72 °C for 20 s.

5.7. Calculate the relative quantification of gene expression by using the $2^{-\Delta\Delta Ct}$ method[15]. Comparatively analyze the gene expression levels in the knockout *vs*. wild-type mice.

REPRESENTATIVE RESULTS:

A DSS-induced IBD procedure was established by administrating 3% DSS in drinking water to α-gustducin-knockout (KO) and wild-type (WT) mice. Compared to WT mice, the knockout mice exhibited more severe colitis with excessive weight loss, diarrhea, and intestinal bleeding (**Figure 1**). After a 7-day DSS administration, the differences in tissue integrity were analyzed using H&E staining as the histological method, and more aggravated tissue damage was found in the proximal, middle, and distal colons of the knockout mice than in WT mice (**Figure 2**). Furthermore, the excessive immune activation led to colitis with infiltration of various inflammatory cells such as macrophages and neutrophils. Immunohistochemical analyses using a number of markers for the immune cells were carried out to determine whether the α-gustducin deficiency affected immune cell infiltration. Comparative analysis showed that the infiltration of leukocytes, neutrophils, and macrophages was significantly increased in the knockout mice compared to WT control mice (**Figure 3**). Finally, some cytokine expression levels in the colons of DSS-induced colitis mice were determined using qPCR with gene-specific primers. Results showed that compared to WT mice, the knockout mice had higher expression levels of TNF and IFN-γ but lower expression levels of IL-5, IL-13, and IL-10; however, no difference was seen in the expression level of TGF-β1 between the knockout and WT mice (**Figure 4**).

FIGURE LEGENDS:

Figure 1: DSS administration renders more severe colitis in α-gustducin knockouts (KO). (A) Percentage of body weight loss: KO mice displayed significantly more body weight loss starting from day 3. (B) Colitis disease index based on the severity of diarrhea and rectal bleeding: KO mice showed significantly greater disease indices than WT controls. (C) Colon (upper panel) and spleen (lower panels) from representative WT and KO mice 7 days post-

DSS administration: KO mice had significantly shorter colons and larger spleens. Error bars represent SEM (*p < 0.05, **p < 0.005, ***p < 0.0005; ANOVA with post hoc *t*-tests). Scale bar = 1 cm. This figure has been modified from a previous publication[13].

Figure 2: α-gustducin KO mice show more severe tissue damage following DSS treatment. (A) H&E staining of colon tissues from WT and α-gustducin KO mice not treated with DSS (water) or treated with DSS for 7 days (DSS). (B) Tissue injury scores based on histological staining of colon tissues from WT and KO mice 7 days after DSS administration. DSS treatment induced some tissue damage in WT colons, which was much worse in the KO specimen. Error bars represent SEM (*p < 0.05). Scale bar = 50 μm. This figure has been modified from a previous publication[13].

Figure 3: α-gustducin KO mice display increased infiltration of immune cells in DSS-induced colitis. (A) Massive immune cell infiltration in the colons of DSS-treated KO mice. (B) Quantification of immune cell numbers: the percentage of immunostained areas divided by the total area of measured tissue based on image analyses. Error bars represent SEM. Scale bar = 50 μm. This figure has been modified from a previous publication[13].

Figure 4: α-gustducin KO mice display different expression levels of immune cytokines in DSS-induced colitis. DSS treatment increased expression of TNF and IFN-γ and decreased expression of IL-5, IL-13, and IL-10 in the KO mice colons compared to WT mice. Real-time quantitative RT-PCR was performed using gene-specific primers. β-actin was used as an internal control gene. Error bars represent SEM (*p < 0.05, **p < 0.005). This figure has been modified from a previous publication[13].

Table 1: List of primers used.

DISCUSSION:

This method can be employed to quantitively determine the effect of mutations of specific gustatory genes on inflammation in a DSS-induced IBD mouse model. To take full advantage, optimal induction of IBD is a key step. The development of colitis is affected by several factors, including mouse strain, housing environment, intestinal microflora, as well as the genes of interest. It is recommended to perform a pilot experiment with a small number of mice to test different dosages and durations of DSS administration. During the pilot experiment, gross symptoms such as weight loss, diarrhea, intestinal bleeding, and some microscopic changes such as tissue damage, inflammation associated with immune cell infiltration, and expression-level changes of cytokines should be analyzed and compared to control groups that have drinking water without DSS[16-19]. Severity of the induced colitis can be monitored by analyzing daily changes in mouse body weight and scores of the collected stool during the DSS-treatment period. After DSS treatment, the tissue damage can be assessed by H&E staining on frozen sections of the colon, whereas infiltration of immune cells and expression levels of cytokines can be identified and quantified using immunohistochemistry and qPCR. DSS

dosage, administration duration, and diet can be adjusted to reveal subtle effects of gustatory gene mutations on the colonic immune responses. However, the sensitivity of this method may still limit its application to studies on the minimal effects of some other genes. In this case, some modifications can be adopted; for example, by minimizing the variables from individual-specific intestinal microflora by administrating antibiotics.

The DSS-induced colitis model has been used the most extensively among chemical agent-induced IBD models, largely due to its simplicity, rapidity, reproducibility, controllability, and most importantly its similarities to human ulcerative colitis, which is useful in evaluating the effectiveness of human therapeutics[2]. One disadvantage researchers must be aware of is that T and B cells are not required for the development of colitis, which is different from development of the disease in humans. However, it may be useful for studying the role of the innate immune system in the development of acute colitis[2].

This study has established a DSS-induced IBD model using α-gustducin-deficient mice, which can be used to investigate novel functions of the G protein α subunit in gut mucosal immunity and inflammation. The results show that mice lacking α-gustducin are more susceptible to DSS-induced colitis and are accompanied by more severe symptoms, including tissue damage, excessive inflammatory responses, and altered expression of potent cytokines[13]. In agreement with recent findings indicating the involvement of taste-like chemosensory pathways in type II immune responses to gut parasites[20], α-gustducin and other taste signaling proteins may have novel and important functions in the intestinal immune balance. However, the exact molecular mechanisms underlying these skewed immune responses remain to be uncovered.

Furthermore, this method can be applied to study effects of other gustatory genes that are expressed in the colon *(e.g.,* the transient receptor potential ion channel Trpm5, which is critical to sweet, bitter, and umami tastes and expressed in human colonic cells[7]). Finally, the same strategy can be used to discover new functions of key proteins and factors in intestinal immunity and help evaluate the effectiveness of novel treatments of certain human diseases.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

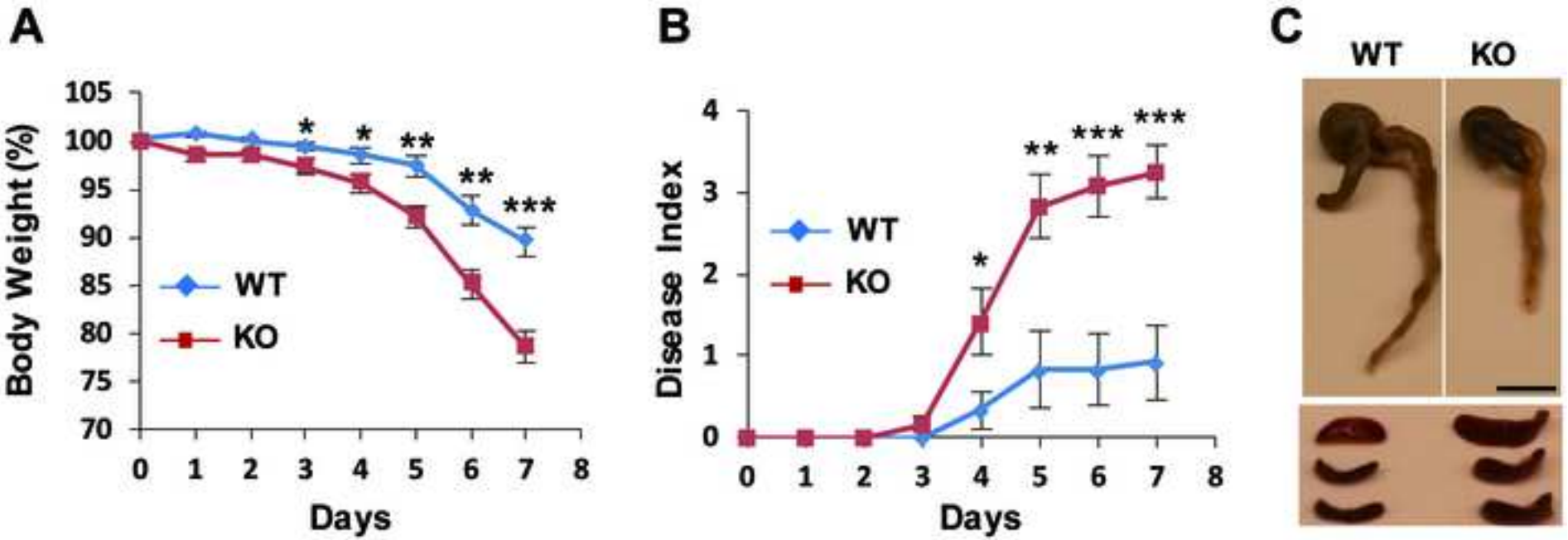

Figure 2

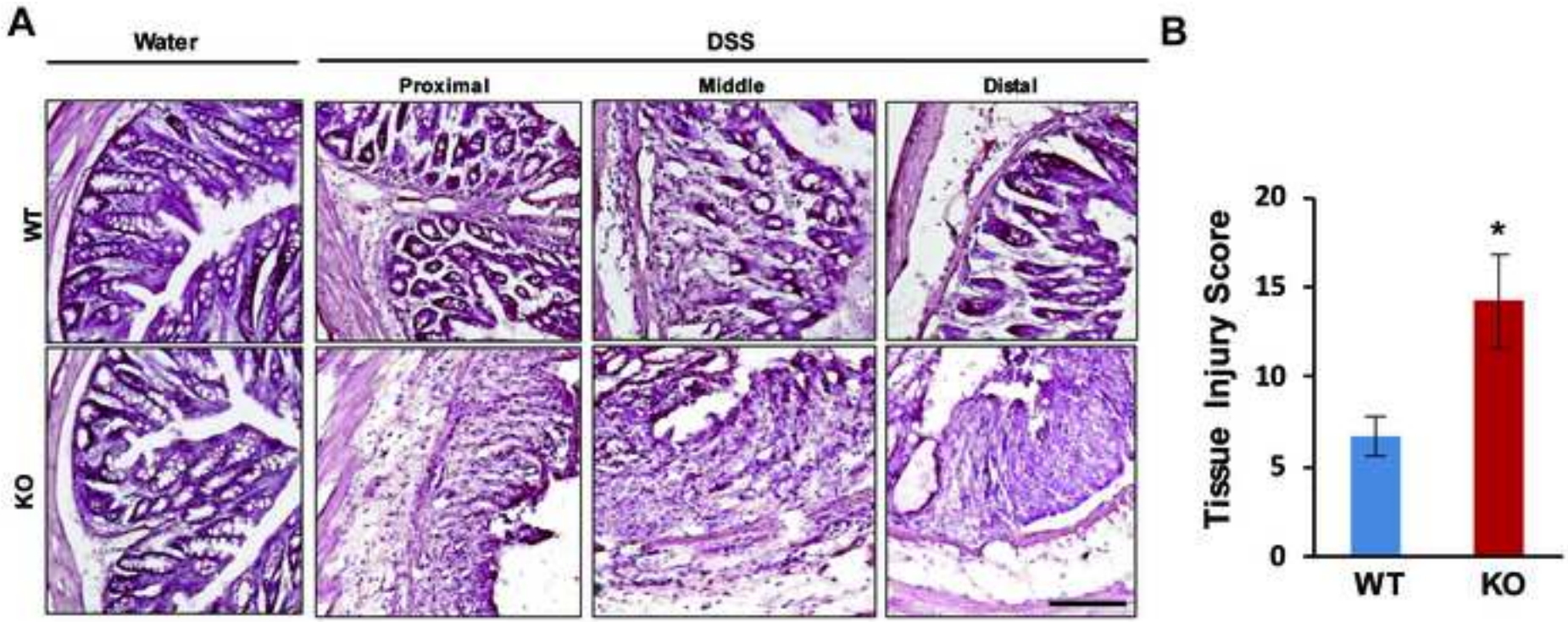

Figure 3

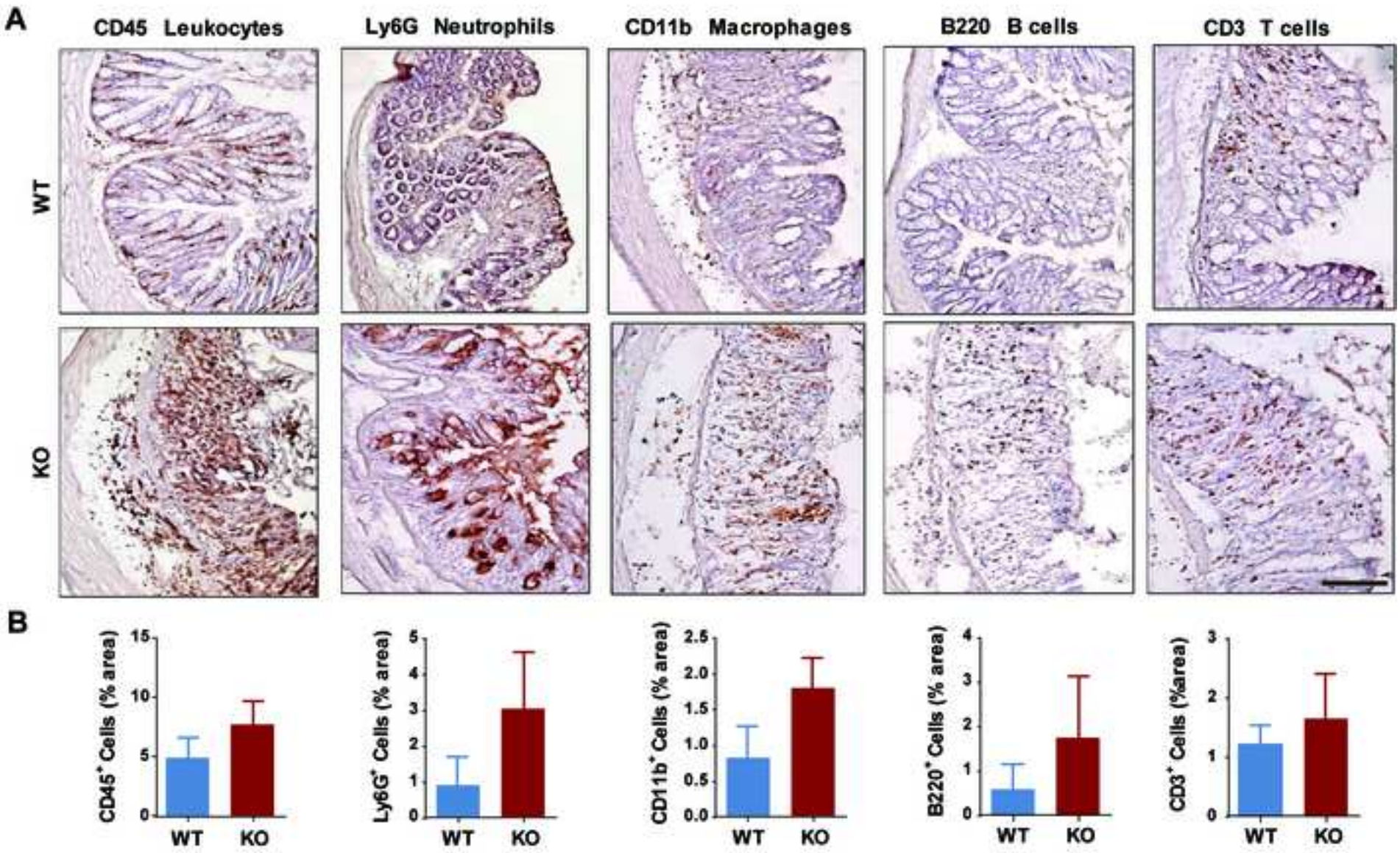

Figure 4

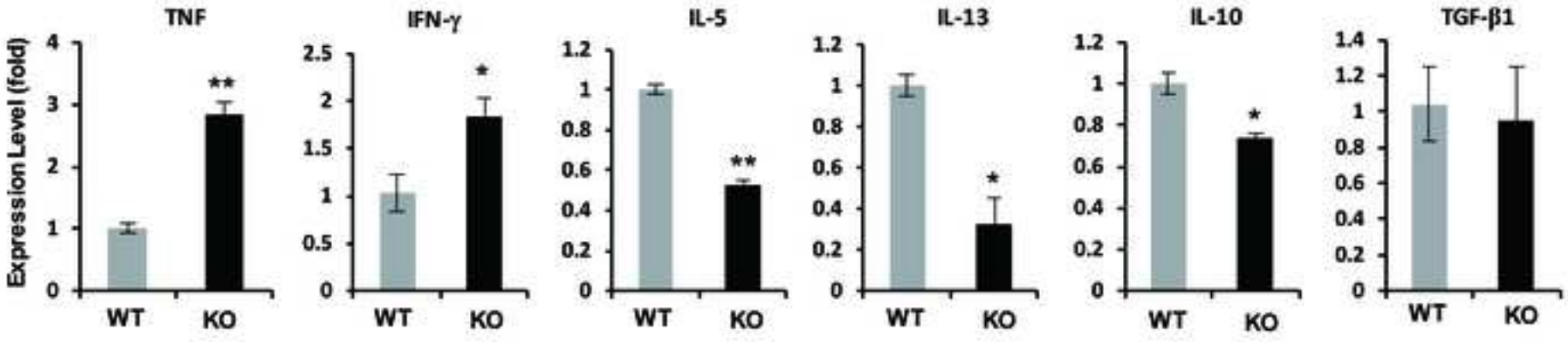

Table 1

		qPCR primers		
Gene	**Acession Number**	**Orientation**	**Sequence 5' to 3'**	**Product size (bp)**
β-actin	NM_007393	Forward	GATTACTGCTCTGGCTCCTA	142
		Reverse	ATCGTACTCCTGCTTGCTGA	
TNF	NM_013693	Forward	CTTCTCATTCCTGCTTGTGG	140
		Reverse	ATCTGAGTGTGAGGGTCTGG	
IFN-γ	NM_008337	Forward	AGCAACAGCAAGGCGAAAA	71
		Reverse	CTGGACCTGTGGGTTGTTGA	
IL-5	NM_010558	Forward	AGCAATGAGACGATGAGGCT	124
		Reverse	GCATTTCCACAGTACCCCCA	
IL-13	NM_008355	Forward	ACAAGACCAGACTCCCCTGT	128
		Reverse	TCTGGGTCCTGTAGATGGCA	
IL-10	NM_010548	Forward	AAGGCAGTGGAGCAGGTGAA	159
		Reverse	CCAGCAGACTCAATACACAC	
TGF-β1	NM_011577	Forward	AGAGAAGAACTGCTGTGTGC	176
		Reverse	GGGTTGTGTTGGTTGTAGAG	

Materials Table

Click here to access/download;Table of Materials;List of reagents.xlsx

Antibody	Company	Catalog
CD45	BD Biosciences	550539
CD3	BD Biosciences	555273
B220	BD Biosciences	550286
CD11b	BD Biosciences	550282
Ly6G	BD Biosciences	551459

Reagent	Company	Catalog
Dextran Sulfate Sodium Salt (DSS)	MP Biomedicals	2160110
Streptavidin-HRP complex	BD Pharmingen	551011
H&E Staining Kit	BBI Life Sciences	E607318
Phosphate Buffered Saline (PBS)	Sangon Biotech	B548117
FastStart Universal SYBR Green Master(ROX	Roche	4913850001
MMLV Reverse Transcriptase, GPR	Clontech,TaKaRa	639574
TaKaRa MiniBEST Universal RNA Extraction k	TaKaRa	9767
BD 10 ml Syringe	BD Biosciences	309604

Instruments and equipment

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scissors
forceps
centrifuge
qPCR machine
staining jars

Software

Imag-Pro Plus	Media Cybernetics, Inc.

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Title of Article: Abolishment of a taste signaling protein exacerbates gut inflammation in a mouse model

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Article Title: Abolishment of a taste signaling protein exaberates gut inflammation in a mouse model

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Dear Dr. Vineeta Bajaj,

Attached please fine our revised manuscript JoVE58668R1 with a new title "Investigating the Effect of Taste Signaling Protein Abolishment on Gut Inflammation in an Inflammatory Bowel Disease Model".

Thank you very much for the very helpful comments. My coauthors and I have revised according to your comments, and also corrected a couple of typos. Attached below is a point-to-point reply to each of your comments.

We hope that the revised manuscript is now acceptable. We are looking forward to hearing from you soon again.

Best regards,

Liquan Huang, PhD
College of Life Sciences
Zhejiang University

Point-to-point reply to editorial comments:

(the line numbers indicated below are those in the newly revised manuscript)

1. The editor has formatted the manuscript as per the journal's style. Please retain the same.

Reply: Thank the editor for formatting the manuscript as per the journal's style. Yes, it has been retained.

2. Please address all the specific comments marked in the manuscript.

Reply: Yes, all the specific comments marked in the manuscript has been addressed. Specifically:

1) Please make the title more concise and please do not use abbreviations.
   Reply: The title has been revised and the new title is more concise and does not have abbreviations, which is: "Investigating the Effect of Taste Signaling Protein Abolishment on Gut Inflammation in an Inflammatory Bowel Disease Model".

2) Mouse model is a bold term since no specific test is done to claim it as a model- please tone it down.
   Reply: Yes, it has been changed to "an inflammatory bowel disease model", which is generated by the dextran sulfate sodium induction and has been referred to as an IBD model in the field. We attempt to combine this prototype model with the gustatory gene knockout technology to make it more powerful.

3) Long Abstract: Please reduce the length as it is more than 300 word limit.

Reply: Yes, the Abstract has been reduced to 299 words.

4) Line 108: Citation for the sentence.
Reply: Yes, a new reference is cited here.

5) Lines 144 and 150: Not in imperative tense, hence converted to a note. Please check.
Reply: Yes, it is fine. Thanks for converting them into two notes.

6) Line 158: Do you have controls as well?
Reply: Yes, we do.

7) Line 162: How-manually?
Reply: Yes, manually.

8) Line 163: How? Is there a citation to refer?
Reply: Yes, a citation has been added.

9) Line 168: Citation?
Reply: Yes, a reference has been added.

10) Line 179: How big?
Reply: 3 cm long.

11) Line 200: So, you do not wear PPE before? Please move this sentence to the beginning of the protocol.
Reply: Thanks for pointing out. Yes, it has been moved to the beginning of the protocol (Line 137).

12) Line 210: We cannot have paragraph of text in the protocol section. Please make substeps so that each individual step has 2-3 action items. Please use imperative tense throughout.
Reply: Yes, it has been divided into 4 substeps. And imperative tense has been used throughout.

13) Line 226: Please move this to the Table of materials.
Reply: Yes, it is in the Table of Reagents.

14) Line 233: Please move the commercial term to the table of materials. We cannot have commercial terms in the manuscript. Please use generic term instead.
Reply: Yes, it is in the Table of Reagents. And a generic term is used instead.

15) Line 257: Is there a reference for the scoring system?
Reply: Yes, two references have been cited.

16) Line 271: Non-ionic detergent?
Reply: Yes, it is.

17) Line 290: Please move the commercial term to the table of materials. We cannot have commercial terms in the manuscript. Please use generic term instead.
Reply: Yes, it is in the Table of Reagents. And a generic term is used.

18) Line 290: Please use imperative tense throughout and use generic term instead. Please also explain how you do the procedure-graphical user interface, button clicks on the software etc.
Reply: Yes, imperative tense and generic term are used throughout. It has been revised to explain how the immunoreactive cells and the tissue areas are identified and measured.

19) Line 303: Please convert to imperative tense.
Reply: Yes, it has been converted to imperative tense.

20) Line 304: Please move the commercial term to the table of materials. We cannot have commercial terms in the manuscript. Please use generic term instead.
Reply: Yes, it is in the Table of Reagents. And a generic term is used.

21) Line 307: Since this is highlighted do you want to show the entire RNA isolation procedure? Else this can be used as a connective statement.
Reply: Yes, it can be used as a connective statement. We do not want to show the entire RNA isolation procedure.

22) Line 312: Is this correct? Changed to generic term. Please check.
Reply: It is mostly correct. More commonly, it is called microspectrophotometer.

23) Line 320: Fluorescent green dye?
Reply: Yes, DNA-binding fluorescent cyanine dye.

24) Line 322: Analyzed or Run? Please use imperative tense.
Reply: "Run" is better. It has been changed to imperative tense.

25) Line 331: Please tone down the novel mouse model claim.
Reply: Yes, it has been changed to "a DSS-induced IBD procedure".

26) A typo is corrected: Line 394: "Scale bar: 1 mm" has been changed to "Scale bar: 1 cm".

3. After formatting, please ensure that the highlight is no more than 2.75 pages including headings and spacings.

Reply: Yes, we have made sure that the highlighted part is no more than 2.75 pages.

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