Responses to Reviewer 2 comments:

Dear reviewer, thank you very much for your timely and valuable comments, please find the point-by-point responses as follows:

Point 1: Abstract, line 13, Introduction, line 58. The statement that "There is no cure for IBD" is not true. As the authors reported in the next lines, numerous methods of treating IBD are used. The problem is the lack of methods for a complete and permanent cure of this disease. Therefore "There is no cure for IBD" should be replaced with "There is no effective therapy for a complete and permanent cure for IBD".

Response 1: We have revised it.

Point 2: Abstract, line 22 and section "Materials and Methods". The authors should at lest 5 experimental groups: (1) Control; (2) Soluble Protein Hydrolysate (SPH) without colitis; (3) Colitis; (4) Colitis +Control peptide (CP); (5) Colitis + SPH. A group SPH without colitis is necessary in order to know whether SPH is causing negative effects in the colon and other organs of the body.

Response 2: We are grateful for the insightful comment. In the pilot experiments, we actually set up 6 groups including 1) Control, 2) Control peptide only (CP), 3) SPH only, 4) Colitis only, 5) Colitis+CP and Colitis+SPH, but we found out that SPH and CP had no obvious negative effects on the intestinal tissues or body weight of these mice. In addition, each mouse needed to be kept in one cage to collect feces for biochemical injury assay, which requiring more space/cost in the animal facility, so SPH only and CP only groups were removed in the real experiments.

Point 3: Abstract, line 24. The authors should provide the form of SPH administration and time relation between TNBS and SPH administration.

Response 3: Colitis was induced by cutaneous sensitization with 1% TNBS on day -8 followed by 2.5% TNBS enema challenge on day 0. Control peptides and SPH were provided to the mice in the Colitis/CP or Colitis/SPH group respectively by drinking water at the final concentration of 2% W/V daily from day -10 to day 4.

Point 4：Abstract, lines 28-29. What does "SPH pre-treatment improved the DAI score and tissue injury" mean? Authors state should unequivocally whether administration of SPH increased or decreased DAI and tissue damage. This comment also applies to the Results and Discussion sections.

Response 4: We have changed "improved" for "decreased".

Point 5： Abstract, line 31 and next parts of the manuscript. All abbreviations should be presented in their full name at the point where they appear for the first time. Full

names of abbreviation presented in the Abstract should be repeated in the body of the manuscript at the place of the first use, as well as in Tables and Figure legends. Tables and Figures should be understandable without carefully reading the text of the manuscript. On the other hand, the number of abbreviations should be reduced. The excess of abbreviations makes the manuscript difficult to understand.

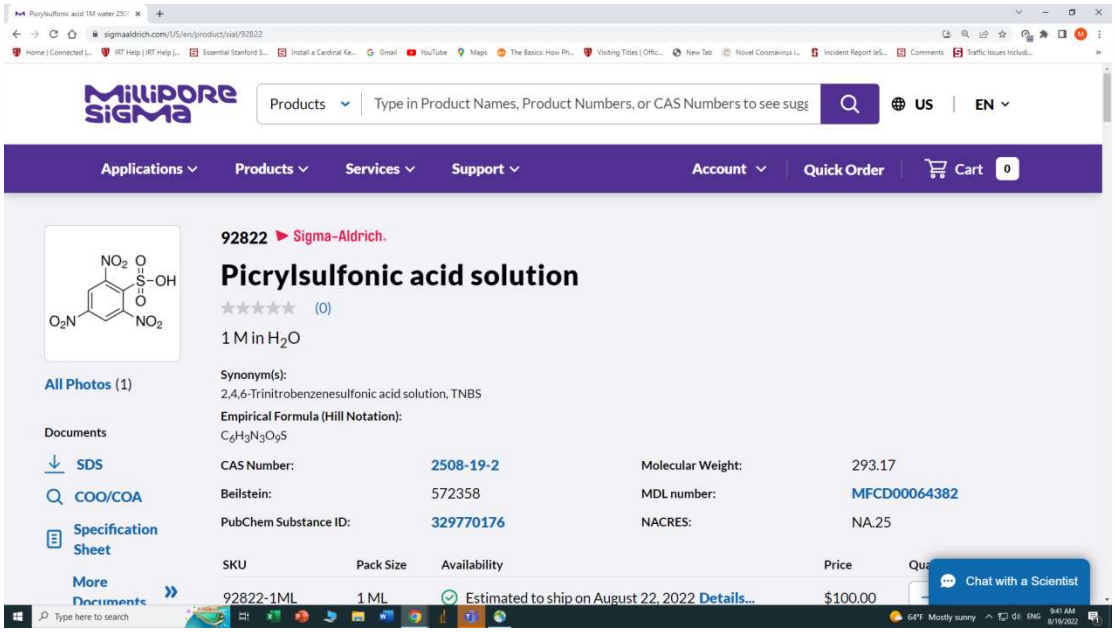
Response 5: We have revised them.

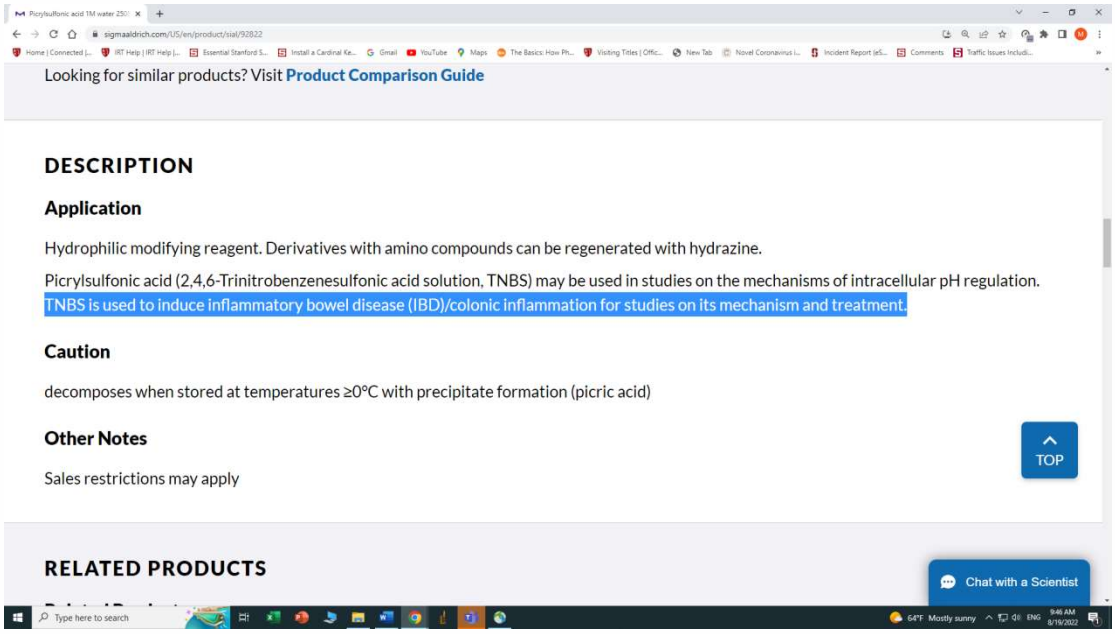
Point 6：Materials and Methods, line 82 and next pats of the manuscript. In the case of all drugs, regents, animals, assays and equipment, the authors should provide general and trade name of the reagent or equipment, manufacturer's name, city, and country.

Response 6: We have replenished some relative information.

Point 7：Materials and Methods, line 87. Since 1975 is not available on the market.

Response 7: Do you mean TNBS? We think it is the most possible reagent you mentioned as we didn't find any reagent in line 87 "shown in Figure 1A, mice were randomly divided into four groups: Control, Colitis" maybe because of wrong line. TNBS (cat# 92822) is purchased from Sigma-Aldrich Corp., St. Louis, MO, USA.





Point 8：Materials and Methods, line 85. The authors stated That “Mice had ad libitum access to water and food”. Were the animals fasted prior to the administration of TNBS enema?

Response 8: The animals were not fasted prior to the administration of TNBS enema.

Point 9： Material and Methods. How did the authors prepare 2.5% TNBS solution? The same vehicle should be used in control and SPH without colitis groups. All these data should be presented in the manuscript.

Response 9: We have replenished and presented the relative description in the methods.

The experimental colitis model was generated through 1% TNBS pre-sensitization and 2.5% TNBS enema stages. Freshly mixed acetone and olive oil in a 4:1 volume ratio by vortexing rigorously. Mixed 4 volume of acetone/olive oil with 1 volume of 5% (wt/vol) TNBS solution in H_2O to obtain 1% (wt/vol) TNBS for presensitization, and mixed 1 volume of 5% (wt/vol) TNBS solution with 1 volume of 70% ethanol to obtain 2.5% (wt/vol) TNBS for enema. The above prepared solution containing TNBS was replaced by the corresponding solution containing Phosphate Balanced Saline (PBS) in the Control group.

Point 10： Material and Methods, line 90. The authors should in detail present haw the animals were euthanized.

Response 10: We have replenished in the manuscript as follow: “Mice were sacrificed in a CO_2-euthanization station chamber equipped with an automatic air flow-regulator that is periodically inspected by the Institutional Animal Care and use committee (IACUC) at the university. ”

Point 11：Material and Methods, line 92. The authors should present more details on control peptides such as their biological source, characteristics, average molecular weight, and form provided by the manufacturer. The same information should be provided for SPH. In addition, in the case of SPH, the authors should declare whether SPH contained only peptides and proteins, or it also contained lipid admixture.

Response 11: Control peptides and SPH (both provided by Hofseth BioCare AS company, Norway) have a similar average molecular weight profile and nutritional calories. The control peptide powder is a dry, water-soluble powder provided by vital protein collagen of bovine source, while SPH is a dry, water-soluble powder which is sourced from salmon. Both powders contain greater than 98% protein and less than 0.5% lipids. Control peptides and SPH were added into drinking water at the final concentration of 2% W/V and provided to the mice in the Colitis/CP or Colitis/SPH group respectively daily from day -10 to day 4.

Point 12：Material and Methods, lines 95-97. Authors should provide the protocol approval number, as well as the date of its issue and the expiry date of its validity.

Response 12: All experiments were conducted under a protocol approved by Stanford University School of Medicine Institutional Animal Care and use committee (IACUC) in accordance with NIH guidelines (Approval code: A3213-01. Approval date: 02/19/2020 (Through 01/20/2023)).

Point 13：Figure 1. Figure 1A concerns study design and for this reason should be presented in Materials and Methods. However, Figure 1B-D presents result of the study and for this reason should be presented as a separate Figure in the section Results.

Response 13: Figure 1 has been rebuilt according to your comment.

Point 14：Figure legends. The authors should provide the number of observations in each experimental group and state how the results are expressed in the bars.

Response 14: We have added relative description in methods and figure legends, such as “31 mice were randomly divided into 4 groups: Control (no colitis, n=7), Colitis (n=8), Colitis/CP (with control peptide treatment, n=8) , and Colitis/SPH (with SPH treatment, n=8) ” in methods to provide the number of observations in each experimental group. Non-normally distributed data were presented with medians and quartiles (Q1:Q3) as shown in Figure 5B, 7A, 7C and quantitative data that fit a normal distribution are presented as means ± SD as shown in other figures. These results have been described in figure legends.

Point 15：Figure 2-6. Figure legends are not for presenting the interpretation of results, but they are for presenting what is shown in the figures.

Response 15: We have revised them.

Point 16： Discussion. The authors should present the importance of animal model of IBD and their limitations (PMID: 27206158; PMID: 34938152). These models are useful in the research concerning etiopathogenesis of IBD and new therapeutic concepts in this disease. It should be noted, however, that there are numerous differences between these models and clinical IBD. Therefore, the results obtained with these models may or may not be consistent with clinical effect.

Discussion. The authors presented some mechanism involved in the protective/therapeutic effect of SPH in TNBS-induced colitis. However, the authors should state that the primary mechanism of protective effect of SPH is unknown. Proteins in the digestive tract are digest into free amino acids, dipeptides, and tripeptides, and in this form are absorbed, which takes place mainly in the small intestine. Thus, there is not direct effect of SPH on the mucosa in the colon. The authors should present the concept that the protective effect of SHP is probably due to the presence of amino acids and short-chain peptides in SPH. Food is essential factor in the regulation of the renewal of gastrointestinal mucosa. Food intake stimulates cell proliferation and the growth and renewal of gastrointestinal mucosa. In the case of fasting, the renewal of gastrointestinal mucosa is inhibited leading to its atrophy (PMID: 8527816; PMID: 3710060), and the degree of weight loss in the digestive tract organs is greater than the body weight loss (PMID: 5659346). Also, in the case of parenteral nutrition, when there is no food in the gastrointestinal tract, the cell renewal of the gastrointestinal mucosa is inhibited, and hypoplasia develops (PMID: 4214726). On the other hand, refeeding leads to a rapid increase in cell proliferation in the gastrointestinal tract and the restoration of its structure (PMID: 1664265; PMID: 6142558). The mechanisms stimulating the renewal of the mucosa after food intake are mainly based the reflex of the enteric nervous system and release of gastrointestinal hormones. Among the gastrointestinal hormones that stimulate the growth of the colon, gastrin plays a special role (PMID: 25716961). Gastrin is produced and released by G cell in the stomach. The presence of small peptides and aromatic amino acids in the stomach is a main factor that acts directly on the G cells and stimulates the release of gastrin (PMID: 6806140; PMID: 25716961). Digestion of proteins in the digestive tract begins within the stomach with participation of pepsin. However, it should be stated that the essential digestion of proteins take place in the small intestine, after leaving the stomach, with the participation of pancreatic and intestinal peptidases. Moreover, pepsin is an endopeptidase that digests internal peptide bonds (PMID: 20522896). Therefore, the release of amino acids and short peptides in the stomach from food is limited. On the other hand, SPH is a product of enzymatic protein hydrolysis and most likely contains a high concentration of amino acids and short peptides, which leads to a significant release of gastrin, thereby significant stimulating the renewal of colonic mucosa and increasing the mucus

production in this intestine. This concept is consistent with previous observations that also ghrelin, another factor that stimulates the growth of gastrointestinal mucosa, exhibits a protective and therapeutic effect in experimental colitis evoked by TNBS (PMID: 16697735; PMID: 19617644), dextran sulfate sodium (DSS) (PMID: 23549326; PMID: 25634696), as well as acetic acid (PMID: 26769837; PMID: 28538694). After presenting this concept with appropriate references, the authors should state this hypothesis should be verified in future research.

Response 16: Thank you very much for your insightful comment and we add some discussion as follows:

This is the first study about the protective role of SPH in IBD mice model. However, the primary mechanism of protective effect of SPH is unknown. As we know, proteins are digested into free amino acids, dipeptides, and tripeptides in the digestive tract, and are absorbed in this form mainly in the small intestine. Food is essential factor in the regulation of the renewal of gastrointestinal mucosa. Food intake can stimulate cell proliferation and the growth and renewal of gastrointestinal mucosa. In the case of fasting or parenteral nutrition, the renewal of gastrointestinal mucosa is inhibited leading to its atrophy or hypoplasia[50,51], while refeeding leads to a rapid increase of cell proliferation in the gastrointestinal tract and the restoration of its structure[52,53] due to the reflex of the enteric nervous system and the release of gastrointestinal hormones that stimulate the growth of the colon. In which, gastrin produced and released by G cell in the stomach plays a special role[54]. The presence of small peptides and aromatic amino acids in the stomach is a main factor that acts directly on the G cells and stimulates the release of gastrin[54,55]. Although digestion of proteins in the digestive tract begins within the stomach with participation of pepsin, the essential digestion of proteins take place in the small intestine with the participation of pancreatic and intestinal peptidases after leaving the stomach. Moreover, pepsin is an endopeptidase that digests internal peptide bonds[56]. Therefore, the release of amino acids and short peptides in the stomach from food is limited. SPH is a product of enzymatic protein hydrolysis and contains a high concentration of amino acids and short peptides which may lead to a significant release of gastrin stimulating the renewal of colonic mucosa, increasing the mucus production in the intestine, and improving the barrier function of the intestinal mucosa. This concept is consistent with previous observations that also ghrelin, another factor that stimulates the growth of gastrointestinal mucosa, exhibits a protective and therapeutic effect in experimental colitis evoked by TNBS[57,58], dextran sulfate sodium (DSS)[59,60], as well as acetic acid[61,62].

Above is our hypothesis about the primary mechanism of SPH that should be verified in future research. There are also other limitations exsiting in this study, such as the difference in the phenotype and function of tissue macrophages between the groups, and the protein expression levels and activity of antioxidant HO-1, SOD, NQO-1 and FTH1. In the future, we can conduct in-depth research from these aspects. In addition,

although the animal models are useful in the research concerning etiopathogenesis of IBD and new therapeutic concepts in this disease, there are numerous differences between animal models and clinical IBD. Therefore, the results obtained with animal models may or may not be consistent with clinical effect and need to be verified in clinical study.