Replies to Editor's Comments (in Tracking Mode)

Comment 1: This study did not utilize animals or animal tissue.

Comment 2: See enclosed signed letter from senior author re: absence of conflicts of interest by all authors.

Comment 3: An Audio Core Tip has been prepared and included as requested.

Comment 4: See enclosed signed letter from senior author re: animal/human use and biostatistics.

Comment 5: A new section on Comments has been prepared and included as requested.

Comment 6: The location of Figure 8 has been marked in the text.

Replies to Reviewer 1's Comments:

1. As the reviewer suggested, we have substituted one-way ANOVA statistical significance testing for Student's t tests wherever experiments (Figures 2, 5, 7, 8 and Table 1) contained multiple conditions. This occasionally nullified conclusions of statistical significance ($P < 0.05$) for a few specific conditions, but did not alter overall conclusions in the study. This is specified now not only in the appropriate figure legends but also in Methods.
2. We pondered combining Figures 1-3 but elected not to because we believe that it will be easier and clearer for the readers as separate Figures. However, in keeping with the theme of the Reviewer's comments, we significantly reduced the verbiage in the legends to Figures 2 and 3 by referencing the legend to Figure 1 in these other Figure legends. The writing is now more concise and streamlined.
3. This was definitely hematoxylin and eosin staining. We have discussed the color issue with a histologist, and we believe that the modifications of true color caused by electronic digitizing of the histology and the further color changes coming from the actual printing process have made it seem as though different staining was performed. However, this was truly H&E. Our microscopic slides do show the characteristic pink staining of cytoplasm and dark purple staining of nuclei that would be the hallmark characteristics of H&E.

Replies to Reviewer 2's Comments:

1. The Reviewer maintains that our model "does not represent the normal clinical situation," because we do not consider the endothelial barrier and PMN migration in our model. But if that was true, it would mean the entire field of gastrointestinal cell culture (CACO-2, HT-29, T84, etc.) is without merit — as far as these models can relate to IBD? That viewpoint would negate 300 papers that have been published since 1980, as indexed in PubMed, i.e., publications utilizing

these epithelial cell cultures for investigations relevant to IBD! The Reviewer also criticizes the *epithelial nature* of our model — as it specifically applies to IBD — because we do not consider white blood movement across the *endothelial* barrier from the bloodstream. It is true that endothelial permeability could be highly important in IBD and is a very underrepresented area in IBD research. However, it would be equally wrong to dismiss the importance of the epithelial barrier in protecting the interstitium AND the vasculature from lumen-based antigens and pathogens in the gastrointestinal tract in IBD. The endothelium has no role in this, since we are talking here about antigen presentation only to the interstitial fluid space. The CACO-2 model we use here is excellently suited for studying this and has been used in almost 200 IBD-oriented studies since the 1980s.

2. The Reviewer maintains that our berberine data lack novelty because they were earlier reported in *PLOS One*. It is very important to point out here that the manuscript he/she refers to *was our own publication*, and it does not report on berberine effects on cytokine or peroxide-induced damage of the epithelial barrier in the exact comparative manner that our current manuscript here does. However, we now highlight the novelty of the findings more explicitly in the revised Discussion.
3. The Reviewer states (without any references from the published literature) that berberine is only active from the bloodstream. We do find evidence for this statement in the biomedical literature, BUT it needs to be stated clearly here that we ourselves — as stated in our current manuscript — also exposed our cell layers to berberine from the basal-lateral (bloodstream) side — a fact that the Reviewer seems to have totally missed. In every experiment we performed with berberine, we exposed our cell layers to berberine simultaneously from both cell surfaces. In our current paper, we make no claims concerning polarity of berberine action, but (for now) forestall this issue entirely by presenting berberine to both cell surfaces, an important aspect of our methodology that we now state even more clearly on pg 9 of the revised manuscript. In a new paragraph in the revised Discussion (pgs 15-16), we now describe these polarity aspects of berberine's action on gastrointestinal epithelia.
4. A minor comment raised by the Reviewer was the potential confusion surrounding the use of the term "cytomix" along with the terms "combination" or spelling out the actual formulation of TNF/IFN/IL1b. For the record, the term "cytomix" is never used in Figure 2. In fact "cytomix" is never used in any figure legend here. The term cytomix is now specified clearly on pg 7 in the revised Methods and again on pg 10 in Results. The word "combination" was used in Figure 3, and we have now changed that to the term "cytomix" since we have now more carefully defined it.
5. As described above in the reply to Reviewer 1, ANOVA followed by Tukey's post hoc testing has replaced Student's t test in Figures 2, 5, 7 and 8, and Table 1, where multiple condition comparisons were in effect. Given that our n value never exceeds 8 cell layers, we feel that it does not warrant a consideration of

Gaussian distribution of the data, as the limited number of cell layers belies such consideration. In other words, our data could be Gaussian, but the limited number of data points may not show it. However, note that our revised statistical treatment occasionally resulted in an inability to claim statistical significance using ANOVA, whereas Student's t allowed it. This was because of occasional failure of the Normality test in ANOVA. However, the essential conclusions of the manuscript were not changed.

Replies to Individual Reviewer Comments

Reviewer 1:

1. "Figure 1 and 2 can be combined because some parts of data appear duplicate."

We ask that Figures 1 and 2 not be combined so as not to complicate the conclusions for the readers. The data used in these figures are not completely the same, and we feel remaining as separate figures helps to emphasize the variability that is seen from experiment to experiment for the effect of TNF alone on mannitol flux.

2. "In exposure to TNF and IFN experiment, dosage of IFN should be fixed not between 100 to 200ng."

The dosage of IFN was fixed in a given experiment. We agree that cytokine doses among experiments should have remained consistent; however, for feasibility we started to reduce concentrations because we were well beyond the concentration needed for maximum effect. Considering we were using saturating levels of cytokines in a receptor-mediated response, and achieving the same result, we concluded that it was reasonable to combine the data. This is now specified in the manuscript.

3. "In exposure to TNF, IFN and IL1B experiment, data of exposure to IL1B alone is lacking. In addition, post-confluent date (21-day not 7-day) and IFN dosage (150ng/ml not 200ng/ml) are different from exposure to TNF and IFN experiment. Why?"

The goal of these studies was not to investigate the effects of individual cytokines, but instead to create a model of graded leak; and therefore we were primarily concerned with the additive effect of IL1B. When used alone we observed that IL1B did not generate leak any greater than TNF or IFN achieved individually; however, when IL1B was included in the cytokine mixture (with TNF + IFN) it opened up the leak to not only Na^+ and Cl^- but also to the small non-electrolyte mannitol. In our studies, we did not observe a difference between the responses of 7- and 21-day post-confluent CACO-2 cell layers to cytokines. Also, as said in response to the second point above, cytokine doses were reduced, but remained above the maximum effect concentration. These points are now included in the manuscript.

4. "In exposure to cytomix and hydrogen peroxide experiment, two different dates (7 and 21-day post-confluent) are presented. Which is the date for Figure 5? And, how about mannitol or lactulose leak in same condition?"

Both 7- and 21-day post-confluent cell layers are represented in Figure 5. As mentioned previously, barrier function at these days are highly similar in CACO-2 monolayers, and their response to cytokine treatment was indistinguishable. Also, given that mannitol and lactulose are much smaller than PEG, cytomix and hydrogen peroxide treatment of CACO-2 cell layers resulted in extreme leak of these molecules, which is pointed out in Table 2.

5. "In transepithelial leak of EGF experiment, the dosage of TNF (50ng/ml not 200ng/ml), IFN (100ng/ml not 200ng/ml) and H2O2 (1mM not 2mM) and exposure time for H2O2 (3hr not 5hr) are different from previous experiment. Why?"

As mentioned previously, we scaled back on time and reduced concentrations to 50ng/ml TNF, 100ng/ml IFN, and 1mM H_2O_2 as these were still concentrations and incubation times considered to achieve maximum effect.

6. "What is the definition of apoptosis in Figure 6? Nucleus concentration and fragmentation cannot be found."

We feel Figure 6D does show evidence of condensed chromatin in certain cells, as well as occasional nuclear fragmentation (apoptotic bodies). We agree that, by itself, this would be insufficient evidence, but together with cytoplasmic blebbing and cell detachment, this seems a reasonable claim.

Reviewer 2:

"The authors should clarify their objectives in the introduction."

We understand the reviewer's concern and have now stipulated individual objections in the introduction as follows:

The objectives of the following study were to: 1) observe the effects of the proinflammatory cytokines, TNF, IFN, IL1B, and H2O2, alone and in combination, on CACO-2 barrier function in order to create an in vitro model of graded leak that can reflect the clinical situation in IBD at different sites along the intestinal mucosa; 2) determine if this leak allows for barrier breakdown to biologically active proteins such as EGF; 3) determine if a previously described nutraceutical capable of barrier protection can in fact reduce barrier compromise under these extreme conditions.

Reviewer 3:

"The manuscript is well written, but there are some uncertainties that are needed to be clarified. In the exposure to cytomix experiment, the group of IL1B exposure alone is lacking. There are inconsistencies between some doses. These issues must be explained or corrected."

As mentioned more thoroughly in response to Reviewer 1's Comments, the IL1B alone condition was not shown, as our focus was not on the effects of individual cytokines, but rather a combination that would generate consistent leak to mannitol, which was ultimately achieved by adding IL1B to TNF and IFN. Also, we understand it would have been favorable for the doses to remain consistent between experiments, and we have now noted in the manuscript that all concentrations used were beyond the maximum effect concentration and therefore achieved the same result.