

A microbiota-IPA axis facilitates intestinal stem cell-mediated regeneration in colitis through a Hopx-associated program

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Version 0:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

In this new report from Zhang et al, the research team highlights a new mechanism involving the microbiota and metabolites (e.g. indole-3-lactic acid, ILA, and indole-3-proponic acid, IPA) role in regeneration of intestinal epithelial cells (IECs) during injury (e.g. colitis/IBD). Using a combination of IBD human data and several murine models involving intestinal injury, results showed a mostly consistent decrease in *Blautia coccoides* (BC), which correlated with decreases in IEC-derived B-hydroxybutyrate (BHB), ILA/IPA, ILA/IPA producers. Using several genetic knockouts and/or conditions (e.g. monocolonization, bacterial metabolite supplementation, inoculation with killed/heat-inactivation of bacteria of interest), researchers concluded that there was a synergistic effect of microbiome (BC and commensal IPA-producers) that converted tryptophan-derived ILA into IPA via bacterial genes (phenylacetate dehydrogenase, *fldH*) that promoted BHB in IECs to activate HOPX+ reserve intestinal stem cells (rISCs) to promote regeneration of Lgr5+ ISC (e.g. epithelial cell repair).

Overall, this is well-designed study with significant amount of data. The authors are commended for consideration and execution of elaborate studies to define the mechanism presented herein (microbiota-IPA/HOPx axis). The manuscript presents an intricate mechanism that is convincing and potentially novel, with significant implications on IBD-related research, in addition to microbiome/intestinal injury related diseases. While at times perhaps oversimplified (e.g. BC is not significantly altered in some study IBD cohorts - suggesting a far more complex system than presented), this study and results are impressive in a well-written report from the research team.

There were some areas of concern that could be addressed, which include:

Major:

1. In Extended Data Fig. 1i (referenced in Line 138 of main text), are you referring to macrophages, neutrophils, and dendritic cells as lymphocytes? If so, this should be corrected. These are examples of leukocytes, but not lymphocytes (e.g. myeloid lineage).
2. In Line 154-155 of main text, it is stated that there are "no baseline differences at D0 (Fig. 2c-e). This seems to be rather misleading statement as it appears there are differences in the genes of interest (e.g. Hopx) at baseline in the two different groups. This should be expected perhaps after two pre-treatments with the bacteria. This statement also seems contradicted later in line 173, as it states constant increase in Hopx across all post-DSS timepoints
3. The overall feeling on the Discussion section is it presents a weak point in the current manuscript. It is suggested that the authors avoid restating results and referring to figures (this is more appropriate only in Results section) and instead focus on highlighting how these data relate to current literature. It is also recommended that limitations be discussed more clearly. While the authors state the limitation of the bacterial gene mutations in results, please elaborate in Discussion on limitation of using *E. coli* instead of the bacterial species focused on in the current report (BC). In addition, while the authors state that they used both sexes and observed no sex differences in studies, some studies only used female mice (e.g. Line 966 - bacterial cross-feeding). It is actually surprising that no sex differences were observed since it is well-known that the colitis model used prominently in the study (DSS) is known to be more severe in male B6 mice than females (PMID: 25962374). Also, IBD is known to have sex differences (PMID: 39962330), so it is concerning that the studies herein do not highlight any of this.

Minor:

1. Line 89/104 - IBD should be abbreviated, as abbreviation was established earlier (Line 44).
2. Line 106 - Should be "previous", not "pervious".

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Reviewer #4 (Remarks to the Author):

In this new report from Zhang et al, the research team highlights a new mechanism involving the microbiota and metabolites (e.g. indole-3-lactic acid, ILA, and indole-3-propionic acid, IPA) role in regeneration of intestinal epithelial cells (IECs) during injury (e.g. colitis/IBD). Using a combination of IBD human data and several murine models involving intestinal injury, results showed a mostly consistent decrease in *Blautia coccooides* (BC), which correlated with decreases in IEC-derived B-hydroxybutyrate (BHB), ILA/IPA, ILA/IPA producers. Using several genetic knockouts and/or conditions (e.g. monocolonization, bacterial metabolite supplementation, inoculation with killed/heat-inactivation of bacteria of interest), researchers concluded that there was a synergistic effect of microbiome (BC and commensal IPA-producers) that converted tryptophan-derived ILA into IPA via bacterial genes (phenylacetate dehydrogenase, fldH) that promoted BHB in IECs to activate HOPX+ reserve intestinal stem cells (rISCs) to promote regeneration of Lgr5+ ISC (e.g. epithelial cell repair).

Overall, this is well-designed study with significant amount of data. The authors are commended for consideration and execution of elaborate studies to define the mechanism presented herein (microbiota-IPA/HOPX axis). The manuscript presents an intricate mechanism that is convincing and potentially novel, with significant implications on IBD-related research, in addition to microbiome/intestinal injury related diseases. While at times perhaps oversimplified (e.g. BC is not significantly altered in some study IBD cohorts - suggesting a far more complex system than presented), this study and results are impressive in a well-written report from the research team.

There were some areas of concern that could be addressed, which include:

Major:

1. In Extended Data Fig. 1i (referenced in Line 138 of main text), are you referring to macrophages, neutrophils, and dendritic cells as lymphocytes? If so, this should be corrected. These are examples of leukocytes, but not lymphocytes (e.g. myeloid lineage).

> We thank the reviewer for raising this important point regarding the precise classification of immune cell types. The reviewer is absolutely correct: macrophages, neutrophils, and dendritic cells belong to the myeloid lineage and should be referred to as leukocytes (or innate immune cells), not lymphocytes. We sincerely apologize for this oversight in terminology and have corrected the text accordingly.

Specifically, in the sentence on Line 138-140, we have revised:

Original:

“Notably, flow cytometric analysis revealed no detectable shifts in colonic lamina propria mononuclear cell populations (cLPMCs), including macrophages, dendritic cells, neutrophils, and CD4⁺/CD8⁺ T-cell subsets at baseline or D7 post-injury (Extended Data Fig. 1i) (cite Manuscript 1 here for gating strategy).”

Revised:

“Notably, flow cytometric analysis revealed no significant alterations in key colonic lamina propria immune cell populations, including leukocytes (macrophages, dendritic cells, and neutrophils) and lymphocytes (CD4⁺ and CD8⁺ T cells), either at baseline or D7 post-injury (Extended Data Fig. 1i) (gating strategy as described in Manuscript 1).”

2. In Line 154-155 of main text, it is stated that there are “no baseline differences at D0 (Fig. 2c-e). This seems to be rather misleading statement as it appears there are differences in the genes of interest (e.g. *Hopx*) at baseline in the two different groups. This should be expected perhaps after two pre-treatments with the bacteria. This statement also seems contradicted later in line 173, as it states constant increase in *Hopx* across all post-DSS timepoints.

> We thank the reviewer for this careful observation and for identifying an inconsistency in our description. The reviewer is correct to point out that the statement “no baseline differences at D0” is misleading, as *Hopx* expression does appear elevated at D0 in the colonized group, an expected effect following bacterial pretreatment. We sincerely apologize for this oversight and have revised the text to clarify the expression patterns.

Our intended meaning was that most of the examined cell populations, including active ISCs (aISCs) and secretory lineages, did not show significant changes at D0, whereas the reserve ISC (rISC) marker *Hopx* was already upregulated at this time point, consistent with its subsequent increase across post-DSS timepoints.

Accordingly, we have revised the sentence in lines 154-155 as follows:

Original:

“Transcriptional profiling further demonstrated that BC colonization selectively amplified markers of active ISCs (*Lgr5/Ascl2*) and reserve ISCs (*Hopx/Clu*) alongside secretory lineage genes (*Ang4/Lyz1* for Paneth cells; *Dclk1/Cd24a/Trpm5* for Tuft cells) at D3 and D7, with no baseline differences at D0 (Fig. 2c-e).”

Revised:

“Transcriptional profiling further showed that BC colonization increased expression of the reserve ISC marker *Hopx* even at D0, while markers of active ISCs (*Lgr5/Ascl2*) and secretory lineages (*Ang4/Lyz1* for Paneth cells; *Dclk1/Cd24a/Trpm5* for Tuft cells) were significantly amplified only at D3 and D7 post-DSS (Fig. 2c-e).”

3. The overall feeling on the Discussion section is it presents a weak point in the current manuscript. It is suggested that the authors avoid restating results and referring to figures (this is more appropriate only in Results section) and instead focus on highlighting how these data relate to current literature. It is also recommended that limitations be discussed more clearly. While the authors state the limitation of the bacterial gene mutations in results, please elaborate in Discussion on limitation of using *E. coli* instead of the bacterial species focused on in the current report (BC). In addition, while the authors state that they used both sexes and observed no sex differences in studies, some studies only used female mice (e.g. Line 966-bacterial cross-feeding). It is actually surprising that no sex differences were observed since it is well-known that the colitis model used prominently in the study (DSS) is known to be more severe in male B6 mice than females (PMID: 25962374). Also, IBD is known to have sex differences (PMID: 39962330), so it is concerning that the studies herein do not highlight any of this.

> We sincerely thank the reviewer for the constructive and insightful critique of our Discussion section. While we recognize that in certain contexts a brief restatement of key findings can help orient readers and clarify connections to prior work, a practice

occasionally observed even in leading journals. But we fully agree that our original version leaned too heavily on restating results rather than synthesizing and contextualizing them. We have therefore substantially revised the Discussion to address this point, focusing on deeper engagement with the literature and clearer articulation of limitations, as detailed below and implemented in the manuscript.

1) Strengthening discussion of mechanisms and literature integration

We have expanded the discussion of our findings in the context of published work. In particular, we have added paragraphs addressing:

- a) The cellular source of BHB in our injury model and how it may differ from steady-state conditions (Cheng et al., Cell, 2019).
- b) Potential epigenetic mechanisms, such as H3K9 beta-hydroxybutyrylation (Terranova et al., Cell Reports, 2021), that could link BHB to HOPX activation.
- c) The apparent discrepancy between our data highlighting HOPX⁺ rISCs and other reports emphasizing CLU⁺ rISCs, noting likely model- and timing-dependent differences in regenerative responses (Ayyaz et al., Nature, 2019).
- d) The emerging relevance of microbial cross-feeding and co-metabolism (particularly tryptophan pathway interactions) in shaping immunomodulatory metabolite output, citing recent work on fiber-dependent modulation of indole vs. IPA/ILA production (Sinha et al., Nature Microbiology, 2024).

We have removed repetitive result statements and focused on interpreting our data in light of current concepts and open questions in the field.

2) Clarifying limitations

a) **Use of *E. coli* as a heterologous expression system:** We have added a paragraph acknowledging that while expressing BC-derived *fldH* in *E. coli* supports the enzymatic potential to generate ILA, it does not formally prove that *fldH* is essential in the native BC genetic background. We note that genetic manipulation in BC remains technically challenging but is an important goal for future studies.

b) **Sex differences:** We appreciate the reviewer's important point regarding known sex differences in DSS colitis severity and IBD (Babickova et al., Inflammation, 2015) and IBD pathogenesis (Tshikudi et al., Nat Rev Gastroenterol Hepatol., 2025). In our study, we used both sexes for the key BC colonization experiments (Fig. 1, Fig. 2 and Fig. 3) and observed no statistically significant sex-based differences in the parameters measured. For the bacterial co-culture and cross-feeding experiments (which did not involve DSS treatment), we used female mice for consistency with earlier protocol setups. We have now explicitly discussed this in the Limitations section, acknowledging that more systematic analyses of sex-dependent effects on BC colonization, metabolite production and stem cell responses represent an important future direction.

All changes have been incorporated into the revised Discussion, which we believe is now more balanced, critical, and informative.

Revised Discussion Section (the changes are highlighted in yellow):

Dysregulation of ISCs impairs epithelial barrier integrity and contributes significantly to the pathogenesis of chronic intestinal disorders. Growing evidence underscores the importance of gut microbiota and their metabolic derivatives in promoting epithelial

regeneration following injury. Nevertheless, the specific microbial metabolites involved and their precise mechanisms of action in ISC-mediated repair remain poorly defined. In this study, we identify BC as a key commensal bacterium that facilitates intestinal epithelial regeneration by activating HOPX⁺ rISCs to restore the LGR5⁺ aISC compartment, a process potentiated through metabolic cooperation with IPA-producing bacteria (Fig. 7h). Our findings advance the understanding of conserved bacterium-host metabolic crosstalk that is essential for epithelial homeostasis and repair, with relevance to IBD, RE, and potentially other conditions involving epithelial damage.

IBD is associated with profound microbial dysbiosis, characterized by altered community structure and metabolic output. Metagenomic analyses of public datasets reveal a consistent depletion of BC in IBD cohorts across diverse geographic regions (Fig. 1d-g). Although establishing direct clinical correlations between BC abundance and disease progression remains challenging (due in part to limited longitudinal sample availability), our controlled studies in DSS-induced colitis models demonstrate an unequivocal protective role for BC. Monocolonization of conventional mice with BC significantly attenuated colitis severity and enhanced mucosal barrier function (Fig. 1i-l). These results are corroborated by earlier work from Holmberg et al., which reported that BC supplementation promotes colonic mucus layer expansion via short-chain fatty acid production (Holmberg et al., *Nature Communications*, 2024), further supporting its role in mucosal protection.

Through the integration of RNA-seq, lineage tracing, and conditional ablation approaches, our study reveals a regulatory mechanism by which BC enhances BHB production in IECs, leading to the activation of a rISC population marked by HOPX expression (Fig. 3 and Fig.

4). Once activated, these HOPX⁺ rISCs demonstrate the capacity to regenerate a LGR5⁺ ISC pool and play a critical role in epithelial repair in both DSS-induced colitis and RE models (Fig. 3). While our data indicate that IEC-derived BHB promotes HOPX⁺ rISC activation, the precise epithelial subpopulation responsible for BHB generation in our injury model remains to be determined. Previous work in homeostatic conditions identified LGR5⁺ CBCs as a major source of BHB, which maintains stemness via HDAC inhibition and Notch signaling (Cheng et al., Cell, 2019). In our DSS-injury setting, however, LGR5⁺ cells are rapidly depleted, making it technically difficult to isolate them for source tracing. Future studies using single-cell metabolomics or in situ activity reporters may help resolve this spatial question. Moreover, the molecular mechanism linking BHB to HOPX induction warrants deeper investigation. Recent studies show that BHB can induce histone H3K9 beta-hydroxybutyrylation (H3K9bhb), an epigenetic mark associated with activation of metabolic and differentiation genes (Terranova et al., Cell Reports, 2021). Whether BHB regulates HOPX through similar chromatin remodeling represents a compelling direction for future work.

Our results are consistent with earlier studies identifying a HOPX⁺-dependent regenerative cell population that contributes to mucosal healing (Wang et al., Cell, 2019). Specifically, we observed that BC treatment in HOPX lineage-tracer mice led to an increase in HOPX⁺ daughter cells co-expressing LGR5 within crypts at D3 post-DSS challenge (Fig. 3f), supporting the plasticity of HOPX⁺ cells in replenishing the injury-depleted LGR5⁺ ISC compartment. It is worth noting that another rISC subset marked by high *Clu* expression has also been shown to restore the LGR5⁺ pool after injury (Ayyaz et al., Nature, 2019). It

reported that low HOPX expression in CLU^{high} rISCs, which may appear discrepant with our findings. However, the injury model (radiation vs. chemical DSS), timing of analysis, and regenerative niches examined differ substantially, likely explaining these phenotypic variations. Together, these observations highlight the context-dependent plasticity and heterogeneity of rISC populations during repair.

Current therapies for IBD primarily target immune suppression often through inhibition of cytokines such as TNF α (Roda et al., Clin Transl Gastroenterol., 2016; Dahmus et al., Clin Exp Gastroenterol., 2020), which carries risks of infections and neoplastic events (Dahmus et al., Clin Exp Gastroenterol., 2020; Singh et al., Clin Gastroenterol Hepatol., 2020). These limitations underscore the need for treatment strategies that promote mucosal healing without broad immunosuppression. Here, we demonstrate that a commensal BC strain expressing *fldH* generates ILA from Trp catabolism, and synergizes with *P. russellii* or *C. sporogenes* to produce IPA both in vitro and in vivo (Fig. 5l-p and Fig. 6c-g). Our work adds to growing evidence that microbial co-metabolism and cross-feeding critically shape host-active metabolite output. A recent study showed that in a defined community, fiber-derived sugars provided by *Bacteroides thetaiotaomicron* suppressed indole production in *E. coli* via catabolite repression, thereby increasing Trp availability for *C. sporogenes* to produce ILA and IPA (Sinha et al., Nature Microbiology, 2024). Similarly, BC may create a metabolic niche that facilitates the growth or activity of IPA-producing partners, possibly through provision of carbon sources or acidification (Kitada et al., Science Advances, 2018), which we will explore in future work. The broader relevance of such metabolic partnerships is further highlighted by a recent report that *Lactobacillus johnsonii* cooperates with *C.*

sporogenes to produce IPA, which enhances anti-tumor CD8⁺ T cell stemness and improves immune checkpoint blockade efficacy across cancer types (Jia et al., Cell, 2024). This underscores the potential translatability of targeting microbial metabolic networks for therapeutic benefit.

In conclusion, our work identifies BC as a commensal bacterium that promotes intestinal epithelial regeneration through a BHB-HOPX⁺ rISC axis, amplified by metabolic interaction with IPA-producing bacteria. These findings highlight the therapeutic potential of targeting conserved microbial metabolic networks to stimulate endogenous repair mechanisms in IBD and other epithelial injury conditions.

Limitations of the study

Our study has several limitations. First, although we demonstrate that BC encodes a functional *fldH* gene and that expressing BC *fldH* in *E. coli* confers ILA production, genetic manipulation in BC remains technically challenging. Thus, we could not generate a BC- Δ *fldH* mutant to formally prove the requirement of this gene in the native bacterium. Developing robust genetic tools for BC will be essential for definitive functional studies. Second, while we used both male and female mice in our main colonization experiments and observed no significant sex-based differences in BC's protective effects, we acknowledge that DSS colitis severity (Babickova et al., Inflammation, 2015) and IBD pathogenesis (Tshikudi et al., Nat Rev Gastroenterol Hepatol., 2025) are known to differ between sexes. Future studies should systematically evaluate potential sex differences in BC colonization dynamics, metabolite production and stem cell responses. Finally,

although our multi-omics and functional data support a role for BC-driven BHB in activating HOPX⁺ rISCs, the downstream epigenetic or transcriptional mechanisms linking BHB to HOPX induction are yet to be fully elucidated.

Minor:

1. Line 89/104 - IBD should be abbreviated, as abbreviation was established earlier (Line 44).

2. Line 106 - Should be "previous", not "pervious".

> We thank the reviewer for these careful corrections. Both points have been addressed in the revised manuscript as follows:

1) On Line 89/104, "Inflammatory bowel disease" has been changed to "IBD" to maintain consistent abbreviation usage throughout the text.

2) On Line 106, "pervious" has been corrected to "previous."

We appreciate the reviewer's close reading, which has helped improve the accuracy and consistency of our manuscript.