Editorial Commentary

Aberrant differentiation of intestinal stem cells due to inflammation-induced mitochondrial dysfunction predicts postoperative recurrence of Crohn's disease

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Previous studies of intestinal inflammation have identified aberrant pathways that disrupt the homeostasis of the intestinal microenvironment. Loss of intestinal epithelial cell (IEC) barrier function, microbiota infiltration, and epithelial regeneration have been well characterized and correlated with dysregulated inflammatory responses in both patients and murine models (1). The role metabolic pathways play during the pathogenesis of inflammatory bowel disease (IBD), however, is not well-defined due to the multifaceted interaction during the disease progression between the host's IECs, microbiota, immune cells, location, and environment (2). Treatment of IBD includes targeting multiple pro-inflammatory cytokines, including TNFα and IL-17A (3). While anti-IL-17A antibody therapy failed in clinical trials, anti-TNFα antibodies, such as infliximab and adalimumab, are now established treatments of IBD (4).

Paneth cells (PCs) play an essential role in maintaining an intestinal stem cell (ISC) niche, and their inclusion in co-culture is sufficient to improve the efficiency of ISC organoid development (5). Furthermore, the addition of a Wnt secretion inhibitor (PORCN) inhibited organoid proliferation, suggesting a role of PCs in shaping the ISC niche through secretory granules (5). PCs also play an important role in regulating microbiota populations through the secretion of essential antimicrobial peptides (AMPs) and lysozyme (LYZ) upon contact with pathogenic microbes (6). In a novel study, Liu *et al.* analyzed the prevalence of PCs in intestinal biopsy specimens of Crohn's disease (CD) patients and concluded that PC abnormalities correlated with the presence of microbiota dysbiosis (7). As such, two major PC functions, maintenance of the ISC niche and antimicrobial activity, are reliant on secretory granule production.

Many studies have also shown the association of impaired intestinal mucosal function in IBD patients with mitochondrial metabolic pathways, such as limited β-oxidation of butyrate (8). Santhanam *et al.* reported an important finding that acetoacetyl CoA thiolase, a mitochondrial enzyme that catalyzes the final step of butyrate oxidation, has markedly reduced activity in the colonic epithelium of ulcerative colitis (UC) patients (9). Many others have reported additional mitochondria-associated features in IBD, including increased reactive oxygen species (ROS), reactive nitrogen species (RNS), and mitochondrial stress, but the role of aberrant mitochondrial function in IBD pathogenesis remains unclear (10).

Mitochondria-related findings have been widely described in several different metabolic diseases. Many of the hallmark phenotypes of these diseases include mutations in the mitochondrial DNA (mtDNA), increased ROS production, and decreased ATP output (10). These mitochondria-related findings are often secondary outcomes of impaired protein folding. As previously published by Berger *et al.*, the mitochondrial unfolded protein response (MT-UPR) resulted in the loss of the mitochondrial chaperone protein Hsp60 and decreased ISC stemness and proliferation (11). Others have shown that the absence of a MT-UPR response in interferon-inducible double-stranded-RNA-activated protein kinase (Pkr)-deficient

mice protects the intestinal epithelium from dextran sulfate sodium (DSS)-induced colitis (12).

The paper by Khaloian *et al.* now provides insight on possible links between mitochondrial and PC dysfunction in CD pathogenesis. As mentioned in the paper, several genetic risk variants of CD-relevant genes involved in autophagy, bacterial-sensing, endoplasmic stress (ER) response, and Wnt signaling have been shown to affect PC function and are particularly associated with ileal CD (13). However, whether ileal CD is a disease of PC dysfunction or is a non-causal, secondary manifestation of CD pathogenesis remains unknown. Although previous works have extensively characterized the morphology of PCs or the pathways associated with mitochondrial metabolism, there is a lack of connection between these two fields in understanding the pathogenesis of ileal CD. Khaloian *et al.* specially addressed this issue.

Leveraging the phenotypic heterogeneity in TNF$^{\Delta ARE}$ mice, the authors found that both presence of LYZ^{+} PCs and percentage of highly granular PCs inversely correlated with the grade of CD-like inflammation. These inflammatory shifts were accompanied by a reduction in ISC stemness, indicated by reduced *Lgr5 and Olfm4* crypt expression.

In order to see if these findings are present clinically, the authors proceeded to stain 70 CD ileal resection tissue margins, classified as inflamed or non-inflamed at the time of surgery, for LYZ protein level and *Lgr5* gene expression. Consistent with the findings in their mouse models, the numbers of LYZ^{+} PCs and highly granular PCs were significantly reduced in tissue margins classified as inflamed at the time of resection. Interestingly, the authors also observed an increase in the number of LYZ^{+} cells in the upper crypt, defined as above the +6 position, in inflamed compared to non-inflamed tissue margins. The LYZ^{+} cells in the upper crypt were accompanied by a reduction of crypts with high *Lgr5* expression, indicating an inflammation-induced aberrant ISC niche structure.

As the authors noted and validated within their own cohort, inflammation in ileal tissue resection margins was predictive of subsequent endoscopic recurrence. Non-inflamed ileal tissue resection margins, however, seemed to carry little prognostic value, as 44% of these patients developed recurrent disease. Motivated by the possibility that non-inflamed regions may already be undergoing pathologic changes on a molecular level, the authors again turn to LYZ staining and *Lgr5 in situ* hybridization. Here, the authors demonstrated that a higher number of LYZ^{+} cells in the upper crypts and decreased PC granularity in non-inflamed-classified samples were predictive of endoscopic recurrence. Although these findings likely need to be validated in an independent and larger cohort, they lend excitement to the potential of utilizing molecular markers to stratify patients on a scale finer than inflammation status and at a time point before such macro changes even develop. It is worth noting that the authors found no further predictive value of these characterizations for inflamed ileal resection margins, indicating that these might be early molecular signs of inflammatory changes.

Phenotypic PC changes have been described in association with impaired mitochondria, and IEC MT-UPR stress signaling has been observed in both IBD patients and mouse models of colitis (12). In agreement with these findings, TNF$^{\Delta ARE}$ mice were found by transmission electron microscope to have PCs that were markedly reduced in numbers with morphologic changes, including secretory granules with broadened halos, intracytoplasmic vacuolations, and dilation of rough ERs. Furthermore, in isolated crypts from TNF$^{\Delta ARE}$ mice, the authors identified increased levels of the MT-UPR proteins, Hsp60 and Pkr, with an increase in transcription of genes indicative of mitochondrial disturbance. These transcriptional changes were accompanied by degenerative mitochondrial alterations, such as mitochondrial swelling with dissolution, disruption of cristae, loss of matrix density, and occasional formation of intramitochondrial electron-dense inclusions, shown by transmission electron microscopy. These observations lend credence to the application of TNF$^{\Delta ARE}$ mice to model and understand IBD-associated mitochondrial and PC dysfunction. Given the earlier observation that inflammatory grade correlates with reduced stemness, in addition to PC absence, in TNF$^{\Delta ARE}$ mice, these findings raise the question of how mitochondrial impairment, PC abnormalities, and reduced ileal crypt stemness might be connected.

To further probe these mechanisms, the authors utilize the *Hsp60*$^{flox/flox}$ × *Lgr5*-eGFP-IRES-CreER$^{T2\text{-}Tg}$ (Hsp60$^{\Delta/\Delta ISC}$) mouse model, wherein tamoxifen administration causes mitochondrial dysfunction within *Lgr5*$^{+}$ ISCs through deletion of Hsp60. Deficiency of Hsp60, the main chaperone of the mitochondrial matrix, leads to disturbed mitochondrial proteostasis and activation of MT-UPR signaling. In line with previous experiments in TNF$^{\Delta ARE}$ mice, Hsp60 ablation in *Lgr5*$^{+}$ ISCs led to decreased proportion of highly *Lgr5* expressing crypts, decreased ISC proliferation, and decreased PC granularity. Additionally, efforts to characterize PCs by LYZ staining and *Lgr5*

in situ hybridization revealed a decrease in *Lgr5*$^{-}$LYZ^{+} cells with an increase in *Lgr5*$^{+}$LYZ^{+} double-positive cells in the crypt base. Staining for Hsp60 revealed that Hsp60^{-} cells originated directly from *Lgr5*$^{+}$ cells that had undergone Hsp60 deletion-induced mitochondrial dysfunction, and the ratio of LYZ^{+} cells within this Hsp60^{-} population increased from day 0 to 2. From this, the authors describe a potential model in which Hsp60 deletion-induced mitochondrial dysfunction within *Lgr5*$^{+}$ ISCs can initiate a transition to a double-positive *Lgr5*$^{+}$LYZ^{+} PC-like phenotype, but cannot complete the differentiation process to mature PCs due to the mitochondrial impairment.

Lastly, the authors observe that crypt cells from inflamed but not non-inflamed TNF$^{\Delta ARE}$ mice fail to grow into organoids. Addition of exogenous Wnt factors into media could not induce organoid formation from inflamed TNF$^{\Delta ARE}$ mice, and the authors reasoned that mitochondrial impairment within these inflamed crypts prevented oxidative phosphorylation necessary for organoid development. To test this, the authors apply dichloroacetate (DCA), which targets the pyruvate dehydrogenase complex to shift ATP generation from glycolysis to oxidative phosphorylation, to culture media and successfully rescued the capacity of inflamed TNF$^{\Delta ARE}$-derived ileal crypt cells to form organoids. These data revealed that interventions shifting cells away from a glycolytic state can help overcome inflammation-induced mitochondrial dysfunction in ileal crypt cells from inflamed TNF$^{\Delta ARE}$ mice to restore stemness and offer support for the potential of metabolic reprogramming therapies for IBD.

The authors, in establishing connections between TNF$^{\Delta ARE}$, Hsp60$^{\Delta/\Delta ISC}$, and CD ileal resection samples, proposed a novel model wherein inflammation-induced mitochondrial dysfunction serves as an upstream initiator for the aberrant PC distribution and phenotypes in colitis. While the study demonstrates the capacity of Hsp60^{-} metabolically disrupted ISCs to give rise to premature, *Lgr5*$^{+}$LYZ^{+} PC-like cells within the crypt bases, the question as to whether these are direct precursors to the upper crypt, LYZ^{+} cells seen in TNF$^{\Delta ARE}$ and CD ileal resection samples persists. Further studies might consider utilizing intestinal ISC mitochondrial dysfunction models, such as Hsp60$^{\Delta/\Delta ISC}$, in concert with lineage tracing to confirm whether these *Lgr5*$^{+}$LYZ^{+} PC-like cells within the crypt bases eventually travel to and replicate the LYZ^{+} upper crypt patterning in colitis. That being said, the authors consider three major features observed in IBD—mitochondrial dysfunction, PC impairment, and dysregulated TNFα—and present a potentially exciting, new mechanistic bridge to be further studied.

In summary, Khaloian *et al.* have utilized the Hsp60$^{\Delta/\Delta ISC}$ and TNF$^{\Delta ARE}$ mouse models to provide novel insight into the role of mitochondrial functions in maintaining homeostasis of the ISC niche. Their novel findings of aberrant PC development due to abnormal ISC mitochondrial function connect part of the gapping knowledge between metabolism and IBD pathogenesis. Furthermore, many of their identified, phenotypic features were also observed within their cohort of CD ileal tissue resection margins. As stated by the authors, many of the widely accepted colitis mouse models, such as DSS- or irradiation-induction, demonstrate an acute response to injury with a regenerative process that is not representative of that during chronic inflammation. The rapid onset of regeneration in these acute colitis models fails to capture the pattern of gradual pathogenesis of the ISC niche and subsequent ISC stemness exhaustion, as is evidenced by the enhanced capacity to form organoids from cells derived from the crypts of DSS-treated mice (14). Furthermore, the authors present a novel finding that shifting the metabolic pathways of the ISC mitochondria towards oxidative phosphorylation can restore stemness within the setting of TNF$^{\Delta ARE}$ organoids and provide potential new avenues from which to target and restore intestinal homeostasis as a treatment for CD.

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