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Intestinal epithelial BLT1 promotes mucosal repair

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Abstract

Acute and chronic intestinal inflammation is associated with epithelial damage, resulting in mucosal wounds in the forms of erosions and ulcers in the intestinal tract. Intestinal epithelial cells (IECs) and immune cells in the wound milieu secrete cytokines and lipid mediators to influence repair. Leukotriene B₄ (LTB₄), a lipid chemokine, binds to its receptor BLT1 and promotes migration of immune cells to sites of active inflammation, however a role for intestinal epithelial BLT1 during mucosal wound repair is not known. Here we report that BLT1 is expressed in IECs both in vitro and in vivo, where it functions as a receptor not only for LTB₄ but also for another ligand Resolvin E1. Intestinal epithelial BLT1 expression is increased when epithelial cells are exposed to an inflammatory microenvironment. Using human and murine primary colonic epithelial cells, we reveal that LTB₄-BLT1 pathway promotes epithelial migration and proliferation leading to accelerated epithelial wound repair. Furthermore, in vivo intestinal wound repair experiments in BLT1-deficient mice and bone marrow chimeras demonstrate an important
contribution of epithelial BLT1 during colonic mucosal wound repair. Taken together, our findings show a novel pro-repair in IEC mechanism mediated by BLT1 signaling.
Introduction

The gastrointestinal epithelium serves as a highly regulated protective barrier against luminal antigens and microbes. Acute and chronic intestinal inflammation is associated with epithelial damage, resulting in mucosal wounds in the form of erosions and ulcers. In response to injury, intestinal epithelial cells (IECs) have a remarkable capacity to migrate and proliferate to cover denuded surfaces and restore the critical epithelial barrier. Such reparative events are orchestrated by the spatiotemporal crosstalk between epithelial cells, infiltrating and resident immune cells including neutrophils, monocytes, macrophages, as well as stromal cells (1). Epithelial and immune cells in the wound milieu secrete mediators including cytokines and specialized pro-resolvin lipid mediators (SPMs) to influence repair. Many SPMs bind to G-protein coupled receptors (GPCRs) and promote resolution of inflammation (2, 3). Recently we reported that the SPM resolvin E1 (RvE1) promotes intestinal epithelial wound repair by increasing migration and proliferation of IECs (4). Receptors for RvE1 include BLT1, a high affinity receptor for leukotriene B4 (LTB4) and ChemR23 also known as CMKLR1 (5). While BLT1 expression
and function in immune cells such as neutrophils has been extensively studied (6-12), a few reports of epithelial BLT1 in the lungs are published but little is known about intestinal epithelial cell expression of BLT1 and associated receptor mediated signaling events (13, 14).

BLT1 agonists LTB₄ and RvE1, trigger distinct responses when binding BLT1 in immune cells. While LTB₄ serves as a chemotactic signal critical in regulation of immune cell migration to sites of active inflammation (15), RvE1 was described as an agonist that binds to the receptor but does not trigger downstream signaling. Enhanced activation of the LTB₄-BLT1 occurs in conditions associated with pathologic intestinal inflammation as observed in inflammatory bowel disease (IBD) and colonic adenocarcinoma (16-18). LTB₄ is increased in the colonic mucosa (16) and supernatant of ex-vivo cultured colorectal biopsy specimens from individuals with IBD (17) and in serum from colorectal cancer patients (18). These findings suggest that the LTB₄-BLT1 path plays an important role in the pathophysiology of a diverse set of intestinal diseases.

In the present study, we investigated the role of BLT1 in regulation of colonic epithelial wound repair.
We demonstrate by *in vitro* and *in vivo* approaches that IECs express BLT1 in a temporal manner, with increased expression after exposure to pro-inflammatory conditions. Using primary cultures of human and murine colonic epithelial cells (colonoids), we show that LTB₄ ligation of BLT1 promotes epithelial migration and proliferation leading to increased wound repair. Furthermore, we show that wound repair is delayed in BLT1 KO (*Ltb4r1−/−*) mice, and bone marrow (BM) transplant experiments demonstrate critical contributions of non-hematopoietic BLT1 expressing cells in colonic mucosal wound repair. We observe that pro-repair effects of the LTB₄-BLT1 axis align with signaling events that regulate cell-matrix focal adhesions and cell migration. Collectively, these data identify an important pro-repair function of epithelial BLT1 signaling in promoting intestinal mucosal wound healing.
Results

1. BLT1 functions as a major epithelial receptor for RvE1

We previously reported that RvE1 functions as a potent pro-repair molecule that promotes intestinal epithelial wound healing (4). To further investigate how RvE1 activates signaling in epithelial cells to facilitate repair, we examined expression of the two known RvE1 receptors: BLT1 and CMKLR1. Given the lack of specific BLT1 and CMKLR1 antibodies, we analyzed spatial expression of these receptors in the human and murine colonic mucosa by RNAscope in situ hybridization. While LTB4R/Ltb4r1 (BLT1 gene name) mRNA was expressed in the colonic epithelium and lamina propria, CMKLR1/Cmklr1 mRNA was detected only in lamina propria cells (Figure 1A and 1B). Given that BLT1 has been reported to be predominantly expressed by immune cells and CMKLR1 expression has been reported in immune and epithelial cells, this was an unexpected finding. To corroborate these results, we performed qPCR on human intestinal epithelial cell lines and primary epithelial cultures (SKCO15, T84 and colonoids grown as monolayers). Such analyses revealed that IECs express 16-fold more LTB4R than CMKLR1,
indicating that CMKLR1 expression is low in IECs (Figure 1C). To determine if BLT1 contributes to the pro-repair activity of RvE1 in IECs, we examined the effect of a BLT1 antagonist on RvE1-induced epithelial wound healing in vitro using primary human colonic epithelial cells (colonoids) cultured as 2 dimensional (2D) monolayers. Time lapse imaging of healing wounds using human colonoids demonstrated that the increased wound repair induced by RvE1 (100 nM) was inhibited by incubation with a selective BLT1 antagonist, CP105,696 (1 µM) (Figure 1D). A similar effect on wound repair was obtained in primary murine colonic epithelial monolayers incubated with this BLT1 antagonist (Figure S1A). Although CMKLR1 is a RvE1 receptor, pretreatment with the selective CMKLR1 antagonist α-NETA (10 µM), did not abolish the pro-repair response triggered by RvE1 in human and murine primary IECs (Figure 1D, S1A). To further determine the interaction of RvE1 and BLT1, we performed a computational docking simulation which is a useful technique to calculate intra- and inter-molecular energies of target receptors and ligands. Results of the analysis supported binding of RvE1 to BLT1 (Figure S1B). Collectively, our findings suggest that BLT1 is expressed in the intestinal epithelia and
functions as a receptor for RvE1 during wound repair.

2. Epithelial BLT1 is upregulated in response to colonic mucosal injury

To investigate the role of BLT1 in intestinal mucosal wound repair, Ltb4r1 mRNA expression and spatial localization was analyzed in healing, biopsy-induced murine colonic mucosal wounds. qPCR analyses of harvested mucosal colonic wounds revealed that Ltb4r1 mRNA was significantly upregulated 24 and 48 h after injury (Figure 2A). Additionally, Ltb4r1 mRNA was detected in the colonic epithelium and in lamina propria cells of murine mucosa by RNAscope in situ hybridization (Figure 2B). Ltb4r1 mRNA expression was increased in the wound bed and epithelium adjacent to wounds 48 h after injury (Figure 2C). We observed Ltb4r1 mRNA was highly expressed in IECs located at the bases of crypts adjacent to healing wounds (Figure 2D). Quantification of these findings revealed Ltb4r1 mRNA was upregulated 6.9-fold 48 h after injury (Figure 2E). Since many mucosal inflammatory diseases such as IBD are associated with mucosal wounds, we examined expression of LTB4R mRNA in tissue sections from samples from individuals with IBD (active ulcerative colitis). Importantly, epithelial LTB4R
mRNA was increased in colonic crypts from IBD biopsy samples (Figure 2G) compared to uninflamed controls (Figure 2F). These results are consistent with the concept of upregulated expression of intestinal epithelial BLT1 in response to mucosal inflammation and injury in vivo.

Our findings suggested that BLT1 is preferably expressed in the base of human and murine colonic crypts, co-localizing with stem cell markers, Lgr5 and HopX (Figure S2). At the crypt base, proliferative crypt epithelial cells differentiate and migrate towards the luminal surface. To further examine BLT1 expression in proliferative crypt-base colonic epithelial cells vs. differentiated luminal epithelial cells, we examined \textit{LTB4R/Ltb4r1} mRNA expression in primary IEC cultures and colonoids differentiated in vitro.

Colonoids in Wnt containing media cultured as 3D cysts are known to contain stem-like/proliferative epithelial cells. Such 3D structures can be dissociated and cultured as differentiated 2D monolayers. As shown in Figure S2, \textit{LTB4R/Ltb4r1} mRNA expression was significantly higher in 3D cultured colonoids compared to 2D differentiated monolayers in both human and murine colonic epithelial cells. These results suggest preferential expression of BLT1 in proliferative colonic crypt base epithelial cells that is
upregulated in response to injury. Unfortunately, these results could not be correlated with protein expression as specific commercial BLT1 antibodies required for these analyses are not available (Figure S3A). We did observe significantly higher levels of LTB₄ in healing colonic wounds compared to intact healthy tissue, indicating that an increase in the receptor mRNA expression correlates with higher ligand secretion in-vivo (Figure S3B).

3. BLT1 regulates intestinal epithelial wound repair

To determine the role of LTB₄-BLT1 axis in regulating epithelial wound repair, we evaluated the effect of LTB₄ using a well-studied and stable agonist of BLT1 on epithelial repair in model human IECs (SKCO-15). As shown in Figure 3A, LTB₄ (1-100 nM) enhanced IEC wound repair in a concentration-dependent manner. Pretreatment with the selective BLT1 antagonist CP105,696 (1 μM) abolished the pro-repair response elicited by LTB₄ (10 nM) (Figure 3A). Furthermore, the response was replicated in healing scratch wounded primary human colonoid cultures (Figure 3B-C). To further verify that epithelial BLT1 activation promotes IEC wound healing, we generated primary colonic epithelial monolayers from
colonoids of WT and BLT1-deficient (Ltb4r1/-) mice. Time-lapse imaging of healing wounds in these cells revealed that LTB$_4$ (10 nM) significantly enhanced wound repair in the IECs from WT mice and was not observed in colonoids derived from Ltb4r1/- mice over a period of 24 hrs (Figure 3D-E). Importantly, wound closure was significantly delayed in primary epithelial cells lacking BLT1 compared with WT control (Figure 3D-E).

It is now appreciated that an inflammatory milieu in wounded mucosa modulates epithelial reparative responses. We have previously reported that the cytokine tumor necrosis factor-$\alpha$ (TNF-$\alpha$) is elevated within inflamed intestinal mucosa where it contributes to wound repair by increasing expression of GPCR$s$ such as PAFR (19). Furthermore, the pro-inflammatory cytokine, interferon-gamma (IFN-$\gamma$) has been observed to upregulate TNF receptor expression in IECs. We investigated if TNF-$\alpha$ and IFN-$\gamma$ modulate BLT1 expression in IECs. The expression of LTB4R mRNA was synergistically increased by combined incubation of IECs with IFN-$\gamma$ and TNF-$\alpha$ in primary human 2D colonoids (Figure 3F) and 3D colonoids (Figure S4). In parallel, we examined the influence of combined stimulation with IFN-$\gamma$ and
TNFα on the pro-repair effect of LTB₄ on IEC monolayers that were scratch wounded. As previously published, stimulation with IFN-γ and TNFα (100 ng/ml each) significantly promoted wound closure in SKCO-15 model IECs (Figure 3G) (19). SKCO-15 cells pretreated with IFN-γ and TNFα (100 ng/ml each) and then incubated with low-dose of LTB₄ (1 nM) further increased IEC wound repair when compared to LTB₄ incubation without cytokine-pretreatment (Figure 3G). We confirmed specificity of the LTB₄ increase in IEC wound healing by treating SKCO-15 cells with the BLT1 antagonist CP105,696 (0.1-1 µM). Importantly, CP105,696 significantly inhibited the IEC enhanced wound healing promoted by LTB₄ in combination with IFN-γ /TNF-α in a concentration-dependent manner (Figure 3G). These results suggest that an inflammatory microenvironment in the intestinal mucosa upregulates intestinal epithelial BLT1 expression that potently promotes wound repair.

4. BLT1 activation promotes migration and proliferation of IECs

Since it is well appreciated that collective IEC migration and proliferation orchestrate repair after injury, we investigated whether the activation of BLT1 promotes intestinal epithelial cell migration during
repair by recording cell movement of wounded monolayers over 12 hours by time-lapse microscopy.

Cell motility was tracked by analyzing centroid location of individual cells during the assay (Figure S5).

As shown in Figure 4A, Plot_At_Origins showed that primary cultures of LTB4-treated colonic epithelial cells derived from WT murine colonoids moved faster and straighter than vehicle-treated cells.

Importantly, there was no difference in cell movement between LTB4-treated and vehicle-treated Ltb4r1-/- primary IECs. Furthermore, the movement of primary IECs derived from Ltb4r1/- murine colonoids was slower than those of WT murine colonoids (Figure 4A). DiPer software-based analyses (20) demonstrated that mean square displacement (MSD), a classic index that provides information about directional persistence and speed, was significantly increased in murine WT IECs treated LTB4 (Figure 4B). However, in Ltb4r1/- IECs, MSD was significantly decreased compared to WT IECs (Figure 4B).

Autocorrelation of cell direction, which reflects cell direction persistence by determining angles of vectors tangent to a cell's trajectory revealed that LTB4 significantly promoted cell direction persistence in WT but not Ltb4r1/- IECs (Figure 4C). Finally, we calculated cell speed during cell migration. Treatment with
LTB₄ significantly increased cell speed in WT but not Ltb₄r1⁻/⁻ IECs indicating that cell speed in Ltb₄r1⁻/⁻ IECs was significantly slower than observed in WT in the presence of LTB₄ (Figure 4D). To explore mechanisms by which LTB₄-BLT1 axis promotes migration of IECs, we analyzed signaling pathways that have been shown to promote epithelial migration and wound repair. Phosphorylation/activation of Src and Focal adhesion kinase (FAK) were examined using murine 2D colonoids. Grid scratched primary IEC monolayers were incubated with LTB₄ for 8 h followed by analyses. We observed increased Src (Y416) and FAK (Y397 and Y925) phosphorylation in IEC treated with LTB₄, consistent with activation of pathways playing important roles in the regulation cell matrix turnover and forward cell movement during migration (Figure 4E). Importantly, increased phosphorylation of Src and FAK at Y416, Y397 and Y925, respectively, was abrogated when the BLT1 antagonist CP105,696 was added in combination with LTB₄. Since wound closure is mediated by epithelial migration and proliferation, we investigated the role of BLT1 in IEC proliferation. The effect of LTB₄ on the incorporation of a thymidine analog EdU in murine 3D cultured colonoids was analyzed. LTB₄ (10 nM for 24h) resulted in significantly
increased proliferation of murine IECs (Figure 4F-G). Importantly, the increase in LTB₄ induced epithelial cell proliferation was significantly inhibited by pretreatment with BLT1 antagonist CP105,696 (Figure 4F-G). To confirm specificity of BLT1 in enhancing proliferation of colonic epithelial cells, we examined the effect of LTB₄ on colonoids derived from Ltb4r1/- mice. Indeed, stimulation with exogenously added LTB₄ did not significantly alter proliferation of colonoids derived from mice lacking the BLT1 receptor (Figure S6).

5. Role of BLT1 in the intestinal mucosal wound repair in vivo

To determine the role of BLT1 in intestinal mucosal wound repair in vivo, we examined intestinal mucosal healing in Ltb4r1/- and WT mice using a well characterized colonic biopsy-induced injury model. As shown in Figure 5A, colonic mucosal wound repair was dramatically delayed in Ltb4r1/- mice compared with WT mice 3 days post injury (46.1 ± 1.9% in WT mice, 27.2 ± 1.6% in Ltb4r1/- mice; p<0.0001). The digitally quantified wound healing data were consistent with histological analyses of healing wounds supporting markedly delayed wound closure 3 days after injury in Ltb4r1/- mice. Since
IECs and immune cells express BLT1 (Figure 1A), we evaluated the relative contribution of these cell types in regulating mucosal wound repair. Irradiated WT or Ltb4r1/- recipient mice were reconstituted with bone marrow cells (BM) from either donor WT or Ltb4r1/- mice to generate chimeric mice (Figure 5B), followed by biopsy-induced mucosal wound repair experiments. As expected, wound closure 3 days post injury was significantly delayed in WT mice reconstituted with Ltb4r1/- BM (Ltb4r1/- > WT) confirming that hematopoietic derived (immune) cell-expressed BLT1 plays a role in regulating colonic mucosal wound repair. However, and importantly, Ltb4r1/- mice reconstituted with WT BM (WT > Ltb4r1/-) also had a similar delay in wound healing responses (Figure 5C), which is consistent with an equivalent non-hematopoietic (e.g. epithelial) derived BLT1 response in regulating intestinal mucosal wound repair.
Discussion

Active and coordinated repair responses that promote migration and proliferation of IECs are essential to cover denuded mucosal surfaces and reestablish intestinal mucosal barrier function. These re-epithelization events are facilitated by interactions between mediators derived from epithelium and immune cells in the injured intestinal mucosa and their receptors (21). This study identifies expression of the RvE1/LTB₄ receptor BLT1 in the intestinal epithelium and demonstrates a critical role of IEC expressed BLT1 and LTB₄ in regulating epithelial wound repair.

It is well appreciated that another important ligand for BLT1 and CMKLR1 is RvE1. In this report, using in vivo RNAscope in situ hybridization we observed that IECs preferentially express Ltb4r1 (BLT1 gene) and not Cmklr1 mRNA, whereas lamina propria cells express mRNA for both these receptors. Human and murine colonic epithelia display robust expression of BLT1 mRNA at the base of the crypt under normal conditions and expression is highly upregulated after mucosal injury in response to the inflammatory milieu in the wound bed. BLT2 but not BLT1 expression by IECs has been reported with
only a few reports showing BLT1 expression by IECs mostly related to carcinoma progression (18). Our expression and pharmacological in vitro studies suggest that ligation of BLT1 and not CMKLR1 by RvE1 mediate intestinal epithelial pro-repair affects, suggesting that BLT1 in IECs acts as an active receptor for RvE1. While we previously reported increased expression of Cmklr1 mRNA in murine repairing colonic mucosal wounds (4) that supports a role of CMKLR1 in mucosal wound repair, these findings are consistent with CMKLR1 playing an important role in immune cell signaling that contributes to intestinal mucosal wound healing.

Spatiotemporal analysis identified expression of LTB4R/Ltb4r1 in both human and murine IECs, supporting a more ubiquitous localization of BLT1 on cell types not previously reported. We observed an enrichment of Ltb4r1 mRNA expression in IECs located at the base of the crypts in murine colonic tissue that is upregulated after biopsy-induced mechanical injury of the mucosa. Using primary human and murine colonoids, and analogous to tissue labeling experiments where BLT1 mRNA was identified in proliferating epithelial cells at crypt bases, we observed increased expression of LTB4R/Ltb4r1 in
proliferative colonoids grown in 3D structures compared differentiated colonoids that recapitulate luminal epithelial cells.

We have previously reported that the pro-inflammatory cytokine TNF-α increases intestinal epithelial wound repair that is in part mediated by the cytokine induced upregulation of pro-repair GPCRs (19). We observed that TNF-α, in combination with IFN-γ stimulates increased expression of LTB4R in human IECs. Importantly, these cytokines also enhance pro-repair effects of epithelial BLT1, suggesting “pro-inflammatory” mediators such as LTB4 and TNF-α, have very important “pro-repair” properties in IECs. Our findings strongly support the current concept that inflammation is not only important for host defense, but also plays a pivotal role in setting the stage for tissue repair. Our studies support a paradigm shift where pro-inflammatory mediators often seen as damaging molecules play a pivotal role in initiation tissue repair. Controlled inflammation is clearly essential for host defense. Pro-inflammatory mediators, often perceived as damaging and detrimental, set the stage for resolution of inflammation and facilitating reparative events which are required for restoring tissue homeostasis.
These highly regulated mechanisms are perturbed in chronic inflammatory diseases that are associated with impaired tissue repair. Thus, an improved understanding of how pro-inflammatory soluble mediators create the bridge to repair will help in the rational design therapies to promote wound healing.

Mucosal tissues obtained from people with chronic IBD have increased expression of both LTB₄ and BLT1 (16-18). We observe that epithelial LTB₄R expression is higher in the crypts from individuals with IBD compared to healthy subjects. The LTB₄-BLT1 axis may thus play a role in the impaired wound repair responses observed in chronically inflamed mucosa as seen in IBD. Here we demonstrate that IEC expressed BLT1 has a beneficial role in promoting acute colonic wound repair, but more work is needed to understand the role of BLT1 chronic intestinal inflammation induced injury.

Given the marked upregulation of BLT1 in healing wounds, we analyzed the specific contribution of BLT1 to IEC repair. Mucosal wound repair requires coordinated migration of epithelial cells from crypts adjoining wounds. During repair, epithelial cells undergo morphologic changes in shape, modify cell-cell
contacts, and migrate collectively to reseal the barrier (1). Given the importance of polarized epithelial cell migration to achieve wound repair, we analyzed the influence of LTB₄ on directional migration of epithelial cells using Diper software (20) (22, 23). These analyses suggest that LTB₄ signaling regulates collective IEC migration through enhanced directional persistence and speed of cell movement. It is also well appreciated that remodeling of the actin cytoskeleton and integrin-containing focal cell-matrix adhesions plays a pivotal role in controlling forward movement of cells (21). Our studies revealed that LTB₄ mediated ligation of BLT1 activates proteins that control remodeling of focal adhesions. Furthermore, we observed that LTB₄ exposure enhances proliferation of colonoids that likely contributes to observed pro-repair properties of LTB₄-BLT1 signaling. Other studies have reported that BLT1 signaling enhances proliferation of other cell types including B cells (24), hepatocytes (25), and smooth muscle cells (26). These reports support our findings that an LTB₄-BLT1 signaling axis likely promotes proliferation of IECs. Importantly, we observed delayed wound healing in 2D colonoids deficient in BLT1, suggesting that epithelial cells may produce LTB₄ in an autocrine fashion to promote wound healing or
that epithelial BLT1 directly regulates expression of molecules involved in the repair process.

Proinflammatory leukotrienes, generated by 5-lipoxygenase (LOX) and the 5-LOX-activating protein (FLAP), initiate, and maintain inflammation while specialized pro-resolving mediators (SPMs) generated by various LOXs promote resolution and repair (27, 28). Since 5-LOX also contributes to SPM biosynthesis, pharmacological manipulation of the 5-LOX pathway and activation of 12-/15-LOXs might cause suppression of leukotriene formation and maintain SPM generation. Previous reports suggest that 5-LOX inhibitors increase wound healing by decreasing LTB4 synthesis and neutrophil recruitment (29, 30). Interestingly, we and others have shown that neutrophil depletion during acute injury causes delayed repair, implying that 5-LO inhibition in early stages of colonic wound healing is detrimental for mucosal repair (31). 5-LO KO mice exhibit faster skin wound healing compared to WT mice (32). However, pharmacological inhibition of 5-LO in vitro inhibits migration and proliferation of keratinocytes (29), suggesting that the role of 5-LO during epithelial wound repair is complex and might depend on the tissue specific molecular interactions in the wound milieu. Furthermore, 5-LO also regulates the
synthesis of anti-inflammatory soluble mediators such as SPMs, and therefore inhibiting these molecules would also impact reparative responses.

Finally, we observed that BLT1 signaling plays an important role in regulating in vivo intestinal mucosal wound repair. Bone marrow transplant experiments and analyses of colonic mucosal wound repair results identified similar contributions of both IEC and immune cell-expressed BLT1 in regulating intestinal mucosal wound repair. It is important to note that neutrophils are the first responders to sites of acute injury in the mucosa (1). In support of this, we observe abundant neutrophils in murine colonic mucosal wounds within 4 to 6 hours after initial injury, with maximum numbers detected between 6 to 24 hours after biopsy induced injury (33, 34). Neutrophils play a critical role in facilitating recovery since their depletion results in impaired mucosal repair and delayed recovery from colitis (35, 36). Neutrophils are also major producers of SPMs and the LTB₄-BLT1 pathway is well known for its function as a chemotactic signal that regulates neutrophil migration to site of inflammation (15). Since we previously showed that LTB₄ levels are increased in acute colonic mucosal wounds compared with intact tissues
(4), and infiltrating leukocytes are a potent source of LTB₄, we therefore suggest that LTB₄ released in
the wound bed engages epithelial BLT1 and triggers intestinal epithelial wound healing. The
concentration of LTB₄ at sites of mucosal injury is much higher than the other BLT1 ligand RvE1.
Interestingly, kinetics of the levels of LTB₄ and RvE1 in wounds are also different. LTB₄ is secreted
during the early stages of inflammation while RvE1 is released at later points when the LTB₄ synthesis
is declining. As mentioned above, Inflammation and repair are complementary events that are initiated
at the same time to orchestrate repair. While LTB₄ promotes migration of immune cells to sites of
mucosal injury, it also enhances migration of epithelial cells. Neutrophils express both BLT1 and
CMKLR1, while IEC only express BLT1. RvE1 signaling through CMKLR1 and BLT1 promotes PMN
apoptosis and in IECs RvE1 sustains the migratory response triggered by BLT1 activation by LTB₄. Our
finding that LTB₄ can signal on epithelial cells and trigger pro-repair responses challenges the long-
standing dogma that LTB₄-BLT1 signaling is exclusively a pro-inflammatory event. Taken together, our
findings highlight a novel intestinal epithelial pro-repair mechanism that is mediated by LTB₄-BLT1
signaling pathway which serves to orchestrate mucosal wound repair and restore the critical mucosal barrier.
Materials and Methods

**Mice:** *Ltb4r1*−/− mice (B6.129Sa-Ltb4r1tm1Adl/J) on a C57BL/6 background (11) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the experimental animal facility at the University of Michigan and were provided free access to food and water. All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health and the University of Michigan.

**Colonic organoid and epithelial monolayer culture:** Human three-dimensional (3D) colonic organoids (colonoids) were provided from Translational Tissue Modeling Laboratory (University of Michigan) and maintained in the laboratory (37). Murine colonoids were created and maintained in culture according to our previous report (38) modified method reported by Sato et al (39). Isolated intestinal crypts from WT or *Ltb4r1*−/− mice were embedded in Matrigel and maintained in LWRN complete media. 2D colonic epithelial monolayers from human or murine 3D colonoids were generated as previously described (40) and maintained in LWRN complete media.
Cell lines: The human IECs, SKCO-15 and T84 were cultured as described previously (19). In some experiments, SKCO-15 cells were stimulated with 100 ng/ml IFN-γ (cat. 285-IF, R&D Systems, Minneapolis, MN) and 100 ng/ml TNF-α (cat. 210-TA, R&D Systems).

RNAscope in situ hybridization: RNAscope was performed on frozen tissue sections of human and murine colonic mucosa. In situ hybridization was performed according to the protocol of the RNAscope Multiplex Fluorescent Reagent Kit v2 (cat. 323100, Advanced Cell Diagnostics, Newark, CA). In this study, positive (Homo sapiens PPIB or Mus musculus Ppib), negative (Bacillus subtilis strain SMY DapB) control probe and 4 different probes (Homo sapiens LTB4R and CMKLR1, and Mus musculus Ltb4r1 and Cmklr1) were used. Images were acquired using a Nikon A1 confocal microscope (Nikon, Japan). Quantification of Ltb4r1 in the murine colonic mucosa was analyzed using QuPath (v0.3.0) as recommended by ACD.

RNA extraction and qPCR: The mRNA expression levels of various genes were measured in human and mouse samples as described previously (41). In brief, total RNA was extracted from the
samples using the RNeasy Mini Kit (cat. 74106, Qiagen, Ann Arbor, MI) according to the manufacturer’s instructions. Reverse transcription was performed using the iScript Reverse Transcription Supermix for RT-qPCR (cat. 1708840, BioRad, Hercules, CA). qPCR amplification was then performed using the iQ SYBR Green Supermix (cat. 1708880, BioRad) in a CFX Connect Real-Time PCR Detection System (BioRad). Target mRNA levels were normalized to those of TBP or Tbp as the internal control in each sample and calculated by the 2^{-DDCt} method. The results are expressed as ratios relative to the average for the control group. The following primer pairs were used: Homo sapiens LTB4R, (forward) 5’-GTTTTGGACTGGCTGGTTGC-3’ and (reverse) 5’-GGTACGCGAGGACGCGGTTGC-3’; Homo sapiens CMKLR1, (ACAGCATCAGTCTACCACCTT) 5’--3’ and (GAGTCCTCAGCAATCAGTC) 5’--3’; Homo sapiens TBP, (forward) 5’-TGCACAGGAGCCAAGAGTGAA-3’ and (reverse) 5’-CACATCACAGCTCCCCACCA-3’; Mus musculus Ltb4r1, (forward) 5’-ATGGCTGCAAACACTACATCTC-3’ and (reverse) 5’-GACCGTGCGTTTCTGCATC-3’; Mus musculus Tbp, (forward) 5’-
GGAATTGTACCGCAGCTTCAAA-3’ and (reverse) 5’-GATGACTGCAGCAAATCGCTT-3’.

**Wound healing assay:** For in vitro experiments, SKCO-1, and primary human and murine colonoids cultured as 2D monolayers were subjected to scratch wounding assays. Monolayers were cultured on 48-well tissue culture plates (Corning Incorporated, Corning, NY) to confluency and scratched using a 10 µl pipette tip. In the case of colonoids, Monolayers were cultured on collagen (cat. C5533, Sigma-Aldrich, St. Louis, MO)-coated 48-well tissue culture plates. Medium was changed after wounding and video quantification of scratch-wound closure was performed by imaging wounds at 1 h intervals in Axio Observer Z1 live cell microscopy system (ZEISS, Oberkochen, Germany). IECs were incubated with LTB4 (Cayman, Ann Arbor, MI) or RvE1 (Cayman, Ann Arbor, MI) for 24h. BLT1 antagonist (CP105,696; Sigma-Aldrich) or CMKLR1 antagonist (a-NETA; Cayman) was applied 30 min before LTB4 or RvE1 treatment. Wound closure was quantified at the indicated time points using ImageJ software (NIH, Bethesda, MD). For in vivo wounding experiments of colonic mucosa, a biopsy-based mucosal wound model was employed using a high-resolution, miniaturized endoscope system.
(Coloview Veterinary Endoscope; Karl Storz, Tuttlingen, Germany) equipped with biopsy forceps to create biopsy-induced injury of the colonic mucosa at five sites along the dorsal aspect of the colon of anesthetized mice (IP injection of 100 mg/kg ketamine and 5 mg/kg xylazine). Wound healing was quantified at 1 day and 3 days after injury. Endoscopic procedures were viewed with high-resolution (1,027 x 768 pixels) images on a flat-panel color monitor. Each wound region was digitally photographed at 1 day and 3 days, and wound areas were measured using ImageJ software.

**Epithelial cell migration assay (Diper):** For time-lapse experiments, cells were imaged for 12h at a time every 30 minutes. Images were exported and stacked to videos. Cellular tracking was performed using 20 cells from each sample (10 cells/each side) using ImageJ software. Data were analyzed via DiPer for Plot_At_Origin (plots cell trajectories emanating from the origin), mean square displacements (MSD), direction autocorrelation, and cell speed (20).

**Immunoblot:** For cell lysis, IEC monolayers were harvested in RIPA buffer as described previously (4). The following antibodies were used: FAK (cat. 610088) BD Biosciences (Franklin Lakes, NJ);
pFAK (Y861) (cat. PS 1008) Calbiochem (Darmstadt, Germany); pFAK (Tyr397) (cat. 3283), pFAK (Tyr925) (cat 3284); Src (cat 2108), pSrc (Tyr416) (cat 2101); Cell Signaling Technology (Danvers, MA)

**Epithelial cell proliferation assay:** Two hours before fixing of cells, 5-ethynyl-2'-deoxyuridine (EdU) was added to the media at a concentration of 100 M. Proliferating cells were detected with the Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 488 dye (cat. C10337, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions and captured using a Nikon A1 confocal microscope.

**BM transplantation:** For total BM transplant experiments, donor BM cells were harvested from WT and *Ltbr1*−/− mice. Recipient mice were sublethally irradiated using 2 times 5 Gy X-rays 4h apart (42). 1x10^6 donor BM cells were transplanted by retro-orbital venous plexus injection into recipient mice. Blood samples were collected from the recipients 8 weeks after BM transplantation to confirm engraftment. Experiments using the recipients were conducted 8 weeks after BM transplantation and
blood samples was collected for engraftment and complete blood cell (CBC) analysis.

**Docking simulation:** For docking studies, BIIL260 was removed from the crystal structure of protein data band (PDB) ID: 5X33 (43) to create apo-BLT1 structure and predicted the binding site of RvE1 to BLT1 using AutoDock Vina (The Scripps Research Institute, La Jolla, CA).

**Statistical analysis:** The data are presented as the means ± SEMs. Statistical analyses were performed with Prism 9 (GraphPad Software, San Diego, CA) using one- or two-ANOVA followed by Bonferroni’s multiple comparison test, Tukey’s multiple comparison test or an unpaired (two-tailed) t test with Welch’s correction. Values of p < 0.05 were considered to indicate significant differences.

**Study approval:** All experimental procedures involving animals were conducted in accordance with NIH guidelines and protocols approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Author contributions:** SH and MQ performed experiments in addition to data analysis/interpretation. SH and MQ wrote the manuscript. MQ, CP and AN oversaw the project design and execution,
provided assistance in writing and editing the manuscript and acquired funding.
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References


15. Subramanian BC, Majumdar R, and Parent CA. The role of the LTB(4)-BLT1 axis in


20. Gorelik R, and Gautreau A. Quantitative and unbiased analysis of directional persistence


38. Muraleedharan CK, Mierzwik J, Feier D, Nusrat A, and Quiros M. Generation of Murine
Primary Colon Epithelial Monolayers from Intestinal Crypts. *J Vis Exp.* 2021(168).


ligands stabilize the inactive state of leukotriene B(4) receptor BLT1. *Nat Chem Biol.*

Figure 1. BLT1 functions as a major epithelial receptor for RvE1. (A) RNAscope staining for LTB4R and CMKLR1 mRNA expression in frozen section from colonic tissue of human. (B) RNAscope staining for Ltb4r1 and Cmklr1 mRNA expression in frozen section from colonic tissue of mouse. (C) qPCR analysis of the expression of CMKLR1 and LTB4R mRNA in the SKCO-15, T84 and human 2D colonoids. The data are presented as the mean ± SEM. (D) Effect of BLT1 antagonist on the pro-repair activity of RvE1 in the scratch wound assay using human primary IECs. After scratch wound was produced, IECs were incubated with RvE1 (100 nM) for 24h. BLT1 (CP105,696; 1 µM) or CMKLR1 (α-NETA; 10 µM) antagonist was applied 30 min before RvE1 treatment. Quantification of wound repair at 24 h after wounding is shown. The data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. *P < 0.05; **P < 0.01, compared with RvE1.
Figure 2. Epithelial BLT1 is upregulated in response to colonic mucosal injury. (A) The changes in the expression of \( \text{Ltb4r1} \) mRNA in 3 mm punch biopsies of intact colonic tissues and colonic mucosal wounds on different days after injury. The data are presented as the mean ± SEM of 4-5 mice. Statistical analysis was performed using one-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. *\( P < 0.05 \); **\( P < 0.01 \), compared with intact tissue (IT). (B-D) RNAscope staining for \( \text{Blt1} \) mRNA in frozen sections from intact tissues and wounded colonic tissues 2 days after injury. W; Wound. Scale bar is 50 µm. (E) The number of \( \text{Ltb4r1} \) mRNA positive dots in the crypt of intact colonic tissues and colonic mucosal wounds (adjacent to wound) on 2 days after injury is shown. The data are presented as the mean ± SEM of 6 mice. Statistical analysis was performed using an unpaired (two-tailed) t test with Welch’s correction. *\( P < 0.05 \), compared with IT. AW; Adjacent to wound. (F-G) RNAscope staining for \( \text{LTB4R} \) mRNA expression in frozen sections from healthy controls and ulcerative colitis patients.
**Figure 3. BLT1 regulates intestinal epithelial wound repair.** (A) Effect of LTB₄ (1-100 nM) in the scratch wound assay using SKCO-15 cells. After scratch wound was produced, cells were incubated with LTB₄ for 24h. BLT1 antagonist CP105,696 (1 µM) was applied 30 min before LTB₄ treatment. Quantification of wound repair at 18h after wounding is shown. Data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. (B-C) Effect of LTB₄ (10 nM) in the scratch wound assay using human colonoid-derived primary colonic epithelial monolayers. After scratch wound was produced, cells were incubated with LTB₄ for 24h. BLT1 antagonist CP105,696 (1 µM) was applied 30 min before LTB₄ treatment. Representative phase-contrast images at 0 and 24h after wounding are shown. Scale bar is 100 µm. (B) Statistical analysis was performed using two-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. **P < 0.01; ***P < 0.001; ****P < 0.0001, compared with Vehicle. ††P < 0.05; †††P < 0.01; ††††P < 0.001, compared with LTB₄. (D-E) Effect of LTB₄ (10 nM) in the scratch wound assay using primary colonic epithelial monolayers derived from colonoids of both WT and Ltb4r1-/− mice. After scratch wound was produced, cells were incubated with LTB₄ for 24h. (D) Representative phase-contrast images at 0 and 24h after wounding are shown. Scale bar is 100 µm. (E) Quantification of change over time in wound repair are shown. Data are presented as the mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. *P < 0.05; **P < 0.001, compared with WT (vehicle). ††P < 0.01; †††P < 0.001, compared with Ltb4r1-/− (vehicle). (F) qPCR analysis of the changes in the expression of LTB4R mRNA in the human 2D cultured colonoid stimulated with IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 24h. The data are presented as the mean ± SEM. Statistical analysis was performed using an unpaired (two-tailed) t test with Welch’s correction. *P < 0.05. (G) Effect of IFN-γ (100 ng/ml) and TNF-α (100 ng/ml) on the pro-repair activity of low-dose LTB₄ (1 nM) in the scratch wound assay using SKCO-15 cells. Quantification of wound repair at 18h after wounding is shown. Data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. **P < 0.01; ***P < 0.001; ****P < 0.0001.
Figure 4. BLT1 activation promotes migration and proliferation of IECs. (A-D) Migration analysis by Diper. (A) Plot at the origin graph of 20 cells. (B) Mean square Displacement (MSD) of 20 cells. The data are presented as the mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. **P < 0.01; ****P < 0.0001, compared with WT (vehicle). ††P < 0.01; †††P < 0.001; ††††P < 0.0001, compared with Ltb4r1-/- (vehicle). (C) Velocity autocorrelation was measured on at least 20 cells. Statistical analysis was performed using two-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. **P < 0.01; ***P < 0.001; ****P < 0.0001, compared with WT (vehicle). ††P < 0.01, compared with Ltb4r1-/- (vehicle). (D) Average cell speed was calculated on 20 cells. The data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. ***P < 0.001; ****P < 0.0001. (E) Immunoblotting was performed on lysates from scratch wounded IEC monolayers treated with LTB4 (100nM) or vehicle. Levels of pSRC (416) and pFAK (Y397, Y925) were compared with total Src, FAK and GAPDH to assess activation. (F-G) EdU incorporation analysis in the murine 3D cultured colonoids stimulated with LTB4 (10 nM) for 24h. (F-G) Effect of BLT1 antagonist. Pictures are showing the representative images of EdU (green) incorporated colonoids. Blue, nuclei. Scale bar is 10 µm. The data are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. *P < 0.05; **P < 0.01.
Figure 5. Role of BLT1 in the intestinal mucosal wound repair in vivo. (A) In vivo intestinal mucosal wound repair in \textit{Ltb4r1}^{-/-} mice. Utilizing a miniature video endoscope and biopsy scissors, 5 wounds were created in the dorsal aspect of the colonic mucosa of anesthetized mice. Digital images of wound surface area at 1 and 3 days after wounding are shown (Left). Points represent the mean value within all wounds from individual mice (Right). The data are presented as the mean ± SEM of 9-10 mice. Statistical analysis was performed using an unpaired (two-tailed) t test with Welch’s correction. **** \( P < 0.0001 \).

(B-C) In vivo intestinal mucosal wound repair in BM chimeric mice. (B) Illustration of BM chimera experiment. (C) Digital images of wound surface area at 1 and 3 days after wounding are shown (Left). Points represent the mean value within all wounds from individual mice (Right). The data are presented as the mean ± SEM of 5 mice. Statistical analysis was performed using one-way ANOVA followed by post-hoc Welch’s t test with Bonferroni’s correction. *** \( P < 0.001 \); **** \( P < 0.0001 \).