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Lymphatic Network Remodeling After Small Bowel Resection

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Abstract

Background: Short gut syndrome (SGS) following massive small bowel resection (SBR) is a major cause of pediatric mortality and morbidity secondary to nutritional deficiencies and the sequelae of chronic total parenteral nutrition use, including liver steatosis. Despite the importance of lymphatic vasculature in fat absorption, lymphatic response after SBR has not been studied. We hypothesize that lymphatic vessel integrity is compromised in SGS, potentially contributing to the development of impaired lipid transport leading to liver steatosis and metabolic disease.

Methods: Mice underwent 50% proximal SBR or sham operations. Imaging of lymphatic vasculature in the lamina propria and mesentery was compared between sham and SBR Prox1 ER^{Cre}-Rosa26^{LSL}-TdTomato mice. mRNA expression levels of lymphangiogenic markers were performed in C57BL/6J mice.

Results: Lymphatic vasculature was significantly altered after SBR. Mesenteric lymphatic collecting vessels developed new branching structures and lacked normal valves at branch points, while total mucosal lymphatic capillary area in the distal ileum decreased compared to both sham and intraoperative controls. Intestinal *Vegfr3* expression also increased significantly in resected mice.

Conclusions: Intestinal lymphatics, in both the lamina propria and mesentery, dramatically remodel following SBR. This remodeling may affect lymphatic flow and function, potentially contributing to morbidities and nutritional deficiencies associated with SGS.

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Disclosures

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Keywords

Short gut syndrome; Intestinal failure; Lymphatics; Remodeling; Small bowel resection; Adaptation

1. Introduction

Short gut syndrome (SGS), the result of extensive surgical loss of small intestine to treat pediatric conditions such as midgut volvulus and necrotizing enterocolitis, occurs in 3 to 5 per 100,000 births annually [1, 2]. SGS patients are burdened with a mortality rate of 27% by 36 months and multiple associated morbidities, one of the most common being the development of intestinal failure-associated liver disease (IFALD) [3–5]. Initially thought to be secondary to prolonged parenteral nutrition, the pathogenesis of IFALD is multifactorial, including alterations in the intestinal microbiome, sepsis, and direct hepatic toxic injury [6–8]. IFALD begins with lipid accumulation in the liver and progresses to hepatitis, fibrosis, and ultimately cirrhosis [9].

Despite the importance of the lymphatics in fat absorption and immunity, little is known about how lymphatic transport is affected in SGS. The intestinal lymphatic vasculature is a one-way network of vessels leading to mesenteric lymph nodes and ultimately to the blood circulation via the thoracic duct. It functions in both nutrient absorption through chylomicrons (fats, vitamins, hormones, and endotoxins) and immune-surveillance (dendritic cells and lymphocytes) [10, 11]. Lymph and immune cells enter lacteals through discontinuous cell-cell button-like junctions between lymphatic endothelial cells (LECs) [12]. Lacteals feed lymphatic capillaries in the submucosal space, which then flow into mesenteric collecting vessels, ultimately leading to the mesenteric lymph node chain [13, 14].

Smooth muscle cells cover lymphatic collecting vessels, aiding in transport, while lymphatic valves prevent back flow [15, 16]. Most nutrient uptake occurs in the proximal small bowel, with lacteal density decreasing from the duodenum to the ileum [17]. Maintenance of lymphatic vasculature, through development and remodeling, relies on the communication of lacteals and their bordering capillaries [18]. Vascular endothelial growth factor C (VEGF-C) is fundamental for intestinal lymphatic maintenance and function, while Prospero homeobox protein 1 (PROX1) stimulates lymphatic budding and valve formation [17, 19, 20]. The expression of PROX1, which governs lymphangiogenesis, has been found to be restricted to LECs [21, 22]. Lymphatic vessel endothelial hyaluronic acid receptor (LYVE-1), expressed by venous endothelial cells and LECs, characterizes lymphatic lacteals [23].

Perturbations in intestinal lymphatics have been shown to lead to severe gut inflammation and have been implicated in the pathogenesis of inflammatory bowel disease [24–27]. While the mechanisms underlying IFALD remain unclear, disturbance in lymphatic vasculature after massive small bowel resection (SBR, a surgical model for SGS) may contribute to this phenomenon. We hypothesize that lymphatic integrity is compromised in SGS, potentially contributing to the development of impaired lipid transport into the systemic circulation,

resulting in diversion of lipid transport to the portal vein, leading to liver steatosis and metabolic disease.

2. Methods

2.1 Animals

Prox1-Cre-ERT² (Jax # 022075; originally generated by R. Srinivasan and G. Oliver [28]) crossed with Rosa26-tdTomato^{fl/fl} reporter (Jax # 007905) 12–16 week male and female mice were orally treated with 20 mg/ml tamoxifen (Sigma, St. Louis, MO) dissolved in corn oil (50 µg/per gram body weight) for two weeks, every other day to induce Cre recombinase activity and downstream tdTomato expression. C57BL/6J 9–12 week male mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed on a 12-hour light-dark cycle in a temperature controlled, specific pathogen-free unit. Food and water were provided *ad libitum*. This study was approved by the Washington University in St. Louis Animal Studies Committee (Protocol 20170252 and 20170154) in accordance with the National Institute of Health laboratory animal care and use guidelines.

2.2 Operations and harvest

All mice either underwent a 50% proximal SBR or sham control operation, as previously described [29]. In brief, bowel was exteriorized through a midline laparotomy and transected 1 to 2 cm distal to the ligament of Treitz and 12 cm proximal to the ileocecal junction. A handsewn end-to-end anastomosis with interrupted 9–0 nylon sutures was then performed. Sham operations consisted of a distal bowel transection 12 cm from the ileocecal junction with re-anastomosis. Intraoperative (IO) resected distal bowel, which served as a non-operative control, was fixed in 10% formalin and embedded in paraffin for histology and immunohistochemistry. Postoperative care included housing in an incubator for temperature stability and fasting for 24 hours before starting standard liquid diet (PMI Micro-Stabilized Robert Liquid Diet LD 101; TestDiet, St. Louis, MO). Small bowel and mesentery were harvested three or seven days postoperatively.

2.3 Immunohistochemistry and immunofluorescence

Intestinal tissue from distal to the anastomosis was fixed in 10% formalin, embedded in paraffin, and processed to generate 5-µm thick longitudinal sections at time of harvest. Villus height and crypt depth were measured to assess for structural adaptation (NIS elements AR 4; Nikon, Melville, NY). Antigen retrieval was performed (Diva Decloaking solution, Biocare Medical, Concord, CA) on deparaffinized slides in a pressurized chamber (10 minutes). Sections were blocked in donkey serum (5%, Sigma-Aldrich), bovine serum albumin (1%, Sigma-Aldrich), and Triton-X100 (0.03%, Pharmacia Biotech) and then incubated overnight at 4°C with primary antibodies diluted in 0.2% bovine serum albumin. Secondary antibodies were added the next day for 1 hour. Antibodies used include rabbit anti-mouse LYVE-1 (1:600, Abcam, ab14917), Cy3-conjugated donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch, 711-165-152) and FITC-conjugated mouse anti-mouse alpha-smooth muscle actin IgG (1:500, Sigma, F3777, Clone #1A4). Images were taken using a confocal microscope (Leica SPE and SP8) and blinded analysis was performed using Imaris (Bitplane, Switzerland) and FIJI (ImageJ) software (National Institute of Health,

Bethesda, MD) of at least 6 mm of intestine for each sample. Mucosal lymphatics were defined as LYVE-1⁺ vessel structures present in the lamina propria.

2.4 Whole mount mesenteric imaging

Prox1-Cre-ER^{T2}xRosa26^{LSL}-tdTomato small intestine with attached mesentery was fixed in 4% paraformaldehyde + 30% sucrose overnight. The mesenteric-gut wall interface distal to the anastomosis in the portion of the ileum undergoing compensatory adaptation was then transferred into phosphate-buffered saline for fluorescent imaging using a stereoscope (Leica M205FA). The gut-mesentery interface was prepared for whole mount imaging by first staining lymphatics (Prox1, 1:1000, rabbit polyclonal, Origene, DF3516) and adipocytes (Perilipin; 1:200, goat polyclonal, Santa Cruz Biotechnology, SC-47322). Tissue was dehydrated and cleared using methyl salicylate (Sigma-Aldrich) as previously reported [30] and imaged using a confocal microscope (Leica SP8). Blinded image analysis, including lymphatic branch and valve counting of the mesentery distal to the anastomosis from the lymph node to the gut wall, was performed using Imaris (Bitplane, Switzerland) and FIJI (ImageJ) software (National Institute of Health, USA).

2.5 RNA extraction and quantitative reverse transcription-polymerase chain reaction

RNA was extracted from post-anastomotic ileal whole tissue from postoperative day (POD) 3 mice using the standard Trizol method. RNA concentration was determined using the NanoDrop Spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE). RNA was converted to cDNA using the qScript cDNA Synthesis Kit (Quanta Bio Beverly, MA). Real time polymerase chain reaction (RT-PCR) was performed using the TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, Cat. 4369016) and Applied Biosystems 7500 Fast Real-Time PCR to determine relative concentrations of the following transcripts: *Flt4/Vegfr3* (Mm01292604_m1), *Prox1* (Mm00435969_m1), and *Actb* (Mm02619580_g1) (endogenous control). All primer probes are from Applied Biosystems.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad-Prism 6 software (La Jolla, CA). Valve to branch ratios, epithelial adaptation (crypt depth and villus height), area of mucosal lymphatic capillaries and lamina propria, and mRNA expression levels were analyzed using the unpaired Student's t test. A p value of <0.05 was considered significant.

3. Results

3.1 Mesenteric collecting vessel change after SBR

SGS is correlated with IFALD, which initiates with improper lipid deposition in the liver. Since lipids are transported from the intestine to the circulation via series of specialized lymphatic vessels, we sought to understand the direct effects of bowel resection on this vascular bed. By POD7, lymphatic collecting vessels present in the mesentery and emerging from the intestinal wall distal to the anastomosis show altered morphology following SBR versus sham mice (Figure 1, A–D). The SBR lymphatic network appeared to be broader and lacked normal valves at branch points. When quantified, we observed a significant 49±1% reduction in valves per branch point (p<0.0001, Figure 1, E–F). Furthermore, many

lymphatic vessels were surrounded by apparently fluid-filled spaces between adipocytes, indicating that the adipose tissue was highly edematous. Moreover, some collecting vessels appeared to have bulbous expansions (Figure 2, A–B). These data demonstrate that bowel resection promotes major downstream lymphatic mesenteric modifications that possibly impact lymphatic drainage and, therefore, its function.

3.2 Intestinal mucosal lymphatic capillaries change after SBR

It is well known that bowel resection promotes structural adaptation within a week in the remaining intestine [29]. To explore upstream intestinal lymphatic alterations after SBR during the adaptation phase, we next investigated the mucosal lymphatic capillaries by immunofluorescence. Both crypt depth and villus height significantly increased in resected mice by POD7, affirming appropriate structural intestinal adaptation (Figure 3, A)[29]. There was a $31\pm4\%$ and $14\pm4\%$ increase in crypt depth at POD3 and POD7, respectively ($****p<0.001$, $**p<0.005$). There was a $19\pm1\%$ increase in villus height at POD7 ($**p<0.005$). Staining for lymphatics was performed on IO, POD3, and POD7 distal ileal tissue to evaluate mucosal lymphatic capillary remodeling (Figure 3, B). Interestingly, image analysis showed no statistical difference in the number of lymphatic capillaries present in the lamina propria; however, their luminal area was significantly changed (Figure 3, B–D). By POD7, sham mice had a significant $19\pm3\%$ increase in mucosal lymphatic capillary area in the lamina propria compared to IO controls ($p<0.05$). By contrast, in resected mice, the area of mucosal lymphatic capillaries in the lamina propria showed a significant $21\pm4\%$ reduction by POD3, which persisted to POD7 ($p<0.05$).

3.3 Intestinal Vegfr3 expression level increases after SBR

Lymphatic remodeling and proliferation is governed by the transcription factor PROX1 and dependent on the VEGFR3 signaling cascade [11, 31, 32]. Hence, in addition to visualizing the structural lymphatic changes in the lamina propria and mesentery of SBR mice, we also measured the relative expression of *Vegfr3*, the gene transcribing VEGFR3, which regulates lymphatic maintenance. We demonstrate a significant 1.6-fold increase in *Vegfr3* expression in SBR compared to sham mice at POD3 in the distal ileum (Figure 4, A, $p<0.05$). There was no difference in *Prox1* mRNA expression between sham and SBR mice (Figure 4, B).

4. Discussion

In the present study, we describe significant changes in the lymphatic vasculature of mice having undergone SBR as compared to sham operation, both in the mesentery and lamina propria network.

Mesenteric whole mount imaging and analysis showed a significant change in lymphatic collecting vessel morphology. In the lymphatic vessels leading from the intestinal wall to the mesenteric lymph node, vessels developed broad extensions into the mesenteric sheath. This early lymphatic remodeling is reminiscent of more primitive collecting vessels normally seen during development in mice [33, 34]. In this setting of amplified lymphangiogenesis, we found a reduction in the ratio of valves per branch point in collecting ducts. Also, many collecting vessels appeared to have bulbous expansions, similar to the vessel dilation seen in

mice with deficient lympho-venous junctions (C-type lectin-like receptor 2), resulting in higher lymphatic pressures and retrograde flow[33]. Moreover, it has been shown that shear stress promoted by lymphatic flow is necessary for the development and maturation of lymphatic valves, which control proper lymph trafficking [11, 33, 34]. Thus, the reduction in valve to branch ratios and presence of bulbous expansions in the collecting ducts suggest that flow is likely compromised after SBR.

Impaired flow may cause lymphatics to function inappropriately. The lymphatic vasculature not only plays a key role in immune surveillance and lipid uptake, but also in maintenance of tissue fluid homeostasis [15]. Many lymphatics in resected mice were surrounded by apparently fluid-filled spaces between adipocytes, suggesting that the adipose tissue was highly edematous from interstitial fluid disequilibrium. In homeostasis, lymphatic absorption of lipids occurs mainly in the proximal bowel [10]. The distal ileum in resected mice significantly increases its expression of *Vegfr3*, which suggests feedback mechanisms are turned on in order to stimulate lymphangiogenesis, maintenance of lymphatic vessels, and lipid absorption [35–37]. However, despite *Vegfr3* elevation, we do not observe mucosal lymphatic proliferation or expansion in SBR at the time points examined, which is supported by the imaging analysis and the unchanged expression of *Prox1* in the intestine. That SBR significantly increases *Vegfr3* expression without increasing total lymphatic area or other lymphatic markers may suggest the existence of a feedback regulation affecting lymphatic adaptation. On the other hand, we have shown increased VEGFR3 is likely necessary following SBR to promote adaptation and sustain the lymphatic network.

In the distal intestine of resected mice, while the number of mucosal lymphatic capillaries sampled remained constant, their total area significantly reduced. Although lymphatic remodeling in the setting of SGS has not been studied, lymphatic changes have been postulated to play a role in the pathogenesis of Crohn's disease [26, 38]. An increase in lymphatic vessel density in intestinal mucosa and submucosa is characteristic of a better prognosis in patients with Crohn's disease [39]. In our POD7 sham mice, there was a significant increase in total lymphatic area. Interestingly, in Crohn's patients requiring ileocolic resections, there is a direct association between postoperative endoscopic recurrence and lymphatic density at the time of surgery: decreased lymphatic density at resection sites increases the risk of postoperative recurrence [40]. This observation correlates with our SBR mice at POD7, which also have a significant decrease in mucosal lymphatic capillary area. We believe there could be two explanations for this change in the SBR mice. Firstly, it could be due to this area of bowel's poor response to inflammation, particularly given the mesenteric remodeling with a loss of valves that we believe results from reduced flow, compromising immunity. Secondly, the distal ileum normally contributes little to lipid absorption, with shorter lacteals than in the duodenum [10, 17]. As such, the proximal small bowel mucosal lymphatic capillaries may increase in size to compensate in SBR with no need for increased lymphatic area in the distal bowel.

Overall, intestinal resection results in early lymphatic remodeling characterized by primitive budding structures in the mesentery, reduced mucosal capillary vessel area, and loss of valves. We initially investigated these lymphatic changes at POD7, when compensatory adaptation that increases the mucosal absorptive surface area has occurred [29]. However,

whether these morphological changes in the lymphatic vasculature persist or if remodeling continues over time remains unknown. We hypothesize that lymphatic remodeling after bowel resection also results in functional changes, such as compromised lymphatic flow resulting in impaired intestinal fat and vitamin transport as well as a weakened gut immunity. We have previously shown that mice develop hepatic steatosis ten weeks after massive small bowel resection in our TPN-independent model[41]. We believe that lymphatic remodeling after SGS could alter intestinal absorption and transport of fats, contributing to this hepatic fat deposition. How these lymphatic changes contribute to the pathogenesis of IFALD is presently unknown. However, it is possible that the absorption of luminal factors that would normally be taken up into the lymphatic system could potentially end up in the portal venous blood. These might include various lipids, endotoxins, or other potentially hepatotoxic factors. Future studies will be directed toward testing these possibilities.

5. Conclusion

These data indicate that intestinal lymphatic vessels significantly remodel following massive SBR. The loss of valves has previously been associated with loss of flow in lymphatics, suggesting that the observed remodeling likely results in impaired intestinal transport of fat via the lymphatic architecture [33, 34]. It is likely that this lymphatic remodeling effects fatty acid uptake and, consequently, these changes may contribute to the development of IFALD, a major morbidity in patients with SGS. Future studies are needed to test the implications that these findings support.

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Discussion

Presenter: Emily J. Onufer MD, MPH

DR. GAIL E. BESNER, COLUMBUS, OH: First of all, that was an exquisite study, congratulations. Your lymphatic staining was gorgeous, I loved it. I'm curious as to first of all, it's so novel to look at these lymphatics, because as you pointed out, it hasn't been commonly done. And I'm wondering if you've thought about looking at the lymphatic distribution or potential abnormalities in other instances in which there is dilated bowel, for instance, proximal to a bowel atresia or the dilated bowel of Hirschsprung disease. A very interesting study, thank you.

DR. ONUFER: That's wonderful. I actually have been helping a lab study this in a Crohn's model as well.

DR. STEVEN FISHMAN, BOSTON, MA: That's really beautiful work, and it warms my heart to see people in our field starting to pay attention to lymphatic, the underappreciated

but extremely important vessel, which I spend my life thinking about. I think that you're on to something then with advances that we have an understanding of lymphangiogenesis and lymphatic control. Hopefully one day this will be an opportunity for addressing this disease.

My question is the seven day mark. Now, I understand we're dealing with rodents and not with humans, but how do you know that this isn't just the early part of the remodeling of lymphangiogenesis, and that if you look at this further on down into adaptation that this would be the same finding, or maybe you've already done.

DR. ONUFER: Are you going to ask this? Hopefully. I've already looked at a long time point, and I can confirm that these are actually increased and more drastic results.

DR. FISHMAN: Terrific, I look forward to that presentation.

DR. MARTIN: I just have one more quick question. Congratulations. So in Warner's lab, traditionally the remnant bowel undergoes adaptation. So a lot of the read outs show that the intestine gets better, more efficient, more proliferative markers, but now you're showing that the lymphatic system is sort of going a maladaptive response. So I'm just sort of curious how a lot of the readouts show positive, and this is a negative in the remnant bowel.

DR. ONUFER: You're right. So I originally thought that too, that the lymphatic structures would turn into this like awesome thing that, especially in the proximal bowel, I thought that they would enlarge and because the proximal bowel is where most fats are absorbed, and there would be hypertrophy there, but you're correct in that the distal bowel actually turns into this more primitive lymphangiogenesis. And so in order to really test this, I don't necessarily think maybe there are these kind of primitive structures, but I do think that maybe the transport of fats are actually improved in the proximal bowel. And to test this we actually have a photoactivatable mouse, which has apo A1 attached to HDL. So you want watch the transport of fat. And the goal would be to cannulate lymphatic ducts that are actually in the proximal bowel compared to the distal bowel, and see if there is a diversion of fat towards the proximal bowel, as opposed to the distal bowel, where most of the work that's been done in short-gut syndrome and on distal essentially epithelium has been in the distal bowel. So I think that there is adaptation. I just think it's more proximal.

Abbreviations:

SGS	short gut syndrome
IFALD	intestinal failure-associated liver disease
SBR	small bowel resection
LEC	lymphatic endothelial cell
VEGF	Vascular endothelial growth factor
PROX1	Prospero homeobox protein 1
LYVE-1	Lymphatic vessel endothelial hyaluronic acid receptor

IO	intraoperative
POD	post-operative day

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Figure 1. Effect of SBR on mesenteric lymphatic vessels by POD7.

(A-B) Whole mount fluorescence stereoscope image of collecting lymphatic vessels (Prox1-tdTomato+) (white) in sham (A) and SBR (B) mice in regions in the ileum-draining mesentery distal to the anastomosis. (C-D) Prox1-tdTomato (red) lymphatics with surrounding adipose tissue (bright field coloration) in sham (C) or SBR (D) mice in mesentery halfway between gut wall and mesenteric lymph node chain. White arrows in B and D show areas of lymphatic vessel sprouting and remodeling. (E) Representative whole mount image of collecting lymphatic vessels (Prox1-tdTomato+) depicting branches (circle arrow) and valves (diamond arrow) in SBR mesentery. (F) Branch and valve points in sham (n=3) vs. SBR (n=7) mice were quantified and compared. ****p<0.0001. Scale bar 400µm.

Figure 2. Whole mount confocal imaging of intestine-mesenteric border distal to SBR anastomosis at POD7.

(A) Representative image of cleared-whole mount tissue stained for lymphatics (Prox1 staining, white) and adipocytes (Perilipin staining, blue) showing increased lymphatic branching and abnormal morphology. Scale bar 100 μm . (B) Higher magnification of region denoted by yellow box showing bulbous lymphatic expansions between adipocytes. Scale bar 30 μm .

Figure 3. Mucosal lymphatic capillary changes in SBR vs. sham mice.

(A-D) Intestinal sections from sham and SBR mice were immunofluorescent stained for lymphatic and smooth muscle markers to evaluate mucosal lymphatic capillary network. (A) Crypt depth and villus height were measured on IO (n=12), POD3 (n=5) and POD7 (n=7) to assess adaptation. (B) Representative immunofluorescence images of sham (left side) and SBR (right side) intestinal tissue sections stained for smooth muscle (alpha-smooth muscle actin, αSMA , green), lymphatics (LYVE-1, red), and nuclei (DAPI, blue) on POD7. Lyve1+ vessels were considered mucosal lymphatics when present in the lamina propria (defined as the space between the basal membrane and muscularis mucosa; αSMA , green). Scale bar 30 μm . (C) The number of mucosal lymphatic capillaries (Lyve1+ vessels as in A) per total area of the lamina propria is shown. (D) Ratio of total measured mucosal lymphatic capillary lumen area to total lamina propria area in intraoperative (n=11), sham at POD3 (n=3), SBR at POD3 (n=5), sham at POD7 (n=6) and SBR at POD7 (n=7) was quantified. *p<0.05, ****p<0.001, **p<0.005.

Figure 4. Lymphatic gene modulation on POD3. mRNA expression levels in whole epithelial tissue from distal ileum in SBR (n=5) vs. sham (n=3) mice at POD3. Relative expression against actin gene for (A) *Vegfr3* and (B) *Prox1* is shown. *p<0.05.