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LYMPHATIC DYSREGULATION IN INTESTINAL INFLAMMATION: NEW INSIGHTS INTO INFLAMMATORY BOWEL DISEASE PATHOMECHANISMS

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ABSTRACT

Alterations in the intestinal lymphatic network are well-established features of human and experimental inflammatory bowel disease (IBD). Such lymphangiogenic expansion might enhance classic intestinal lymphatic transport, eliminating excess accumulations of fluid, inflammatory cells and mediators, and could therefore be interpreted as an 'adaptive' response to acute and chronic inflammatory processes. However, whether these new lymphatic vessels are functional, unregulated or immature (and what factors may promote 'maturation' of these vessels) is currently an area under intense investigation. It is still controversial whether impaired lymphatic function in IBD is a direct consequence of the intestinal inflammation, or a preceding lymphangitis-like event. Current research has uncovered novel regulatory factors as well as new roles for familiar signaling pathways, which appear to be linked to inflammation-induced lymphatic alterations. The current review summarizes mechanisms amplifying lymphatic dysregulation and remodeling in intestinal inflammation at the organ, cell and molecular levels and discusses the influence of lymphangiogenesis and intestinal lymphatic transport function as they relate to IBD pathophysiology.

Keywords: inflammatory bowel disease, lymphatic remodeling, lymphatic pumping, VEGFR-3, VIP, histamine, intestinal lymphangiogenesis, NF-κB, TLR, endothelin

The two most prominent forms of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), remain serious and globally increasing medical conditions, now affecting ~1.4 million Americans (1). Currently, the precise etiology of IBD has not yet been elucidated whereas it is commonly accepted that in genetically susceptible individuals, altered and inappropriate intestinal interactions between unspecified environmental factors and host immunity (e.g., NOD2 /ATG16L1 polymorphisms) contribute to the fundamental pathophysiology of IBD (2). Rather than additive factors, mechanisms contributing to IBD are understood to represent multiple pivotal factors, which interact closely and modify each other synergically. Therefore, understanding complexities in IBD pathogenesis requires identification and integration of host related processes, effects of individual microbiomes and environmental influences, which when combined, lead to improper and intense host immune responses (3,4).

However, despite different individual ranges of clinical manifestations and courses

in IBD, the primary and cardinal characteristic symptom of these two idiopathic inflammatory disorders still remains robust intestinal inflammation, whose attendant pathological origins have now been widely investigated (3,5). The gastrointestinal tract, more specifically the gut mucosa, is the principle 'frontier barrier' to bacterial flora and foreign antigens. Consequently, the initial regional mucosal immune response likely originates at the mucosal border and represents the primary initiator for subsequent disruptions in the intestinal immune homeostasis and accompanying chronic bowel inflammation in the pathophysiology of IBD (6,7).

Within the mucosal immune system, several key factors contributing to IBD pathology have thus far been identified. Contemporary research has been primarily focused on components of the innate and acquired arms of the immune system, whereas non-immune cells have received less but increasing attention (8). It is now widely established that the formation of *new blood vessels* (*hemangiogenesis*) in IBD contributes to increased intestinal inflammation, since both angiogenesis and inflammatory mediators (e.g., VEGF-A, TNF-α) help to mobilize leukocytes and increase premature fibrotic tissue remodeling (9,10). Where different components of the intestinal microvasculature (arterioles, capillaries, venules) have been extensively studied for their response towards mediators and mechanisms of leukocyte recruitment and extravasation, the removal of infiltrated immune cells and transport of antigen-presenting cells to appropriate downstream compartments through the lymphatic system has been comparatively neglected (11). For example, accumulating evidence now indicates that intestinal edema (a major clinical sign in IBD) is not only caused by inflammatory mediators which increase vascular solute permeability (*afferent arm*) but also reflects negative influences of these mediators on intestinal lymphatic transport function (*efferent arm*) contributing to both edema initiation and perpetuation. Therefore, besides blood vessel angiogenesis, disturbances in intestinal lymphatic vessel function and remodeling are important vascular contributions to the pathophysiology of IBD (11).

In 2008, van Kruiningen and Colombel reintroduced the lymphatic system as a '*forgotten*' factor in IBD pathophysiology when they, among others, reminded the IBD community that alterations in lymphatic vessels have been observed since the first description of regional ileitis by Crohn et al in 1932 (12,13). Over ensuing decades surgeons, pathologists and researchers have repeatedly recognized increased lymphatic vessel density as a characteristic feature in CD, and more recently UC, with evidence of lymphatic expansion in both the inflamed and non-affected areas of the gut. Importantly, the key functional impact of lymphatics in maintaining gut immune homeostasis has at least been underrated in the pathogenesis of IBD (11,13-15). New experimental methods and recent findings now have re-positioned intestinal lymphatics as an important component of the mucosal immune response, whereas lymphatic failure is closely and causally linked to intestinal inflammation. By now, disturbances in the intestinal lymphatic transport system have been equally documented as important factors in the initiation and development of IBD (11,16,17).

Acute inflammatory conditions have been traditionally recognized to increase lymphatic flow to balance the associated profound tissue edema through an active and unidirectional transport at least under acute conditions (18,19). However, accumulating reports reveal disturbances in intestinal lymphatic transport function (and even retrograde lymph flow) in IBD, which may be caused by obstruction or pumping failure. Recently, Colombel et al 2011 studied regional obstruction of lymphatics in CD, reporting increased inflammatory lymphoid follicles, lymphangiectasia and lymphocytic

perilymphangitis in all CD samples, demonstrating the strong correlation between inflammation and lymphatic transport failure caused by obstruction (20). This concept is supported by observations in experimental models of lymphatic obstruction: deliberately impaired intestinal lymphatic transport capacity **alone** was found to be able to recapitulate the anatomic and clinical fundamental features of CD (e.g., leukocyte extravasation, edema, and even the development of enteroenteric and enterocutaneous fistulae) (21). It is therefore possible that lymphatic obstruction and subsequent remodeling may further impair lymphatic transport capacity, leading to sustained lymphangiogenesis (11). Interestingly, and in agreement with this, Zampfell et al have demonstrated that lymphatic stasis is a highly potent inducer of lymphatic expansion (22). Additionally, von der Weid et al found that heterogeneous factors like cytokines or reactive oxygen/nitrogen species can suppress lymphatic pumping (16,23,24). Therefore, it seems as though lymphostasis promotes the accumulation of several diverse heterogeneous lymphatic pump-suppressing factors in IBD, some of which may provoke lymphangiogenesis. Still, the gap between cause and consequence in IBD pathophysiology and whether increased lymphangiogenesis should be considered as pathological or protective is still debatable.

An important challenge for future lymphatic endeavors will be to characterize the nature of these observed alterations in lymphatic vessel function and formation in IBD. The lymphangiogenic expansion in IBD might contribute to classic intestinal lymphatic transport, eliminating accumulations of fluid, inflammatory cells as well as mediators and could therefore be interpreted as adaptive to the inflammatory process. Whether these new lymphatic vessels are functional, unregulated or immature (and what factors are needed to 'mature' these vessels) is currently an area under concentrated investigation, and inflammatory cytokines may even disturb junction architecture to increase permeability in 'mature' lymphatic vessels (11). Whether impaired lymphatic function in IBD is a direct consequence of the intestinal inflammation or a lymphangitis like preceding event is still up for prospective debate. This review summarizes different factors amplifying lymphatic dysregulation in intestinal inflammation and discusses the influence of lymphangiogenesis and intestinal lymphatic transport function on IBD pathophysiology.

Intestinal Lymphatic Transport Function

Intestinal lymphatic vessels are the central conduit for efferent removal of interstitial fluid, perivascularly infiltrated immune cells, lipids and cholesterol from digestive visceral organs (25-28). Lymph collection begins at blind-ended initial lymphatics which are structurally adapted to be 'pulled open' by increased tissue turgor allowing lymphatic entry of interstitial fluid, particularly during acute edema (11). In contrast to the structurally unique, discontinuous "button"-like junctions in initial vessels, which contain adherens and tight junction proteins (VE-cadherin, occludin, claudin-5, zonula occludens-1, junctional adhesion molecule-A, and endothelial cell-selective adhesion molecule) (29), adherens and tight junctions in collecting lymphatics are relatively continuous with zipper-like junctions. Intestinal lymphatic transport is at least partially an active process, accomplished by the 'lymphangions' (literally lymph-'hearts'), which are valve-containing contractile subunits of lymphatic vessels invested with lymphatic medial smooth muscle cells in close approach with nerve terminals which can influence lymphangion contractility and periodicity (30). Accordingly, the ability of intestinal lymphatic vessels to adequately remove interstitial fluid and cells from the gut interstitium depends on both adequate patent lymphatic conduits and active pumping of these lymphangions (24).

Besides autonomous, spontaneous myogenic contractions which actively maintain unidirectional lymph outflow, the contractile behavior of lymphangion pumping is also modified by multiple extrinsic influences including external tissue compression, intraluminal pressure and fluid load, nervous inputs as well as contributions by many local and circulating autocrine and paracrine signal mediators (e.g., cytokines or chemokines) (16). Consequently, lymphatic pumping represents an active and highly regulated process whose failure interrupts lymphatic transport function, often with disastrous consequences. However, lymphatic vessels exhibit an astonishing ability to adapt to enormous changes in the interstitial environment, whose major challenge to these mechanisms is inflammation.

The intestinal inflammation occurring in the clinical course of IBD is not only accomplished by a persistent imbalance between pro- and anti-inflammatory but is also associated with a distinct disturbance in intestinal fluid load, which both affect lymphatic transport function in UC and CD. Whereas it is well described that lymph flow and intestinal morphologic lymphatic expansion (lymphangiogenesis) are consistently increased in IBD, how intestinal lymphatic contractile function is altered under inflammatory conditions still remains unclear (11). However, the malfunction of intestinal lymphangions, either congenitally or acquired through blockade, distension or lymphatic medial smooth muscle disturbances, influences the onset and persistence of lymphostasis, and may contribute to the etiology of IBD (16,23,24,31).

Prostanoids

Among the various paracrine signal mediators composing the intestinal mucosal immune system, prostanoids [(cyclooxygenase (COX) derived metabolites of arachidonic acid (AA)] are exceptionally relevant to IBD pathogenesis. Members of the prostanoid family include Prostaglandin-H2 (PGH_2), -F2 (PGF_2), -D2 (PGD2), -E2 (PGE_2), -I2 (PGI_2 'Prostacyclin') and Thromboxane (TXA). These agents modulate numerous pathways in both normal physiology and pathological conditions (especially inflammation) including vascular permeability, leukocyte infiltration, T-cell activation or nociceptor sensitization (32,33). Accordingly, it is well documented that elevated prostaglandin levels are found in UC, CD and in experimental models of intestinal inflammation (34,35). Whereas the main source of AA derivatives seems to be inflammatory cells, studies also demonstrate that lymphatic vessels themselves are able to release prostaglandins, regulating lymphatic contractility with a more significant role in increasing rhythmical constriction for PGH_2, PGF_2 and TXA_2 whereas PGI_2 and PGE_2 were found to decrease lymphatic pumping (36-40). However, this regulatory mechanism seems to be disturbed in inflammation.

Recently, the influence of prostaglandins on intestinal lymphatic pumping under inflammatory conditions has been most exhaustively studied by the group of von der Weid et al (16,40-42). Using the trinitrobenzene sulfonic acid (TNBS) induced ileitis model in guinea pigs, the group likewise identified EP_4 and IP as associated receptors in the PGI_2, and PGE_2 mediated decrease of intestinal lymphatic vessel contraction frequency and established mechanistic links to protein kinase A activation and ATP-sensitive K^+ channels (41). Although now several coincident reports have found that PGI_2 and PGE_2 formed by endothelial cyclooxygenase significantly impair normal lymphatic pumping (promoting edema and lymphostasis), cyclooxygenase inhibitors are ***not*** beneficial in IBD, more often intensifying bleeding, protein- losing enteropathy and dysregulating epithelial barrier, which is normally enhanced by PGE_2 (23,42-48).

Nitric Oxide

Among the numerous mediators released in response to the dysregulated intestinal immune response in IBD, the ubiquitous intercellular messenger and potent vasodilator nitric oxide (NO), has been closely linked to impaired lymphatic transport function. NO• (a small gas gasotransmitter) is enzymatically produced by several nitric oxide synthases (eNOS, iNOS) (49). NO• flux closely reflects NOS isoform expression: inducible NOS (iNOS) produces high tissue levels of NO•, whereas eNOS produces low, calcium regulated bursts of NO• (50). Interestingly, iNOS is expressed in a species and anatomy-specific fashion by endothelial, smooth muscle and various immune cells (macrophages, neutrophils) and can be induced by inflammatory cytokines like IL-1β, TNF-α or IFN-γ. Furthermore, it has been established that elevated tissue levels of iNOS are present within inflamed regions in intestinal samples from IBD patients, and that increased NO• production contributes to tissue injury, mucosal vasodilation or enhanced vascular and epithelial permeability at these iNOS+ foci (51). Conversely, iNOS may paradoxically be beneficial in the setting of IBD as intestinal microvessel endothelium basally express iNOS, which appears to suppress inflammation induced leukocyte adhesion (52,53). Importantly, NO• is known to hyperpolarize lymphatic smooth muscle cells and reduces lymphatic contractility, strongly inhibiting spontaneous pumping (39,54,55). Recent studies in animal models of experimental erosive e.g. TNBS-colitis, (known to robustly and reproducibly depress intestinal lymphatic function), shows highly upregulated iNOS expression in intestinal lymphatic smooth muscle cells themselves. This iNOS mediated NO•-flux accompanies increased smooth muscle hyperpolarization and consequently, impaired lymphatic contractility (40). Mechanistically, K_{ATP} channel activation has been shown to be an important mechanism underlying NO•-induced lymphatic relaxation and hyperpolarization. Therefore, the therapeutic usage of K_{ATP} channel blockers might be able to restore lymphatic pump activity (as has been shown *in vitro*) (56).

Histamine

As already stated, gut interstitial fluid and immune cell overload not only depends on afferent influx from blood vessels, but is also linked to efferent intestinal lymphatic transport function. Both pathways, vascular permeability and active lymphatic transport, are adjusted by various forms of paracrine and systemic signaling. Among these, histamine is a mediator which influences both arms of this tightly regulated system (57). Histamine storage and release by mast cells plays an important role in gastrointestinal secretion and immune modulation in allergic diseases (58). The response to histamine reflects the type and distribution of histamine receptors (H1R-H4R) (59). Interestingly, the first description of physiological effects of histamine in 1910 by Dale et al included intestinal smooth muscle contraction and vasodilatation (60). In IBD, it is well established that increased mast cell recruitment (leading to elevated, potentially degranulating, local histamine levels) within the mucosa, is a characteristic sign of intestinal inflammation in CD and UC (61,62). Histamine levels are greatly increased in IBD patients, and correlate with disease severity (63,64). Since histamine secretion and H-receptors are found in macrophages, dendritic cells, T- and B-lymphocytes, histamine may not only mediate vasodilatation, but also immune processes in inflammatory disorders (65). Recently, studies have investigated the effects of histamine on intestinal lymphatic smooth muscle function. Direct application of histamine decreases mesenteric lymphatic contractility (via H_1 receptors expressed on lymphatic smooth muscle), whereas histamine binding to H_2 receptors can induce lymphatic contractility. However, based on the observed receptor distributions on intestinal lymphatics, the dominant effect

appears to be an increase in contractility (66). Interestingly, other studies showed that low concentrations of histamine increase lymphatic contractility by binding to smooth muscle H_1 receptors which are highly sensitive to histamine, while higher concentrations of histamine decrease lymphatic contractility through H_2 receptors (expressed on lymphatic endothelial cells) via stimulation of the NO• production (66,67). In conclusion, effects of histamine on gut edema formation may reflect tissue histamine receptor expression and histamine abundance, which modulate both vascular permeability as well as lymphatic transport function.

Vasoactive Intestinal Peptide (VIP)

The wide spectrum between physiological function and inflammation-induced disturbances in intestinal lymphatic transport are illustrated by responses to vasoactive intestinal peptide (VIP), a 28-amino acid immunomodulating and anti-inflammatory neuropeptide (68-72). VIP is usually released by peptidergic nerves, but also can be released (infrequently) by immune cells. In the setting of inflammation, VIP has been shown to inhibit LPS induced release of TNF-α and IL-6 by macrophages (71,73) suppresses the release of IL-2, -4 and -10 in peripheral T-cells (74), and decreases GI mucosal injury (75), possibly through enhancement of barrier function (76). In the context of lymphatic homeostatic regulation, VIP is mainly secreted by nerve fibers in the adventitia of lymphatics, which modulate both vessel relaxation and contractility in the control of interstitial fluid balance (77,78). Von der Weid et al studied the effect of VIP on porcine lymphatic pumping capacity in an *in vitro* model and found that topical VIP administration diminished lymphatic pumping by decreasing the frequency of lymphatic contractions and by hyperpolarizing lymphatic muscle membrane potentials (77).

Conversely, Yang et al found that systemic (i.p.) administration of VIP (0.2pmol/g) in an *in vivo* model of intestinal ischemia reperfusion (I/R) injury *increased* intestinal lymph flow (by 80%) (76). Interestingly, their group found that the increase in lymph flow produced by 0.2pmol/g VIP (i.p.) was also associated with a significant decrease in lymph and serum concentrations of LPS, TNF-α as well as reduced gut histopathology, ALT, D-lactate and creatinine levels. Importantly, this did not appear to reflect a lymph "dilution" effect. Most importantly perhaps was the observation that VIP administration led to a reduction of T-cells in Peyer's patches, mesenteric lymph nodes, stomach and large intestine (76). As previously stated, it is worth noting that under inflammatory conditions VIP can be secreted by several immune cells (characteristically elevated in the intestinal inflammatory mucosa and in lymph in IBD). Therefore, it could be anticipated that increased intestinal VIP production by mucosal resident cells might have paracrine effects on lymphatic vessels to modulate lymphatic transport function in a time and spatially regulated fashion. There appears to be a marked decrease in VIP-immunoreactive nerve fibers in the lamina propria and submucosa of patients with IBD, indicating that neutrally-derived VIP levels may be depressed in these patients (79). However, in addition to the aforementioned vascular effects, it has also been unexpectedly reported that VIP can act as a potent anti-inflammatory agent which beneficially modulates innate and adaptive immunity, suggesting its potential therapeutic use in immune and inflammatory disorders (69). Interestingly, systemic VIP treatment showed broad prophylactic and therapeutic benefit in an *in vivo* murine model of TNBS-induced colitis (80,81). In this model, VIP treatment showed potent local and systemic effects e.g., attenuating the severity of colitis, reducing systemic and colonic pro-inflammatory cytokines and increasing anti-inflammatory cytokines and reducing inflammatory induced TLR2/4 overexpression (80,82). However, it is also worth noting that

Newmann et al were not able to perceive this protective effect of VIP in a similar model of experimental TNBS-colitis (83). Interestingly, when VIP treatment increased intestinal lymph flow (after experimental intestinal I/R injury), there was an observed decrease in the concentration of inflammatory gut-derived mediators, including endotoxin and TNF-α (76). The apparently contradictory results of increased lymph flow with a concomitant decrease in the concentration of inflammatory mediators (76) might reflect local immune modulating and anti-inflammatory effects of VIP. However, these optimistic results of therapeutic VIP usage in experimental models of inflammation (especially in models of experimental colitis) demonstrate the need for additional interpretive research and translational studies. So far, VIP has been positively tested in one clinical trial for sarcoidosis, a related inflammatory-immune disorder, with good effect (84). Further efforts are also needed to clarify the role of VIP in intestinal lymphatic transport function in the setting of IBD. Endogenous VIP might therefore play an important regulatory role in controlling intestinal inflammation and represent a potential treatment for IBD. The effects of VIP in these models may also maintain lymph flow through several possible mechanisms unrelated to lymphatic pumping (e.g., barrier function and immunomodulation). Paradoxically, Yang et al showed that in I/R injury *increased* lymph flow is associated with improved clinical features in the intestine, although lymph has been shown to transfer inflammatory cells and mediators from sites of injury to the general circulation, e.g., during shock ("*shock lymph syndrome*"), often provoking systemic inflammatory response syndrome/ multi-organ dysfunction syndrome (SIR/MODS) (76). One interpretation of the findings by Yang et al is that a *reduction in the residence time* of lymph within the inflamed gut may reduce the content (or composition) of inflammatory mediators in the interstitium or lymph, which favorably affects clinical outcomes. It is also possible that decreased lymph flow during inflammation could be beneficial by limiting transfer of toxins into the general circulation. The apparent disconnect between the findings of von der Weid et al and Yang et al could indicate differing effects between direct VIP actions on lymphatic smooth muscle (decreased lymphatic smooth muscle contractility) compared with indirect smooth muscle cell-independent effects of VIP (endothelial, immune cells).

Alcohol

In addition to the above-listed 'endogenous' factors, exogenous and environmental factors (e.g., Western diet) may also modulate extent and activity of intestinal inflammation in IBD (85,86). However, the role of environmental factors as modifying "triggers" in IBD is often complex and state dependent. For example, cigarette smoking decreases the extent of disease in UC, but worsens symptoms in CD (87). Another environmental factor that increases abdominal symptoms in IBD and that affects intestinal lymphatic transport capacity (and also shows contradictory results), is alcohol consumption. Cohort studies of IBD patients have shown that alcohol consumption generally worsens intestinal symptoms in CD and UC (65,88), apparently via enhanced immune responses. In particular, alcohol consumption can increase intestinal epithelial permeability, an established pathomechanism in IBD (89). The effects of alcohol consumption on lymphatic transport function are more complex in IBD. Mesenteric lymph flow in rats and thoracic duct lymph flow in human are both increased by alcohol consumption (90,91). However, other *in vitro* studies show that alcohol administration directly onto mesenteric lymphatic vessels inhibits lymphatic myogenic responses. Conversely, "digestive" alcohol consumption (possibly mediated by acetaldehyde) may indirectly increase myogenic contraction and lymph flow in mesenteric lymphatic vessels, which

would be expected to have beneficial effects on ISF conduction or intestinal immunity (92). However, it remains unclear if alcohol related modulation of intrinsic lymphatic pumping significantly impacts tissue edema and inflammation or negatively regulates gut barrier function in IBD. In spite of the controversy resulting from differences in *in vivo* and *in vitro* studies, recent patient targeted dietary recommendations still recommend alcohol abstinence for CD and UC patients (93).

Beyond the aforementioned results (mostly generated under experimental conditions), a translational clinical study reexamining the role of deliberately impaired intestinal lymphatic transport function in IBD was accomplished by Tonelli et al (94). Based on the proposed role of intestinal lymphatics in the pathogenesis in CD, those authors measured the intestinal lymphatic transport capacity to distinguish between inflammation-affected and 'healthy' areas in the small intestines of CD patients. The functional capacity of lymphatic integrity and transport was established by subserosally injecting Evan's blue (a potent marker of lymphatic vessels) into the intestinal wall of patients undergoing surgery (95). Preliminary data showed that slowed lymphatic outflow (requiring >2 minutes) is correlated with CD-like macroscopic features like fat-wrapping, wall thickening, wall edema or ulcers while an outflow time of ≤ 2 minutes was correlated with macroscopically normal findings. As of yet, the long-term goals of the study, i.e., establishing rates of lymphatic outflow as a predictive marker of recurrence after surgery for CD, still await further validation (94).

Intestinal lipid clearance

While the previously described signs of inflammatory-induced intestinal lymphatic transport failure concern fluid removal (edema) and immune cell clearance (leukostasis), disturbances in lymphatic vessel integrity and transport function might also affect the unidirectional lipid clearance. 'Fat wrapping' (creeping fat) at the surface of the large and small bowel plus mesenteric fat deposition are characteristic findings in CD, which are accompanied by increased numbers of mesenteric adipocytes (96,97). However, this condition is accompanied by increased abdominal but not total body fat (BMI is usually reduced in forms of IBD) (98). Since the intestinal localization of accumulating fat correlates with transmural inflammation, ulceration and strictures in CD, a direct contribution of fat wrapping in IBD pathophysiology has been suggested (99). The role of intestinal fat as an important extraluminal factor in IBD is also supported by observations that increased gut adiposity increases local and systemic levels of TNF-α, leptin, adiponectin, macrophage colony-stimulating factor (M-CSF), macrophage migration inhibitory factor (MMIF) and C-reactive protein (CRP), therefore influencing intestinal inflammatory responses in IBD (96,98,100,101). Interestingly, fat deposition found in CD also resembles fat accumulation in experimental models of impaired lymph transport and lymphedema (22,102). Moreover, fat deposition in these settings is often accompanied by fibrosis, another common finding in IBD (103). Importantly, related studies showed that disturbances in lymphatic specification genes like Prox-1 can trigger a loss of lymphatic integrity, which reciprocally induces fat leakage from lymphatics to exacerbate lymphatic structural defects in IBD, and may be an underlying cause of mesenteric fat (102). In addition, fatty acids also differentially modulate the activity of NOD2, a signaling module commonly dysregulated in CD, which controls NF-κB activity and alpha-, beta-defensin expression (104,105). Thus, it is worth noting that it is still unknown whether changes in the intestinal lipid milieu might influence human IBD activity through altered regulation of NOD2 signals.

Lim et al demonstrated that cholesterol also might interfere with lymphatic

functioning (106). Hypercholesterolemic mice exhibit disturbances in lymphatic vasculature and lymphatics actually undergo degeneration in response to elevated cholesterol (107). Considering the complex gap between lymphatic rarefaction and accumulation of intestinal fat, cholesterol accumulations might significantly deteriorate lymphatic functioning in IBD through several molecular mechanisms, especially lipid rafts. Therefore, anticholesteremic HMG-coA reductase inhibitors (statins) have been tested in models of intestinal inflammation and have been shown to prevent cholesterol synthesis and moreover, improve experimental IBD (108). Following these results, Pravastatin has been studied in clinical trials for IBD (NCT00599625, B. Behm, MD, Univ. of Virginia) but so far not yet been shown to directly influence or stabilize lymphatic structure or function in IBD.

Adequate intestinal lymphatic transport may depend on maintenance of lymphatic vessel 'specification,' which is also responsible for lymphatic-blood vascular partitioning during lymphangiogenesis. In the intestine, fasting induced adipose factor (Fiaf) is an important factor, which helps to maintain postnatal lymphatic vessel specification through induction of Prox-1 (109). Fiaf is an adipocytokine lipoprotein lipase inhibitor, expressed by adipocytes as well as several other cell types including nerves, epithelial cells, which acts as a stimulus for fatty acid oxidation and uncoupling in fat metabolism (110). Since Fiaf suppression by locally accumulated fat might secondarily repress lymphatic specifying programs (via Prox-1 pathways), disturbances of gut lipid metabolism in IBD might provide an additional mechanistic basis for lymphatic dysregulation. Furthermore, a diminished capacity of lymphatics to clear intestinally absorbed fat might also further suppress Fiaf expression and Fiaf-dependent lymphatic vessel expansion.

Intestinal lymphangiogenesis

These observations of lymphatic vessel expansion in IBD have been recently confirmed by studies showing that intestinal inflammation in UC, CD and experimental IBD is correlated with extensive gut lymphangiogenesis (111-114). Interestingly, Rahier et al, pointed out that lymphatic density increases transmurally in CD, but is limited to the inflamed mucosa in UC, and closely parallels the extent of inflammation in these conditions (15). Geleff et al concluded that lymphatic proliferation in both forms of IBD is activated by inflammatory processes that affect all regions of the gut, irrespective of the disease process, and also stated that lymphatic expansion can also be observed in fibrotic end-stage IBD (111). This widespread induction of lymphatics (now commonly visualized using lymphatic biomarkers like podoplanin) is seen even in 'quiescent' segments of the ileum and colon in CD, indicating that processes and mediators promoting lymphatic expansion may be present throughout the intestine before clinically active disease can be recognized (15).

Lymphatic vessels are also very rapidly increased in experimental colitis and may represent an adaptation, at least in acute inflammation (14,111,114,115). We found that acute experimental colitis (induced by DSS 3%) in mice was characterized by a dramatic expansion of lymphatic vessels at 7 days (114). The inability to remodel these vessels (produced by a genetic deficiency in Angiopoetin-2 (Ang-$2^{-/-}$)) was found to produce greater disease activity, despite lower levels of intestinal leukocyte (neutrophil) infiltration, also revealing an important role of Ang-2 in gut leukocyte recruitment. These findings suggest that defects in the ability to remodel lymphatic alterations, as well as their normal distribution or abundance, may increase gut susceptibility to injury. Whether increased lymphatic density (present prior to active disease) is a basis of disease or a compensatory physiological response that suppresses IBD development remains controversial and complex. Although there is

clearly an increase in the abundance of mucosal lymphatic vessels in human IBD and experimental erosive intestinal injury, it is unclear whether, and when, lymphatic expansion is adaptive (or not). For example, ovarian cancer induces a remarkable increase in lymphangiogenesis (in response to $CD11b^+$ macrophage recruitment), which is, however, largely dysfunctional in terms of fluid drainage (116).

Vascular Endothelial Growth Factor Receptor-3

Postnatal lymphangiogenesis is mainly mediated through stimulation of Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3) which is expressed principally on lymphatic endothelial cells (LEC) and activated by growth factors VEGF-D and VEGF-C (117,118). Stimulation of VEGFR-3 signaling increases LEC proliferation, migration, and survival, whereas blocking the VEGFR-3 pathway inhibits both inflammation-induced and tumor mediated lymphangiogenesis (117,119-121).

Several additional signals can epigenetically control LEC phenotype through VEGFR-3. Capillary tube formation by LEC depends on mTOR (mammalian target of rapamycin), and the mTOR inhibitor rapamycin diminishes LEC tube formation in vitro, an effect which was blocked in cells expressing rapamycin sensitive mTOR. Mechanistically, rapamycin decreased VEGFR-3 synthesis and lability in LEC (122). This effect was recapitulated in LEC by intentional suppression of VEGFR-3, and demonstrates a role of VEGFR-3 in lymphatic 'capillary' tube formation. This finding has important implications for lymphatic suppression in Rapamycin ('sirolimus') therapy for cancer. It is unclear if or how this lymphatic suppression modulates rapamycin treatment of refractory CD (123). Similarly, soluble VEGF receptors like sVEGFR-2 also appear to represent a class of important emerging endogenous modulators of lymphatic vascularity (124). sVEGFR-2 levels regulate transplant success and represent both mediators and potential therapeutic modalities in IBD and other chronic inflammatory diseases.

Lymphangiogenesis and LEC capillary tube formation critically depend on signaling mediated by VEGFR-3 activity, ERK and small GTPase activity (125,126). A soluble VEGFR-2 variant of 'sVR2' was found to not affect hemangiogenesis in tumors but suppressed lymphangiogenesis, an effect thought to represent VEGF-R2 binding of VEGF-C (124). In fibroblasts obtained from strictured segments of bowel from Crohn's disease, VEGF-A production was higher in the strictured segments than in the adjacent non-strictured segments, or normal controls; how VEGF's types and levels are altered in IBD to affect both blood and lymphatic vessels is an important area of investigation (127).

As the central regulator of lymphangiogenesis, VEGFR-3 activity depends on its level of expression, availability of ligand (VEGF-D and C) and downstream signals. VEGFR-3 is not only a growth factor, but serves as a potent signal modulator affecting barrier function and lymphatic pumping. As in blood vessels where VEGF-A decreases blood vessel barrier, VEGFR-3 activation of lymphatic endothelial cells decreases their barrier function, potentially through changes in monolayer or junctional organization (128). Most interestingly, while VEGFR-3 stimulation increases permeability, laminar fluid shear in lymphatics improves lymphatic endothelial barrier, a phenomenon that involves Rac-1-actin microfilament dynamics (129). Therefore chronic lymphostasis in IBD would be anticipated to increase lymphatic permeability and interfere with containment (and hence) transport of lymph.

Nuclear Transcription Factor Kappa-B (NF-κB)

The nuclear transcription factor kappa-B (NF-κB) has been comprehensively described

as a key regulator in the physiological perpetuation of innate and acquired immunity. Multiple intracellular signaling cascades rely on NF-κB to orchestrate the transcriptional control of a variety of pro- and anti-inflammatory mediators (e.g., chemokines, cytokines, adhesion molecules) shaping inflammatory responses and homeostatic processes like growth control and apoptosis (130,131). At the molecular level, the main mediators of an appropriate inflammatory response through rapid gene expression reprogramming are members of the NF-κB family (present in virtually every cell type), consisting of five mammalian proteins NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel, which all share a highly conserved 300-amino acid Rel homology region (RHR) responsible for homo- and hetero-dimerization and sequence-specific DNA binding (132). In unstimulated cells, NF-κB is sequestered in an inactive form in the cytoplasm bound to specific inhibitory proteins of the inhibitor (I)-B family. Following stimulation through TLRs, proinflammatory cytokines or antigen receptors I-κB are phosphorylated, ubiquitinated and finally proteolytically degraded, leading to de-repression of NF-κB, which rapidly translocates to the nucleus and activates the transcription of several target genes (130,133). It has also been thoroughly established that intestinal inflammation occurring in IBD is accompanied by an NF-κB driven overexpression of pro-inflammatory adhesion molecules and mediators, leading to disturbances in mucosal immunity (134). Beside classic targets (chemokines and cytokines), it has been recently described that inflammation induced NF-B directly upregulates two major transcripts involved in lymphangiogenesis, vascular endothelial growth factor receptor (VEGFR)-3 and its key transcription factor Prospero-related homeobox-1 (Prox1), resulting in a sustained inflammatory-induced lymphatic formation (135).

Unlike most cells, NF-κB is constitutively active in the lymphatic endothelium in most organs including the intestine, where lymphatic endothelial cells (LEC) persistently express nuclear NF-κB (136). NF-κB expression in LEC is necessary for maintenance of lymphatic endothelial programming, survival and lymphangiogenesis through the essential induction of Prox1 and VEGFR-3, particularly during episodes of active inflammation (135,137). Lymphatic endothelial NF-κB (p50) interacts with the transcription factor Prox1 to activate VEGFR-3 transcription, supporting VEGFR-3 dependent phenotype and lymphatic survival signals. Conversely, silencing of NF-κB reduces Prox1 and VEGFR-3 expression in cultured LEC (137). Because NF-κB is normally persistently expressed, nuclearly localized, and active in lymphatic endothelial cells to maintain their normal physiological functions, disturbances in NF-κB function or signaling (which coincides in the setting of intestinal inflammation in IBD) might lead to alterations in lymphatic character and formation, which could exaggerate forms of IBD (136,138). Therefore, while inflammation promotes lymphangiogenesis, suggesting that increased lymphatics are adaptive, inflammatory factors (IL-1β, TNF-α, IFN-γ) also present during active IBD might limit the full functionality of inflammation induced lymphatic networks, (at least as long as they are present) (114).

Thus, whereas an increase in lymphatic vessel density (LVD) has been concordantly described as a constant feature of CD and UC, the exact molecular triggers have remained unidentified (13,15). The NF-κB pathway presents an auspicious mechanistic connection between a variety of inflammatory stimuli and inflammation-induced lymphatic hyperplasia as well as lymphangiogenesis through a Prox-1 regulated increase of VEGFR-3 expression, amplifying cellular responses to local growth factors like VEGF-C/D (135,137,139). These interactions also underscore the independence of lymphangiogenesis in the pathophysiology of IBD because paradoxically, proliferation of blood vessel endothelial cells in angiogenesis may

in some cases be suppressed by activation of NF-κB (140-142).

However and intriguingly, the previously mentioned main lymphangiogenic growth factors (VEGF-C/D) actually appear to be depressed in IBD, while VEGFR-3 appears to be increased. Rahier et al suggest that VEGF-C and VEGF-D expression was absent or greatly decreased in inflamed as well as in non-inflamed intestinal tissues specimens of IBD patients whereas the abundance of VEGFR-3 in Crohn's disease and ulcerative colitis is less clear (15,17). The upregulation of VEGFR-3 expression by inflammatory cytokines through NF-κB and Prox-1 should result in an increased receptor availability, but a lower net availability of local VEGF-C/D might potentially contribute to "aberrant" IBD associated intestinal lymphatic characteristics (14,17,135,137). This might lead to a shift in IBD toward deficient VEGFR-3 signaling. Although there is clearly an increase in lymphatic vessel density in IBD (14,15,111), the mechanisms which account for lymphangiogenesis in IBD may therefore differ somewhat from those active in normal physiology (and suggest eminent roles for the NF-κB pathway). This finding suggests that the sources of lymphangiogenic growth factors in IBD may be suppressed or blocked (whereas the corresponding receptor is sensitized). It is furthermore still uncertain whether and when lymphatic vessel expansion in IBD is accompanied with impaired or improved lymphatic function. These findings may indicate that an IBD associated depression in lymphatic activation (possibly through NF-κB pathway suppression) could lead to either insufficient lymphatic density, loss of lymphatic specification, inappropriate patterning or remodeling, any of which would be anticipated to intensify IBD disease activity (15,24).

Recent reports also connect the NF-κB pathway with another aspect of IBD pathophysiology. Whereas genetic mutations associated with the susceptibility for IBD might interfere with the normal tonic activation of lymphatic NF-κB, it is interesting to speculate that lymphatic disturbances in CD (and perhaps also UC) might increase IBD activity through lymphatic suppression as described by Flister et al and in some experimental IBD models (11,137,143).

As shown above, the role of NF-κB in IBD related intestinal inflammation is by now clearly established, although there are gaps in our knowledge still regarding inflammatory induced lymphangiogenesis through Prox-1 and VEGFR-3 and their activating growth factors (VEGF-C, VEGF-D), which remains a new and promising area. Besides the sometimes conflicting findings for lymphangiogenesis in IBD, it is also still unclear what effects standard IBD therapies (corticosteroids, sulfasalazine or methotrexate, etc.) have on NF-κB mediated lymphatic vessel function and formation (since some are known to at least partially inhibit NF-κB activity) (134,144,145). In conclusion, these findings suggest that identification of mechanisms which support and induce expression of lymphatics in the inflamed intestine may reveal novel therapeutic approaches for treating IBD.

Transforming Growth Factor (TGF)-β1

The alterations in lymphatic integrity, remodeling, and especially function, found in CD and UC, find their basis to some extent in unbalanced equilibrium in pro/anti-lymphangiogenic factors (22,146). In addition to members of the vascular endothelial growth factor family, which support lymphatic proliferation and capillary formation, recent studies identify potent anti-lymphangiogenic mediators in the process of lymphangiogenesis, for instance, interferon-γ (IFN-γ), transforming growth factor-β and endostatin (147,148). Among these, the cytokine TGF-β1 appears to play an important role in blocking lymphatic vessel regeneration and reorganization, and is linked closely to several events in the pathophysiology of chronic intestinal disorders such as IBD (146,149-151).

TGF-β1, the canonical member of the transforming growth factor superfamily, is secreted by various immune cells (e.g., T-cells) and transduces its intercellular activity through transmembrane receptors TGF-βR-I/II, activating multiple downstream signals (e.g., Smad protein phosphorylation), ultimately regulating transcription control of multiple target genes (152).

Besides different signaling pathways in anti-inflammatory immunity and growth control, TGF-β1 is known as a potent inducer of fibrogenesis in multiple organs (153,154). The induction of fibrosis through TGF-β1 via epithelial-mesenchymal transition (EMTs) has been well-described in CD, where fibrosis and disturbances in anti-inflammatory pathways represents intestinal key features in inflammatory pathophysiology (150,155-157). Accordingly several groups report increased levels of TGF-β isoforms in active IBD, with clear elevation of TGF-β1, TGF-β3 and TGF-BR-I, whereas phosphorylation of downstream proteins was considerably reduced (149,158). However (and paradoxically), it seems likely that deficiency in TGF-β and Smad signaling contributes to development and maintenance of UC and CD (159).

Currently, an accumulating experimental database introduced a possible gap between TGF-β1, IBD and lymphatic reorganization. Avraham et al (2010) showed that TGF-β1 intensifies tissue fibrosis by suppressing both lymphangiogenesis and lymphatic function and that local blockade of TGF-β1 reduced tissue fibrosis, decreased chronic inflammation including Th2 cell migration, and importantly improved lymphatic function in an experimental model of wound repair (160). Similarly, Yan et al (2011) showed that adipose derived stem cells stimulate lymphangiogenesis by inhibiting TGF-β (161). Zampell et al found that TLR-4 and -9 knockout mice showed elevated deposition of type I collagen (a marker of fibrosis) and importantly, higher levels of TGF-β1 expression, which appear to both promote fibrosis and interrupt lymphatic regeneration to exacerbate lymphatic dysfunction (151).

Given the precise and balanced role of TGF-β in regulation of lymphatic function and formation modulated by pro/anti-lymphangiogenic stimuli, it seems reasonable to suggest an essential participation of TGF-β signaling, leading to disturbed ligand and receptor distributions, in the established dysfunction of intestinal lymphatic vasculature in IBD.

Toll Like Receptors (TLRs)

The gut is continuously exposed to an enormous load of commensal and pathogenic stimuli which help to regulate immunity. Within the immunity related mechanisms, toll like receptors (TLRs) are essential pattern recognition receptors (PRRs) which respond to pathogen and danger associated molecular patterns (PAMPs and DAMPs), transducing intestinal immune and inflammatory responses at several cellular and molecular levels and affecting various cell types, including lymphatic endothelial cells (LEC). So far, several TLRs (TLR-1 to 13) have been discovered, which show differential expression and ligand binding specificities. TLR-1 binds the acylated NH_2 termini in bacterial lipoproteins (as well as the TLR-1/2 mimetic PAM3CSK4). TLR-2 can dimerize with TLR-1 or TLR-6 to recognize bacterial vs. mycoplasma lipoproteins, respectively (162,163). TLR-3 recognizes viral double-stranded RNA (164) while TLR-4 binds lipopolysaccharide (LPS) (165). TLR-5 binds flagellin (166); TLR-7 and TLR-8 react with single-stranded RNA (and the synthetic immunomodulator ligand drug 'Imiquimod') (167,168). Lastly, TLR-9 reacts with unmethylated CpG motifs present in bacterial and viral DNA.

A comprehensive study by Pegu et al evaluated TLR expression in several lymphatic endothelial cell (LEC) models from primary (1°) skin, 'stretched' skin and 1° lung cultures, evaluating LEC inflammatory

responses after exposure to TLR agonists *in vitro*. All LECs expressed mRNAs for TLRs 1-6 and 9; TLR-4 mRNA was most abundant (followed by TLRs 1-3 and 6, expressed at equivalent levels). Only low levels of TLR-5 and -9 were found; (mRNAs for TLRs-7, 8, and 10 were not observed) (169). This expression pattern is similar to that reported by Garrafa et al who found mRNAs for TLRs 1-4, 6, and 9, but not TLR-5 (170); thymic LECs were seen to express mRNAs for TLRs 1-6 and 9. TLR mRNA and protein expression were not however uniform.

At the protein level, Pegu et al also found that TLR-4, -5 and -6 were expressed at the LEC surface in skin and lung LEC, with lower expression on 'stretched' LECs, suggesting potential links between loss of growth control regulation and LEC phenotypic maturity. TLR-5 and 6 were highly expressed at similar levels and were even more abundant than TLR-4 (at the protein level).

Interestingly, TLR-3 and -9 were expressed on LEC outer cell membranes as well as intracellularly, (except TLR-1 and 2, which were only intracellular) (169). Intracellular vs. extracellularly presented TLRs likely regulate responses towards lymphatically filtered antigens, or assist LEC responses to internalized antigens.

With respect to these signals, selective LEC TLR ligand stimulation also activated downstream signaling programs related to inflammation including chemokines, cytokines, growth factors and adhesion molecules. Pegu et al found strongest LEC TLR responses following activation of TLR 1/2, -3, -4, -5 and -8. mRNAs for the CXCR3 ligands CXCL9, IP-10 and CXCL11 were among those most highly and consistently induced in all LEC. TLR-3 was seen to be the most active inducer, followed by TLR-8 and then -4. In 1° skin LEC, TLR-2/6 stimulation also increased mRNAs for these ligands (an effect not seen in lung or stretched LECs).

In general, TLR ligands increased mRNA expression of the lymphocyte chemoattractant and CCR6 ligand CCL20 in skin and lung LEC, with TLR-1/2, -3, -4 agonists potently increasing CCL20 mRNA; TLR-2/6 stimulation increased CCL20 in 1° skin LECs. CCL20 protein was expressed by skin and lung LEC following TLR-1/2, -3, -4, TLR, 1° skin LECs also increased CCL20 after TLR-6/2 activation. RANTES and IL-8 were increased in all LECs after TLR-1/2, -3, -4 stimulation. TLR-6/2 stimulation also increased RANTES and IL-8 expression (in 1° skin LECs). TLR-3 stimulation increased IP-10 in all LECs and TLR-1/2, TLR-4 and TLR-6/2 stimulation inducing IP-10 in 1° skin LEC alone. TLR-8 also increased IP-10 in stretched LEC alone.

mRNAs for IL-1β, TNF-α, and IL-6 were increased in skin LEC in response to stimulation of TLRs-1/2, 3, 4; TLR-5 activation also increased cytokines in 1° skin LECs. Garrafa et al found that lymph node (LN) LEC also mobilize ICAM-1 and VCAM-1 after TLR-3 and -4, and after TLR-3, respectively. Thymic LEC only expressed ICAM-1 after TLR-3, -4 activation. Interestingly, Pegu et al described only moderate increases in VEGF-C (mRNA) in response to TLR-1/2,-3, -4 and -5 in 1° skin LEC and in stretched skin LEC (following TLR-1/2, -3 and -8); strikingly, no changes in VEGF-D were observed (169). TLR-1/2 -3, -4 and 6/2, also increased VCAM-1 and ICAM-1 mRNAs in both skin and lung LECs; lung LEC increased VCAM-1 mRNA in response to all TLRs. TLR-3 and -4 stimulation increased VCAM-1 and ICAM-1 expression on cell membranes of both skin and lung LEC. TLR agonists had little effect on D6 scavenger receptor expression except for lung LEC (D6 was modestly induced by LPS and PAM3CSK4). Garrafa et al reported that in LN LEC, TLRs-1, 3, and 4 induced IL-8, a potent chemoattractant for lymphangiogenic immune cells. In thymic LEC, IL-8 was also stimulated by TLRs 1-6 and 9. RANTES was induced in LN EC by TLRs 1, 3, and 4 ; in thymic LEC, RANTES was induced by TLRs-2 and 3. In LN LEC TNF-α was released by TLRs-1-4 and by TLRs 1-6 and 9

(thymic LEC). IL-6, (another potent immune cell attractant) was also induced by TLRs-3, 4 in LN LEC, and by TLRs 1-6 and 9 in thymic LECs. Monocyte chemo-attractant protein-1 (MCP-1) was also released by LN LEC after TLR 1-4 and 6 activation, but only TLR-3 induced MCP-1 in thymic LEC. Lastly, TLR-3 stimulated IP-10 in both LN and thymic LEC, with a much more prominent effect on thymic LECs. Garrafa also found that NF-κB p65 was activated by TLRs 1-4, 6 and 9 (in LN LEC) and by TLRs 1-6 and 9 in thymic LECs. Because NF-κB is persistently active in LECs to maintain their phenotypic specification, TLRs may provide important links between pattern recognition, lymphatic structure, maturation and function.

These studies show some heterogeneity among lymphatic endothelial cells from differing anatomic sources, which still share a common ability to respond to PRRs. In different tissues, TLRs may modulate LEC activation, immune cell binding and guidance molecule expression. Although these molecular responses appear to be well-developed in lymphatic endothelial cells, inappropriate or excessive activation of LEC adhesive induction could promote lymphoid retention of immune cells and increase the potential for obstruction. We have found that experimental elimination of gut flora using triple-antibiotic treatment reduced gut injury in experimental (DSS) colitis (171). Despite an apparent reduction in gut epithelial barrier in this model, the suppression of gut flora significantly prevented the development of histopathology (crypt damage, epithelial injury), weight loss, stool bleeding, etc. Importantly we found that elevations in blood vessel density were apparently prevented by gut 'sterilization,' while lymphatic density was increased over control, albeit lower than in 'non-sterile' DSS treated mice. This suggests that factors released by gut flora which activate PRRs help to induce lymphangiogenic programming. The relatively high lymphatic to blood vessel ratio seen in gut 'sterilized' mice associated with protection might support the use of antibiotics in IBD therapy (172). We found that TLR-4 expression in the gut was increased by fat feeding and increased NF-κB p65 phosphorylation, while simultaneously reducing SIRT-1 expression, effects which were eliminated in antibiotic treated or TLR-4 -/- mice (86). These results with TLR-4 parallel our other findings in Patel et al (using gut sterilization), which also showed a suppression of inflammation and lymphangiogenesis (171). While not yet fully explored, the inverse relationship between SIRT-1 and NF-κB in inflammatory lymphangiogenesis is intriguing.

Chemokine Scavenging Receptor (D6)

Recently, a basis for lymphatic obstruction in IBD involving the D6 chemokine scavenging receptor has been described (173). D6 is a CC chemokine 'clearing' receptor expressed by lymphatic endothelial cells as well as stromal and B-cells (174). D6 binds to, and eliminates inflammatory CC chemokines, (especially CCL2) while sparing CCL19 and CCL21. D6 was upregulated by the GATA1 transcription factor, (in hemopoietic cells) , suppressed by LPS (via TLR-4) and upregulated by TGF-β1 (175). Importantly, Lee et al demonstrated that D6 knockout mice exhibit dysfunctional lymphatics with poor interstitial fluid clearance, entrapment of inflammatory cells and lymphatic congestion (173). Bone marrow transfer studies in these mice showed that D6-knockout mice were more vulnerable to experimental colitis strongly supporting links between lymphangitis and D6-deficiency as a potential exacerbating event in IBD (176). However, currently it is unclear if D6 is also dysfunctional in IBD, but experimental studies support accumulation of chemokines as an important exacerbating event in colitis.

Endothelin

Besides NO• and prostanoids, primary endothelial-derived factors e.g., endothelins

(ET-1,-2,-3) and their apposite receptors (ET_A, ET_{B1}, ET_{B2}, ET_C) also might influence lymphatic functioning in IBD. Reeder and Ferguson and later, Marchetti et al found that lymphatic endothelial cells (like blood vascular endothelium) release the potent vasoconstrictor ET-1 (177,178). Interestingly, ET_B receptor activation on lymphatic endothelial cells mediates the release of two potent vasodilators, NO• and PGI_2 (possibly a contractile feedback mechanism), while ET_A receptors on lymphatic vascular smooth muscle can mediate contraction (179). Although ET_A receptors appear not to be expressed by lymphatic endothelial cells, Zhao and Van Helden report that low levels of ET-1 ($\leq$ 10nM) augmented lymphatic pumping while higher levels ($\geq$ 100 nM) triggered vasospasm, which would be expected to impair lymph flow (180). In lymphatic vessels, blocking ET_A receptors (using BQ-610), or ET_B receptors (BQ-788) prevented ET-1 induced contraction responses with ET_A blockade more effective than ET_B (180). Because ET-1 mediated lymphatic contraction was found to be increased by endothelial injury, but not by NO synthase inhibition, endothelial derived hyperpolarizing factors were also proposed to modulate these responses. Therefore, coordinated interactions between ET_A and ET_B receptors (on lymphatic smooth muscle and endothelium) could have important effects on lymphatic integrity and function in IBD. With regard to lymphatic remodeling, ET-1 has also been reported to promote lymphatic vascular invasiveness and control lymph flow (via ET_B receptors), a process which appears to involve matrix metalloproteinases (MMP)-2 and -9 (181). Endothelin-1 can further promote endothelial proliferation, migration and invasion through ET_B receptors (182,183). Based on the ability of ET antagonists to block Kaposi sarcoma cells (which share lymphatic endothelial programming), lymphatic endothelial proliferative responses to ET_A may be at least partly autocrine in nature. Spinella et al also suggested that ET-1 increases lymphatic density by enhancing expression of VEGF-C and -A. In 2010, they showed that ET-1 binding to ET_B receptors increased the activation of MMP-2 and -9 proforms, a step which may be necessary for the development of fully functional lymphatic vessels (181,184,185). Doboszynska et al indirectly showed the relationship of ET to lymph flow based on immunostaining of different lymphatic regions (184,185). Because the homeobox transcription factor Nkx 2-3 has been shown to be dysregulated in IBD (which downregulates ET expression in both UC and in CD), endothelin effects on lymphatics might be a novel and important mechanism in IBD pathogenesis (186).

Lymphatic Restoration

Several lines of evidence support that the expansion and normalization of lymphatic structure and function will improve the intestinal inflammation seen in IBD. Tabibiazar et al suggest lymphatic specific growth factors might attenuate edema, while blood vascular VEGFs intensify edema (187). Similarly, suppression of lymphatic vessels in mice expressing sVEGFR-3 Ig intensifies edema, particularly in neonates. In support of this finding, Huggenberger et al have shown that induction of lymphatics improve edema in the skin (188,189). Interestingly anti-TNF-α treatment with Infliximab in arthritis (both murine and human) has been associated with an increase in lymphangiogenesis, suggesting that TNF-α may interfere with lymphangiogenesis and inflammation through decreased LEC proliferation, barrier function and capillary network development (114,190). Whereas the immunomodulating effects of Infliximab in human IBD and experimental colitis are well established and widely used, the effect on intestinal lymphatic vessels for now remains unclear.

CONCLUSIONS

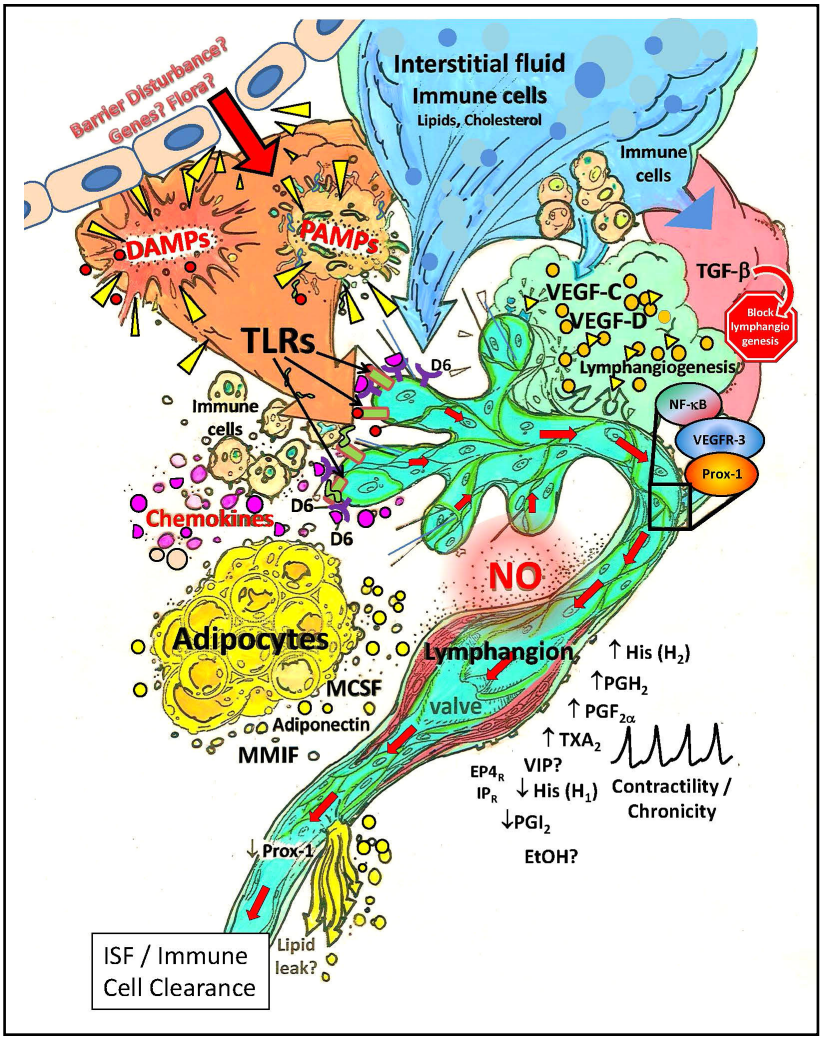

Fig. 1. Intestinal lymphatics normally transport interstitial fluid, immune cells, their mediators, antigens, digested lipids and cholesterol back to the central circulation and thereby prevent their accumulation in the interstitium. Lymphatic flow starts in initial lymphatics through specialized button-like junctions, and its unidirectional flow is maintained through semilunar valves and an active pumping capacity in collecting lymphatics. In IBD, disturbances in the intestinal epithelial barrier (in conjunction with gene polymorphisms and altered microbiota) provoke activation of inflammation through danger and pathogen associated molecular patterns, triggering immune cell infiltration which produces persistent extensive tissue injury. Tissue infiltration by immune cells can be initiated and intensified by chemokines and cytokines, which are cleared by lymphatic outflow as well as scavenger receptors present on lymphatic endothelium. Transmural inflammation activates the release of lymphangiogenic growth factors VEGF-C and VEGF-D, which induce new lymphatic growth. Transforming growth factor-beta released by immune and interstitial cells can oppose lymphangiogenic signaling, which is mediated by Prox-1, VEGFR-3 and NF-κB downstream signaling. Lymphangion contractility actively supports centripetal lymph flow and is positively modulated by prostanoids (PGH_2, $PGF_{2\alpha}$, TXA_2), histamine (H_2 receptors) and negatively modulated by NO, histamine (H_1 receptors), prostacyclin (PGI_2) whereas Vasopressin and ethanol have controversial effects on lymphatic pumping (contractility/chronicity). Adipocyte derived factors (MCSF, adiponectin, MMIF) also influence lymphatic integrity. Downmodulation of Prox-1 in response to inflammatory mediators may lead to lymph leakage or reflux. The cumulative influences of these factors and conditions may lead to development of a chronic inflammatory state which impairs the outflow of interstitial fluid and immune cells thereby perpetuating gut inflammation.

As in the blood vasculature, many inflammatory signaling mechanisms mediate the disorganization, integrity and hypofunction of intestinal lymphatics in experimental and human forms of IBD (Summary *Fig. 1*). These changes may contribute to disturbances of normal gut homeostasis and restitution, which initiate and maintain disease activity in IBD. Studies of lymphatic mechanisms identified in CD and UC not only reveal mechanistic bases but may also provide novel and important targets for the therapeutic intervention in IBD and maintenance of its remission.

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