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In the current funding period, we completed studies on the protective effects of adenoviral induced VEGF-D and compared this effect to VEGF-C. These findings were correlated with lymphatic and blood vessel density and support our hypothesis that lymphatic vessel induction is protective in IBD. These findings support lymphatic expansion as a controlling element of blood vessel expansion. We next examined the effects of VEGFR-2 kinase inhibitor, SU1498 and a VEGFR-2 competitor antibody on acute erosive colitis. VEGFR-2 blockade was protective in some phases of colitis and appears to reflect suppression of blood vessels. We also explored whether and how blockade of the pro-lymphangiogenic VEGF receptor, VEGFR-3 (using MAZ51, a VEGFR-3 kinase blocker) would affect acute erosive colitis. We found that VEGFR-3 blockade lead to significant increases in gut tissue injury, particularly when given during the ‘recovery’ phase of the DSS model. This suggests to us that lymphatics may exert protective influences colitis by hastening recovery from disease rather than preventing its induction.

**VEGFR – vascular endothelial growth factor receptor, MAZ51, VEGFR-3 kinase blocker, SU1498, VEGFR-2 kinase blocker**
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Introduction

Crohn’s disease and ulcerative colitis represent the two most important forms of inflammatory bowel disease (IBD). Recent studies have renewed the original interest of these conditions as an obstructive lymphangitis, as was originally reported by Crohn, Ginsberg and Oppenheimer in 1932; similar disturbances in lymphatic structure and functioning have been remortpered in ulcerative colitis. Therefore, the restoration of normal (or adequate) lymphatic functioning may be able to relieve some of the symptomatology of IBD, at least during some phases of the disease. This research project ‘Lymphatic Therapy in IBD’ was initiated to investigate how lymphatic structure is disturbed in forms of IBD, how lymphatics and blood vessels are remodeled in human and in animal IBD models and how the administration of lymphatic selective growth factors can restore lymphatic density and function to diminish the inflammatory features of IBD. The successful application of these findings could allow for extension or maintenance of periods of remission in human disease. The most important findings in the current period of research have been the demonstration that adenovirally induced VEGF-D leads to a dramatic improvement in disease activity, weight maintenance, stool form and reduction in stool blood. These features of disease were also linked with a reduction in blood vessel density and most importantly, infiltration of neutrophils (see appendix ‘Differential Roles of Lymphangiogenesis and Angiogenesis in Experimental Colitis.’) This last disease feature, neutrophil entrapment is strikingly important and suggests that accumulation of microbial factors in the lamina propria reflects a failure of lymphatics to clear these factors, precipitating immune system activation and injury. This inflammation can trigger an inflammatory expansion of blood vessels which is not always adaptive and can contributes to permeability edema. Several manipulations including the use of VEGFR-2 antibody and VEGFR-2 kinase blockade show some protection against this type of injury.

Conversely, the use of a VEGFR-3 antibody exacerbates experimental injury (see appendix) and a VEGFR-3 kinase blocker, MAZ51 was seen to exacerbate gut injury, particularly in the recovery phase of acute erosive colitis (see Figs 18, 19 and 20) but not during the induction phase where they may actually promote worsening of disease. In chronic models of mouse IBD, we found that the Samp-Yit model of IBD shows a progressive development of disease which is associated with a decrease in lymphatic vessel density in the colon (Fig 21), but not in the small intestine. These findings recapitulate our previously published findings in the CD45RBhi / SCID mouse (Alexander et al., Ann N Y Acad Sci. 2010 Oct;1207 Suppl 1:E75-85.).

Therefore, while there is clear evidence for expansion of lymphatic vessels in IBD, our current findings indicate that lymphatic expansion may not always ‘keep pace’ with inflammation or with blood vessel expansion which leads to an intensification of injury. This appears to be especially true in chronic inflammation, where some cells/mediators may inhibit lymphangiogenesis leading to a vicious cycle of impaired interstitial clearance and immune system activation. The administration of pro-lymphangiogenic growth factors especially during remission of disease may enhance recovery or maintain the length of disease free periods.

In the next funding period, we will investigate how VEGFC-156s, a selective lymphangiogenic factor may suppress IBD disease as a more selective lymphangiogenic modifier than VEGF-D, currently the most specific lymphangiogenic growth factor tested. Mechanistically we are now focusing on how limitations to lymphatic clearance trigger leukocyte infiltration. Similarly, it is possible that once infiltrated into inflamed tissues, lymphatic failure may restrict the egress of several classes of pro-inflammatory immune cells which exacerbates tissue injury. Additionally, we will accomplish studies on myeloperoxidase content in gut tissues as modified by VEGFR-2 and VEGFR-3 blockers, and also measure the infiltration of monocyte/ macrophage. These studies will be correlated with immunostaining for leukocyte chemoattractant cytokines and chemokines to document how factors trapped in the static gut interstitial compartment activate IBD disease. These studies will be compared with control and IBD human gut tissue to determine which factors are enhanced in both and might be relevant targets and mediators of disease. We are currently negotiating with a donation of VEGF-D from Circadian Technologies, Toorak Australia which will allow us to perform many more VEGF-D studies in these models.
Body

Task 1: Determine colon lymphatic and blood vessel densities and proliferation and abundance of lymphangiogenic and angiogenic mediators in experimental and human IBD (Months 1-36) (JSA Lab). Human colon specimens. Working with national direct research interchange we have now obtained 7 control human colon samples which have been frozen, fixed and paraffin embedded for evaluation of lymphatic and inflammatory biomarkers. We have successfully immunostained lymphatic and blood vessels in these tissues using D2-40 (podoplanin).

We have also arranged to obtain IBD and control serum samples from Plasma services group.

1) SU1498 VEGFR-2 kinase inhibitor used as pre-treatment, co-treatment and post treatment in DSS colitis.
In this series of studies, mice were administered 3% DSS in their drinking water for 7 days and SU1498, a VEGFR-2 kinase blocker administered prior to, at the beginning and following induction of acute colitis. This drug interferes with normal VEGFR-2 signaling which is believed to modulate signaling for both angiogenic and lymphangiogenic programs. The concept for these studies was that if either angiogenic or lymphatic remodeling was important during initiation or maintenance phase of erosive colon injury, then SU1498 would prevent or limit gut injury and lead to significantly lower disease activity in acute colitis.
Disease activity (DAI). The VEGFR-2 kinase blocker SU1498 does not affect recovery of disease activity from acute (3% DSS) colitis. We investigated the effect of the VEGFR-3 blocker SU1498 given prior to, at the induction and during active acute colitis. Compared to 3% DSS or a vegetable oil control + 3% DSS there was no apparent difference in disease activity when SU1498 was administered as a pretreatment, at d=0 or after 4 days by i.p. injection. (Repeated measures ANOVA testing showed changes in DAI in DSS, vegetable oil + SU1498 + DSS). This suggests that block of VEGFR-2 did not dramatically affect DAI in experimental IBD.

Weight changes in SU1498 + DSS treated mice. The VEGFR-2 kinase blocker SU1498 also did not dramatically affect weight changes, when given prior to, at d=0 or during active disease in acute (3% DSS) colitis. We also found that SU1498, a VEGFR-2 blocker itself did not significantly affect body weight in the acute DSS model. We did however, see a slight but significant improvement in weight loss on day 7 with SU1498 with oil vehicle control + DSS control, *p<0.05). This finding suggests that weight loss was affected to a small degree by VEGFR-2 signaling blockade.

Stool blood in SU1498 + DSS colitis. The VEGFR-2 kinase blocker SU1498 did not affect the presence of stool blood in acute (3% DSS) colitis. The appearance of stool blood as an index of colon injury in the acute DSS model of colitis found that this treatment did not significantly reduce presence of stool blood in DSS colitis when given at any time point.

Stool form in SU1498 + DSS colitis. Similarly, stool form was not affected in mice given SU1498 at any time in this study. This indicates that VEGFR-2 signaling did not significantly protect the colon against losses in intestinal water resorption. Most importantly, we found that pre-treatment with SU1498 lead to a significant shortening in colon length compared to the DSS treated mice. Because colon shortening is an indication of tissue injury, this finding indicates that VEGFR-2 signaling may play a protective role when activated prior to injury, but that this effect is lost when given at later time points. This may suggest that VEGF may be beneficial prior to injury in the colon. Furthermore, when we measured the colon weight to length ratio in these studies, we found that SU1498 significantly increased this ratio compared to DSS treated mouse colons. This indicates that the SU1498 treated colons are both heavier and shorter when colons are pre-treated to block VEGFR-2 compared with DSS treated colons or other later treatment schedules. Therefore, VEGFR-2 may be essential to the colon prior to injury and that VEGFR-2 block can lead to greater injury. However, after injury is induced, VEGFR-2 kinase blockade made no difference in development of disease. Lastly, we found that spleen weight, an indicator of ongoing systemic inflammation was not altered by SU1498 given at any schedule in this study.
2) MAZ51 pre-treatment, co-treatment and post-treatment in progressive DSS colitis. In this series of studies, we investigated how MAZ51, a VEGF-2 kinase inhibitor used as a pre-treatment, as a co-treatment at t=0 and treatment after 4 days of DSS.colitis affected various parameters of IBD disease activity.

Fig. 9. Effect of MAZ51 pre, same day or post-treatment on weight change in DSS colitis

Fig. 10. Effect of pre, same day or post treatment with MAZ51 on stool blood in DSS colitis. Mean +/- SE

Fig. 11. Effect of pre, same day or post treatment with MAZ51 on stool form in DSS colitis. Mean +/- SE

Fig. 12. Effect of pre-, same day or post-treatment with MAZ51 on spleen weight in DSS colitis. Mean +/- SE.
Disease activity in MAZ51 treated mice in DSS colitis. We found that Day 0 treatment resulted in a slowing of significant disease progression by about two days (from day 2 on DSS alone to day 4 on day 0 treatment + DSS). No other change were found to be significant. However, there appears to be a trend towards worsening of DAI in MAZ51 post-treated groups and a lessening of DAI in day 0 and pretreatment groups. These data may suggest that once colon injury exists, lymphatic remodeling may improve intestinal recovery, but that earlier lymphatics may help to drive disease.

Weight changes in MAZ51 treated mice in DSS colitis. We found that significant weight loss occurred one day earlier in the MAZ51 post-treated group and one day later in day 0 and pretreatment (DSS-day 6, post-day 5, pre & d0-day 7). Again these data suggest that once disease is initiated lymphatics are protective but that they may help to initiate disease in early phases of IBD. This suggests that different lymphatic active drugs may be useful in different phases of IBD activity and remission with lymphangiogenic growth factors being useful to promote remission, but needing to be discontinued thereafter.

Stool blood in MAZ51 treated mice in DSS colitis. In stool blood experiments, we found that day 0 MAZ51 treated mice had slightly delayed stool bleeding scores compared to DSS (DSS-Day 3 & Day 0- Day 4) consistent with MAZ51 being slightly protective in early phases of disease. No effect was seen with later phases.

Stool form in MAZ51 treated mice in DSS colitis. In stool form experiments, pre treated and Day 0 treated mice showed delayed worsening of stool form compared to DSS (DSS-Day 1, PRE-Day 6, and Day 0-Day 4). Again consistent with early protection with MAZ51.

Spleen weight was not significantly affected by MAZ51 in these studies.

![Fig. 13. Effect of pre-, same day or post treatment with MAZ51 on spleen weight in DSS colitis. Mean +/- SE.](image1)

![Fig. 14. Effect of pre-, same day or post treatment with MAZ51 on spleen weight in DSS colitis. Mean +/- SE.](image2)
Colon shortening in MAZ51 treated mice. We cannot know at this point if there was significant colon shortening of day=0 treated mice due to MAZ or VO+DSS because in this series of studies we did not yet perform an appropriate vehicle oil (VO) control +DSS. However, so far all MAZ51 treated groups were shorter than the DSS treated group. There were no groups which were lengthened by MAZ51 indicating that the general effect of this agent is to shorten colon length.

3) MAZ51 effects on recovery in reversible DSS colitis. In this series of studies, mice were administered 3% DSS in their drinking water for 5 days and then DSS was discontinued and mice were restored to normal tap water and chow. At the time as the DSS was discontinued, mice then received an intraperitoneal injection of the VEGFR-3 kinase blocker MAZ51.
Because this drug interferes with normal VEGFR-3 signaling, which is understood to modulate lymphangiogenic signaling (necessary to initiate or sustain lymphangiogenesis) this treatment protocol may allow us to evaluate how lymphatic remodeling might contribute to **gut restitution following acute erosive injury**. The concept for these studies was that if lymphatic remodeling was important during the recovery from colon injury, then MAZ51 should intensify or exacerbate gut injury and lead to more persistent disease or prevent recovery from injury.

**Fig. 19.** Significantly higher colon weight to length ratio in MAZ51 treated mice compared to vehicle-oil treated mice (mean +/- SE).

**Fig. 20.** Increased spleen weight in MAZ51 + DSS treated mice compared to DSS (mean +/- SE).

**Fig. 21.** Lymphatic density in the colon (left) and small intestine of Samp Yit mice (middle). There is a trend for a time dependent reduction in **colon lymphatics** in the Samp Yit mouse, this is not seen in the **small intestine**. Staining is for lymphatic vascular hyaluronan receptor (LYVE-1). Right – HRP -LYVE-1 staining of lymphatics.

**Weight change in MAZ51 recovery.** The VEGFR-3 kinase blocker MAZ51 did not affect recovery of weight in acute (3%DSS) colitis. However, it is worth noting that weight loss did not reverse to a great extent in...
this model. We currently believe that a lower level of DSS (1 or 2% instead of 3% DSS) might reveal changes in a more reversible model.

By itself, we also found that MAZ51, did not significantly affect body weight in the acute DSS model. There was a slight and significant improvement in weight recovery on day 7 and on day 9 (measured by 2-way ANOVA comparing MAZ51 recovery with oil vehicle control + DSS control, *p<0.05). This finding suggests that recovery of weight loss was affected to a small degree and on a few days by blockade of normal lymphangiogenic signaling.

Disease activity in MAZ51 treated mice in DSS recovery. The VEGFR-3 kinase blocker MAZ51 does not affect recovery of disease activity from acute (3%DSS) colitis. We investigated the effect of the VEGFR-3 blocker MAZ51 given after the induction of acute colitis to modify the course of disease recovery. Compared to the 3% DSS or a vegetable oil control + 3%DSS there was no apparent difference in disease activity when MAZ51 was administered by i.p. injection. (Repeated measures ANOVA testing showed changes in DAI in DSS, vegetable oil + MAZ51 and MAZ51 given during recovery although no significant changes between these treatment groups were found). These findings suggests that interruption of

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**Fig. 22.** Effect of pre or post treatment with VEGFR-2 antibody on disease activity in DSS colitis.

**Fig. 23** Effect of pre or post treatment with VEGFR-2 antibody on weight change in DSS colitis.

**Fig. 24** Effect of pre or post treatment with VEGFR-2 antibody on stool blood and stool form in DSS colitis.
lymphatic signaling may not exacerbate these parameters of IBD recovery.

**Stool bleeding in MAZ51 recovery.** The VEGFR-3 kinase blocker MAZ51 did not affect the presence of stool blood in acute (3% DSS) colitis. We measured the appearance of stool blood as an index of colon injury in the acute DSS model of colitis but found that treatment with MAZ51 appeared to slightly, but not significantly affect the presence of stool blood following withdrawal of DSS on day 4.

**Stool forms in MAZ51 recovery.** Similarly, we also studied how stool form was affected in mice in which MAZ51 was given during the recovery phase. We found that there was no significant effect on the stool form scoring in mice in which MAZ51 was given, therefore interruption of normal lymphangiogenic signaling at this dosage did not appear to have an exacerbating effect on intestinal water resorption, nor was this treatment seen to be protective compared to the oil-vehicle controls.

**Colon shortening in MAZ51 recovery.** Although mouse weight, disease activity and stool condition was not affected by

![Fig. 25. Lymphatic (left) and blood vessel remodeling in DSS colitis. There was similar remodeling in WT and FOXC2 KO mice.](image)

![Fig. 26. Dependence of weight loss, disease activity, stool form and leukocyte infiltration on gut flora in 3% DSS induced colitis.](image)
lymphangiogenic inhibition, the condition of the colon itself appeared to be affected. Most importantly, we found that treatment with MAZ51 lead to a **significant shortening in colon length** compared to the vehicle-oil control + DSS treated mice. Because colon shortening is an indication of tissue injury, this finding does indicate that MAZ51, a lymphangiogenesis blocker, produced or maintained colon shortening compared to DSS treated mice when they were in a recovery phase. Therefore, lymphatics may be important in restoring gut repair (and restoration of normal length) which is blocked by VEGFR-3 blockade.

Colon weight to length ratio in MAZ51 recovery. Furthermore, when we also considered the colon length in these studies in terms of the colon weight (weight to length ratio), we found that MAZ51 significant increased this ratio compared to vehicle oil + DSS treated mouse colons. This is a highly sensitive indicator of gut condition and strongly indicates that the MAZ51 treated colons are both more massive and shorter during recovery than DSS treated colons consistent with persistent colon inflammation as a result of VEGFR-3 kinase inhibition.

**Spleen weight in MAZ51 recovery.** Spleen weight, an indicator of persistent immune system activation was also seen to be significantly increased in MAZ51 + DSS treated mice compared to DSS. Therefore, although weight and disease activity were not seen to be significantly exacerbated by lymphatic inhibition, several other more colon and immune system sensitive and relevant markers were shown to be worsened by MAZ51 during the recovery phase. Therefore, lymphatic expansion does appear to be important during the recovery phase following gut injury and that inhibition of lymphatics leads to worse injury.

4) **Samp Yit mouse chronic colitis model.**

Using the Samp Yit model of progressive immune mediated chronic colitis we have begun to investigate how lymphatic density and IBD disease activity in this progressive colitis model may be related. We found that there appears to be a net reduction in the amount of colon lymphatics in this model at 16 weeks while there was not an equivalent reduction in the number of small intestinal lymphatics. This reduction in lymphatics was associated with an increase in disease activity (stool bleeding, form). This relative ‘rarefaction’ of lymphatics has been previously described in chronic colitis (Alexander JS, Chaitanya GV, Grisham MB, Boktor M,

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Fig. 27. Exacerbation of weight loss (top left) and disease activity (top right) as well as stool bleeding (lower left) and stool form (lower right) in Helicobacter mice receiving 3% DSS. These changes were accompanied by lymphatic hyperplasia.
2010, Emerging roles of lymphatics in inflammatory bowel disease. Ann N Y Acad Sci. 2010 Oct;1207 Suppl 1:E75-85 where lymphatics were expanded, but did not keep pace with blood vessel expansion. This is a second instance where this appears to be the case. Blood vessel densities are currently being evaluated in this tissue to determine if this is true of this model as well.

5) VEGFR-2 antibody antagonism on DSS colitis. In addition to studies using a VEGF-2 kinase blocker to inhibit function of this receptor, we also used an antibody directed against the receptor administered early and late in DSS colitis to examine how this would affect the course of experimental colitis (Task 2: Determine roles of VEGF receptors in mediating protection and injury in acute experimental IBD).

Effect of VR2 antibody on disease activity in DSS colitis. We found that VR2 pretreated mice had a significant disease activity one day later than the rat IgG group (PRE-day 2 *, day 3 *, day 4 **; Rat IgG- **on day 1). On day 7, the VR2 pretreated group had a significantly lower DAI than the Rat IgG group, albeit this was a relatively small change in the net DAI (although significant).

Effect of VR2 antibody on weight change in DSS colitis. We found that the VR2 pretreated group had a slower reduction in weight loss by about one day (pretreated group- sig. at day 6; Rat IgG- *sig. at day 5). By comparison, on day 7, the percent of original weight was significantly greater in the pretreated group than the rat IgG group. These data show protection when the VEGFR-2 receptor is blocked by this antibody.

Effect of VR2 antibody on stool blood in DSS colitis. We next studied how stool blood was affected by VR2 antibody administration. No changes were noted.

Effect of VR2 antibody on stool blood and stool form in DSS colitis. We found that VR2 antibody administration had no effect on stool bleeding or diarrhea scores (form) induced by 3% DSS. Colon length was reduced in all treatment groups containing DSS (no change with VR2 – data not shown, similiary weight to length ratios were not affected by VR2 antibody – data not shown).

6) Effects of Adenoviral induced VEGF-D and VEGF-C are summarized in the attached manuscript (appendix A). 7) Effect of 3% DSS colitis in FOXC2 knockout mice. Task 6: Determine the extent of disease enhancement / blockade in lymphodysplasia (Chy-3, ap-FOXC2, FOXC2, Ang-2 gene knockout) mouse models. (Mos. 1-36) (Witte Lab / JSA Lab). FOXC2 KO mice in DSS colitis. We examined the development of DSS colitis in mice with a genetic deletion of FOXC2 which is understood to induce a state of lymphatic disturbance in the intestine which lead to a failure in normal intestinal lymphatic patterning and may be a model of human lymphedema distichiasis. These mice are thought to have fewer lymphatics and would be expected to exhibit greater sensitivity to injury which is relieved by lymphatic drainage. Curiously, this strain of mouse failed to exhibit a greater injury in the 3% DSS model of IBD with respect to weight change, DAI, stool form or stool blood. (We are repeating studies on colon length and weight in collaboration with Dr. Marlys Witte, U.AZ.). Because Foxc2 indirectly controls angiogenesis as well as remodeling and maturation of the vasculature by inducing Ang-2 expression in adipocytes (Xue et al. 2008, Kume 2008). patterning, failure of lymphatic valve formation and lymphatic dysfunction (Kriederman et al. 2003, Dagenais et al. 2004, Petrova et al. 2004). However, a GI phenotype in lymphedema distichiasis (humans) is not apparent, this is not seen in this model either. There is a reported apparent greater engorgement of lymphatic vessels in Foxc2 +/- animals, suggests lymphatic drainage may impeded by absence of valves. Foxc2 and Ang-2 mice both lack lymphatic valves and have improper recruitment of smooth muscle cells into lymphatic vessels (Gale et al. 2002,Petrova et al. 2004, Shimoda et al. 2007,Dellinger et al. 2008). It is possible that because FOXC2 affects both lymphatic and blood vessel development genetic ablation of FOXC2 may make it difficult to separate effects mediated by this knockout.
8) Dependence of gut injury and vessel remodeling in IBD on gut flora. Because spleen weight and immune system activation largely reflect the responses to epithelial barrier failure induced by DSS, we investigated whether DSS colitis could be active without gut flora. We found that it was not and importantly, have shown that the injury in this model may largely reflect the infiltration of the gut by immune cells including neutrophils which respond to the presence of chemoattractants released during gut barrier disturbances (Fig. 26, lower right). These changes were accompanied by significant suppression in lymphatic vessel expansion (This study is being resubmitted to European J. of Gastroenterology and Hepatology).

9) Exacerbation of DSS colitis by Helicobacter species. We also found that prior infection with Helicobacter species (in this case muridarum) leads to a greater disease activation in DSS colitis (Fig. 27) and may show a novel risk factor in human IBD. These changes were accompanied by significant changes in lymphatic vessel expansion (data in preparation).

Key Research Accomplishments

• Demonstration that VEGF-D is protective in acute erosive colitis. A key accomplishment in the current funding period have been the demonstration that VEGF-D is protective against the induction of acure erosive colitis. The summary of these findings is found in the attached manuscript ‘Differential Roles of Lymphangiogenesis and Angiogenesis in Experimental Colitis’. These findings support our overall hypothesis and indicate that our concept is relevant at least with respect to animal models. These findings will need to be correlated with findings in human tissue and plasma samples to better define how VEGFR-3 agonists might be used in IBD (and other acute / chronic inflammatory phenomena).

• Demonstration of suppression of leukocyte infiltration as a mechanism of VEGF-D mediated protection in acute IBD. This is an important finding which we plan to explore in depth as this was largely unanticipated but may be central to the development of gut injury.

• We showed that VEGFR-3 blockade intensifies colon injury during the recovery phase of acute erosive colitis. While we expect that the induction of VEGF-D is in fact mediated by VEGFR-3, it is also possible that VEGFR-2 might also help to mediate these effects. Therefore to investigate this possibility, we attempted to block the signalling mediated by VEGFR-3 during acute erosive colitis using MAZ51. We found that during the recovery phase that administration of MAZ51 lead to significant shortening of colon length, and increase in colon weight. These findings support VEGFR-3 as being essential for the recovery from colon injury. We therefore anticipate further experiments in the next funding period in which VEGF-D mediated protection of the colon against erosive colitis might be blocked by treatment with MAZ51. Interestingly MAZ51 administration in the absence of VEGF-D did not show a dramatic effect to exacerbate disease and in some cases actually seemed to be slightly protective. Therefore we feel that use of VEGFR-3 ligands should be explored for phase dependent actions in disease.

• Evaluation of suppression of lymphatic vessel density in chronic Samp-Yit colitis. We previously reported a suppression of lymphatic expansion in the chronic colitis model of CD45 RBhi / SCID mice compared to acute lymphatic expansion in DSS. In the current funding period we again found that in a second set of experiments that when Samp-Yit mice (another model of chronic progressive colitis) were followed over time, (up to 16 weeks) that there was a gradual loss in lymphatic vessel density. Importantly, this net decrement in lymphatic vessel density would be expected to contribute
to the development of progressive interstitial fluid accumulation which is a suspected mechanism in all of our colitis models. We are currently studying blood vessel densities in this model to see if there is a reciprocal increase in blood vessel density and whether these changes can be related to gut histopathology, leukocyte infiltration, etc.

**Reportable Outcomes**


**Conclusion**

The research accomplished to date has demonstrated the ability of VEGF-D administration to reproducibly limit the development of acute erosive colitis in the 3% DSS model. This model provides a highly reproducible test in which VEGF-D appears to be active, however this may not recapitulate all features of both forms of human IBD. The research accomplished to date has also demonstrated that this protection was also associated with a net reduction in the amounts of infiltrated leukocytes which are present in the gut (at the end of the study). Mechanistically these data suggest that whatever processes are activated by VEGF-D, they may limit the accumulation of immune cells which induce coliA major problem encountered to date has been simultaneously measuring the blood and lymphatic vessels in the same tissue sample. This has now been accomplished in the current proposal and will allow us to compare lymphatic and blood vessel supplies as they may be related to injury. This is key to demonstrating that lymphatic expansion contributes to protection in IBD. Our studies during year 2 of the proposal will involve more extensive evaluation of the chronic Samp-Yit model using some of the treatments which appear to protect in the ‘acute’ model. The banked tissue catalogs that we have produced from the defined colon specimens should help to demonstrate how lymphatic and blood vessels are related in normal and in IBD tissues. The overlap of mediators and processes in human specimens and in acute / chronic mouse IBD models should help to define how lymphatic remodeling in the pathogenesis of IBD and how VEGFR-3 agonist may be used as drug/therapeutic targets. Several candidate VEGFR-3 agonists (VEGF-D, VEGFC-165S) are currently under study.

**References.** None.

**Appendix** (see below – starts on page 18)
Differential Roles of Lymphangiogenesis and Angiogenesis in Experimental Colitis. Alexander, J.S. 1; Chaitanya, V.G. 1; Jennings, M.H. 1; Ando, T. 1; Specian, R.D. 1; Granger, D.N. 1; Karlsson, F. 2; Podduturi, J. 2; Cromer, W. 2; Mathis, J.M. 2; Wang, Y. 3; Minagar, A. 4; Memet, S. 5; Pytowski, B. 6; Rissanen, T.T. 7; Tammela, T. 7; Ylä-Hertuala, S. 7; Alitalo, K. 7; 1LSUHSC-Shreveport, Molecular and Cellular Physiology, 2LSUHSC-Shreveport Cell Biology Anatomy/Gene Therapy Institute of NW Louisiana, Shreveport, LA, 3LSUHSC-S Obstetrics/Gynecology, Shreveport, LA., 4LSUHSC-S Neurology, 5Pasteur Institute, Paris, France, 6Imclone, NYC, NY, 7Univ. Helsinki, Finland. Correspondence should be addressed to J.S.A. (jalexa@lsuhsc.edu).

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Abstract. Angiogenesis contributes to the pathophysiology of chronic inflammation, but whether or how lymphangiogenesis contributes to the etiology of chronic inflammation is not understood. To compare angiogenic and lymphangiogenic responses during gut inflammation, we investigated the effects of VEGF receptor blockade and activation in experimental IBD. Antibody blockade of VEGFR-2, (but not anti-VEGFR-1 or VEGFR-3), attenuated weight loss, disease activity and colon histopathology in DSS colitis. Adenoviral induction of VEGF-D (but not VEGF-C) in the distal colon prior to colitis induction reduced wasting, disease activity, gut shortening, neutrophil infiltration and gut histopathology. Although adeno-VEGF-C somewhat reduced evidence of histopathological injury, it did not prevent weight loss or disease activity. Lymphangiogenesis and angiogenesis were both elevated in experimental colitis and adeno-VEGF-D protection was associated with increased lymphatic vessel expansion. Experimental gut inflammation may be mediated by VEGFR-2 but that VEGF-D/VEGFR-3 mediated lymphatic expansion may protect against experimental colitis. The maintenance or induction of lymphatics may thus be a useful therapeutic strategy in inflammatory injury in IBD and other conditions such as arthritis and psoriasis.

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Introduction

Inflammatory bowel diseases (IBD) are characterized by structural and functional changes in the microvasculature which include leukocyte adhesion and extravasation into tissues leading to extensive injury. Recently angiogenesis has also been recognized as an important event in IBD etiology [1-3]. Although angiogenesis is essential during development and wound healing [4], inflammatory angiogenesis may intensify injury in IBD [5] and forms of chronic inflammation (e.g. rheumatoid arthritis) [6]. In IBD the induction of angiogenesis in response to the delivery and local synthesis of VEGFs and angiogenic cytokines initiates and sustains vascular leakage, ICAM-1 induction, entrapment of leukocyte leading to gut injury [2; 3; 7; 8]. VEGF-A, like TNF-α, drives both angiogenesis, inflammation and injury in IBD models. Both mediators up-regulate ICAM-1 expression and support leukocyte-endothelial adhesion [9]. The roles played by VEGFs and VEGF receptors may change during different phases of IBD-associated angiogenesis while still controversial, are becoming better understood.

Lymphangiogenesis, the development of lymphatic vessels is less well understood. Lymphangiogenesis has been described in human IBD (Crohn's disease and ulcerative colitis) [10-12], but how and whether the induction of lymphangiogenesis modulates disease activity in IBD is unclear. Because lymphatics play important roles in the regulation of interstitial fluid balance their expansion in IBD may be adaptive. Conversely, by transferring immune cells and bacterial antigens from the site of inflammation to lymph nodes and gut-associated lymphoid tissues, abundant lymphatics might intensify immune responses underlying injury. Lastly, while abundant, newly formed lymphatics may not exhibit fully mature barrier or transport properties, or might be dysregulated by the abundance of Th1 cytokines present in the IBD-inflamed colon [13]. Since colon lymphatics are increased and reorganized in IBD, understanding and controlling their behavior might represent an important novel avenue in IBD therapy.

Lymphangiogenesis is mediated by the lymphatic vascular endothelial selective growth factors VEGF-C, D binding to VEGF receptors-2 and -3 [14; 15]. Lymphangiogenesis increases in parallel with angiogenesis in acute and chronic inflammation (like IBD) but, whether it is adaptive, or exacerbating remains unclear. For example, although lymphangiogenic factors can be found in inflammatory CD11b macrophages [16], by providing conduits for interstitial fluid and immune cell clearance the induction of lymphangiogenesis may protect against inflammation by helping to balance edema generating effects of angiogenesis.

We examined how experimental colitis was modified by VEGF receptor blockade, and by adenoviral induction of VEGF-C and D. VEGFR-2 blockade prevented weight loss and injury in colitis; antibodies against VEGFR1 or VEGFR3 had no effect. Importantly, the delivery of VEGF-D delivery to the intestinal mucosa provides significant protection against colitis. Adenoviral expansion of lymphatic vessels correlates with protection against weight loss, histopathology, and gut inflammation, blood vessel expansion matches disease progression. Therefore the expansion of the lymphatic vasculature during inflammation may be protective, apparently reflecting clearance of interstitial fluid and immune cells.

Results
VEGFR-2 antibody reduces weight loss and disease activity in DSS Colitis. VEGF receptors (VEGFR-1, -2 or -3) mediation of DSS colitis was studied in mice given antibodies against murine VEGF receptors prior to and during DSS colitis. Neither anti-VEGFR-1 (mAb MF-1) non anti-VEGFR-3 (mAb mF4-31c1) prevented DSS induced weight loss (Fig. 1A) or disease activity (Fig. 1B) (DSS vs. VEGFR-1+DSS = 80±3.2% vs. 86.4±5.9%; DAI: DSS vs. VEGFR-1+DSS = 3.9±0.83 vs. 3.11±.73) (DSS vs. VEGFR-3-DSS = 80±3.2% vs. 81.4±3.8%, DAI DSS vs. VEGFR-3-DSS: 3.92±0.83% vs. 3.46±.0.23). Mice treated with anti-VEGFR-2 showed significant protection against weight loss (DSS vs. VEGFR-2 Ab 80±3.2 vs. 88.6±3.8, p<0.05) and disease activity (3.92±0.8 vs. 2.5±0.2, p<0.05).

In these studies, colon-cecum length was significantly reduced by DSS (from 8.16 ± 0.25 cm control vs. 5.48 ± 0.17 DSS). Colon shortening produced by DSS was not significantly reversed by anti-VEGFR-1 (6.21±0.35 cm) or anti-VEGFR-3 (5.66 ± 0.18 cm), but was significantly reversed by anti-VEGFR-2 (6.2 ± 0.16 cm, *p<0.01) on day 10. Gut histopathology was significantly increased in DSS and was significantly attenuated by treatment with anti-VEGFR-2 treatment (Fig. 1C). Myeloperoxidase content of colon tissue was significantly increased in DSS colitis (Fig. 1D) and was significantly reduced by VEGFR-2 antibody (p<0.05), but not by other antibody treatments.

kB-lacZ lymphatics vessels increase in DSS colitis. Lymphatics were seen to expand dramatically during DSS colitis using the kB-lacZ reporter mouse. kB-lacZ lymphatic vessel staining increased in a time-dependent manner in the gut mucosa and submucosa, which was correlated with disease activity and weight loss during DSS disease. Lymphatics marked by kB-lacZ were not abundant in controls, but submucosal lymphatics significantly increased over 10 days of DSS administration. Lymphatics in the submucosa increased from nearly absent on day 0 to 11.7 ± 12 / section on day 3 to 33.0±18.68 / section, * p<0.05 on day 6 and to 62.7±5.5 / section, ** p<0.01 on day 9. In the muscular layer, lymphatics increased from (0.0±0.0 day 0) to 3±3.6 on day 3 to 11.7±3.8, ** p<0.01 on day 6 and to 18±4.6, **, p<0.01 on day 9. In the adventitia, lymphatics increased from (0.0±0.0) on day 0 to (8±3.6* p<0.05) on day 6 and to (18.7±5** p<0.05) on day 9. Disease activity index increased from (0.0 ± 0) on day 0 to (1.13 ± 0.2) on day 3 to (1.7± 0.6) *p<0.05, on day 6 and to (3.0 ± 0.3) on day 9, *p<0.05. This was also paralleled by weight loss over 10 days. Intraperitoneal administration of adenoviral VEGF-C or VEGF-D does not protect against DSS colitis. Pilot studies were conducted in which adenoviral particles encoding VEGF-D (2X10^5) were injected intraperitoneally into mice to induce VEGF-D protein expression in the abdominal cavity near the gut. Intraperitoneal administration of VEGF-D or -C adenoviruses did not lessen the development of DSS colitis; weight loss and cumulative disease activity in these mice were indistinguishable from mice receiving DSS alone (data not shown). However, it was noted on autopsy that i.p. VEGF-D treated mice showed chylous exudate in the thoracic cavity. Food and water consumption. Control mice each consumed 3.2 ± 0.05 g pellet/day and 4.1 ± 0.3 ml water /day. 3% DSS treatment gradually reduced drinking and feeding behavior, so that by day 10, 3% DSS treated mice each consumed only 1.3 ± 0.3 gm pellet/day, and 2.2 ± 1.2 mls liquid (3% DSS)/day. This reduction in food and liquid consumption was similar to that in the adeno-VEGF-C + DSS on day 10 (food consumed= 1.8 ± 0.8 g /day; liquid = 2.5 ± 0.7 ml/day). Similarly, each adeno GFP + DSS treated-mouse consumed 1.2 ± 0 g food and 2.0 + 0 ml liquid/day on day 10. Conversely, adeno-VEGF-D + DSS treated mice maintained normal feeding (2.8 ± 0.3 g/day), despite consumption...
of a control level of liquid 3% DSS (4.8 ± 0.4 ml/day) on day 10, significantly greater than either adeno-GFP + DSS (p<0.05), or adeno-VEGF-C + DSS treated groups (p<0.05).

**VEGF-D blocks colon shortening in experimental IBD.** Colon length (measured cecum-rectum) was significantly shortened in DSS colitis compared to controls (**Fig. 2A**, control: 7.75±0.35 cm vs. DSS: 4.66±0.58 cm, *p<0.05). Similar colon shortening was also seen in ad-GFP/Luc+DSS treated mice (4.66±0.50 cm, p<0.05) and AdVEGF-C+DSS treated mice (4.83±0.76 cm, p<0.05). However, VEGF-D significantly blocked DSS-induced colon shortening (6.73±0.68 cm); these colons were significantly longer than DSS-treated colon (and not different from controls).

**VEGF-D reduces myeloperoxidase in DSS colitis.** We found that MPO content, a marker for polymorphonuclear leukocytes (PMN), measured as \(\Delta OD_{650}/mg \text{ tissue weight}\), was significantly elevated in gut specimens collected from DSS treated mice compared to untreated controls (0.0073± 0.004 vs. 0.075 ± 0.02, **p<0.01, **Fig. 2B**). Administration of adenoVEGF-D to DSS treated mice significantly reduced colon MPO to 0.02 ± 0.002, *p<0.05). AdenoVEGF-C also reduces MPO content, although not significantly compared to DSS (0.035 ± 0.009). Similarly, colon MPO content in adenoGFP + DSS treated mice was not significantly different from DSS (0.074 ± 0.016).

**Histopathological comparison of DSS / adeno-hVEGF-C, adeno-VEGF-D mice.** Colon histopathology was also affected by DSS / adenovirus treatment. Compared to untreated controls (**Fig. 2C**), colon structure in 3% DSS-treated mice showed moderate inflammation, which involved >50% of the tissue sections analyzed. DSS produced transmural tissue injury, which involved >75% of the tissue (**Fig 2C**). These figures show an extensive loss of epithelial cells and absence of crypts characteristic of this model. This appearance was seen in all DSS sections on day 10. Some improvement in tissue structure was seen with adenoviral VEGF-C (**Fig. 2 C**). Adeno-VEGF-D treated mouse colons show significant improvement in epithelial integrity and crypt structure.

**Gut histopathological score in DSS colitis is improved by adeno-VEGF-D.** Pictures of gut histology were quantitated on the basis of extent involvement, inflammation and crypt injury (**Fig. 2D**). Cumulatively, DSS treated colons showed significantly worse disease than controls (DSS: 34.7 ± 2.3 vs. control: 0.3 ± 0.7, ***p<0.001). Histopathological injury produced by DSS was significantly improved in adenoviral-VEGF-D + DSS treated mice which scored 14.4 ± 5.4, (*p<0.05 vs. DSS). Despite a significant improvement in tissue histopathology, this tissue injury was still significantly higher than controls (****p<0.001). Adeno-VEGF-D + DSS injury consisted of slight to moderate tissue infiltration by leukocytes that involved ~25-100% of the sections. Adenoviral VEGF-D protection also represents a reduction in the depth of injury (mucosal – submucosal but not transmural) and was seen in 75-100% sections examined. Most importantly, crypt damage and epithelial injury was reduced by adeno-VEGF-D, which shows a significant preservation of crypts and epithelium. Adenoviral VEGF-C + DSS also significantly reduced histopathological injury produced DSS but to a lesser extent than VEGF-D (*p<0.01 vs. DSS) (26 ± 1.7,
significantly greater than control, ***p<0.001). This protection reflected reduction in inflammatory infiltrates, depth and extent of injury (limited to mucosal / submucosal injury). In VEGF-C samples, crypts and epithelial cells were less preserved than in VEGF-D. Adenoviral GFP + DSS did not provide significant protection against tissue injury (29 ± 2.3, not significant different vs. DSS, significantly greater than control, ***p<0.001) showing moderate inflammation, mucosal to transmural injury and loss of crypts similar to DSS.

Colonic administration of adeno-VEGF-D prevents weight loss in DSS colitis. The administration of adenoviruses encoding human VEGF-D (but not VEGF-C) to the colon lumen significantly attenuated weight loss, a major marker of disease activity in 3% DSS colitis (Fig. 3A). 3% DSS produced a significant reduction in body weight (21.2 ± 4.8% weight loss) on day 10. Adeno-GFP (encoding green fluorescent protein, a control adenovirus) +DSS lost nearly an identical amount of weight over 10 days as mice on DSS alone (20.7 ± 7.5%, not different from DSS treatment alone). Similarly, adeno-VEGF-C + DSS treated mice also showed a gradual weight loss nearly identical to that produced by DSS alone, (19.9 ± 8.3% by day 10). However, adeno-hVEGF-D + DSS treated mice showed complete protection against weight loss induced by DSS, with no significant loss of weight over the 10 day course of the study (loss of 1.7± 3.2% by day 10) (Fig. 3A).

Disease activity index is improved by adeno-VEGF-D. The disease activity index (DAI) in this model, (a combinatorial score including stool blood, form and body weight) showed a significant improvement with adeno-VEGF-D (Fig. 3B). At day 4, blood was not detected in any control stools, but was evident in all DSS-treated groups. Disease activity in DSS groups gradually increased over 10 days, and reached a maximal level of (3.9 ± 0.1) in the DSS alone group. Similarly, adeno-VEGF-C + DSS also gradually increased to 3.8 ± 0.2 (day 10). Ad-GFP +DSS also reached a DAI 3.9 ± 0.2 on day 10. Disease activity in DSS colitis was also improved by adeno-VEGF-D. Stool form in Ad-VEGF-D + DSS mice remained normal through day 7. The ad-VEGF-D + DSS group also significantly increased disease activity after day, (p<0.05), but was still significantly lower than DSS, reaching a maximum of 2.3 ± 0.4 on day 10; this level of disease activity was still higher than that of untreated controls (0.0±0.1, day 10).

Expression of adenoviral proteins in mouse intestinal epithelial cells. To characterize induced VEGF expression by target tissues, VEGF-C and D adenoviruses were transfected in vitro into mouse ‘YAMC’ (young adult mouse epithelial cells (YAMC a gift from Dr. Robert Whitehead, Vanderbilt University) at a ratio of ~55:5 virion: YAMC cells. As colon epithelial cells, YAMC resemble the in vivo cell target of the adenoviruses used in this study (when given intestinally). Supplementary Fig. 1A shows that YAMC induced to express hVEGF-D produce a 22 kD protein similar to the recombinant protein standard (~20 kD). Similarly, YAMC induced to express hVEGF-C yield a 27kD protein in vitro close to the reported value of 23 kD. Induced VEGF-D protein was found within the cytoplasm of transfected cells and in culture supernatant from transfected cells.
Intracolonic administration of adenoviral GFP vector: expression with colon and remote organs. Using in vivo imaging (IVIS), we tracked organ expression of adenovirally induced green fluorescent protein (GFP). Intra-intestinal delivery of adenovirus encoding GFP showed maximal intestinal expression on day 4; other organs (liver, brain, kidney, spleen, and lung) showed no measurable GFP expression on day 4. By day 10, GFP expression within the colon was not detected, but GFP expression was seen in the liver (supplementary Fig. 1), suggesting that protein translated both within the gut and possibly the liver may have mediated therapeutic effects of the VEGFs in these studies.

Cross-sectional area of colons. H/E stained sections of distal colon were analyzed for total surface area to determine how cross-sectional area is changed during DSS and experimental treatments. Control colons have a cross-sectional area of 12.3 ± 2.6 mm$^2$; the cross-sectional area of DSS colons was 22.0 ± 7 mm$^2$, significantly greater. This value was reduced, but not significantly so, by VEGF-C (18.1 ± 5.8 mm$^2$). VEGF-D significantly decreased cross-sectional area (to 16.4 ± 4.7 mm$^2$).

Lymphangiogenesis increased in DSS colitis is enhanced by VEGF-D. Control colons showed low numbers of VEGFR-3$^+$ vessels per mm$^2$ (Fig. 4A); this which was significantly increased in DSS colitis (2.94 ± 0.24 vessels/mm$^2$) (Fig. 4A). This increase in VEGFR-3$^+$ lymphatic vessels is similar to that reported in human IBD.[12,13]. VEGFR-3$^+$ lymphatic vessels also increased in VEGF-C + DSS-treated colons, (Fig. 4A) (3.54 ± 0.19 vessels/mm$^2$, p<0.001, and in VEGF-D + DSS treated mice, (3.73 ± 0.2 vessels/mm$^2$). VEGF-C treatment did not increase lymphatic density over that of DSS alone, but lymphatic density in VEGF-D + DSS treated mice was significantly higher than in DSS (*, p<0.05). Angiogenesis increases in DSS colitis: effects of VEGF-D. MECA-32$^+$ staining for blood vessels in controls (Fig. 4), revealed 0.56 ± 0.07 blood vessels/mm$^2$; blood vessel density was significantly increased by DSS (Fig. 4) to 1.37 ± 0.19 vessels/mm$^2$ (p<0.01). MECA-32$^+$ blood vessels were also elevated in VEGF-C + DSS treated colons (Fig. 4) (1.5 ± 0.2 vessels/mm$^2$). Blood vessel densities in VEGF-D + DSS treated colons (Fig. 4) (1.01 ± 0.2) were not significantly different from controls, VEGF-C or VEGF-D.

Discussion

The pathobiology of IBD is complex, but clinical and research findings suggest that multiple risk factors including genetic (Nod2, CARD15), environment - diet and bacterial flora are needed to develop the different forms of IBD.[17].Regardless of the initiating causes of IBD, ‘lymphangitis’, (inflammation, obstruction or obliteration of lymphatic channels) remains an important and frequently neglected contributor to several forms of inflammation including that of the intestine in IBD. Lymphatic expansion and inflammation have been linked since 1867 (based on historical reviews by Pullinger and Florey (1937), and suggest that lymphatic density is adaptively increased during inflammation to handle increased formation of interstitial fluid resulting from increased capillary filtration. The clinical
and experimental correlates between lymphangitis and IBD have known since 1939. In 1939 Blackburn described lymphatic dilation and lymphostasis in Crohn's which was confirmed and expanded by a 1952 study by Van Patter et al., describing lymphatic proliferation and congestion in Crohn's and more recently, Morson in 1972 described that the granulomas which are pathognomonic for Crohn's disease occurs near or within lymphatics of the colon [18]. These studies all document lymphatic reorganization in association with inflammation, but do not address how it relates to Crohn's pathobiology. The current availability of lymphatic-specific growth factors, receptors and biochemical markers currently permits consideration of how lymphatics affect the course of IBD. Kaisserling et al. found an enlargement of mucosal associated lymphoid tissues with an increased density of dilated podoplanin+ mucosal lymphatics in ulcerative colitis [19]. Fogt et al. [10] describe expansion of D2-40+ lymphatics in ulcerative colitis. In normal colon tissue, lymphatics lie below the muscularis mucosa with few branches extending to the lamina propria. D2-40+ lymphatics were elevated in ulcerative colitis, expanding into lamina propria. Crohn's disease (CD) was also characterized by dramatic increases in mucosal lymphatics (increased density of podoplanin+ lymphatics in active CD) [11]. Clearly, lymphatics increase during active IBD, but whether this is beneficial remains unclear; however animal models of lymphatic obstruction suggest lymphatic expansion is adaptive. Deliberate sclerosing of gut segments causes injury with features of IBD including lymphocytic/granulocytic accumulation, gut thickening and development of several types of fistulae [20]. Van Krungen and Colombel (2008) [18], conclude that lymphatic dysfunction is a likely cause of tissue injury in IBD, and normalizing gut lymphatics may lessen IBD disease activity.

Our VEGF receptor blocking studies show that antibody blockade of VEGFR-2, protects against colitis, while blocking VEGFR-3 or VEGFR-1 was ineffective. VEGFR-2 blockade also reduced weight loss and disease activity compared to DSS (Fig. 1) and lowered histological evidence of tissue injury. Interestingly, VEGFR-2 receptor blockade also decreased neutrophil infiltration (tissue myeloperoxidase content), potentially a new role for this receptor in inflammation. We have previously shown that VEGF-A activates colon microvessel endothelial ICAM-1-dependent neutrophil adhesion [9]. Our findings here using VEGFR-2 antibody blockade are consistent with this model, and suggest that colon neutrophil infiltration in DSS is mediated via VEGF-A:VEGFR-2 activation. Recombinant VEGF-D protein did not provide significant protection when administered i.p., and i.p. administration of adenoviruses encoding human VEGF-D or VEGF-C (10^8 infectious units) did not protect against colitis, but did promote thoracic chyle accumulation suggesting that general, systemic induction of VEGFs is less desirable for the treatment of inflammation.

Karpanen et al. (2006) [21] suggest that localized induction of lymphatic VEGFs may be therapeutic in diseases characterized by lymphatic hypofunction. We found that localized adenoviral induction of VEGF-D in the gut blocked acute colon injury in the DSS colitis model; VEGF-C did not provide equivalent protection. VEGF-D protected against macroscopic (weight loss, diarrhea, stool bleeding, and colon length) and histological evidence of injury (myeloperoxidase, leukocyte infiltration, epithelial injury). While VEGF-C and D are both reported as angiogenic and lymphangiogenic, they do not produce identical levels of protection. One possibility suggested by Benest et al. (2008) [22] is that when lymphatics are in close proximity to blood vessels they can suppress blood
vessel growth. In the absence of lymphatic vessels, VEGF-C is angiogenic [23] but when lymphatic and blood vessels interact there appears to be a preferential lymphangiogenic response [24-26]. Therefore, early adenoviral induction of more selectively lymphangiogenic factors, e.g. VEGF-D might more potently activate lymphatic VEGFR-3 to induce lymphatic growth and possibly also inhibit angiogenesis.

Interestingly, protection against DSS mediated weight loss and disease activity seen with induction of VEGF-D did not reflect diminished consumption of DSS (i.e. DSS ‘load’) [27]. VEGF-D treated mice consumed as much DSS as control mice consumed water; groups with disease actually decreased consumption. Thus despite an increased DSS consumption, VEGF-D + DSS treated mice still had less disease than DSS mice, confirming that VEGF-D protects in this model by mechanisms unrelated to DSS ‘load’.

Lymphangiogenesis and angiogenesis are complex phenomena linked by overlapping VEGFs and receptors [28]. VEGFs A, C and D support both angiogenesis and lymphangiogenesis. While VEGF-C and D are potent lymphangiogenic VEGFs in humans, they also have angiogenic activity. Mouse VEGF-D is a selective ligand for mouse VEGFR-3. Postnatally, VEGFR-3 expression is restricted to lymphatic endothelial cells [29; 30]. VEGF-D is dispensable for fetal lymphatic development, it can rescue VEGF-C deficient functions and may play roles in adult lymphatic and blood vascular remodeling.

Since angiogenic growth factors (VEGF-A) and angiogenesis are upregulated in IBD [2; 3; 5] it has been proposed that angiogenic mechanisms contribute to IBD pathology. In IBD, VEGF-A acting as an inflammatory cytokine may promote injury by inducing ICAM-1 dependent leukocyte adhesion and migration [9]. VEGF-A may also cause pathological changes in lymphatic structure and function, and diminished lymphatic clearance of angiogenic cytokines e.g. VEGF could increase gut injury in IBD.

Besides the amount of VEGF-C and D expression, VEGF-C and VEGF-D biological responses also differ between species, and as a result of proteolytic processing. Despite VEGF-D binding to both VEGFR-2 and -3, depending on the system it can produce angiogenesis and lymphangiogenesis [28; 30], or lymphangiogenesis with suppression of angiogenesis [31]. This could represent different tissue-specific patterns of VEGFR-3 receptors expressed in lymphatic vessels, and blood vessels.

Fully processed hVEGF-D binds both mouse VEGFR-2 and VEGFR-3; unprocessed VEGF-D at physiological concentrations binds VEGFR-3, but is a weak VEGFR-2 agonist [32]. VEGF-C also requires processing for full biological activity particularly VEGFR-2 activation [33]. Therefore, full proteolytic VEGF-C and –D processing must occur to observe maximal VEGFR-2 effects; immature forms would tend to produce VEGFR-3 dominant biological effects. Figure 9 shows that both adenoVEGF-C and D forms appear at the anticipated MW sizes (VEGF-D ~22 kD, VEGF-C~27 kD). VEGFR-3 is necessary for embryonic and neonatal lymphatic development, but >4 wks, lymphatics become stable despite VEGFR-3 blockade (with VEGFR-3-Ig). VEGF-C hyper-expressing mice show lymphatic hyperplasia before birth; VEGF-D hyper-expressing mice develop normal lymphatics pre-natally and show lymphatic hyperplasia post-natally. The differences in these responses do not reflect different levels of VEGFR-3 but rather the development of responses to VEGF-D under the influence of alternate growth factors (VEGF, HGFs, angiopoietins), matrix, or integrin signals [21]. Kopfstein et al., also propose that stimulation of newly formed
lymphatic vessels with VEGF-D or VEGF-C yields divergent endothelial phenotypes which release different paracrine mediators [31] to produce different responses.

VEGFR-3 expression is elevated in the inflamed colon, consistent with elevated lymphatic density which may be adaptive in gut inflammation. It has been reported that in the large intestine, the only vascular structures which express VEGFR-3 are lymphatics [29]; therefore, VEGFR-3+ was selected as the marker for immunolocating lymphatics in experimental IBD. The density of VEGFR-3+ lymphatic vessels was increased in response to DSS colitis, as found in human IBD [10-12]. Lymphatics were also increased in colons of DSS treated given VEGF-C or VEGF-D. Only VEGF-D treatment further increased lymphatic density (relative to DSS). VEGF-D treated mice also showed significant protection against weight loss and disease activity. Similarly, blood vessel (MECA32+) density was also increased in DSS colitis. Blood vessel density was also elevated in VEGF-C + DSS treated colons. Since angiogenesis promotes inflammation, VEGF-D mediated suppression of angiogenesis in DSS colitis might contribute to protection in this model. VEGF-D + DSS treated tissue showed a reduction in blood vessel density, which was not significantly different from controls, (or from DSS). Therefore, despite some reduction in angiogenesis, our data are most consistent with protection mediated by increased lymphatic density. The relative inability of VEGF-C to block disease may reflect VEGF-C processing, lower net expression (compared to VEGF-D), or both.

TNF-α has been reported to reduce VEGFR-3 and VEGFR-2 expression in venous endothelium [34] while TNF-α antibody blockade (infliximab™), increases lymphangiogenesis while reducing inflammation in experimental and human arthritis [6] suggesting that TNF-α antagonizes lymphangiogenesis. Whether inflammatory cytokines e.g. TNF-α suppress VEGF-D is not known. We found that VEGFR-3+ lymphatic vessel density is increased DSS colitis, and further increased by VEGF-D, possibly reflecting suppression of TNF-α effects by VEGF-D. Besides VEGF-D, VEGFR-3 selective agonists like VEGF-C156s, may have lower risks for edema and angiogenesis in therapy than use of VEGF-C supported by our findings.

Besides expanding lymphatics in non-vascular tissues, (e.g. corneal epithelium), VEGFR-3 could act as a decoy for VEGF-C/D to limit inflammatory angiogenesis. Chidlow et al. (2006) [1] reported that VEGF-C and D mRNAs were reduced in DSS colitis [1], but did not determine VEGF-C/D protein expression in that model, or if these levels were adaptive or exacerbating. In addition to altering lymphatic function and density, VEGF-D may in some cases be anti-angiogenic [31], and block angiogenic inflammation. It is also possible that VEGF-D binding to heterodimer of VEGFR-2 and VEGFR-3 could also shift signaling from an angiogenic (inflammatory) to (lymphatic) protected phenotype [35]. Therefore, in addition to lymphatic expansion, the VEGFR-3 / VEGF-D system may be anti-inflammatory through mechanisms e.g. suppression of angiogenesis, growth factor scavenging or VEGF receptor competition. Genetic deletion of VEGF-C is lethal, VEGF–D is not, and VEGF-D−/− embryos survive and develop apparently normal lymphatics. While VEGF-D is not essential for development, it can regulate lymphatic expansion after birth [36]. Besides effects on lymphatic abundance and area, other factors like lymphatic pumping or endothelial barrier function may also mediate VEGF-D protection.

Active colon inflammation is associated with increased angiogenesis and lymphangiogenesis [10-12; 37]. Unlike the inflammation-intensifying events of angiogenesis, lymphatic expansion may represents a physiological
response to clear accumulated interstitial fluid and immune cells and exerts an anti-inflammatory response. In this model of initiation of intestinal inflammation, lymphangiogenesis was protective at several levels. Therefore the failure to appropriately balance angiogenesis with lymphangiogenesis may represent an important underlying cause for chronic inflammation failing to resolve. The therapeutic expansion of lymphatics by manipulation of the levels of lymphangiogenic growth factors and receptor signaling is protective, and may provide a novel method for relieving injury in forms of chronic inflammation like colitis, shown here, as well as arthritis, diabetes, lupus and psoriasis.

Methods
Mice. Mice used in this study were male or female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) or κB-lacZ mice (provided by Dr. Sylvie Memet, Pasteur Institute, Paris, France) [38]. Mice were used at 8-12 weeks of age in colitis studies and had an average initial weight 23 ± 2 g (on day 0). Mice were housed in the LSUHSC-S vivarium (@ 25°C with a 12h/12h light/dark cycle), allowed free access to standard pellet diet and either tap or acid-treated water. All protocols were approved by the LSUHSC-S animal care and use committee.

Induction of experimental colitis. Experimental colitis was induced in mice by feeding 3% dextran sulfate sodium (DSS, MW= 36-50 kDa; ICN Biomedicals Inc.) ad libitum in drinking water as described [27]. Control mice (n = 5) group received tap water without DSS. 3% DSS administration produces an erosive colitis with an initial onset of 4-5 days which is characterized by progressive weight loss, diarrhea, occult blood, leukocyte inflammation, colon shortening, histopathological changes in colon structure and loss of intestinal epithelial barrier. Mice typically lose up to 20% body weight over 10 days of DSS administration. On day 10, mice under ketamine/xylazine anesthesia are sacrificed by cardiac puncture. Histology samples are fixed in cold 3.7% phosphate buffered formalin; or snap frozen at -80°C for myeloperoxidase and western blotting analysis. Disease Activity Index. Mouse weight, feces and blood were scored daily as described [37]. Disease activity index (DAI) was calculated as the average of weight loss, stool form, and occult blood scores. Scores were determined as: weight change (0 :< 1%, 1: 1–5%, 2: 5–10%, 3:10-15%, 4 :> 15%), occult blood (0: negative, 2: positive, 4: gross bleeding), and stool form (0: normal, 2: loose stools, 4: diarrhea). Occult blood was detected chemically using 'Coloscreen' test kits, (Helena Labs, Beaumont, TX).

VEGFR-1, VEGFR-2 or VEGFR-3 antibody blockade. To determine the contributions of the 3 VEGF receptors to the development of DSS colitis, VEGF-receptors were blocked by daily i.p. injections of 50 ug of anti-mVEGFR-1 [clone MF1:VEGFR1], anti-mVEGFR-2 [clone DC101], or anti-mVEGFR-3 [clone 31C1] (ImClone Systems) in 0.1 ml of PBS 3 days prior to, and then during induction of colitis daily (10 days). Body weight and disease activity score were monitored daily; all mice were sacrificed on day 10.

Adenoviral vectors for VEGF-C and D. Adenoviral constructs encoding full-length hVEGF-C cDNA and hVEGF-D were expanded as described20. Replication-deficient E1-E3 deleted adenoviruses were cultured in 293 cells and concentrated by ultracentrifugation. Prior to use, adenoviral preparations were determined to be free from
contamination with helper viruses, bacteria or lipopolysaccharide. **Adenovirus administration.** 24 h prior to the induction of experimental colitis, mice were fasted for 12h (food only withheld). Mice were anesthetized with ketamine/xyloazine, and a 16 gauge silicone rubber tipped probe (Popper and Sons, New Hyde, NY), was inserted 3 cm into the colon. 2X10⁸ infectious particles were administered to the colon mucosa in 50 ul of PBS. Mice were held in a 45° tail-elevated position until they regained consciousness (~15 mins). For the next 10 days, mice were given unrestricted access to pellet diet (Purina), and tap water (control), or tap water containing 3% dextran sulfate sodium (DSS) in colitis groups[27]. Mouse weight, stool form, occult blood, food and liquid consumption were recorded daily.

**Histopathological analysis.** Colon sections were formalin-fixed and paraffin-embedded. 10um sections were stained with hematoxylin eosin slides and analyzed for histopathological injury (edema, extent of injury, leukocyte infiltration, crypt abscesses and loss of goblet cells) [27]. A maximum possible histopathology score of 40 is based on a combined score of 1) inflammation severity (a 0-3 scale, (0-none to 3-severe), 2) extent of injury (a 0-3 scale (0-none, 1-mucosal, 2-mucosal + submucosal, 3-transmural), and 3) crypt damage, a 0 - 4 scale (0-none, 1- basal 1/3 damaged, 2- basal 2/3 damaged, 3- only surface epithelium intact, 4- entire crypt and epithelium lost). Each value was multiplied by a factor of 1-4, reflecting the extent of involvement from 0% (X1) to 100% (X4).

**Measurement of tissue myeloperoxidase content.** Myeloperoxidase activity was measured as described [27]. ~20 mg samples of colon tissue were frozen in N₂(l) crushed, and freeze-thawed in 0.5% HETAB buffer 3X, sonicated (10s @ 50% max power), and cleared by centrifugation at 10,000 x g before measuring MPO activity using o-dianisidine substrate. Activity is reported as the change in absorbance at 650 nm/min/mg tissue.

**Lymphatic kB-lacZ.** To visualize lymphatic kB-lacZ staining, 1% formalin / 0.2% glutaraldehyde fixed kB-lacZ tissues (30 min) were incubated in 4 mM K₄(Fe(CN)₆), 4 mM K₃(Fe(CN)₆), 2 mM MgCl₂, and 400µg/ml X-gal, (Sigma) in PBS (16 h) at 30ºC as previously described [38]. After staining, tissues were post-fixed 24h in 3.7% formalin, paraffin-embedded and sectioned (10um) and eosin counterstained. **Immunohistochemistry.** Formalin-fixed tissue samples were immunostained using DAB/HRP staining for VEGFR-3 staining (lymphatic vessels) and MECA-32 (mouse endothelial cell antigen-32). Colon sections were embedded in paraffin and 10um sections collected on Superfrost-Excell slides. Slides were deparaffinized antigen retrieved and incubated in 1° antibody, (1:125, 1hr), washed in 0.1% BSA and reacted in HRP-conjugated 2° antibody (1:2,500, 1h), and reacted with DAB/H₂O₂ (5 mins). Slides were hematoxylin-stained and sealed w/ Permount. **Immunofluorescent staining.** Antigen-retrieved slides were also fluorescently co-stained for blood vessels (MECA-32) and lymphatics (LYVE-1) using fluorescent antibodies tagged with Alexa-fluor488 (green), or Cy3 (red) as well as DAPI staining (blue, DNA).

**Adenoviral VEGF-C VEGF-D transfection of intestinal epithelia.** The receptor specificity and bioactivity of VEGF-C and VEGF-D are regulated through the type and extent of VEGF post-translational processing [33; 36]. Therefore, we determined the molecular weight of VEGF-D and C isoforms synthesized by adenoviral transfected gut epithelia.
Young adult mouse colon epithelial cells (‘YAMC’), a conditionally transformed epithelial cell line, (provided by Dr. Robert Whitehead and Jen Fenton,, Vanderbilt University), was used as ‘host’ for in vitro adenoviral protein expression. Confluent YAMC cultures (35 mm dish, 2.5X10⁶ cells) were transfected with 10⁷ infectious particles for 48 h. Samples were western blotted for hVEGF-D or hVEGF-C (R&D, Minneapolis, MN) and visualized by ECL.

**Microphotography.** Images of stained tissue sections were photographed using a Nikon coolpix 900 and analyzed for number of vessels and vessel dimensions using Image-J (NIH). Immunofluorescent confocal images were taken using a Leica DMI 6000CS with Leica TCS SP5 confocal system using the LAS AF acquisition software package.

**Statistical Analysis.** Weight changes and disease activity studies were evaluated using repeated measures ANOVA with Dunnett’s post-testing (Instat3, Graphpad Software). All other data (MPO) were analyzed by one-way ANOVA with Dunnett’s post-testing.

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Reference List


Figure 1. Intraperitoneal (i.p.) administration of anti-VEGFR-2 antibody significantly reduces weight loss (A) and disease activity (B) in experimental colitis. Figure 1A - Disease activity index in mice receiving no treatment compared to 3% DSS (●, filled circles) over 10 days. Antibody treatments were administered i.p. 3 days prior to and during colitis. Animals receiving 3% DSS + VEGFR-2 antibody show protection from day 5 onward (*p<0.05 - DSS vs. VEGFR-2 Ab/DSS). VEGFR-3 and VEGFR-1 did not significantly improve DAI. Figure 1B. Weights of mice receiving normal water vs. mice receiving 3% DSS (●, filled circles). Mice receiving VEGFR-2 antibody showed less weight loss (▼, filled triangles), neither VEGFR-1 nor VEGFR-3 antibody modified disease vs. DSS. (C) VEGFR-2 blockade reduced histopathology (C) and myeloperoxidase (neutrophil content) in experimental colitis. Colon histopathology (C) was increased by DSS colitis (*p<0.05 vs. control); histopathology score was not increased when VEGFR-2 was administered (#p<0.05 vs. DSS). (D). Colon MPO content was significantly increased by experimental colitis (*p<0.05 vs. control); myeloperoxidase was not increased in DSS + VEGF-2 treated (p<0.05 vs. DSS).

Figure 2. (A) Colon shortening in experimental colitis is prevented by adenoviral induction of VEGF-D. Compared to controls (1st bar), colitis reduced colon length (2nd bar), p<0.001. Mice receiving adenoviral VEGF-D prior to DSS did not significantly change colon length (3rd bar, not sig. from control). Neither adenoviral VEGF-C (AdVEGF-C) or green fluorescent protein (adGFP) administration prior to DSS were able to prevent DSS induced colon shortening (4th and 5th bar, ***, p<0.001 vs. control). (B). Compared to untreated controls, myeloperoxidase activity was increased in colons of mice treated with DSS, ** - p<0.01). Intestinal administration of adenoviral human VEGF-D reduces colitis induced neutrophil accumulation. Mice treated with adenoviral VEGF-C show lower, but not significantly reduced MPO. (C) Histopathology reduction by administration of adenoviral VEGF-D. Control colons (a) show normal tissue structure with intact epithelial lining and normal crypt structure and distribution. DSS (b) shows extensive leukocyte infiltration, loss of epithelia and crypt structure. Colon administration of AdenoVEGF-C + DSS (c) shows significant improvement in tissue structure with maintenance of crypts and epithelial lining, some edema and infiltration is still noticeable. Administration of AdenoVEGF-C (d) shows less improvement in tissue structure, some crypts and epithelia persist but less so than in AdenoVEGF-D. (D) Adenoviral VEGF-D reduces histopathology in colitis. Histological evidence of injury measured on a 0-40 score shows that controls score 0.29 ± 0.76; histopathological injury was maximal in colons from experimental colitis group (34.7 ± 2.3, *** - p<0.001 vs. controls). There was a non significant reduction in histopathology in VEGF-C + DSS (26 ± 1.7). Mice receiving adeno VEGF-D + DSS showed significant protection against histopathologic injury (* p<0.05 vs. DSS) with the least injury of treatment groups (14.4 ± 5.4).

Figure 3. Administration of adenoviral VEGF-D prevents weight loss in DSS Colitis. (A) Mice were fasted for 24 h prior (day -1) prior to the administration of either adenovirus expressing GFP (filled triangles, ▼), adenoviral VEGF-C (open triangles, △), or adenoviral VEGF-D (filled squares, ■). On day 0, control mice were fed either water (filled circles, ●), and the remaining groups given 3% DSS in drinking water (open circles, ○). Mice drinking water showed no weight loss over the following ten days (●). Mice on 3% DSS exhibited a progressive loss of body weight compared to controls (a** - p<0.01 vs. control, a* - p<0.05 vs. control). Similar weight losses were seen in adenoGFP (▼, c** - p<0.01 vs. control, c* - p<0.05 vs. control) and adenoVEGF-C (△, b**, p<0.01 vs. control, b* p<0.05 vs. control). Mice administered VEGF-D and also consuming 3% DSS (■) did not lose weight over 10 days and were not significantly different from controls at any day. (One-way ANOVA with Dunnett's post-testing). (B). Intestinal administration of adenoviral VEGF-D significantly reduces disease activity in colitis. Disease activity measured as an index incorporating weight change, stool blood and diarrhea showed that control mice did not exhibit significant disease activity over 10 days (filled circles, ●), but mice fed 3% DSS showed a progressive increase in disease starting on day 2 and progressing through day 10. Nearly identical disease activity was seen in mice given adenoviral VEGF-C + DSS (△) or adenoviral GFP + DSS (▼) were all significantly different from controls after day 2). Mice receiving adeno VEGF-D + DSS (■) showed significant protection compared to DSS alone, (e – p<0.001 vs. DSS), but still showed significant increased disease compared to untreated controls (d - p<0.001 vs. controls, d**, p<0.01 vs. control). (‘a-e’ alone – p<0.001, ** - p<0.01, *p<0.05, One-way ANOVA plus Bonferroni post-testing).
Figure 4. Changes in VEGFR-3+ and MECA32+ vessel density in DSS colitis. Left panel: VEGFR-3 (a-d) and MECA-32 staining (e-h) in control (a, d), DSS-treated colon (b, f), VEGF-C + DSS treated (c, g) and VEGF-D + DSS treated colons (d, h). Bar = 100 um, 200 X magnification. Vessel density analysis VEGFR-3+ (lymphatic vessel) and MECA32+ (blood vessel) densities compared in control, DSS-treated, VEGF-C + DSS-treated and VEGF-D + DSS treated colons. (*** p<0.001 vs. control, * p<0.01 vs. control).

Figure 5. Immunolocalization of lymphatics and blood vessels in adenovirally treated colon tissue. A. Control, lower power localization of blood vessels (MECA-32) and lymphatic vessels (LYVE-1), B. lower power DSS treated colon tissue. C. Adenovirally induced VEGF-C + DSS treated colon tissue. D. Adenovirally induced VEGF-D + DSS treated colon tissue. E, F, G and H are high power photomicrographs of control, DSS, adenoviral VEGF-C + DSS and adenoviral VEGF-D + DSS treated colon tissue.

Supplementary figure 1. Adenoviral protein induction. A) Expression of adenoviral proteins in mouse colon epithelial cells. Transfection of adenovirus encoding human VEGF-C and VEGF-D in mouse colon epithelial cells shows anticipated MWs of these proteins compared to standards of recombinant proteins (expressed in insect cell lines). VEGF-D adenoviral transfection leads to production of 22 kD VEGF-D in YAMC similar to standard. VEGF-C adenoviral transduction leads to production of 27 kD VEGF-C in YAMC similar to standard, 23 kD. Adenoviral GFP (control) does not induce any signal. B) IVIS imaging of adenoviral transfection. Left panels show normal non-transfected colon and liver (bottom); middle panel shows colons and liver transfected with Adeno-GFP at 4 days (4d). Colons show GFP expression, livers do not GFP show signal. Bottom panel shows colons and livers at 10 days (10d); colon GFP expression is decreased, GFP expression is seen in the liver.
Fig. 1

(A) PERCENT WEIGHT CHANGE vs. Days

(B) DISEASE ACTIVITY INDEX vs. Days

*p<0.05 DSS vs. DSS + VEGFR-2 Ab

*p<0.05 DSS vs. DSS + anti-VEGFR2 Ab
Fig. 1
<table>
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*** ns

Fig. 2A
Fig. 2B
Fig. 2D

Histopathology Score (Scale 0-40)

Control  |  DSS  |  VEGF-D +DSS  |  VEGF-C +DSS

Treatment

***  |  *  |  ns  |
Fig. 3A

a** - p<0.01, DSS different than control
b** - p<0.01, VEGF-C different than control
c** - p<0.01, GFP different than control

a* - p<0.05, DSS different than control
b* - p<0.05, VEGF-C different than control
c* - p<0.05, GFP different than control
Fig. 3B

- a- p<0.001, DSS different than control
- b- p<0.001, VEGF-C different than control
- c- p<0.001, GFP different than control
- d- p<0.001, VEGF-D different than DSS
- e- p<0.001, VEGF-D different than control
Fig. 5