Title: Recombinant VEGF-C (Cys156Ser) improves mesenteric lymphatic drainage and gut immune surveillance in experimental liver cirrhosis

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Introduction:

Chronic liver disease (CLD) can progress from mild fibrosis to cirrhosis which may further progress to end-stage liver disease with the onset of decompensation, such as ascites, hepatic encephalopathy, and variceal bleeding. In addition to other factors, the deranged gut-liver axis plays a significant role in the progression of liver disease (Albillos, de Gottardi, and Rescigno 2020; Fukui 2021).

The intestinal lymphatic vasculature comprises the longest-studied lymphatic vessel (LVs) bed. It plays a major role in dietary fat uptake and transport, viscera fluid balance, and gut immunosurveillance (Bernier-Latmani and Petrova 2017a). The intestinal lymphatic capillaries (lacteals) and associated collecting vessels in the mesentery form the drainpipe that clears interstitial fluid, inflammatory cells and molecules, and microbial debris from the abdominal viscera. The intestinal lymphatic system has been associated with pathologies, including inflammatory bowel disease and obesity (D'alessio et al., 2014; Cao et al., 2021). Intestinal inflammation is correlated with extensive intestinal lymphangiogenesis, and specific loss of intestinal lymphatics results in severe gut inflammation, sepsis, and complete short-term lethality, highlighting the crucial role of intestinal lymphatics in inflammation (Jang et al., 2013). LVs in the gut bypass the portal circulation and directly connect the gut to systemic circulation via the thoracic duct (Cifarelli and Eichmann 2019). Mesenteric lymphatic vessels (mLVs) prevent edema and abdominal ascites by facilitating removal of interstitial fluid and returning it back to blood circulation (Bernier-Latmani and Petrova 2017b). Gut lymphangiogenesis compensates for lymphatic insufficiency in patients with inflammatory bowel disease (IBD) (Danese 2011; Hong et al. 2019). During cirrhosis, the production of abdominal lymph increases 30-fold, and there is a positive correlation between increased lymph flow and portal pressures (Chung and Iwakiri 2013; Dunbar et al. 1989). In CCl4-induced cirrhosis models, an elegant study reported that gut LVs show an impaired phenotype and reduced contractility (Ribera et al. 2013). However, no studies have characterized gut LVs in clinical studies. An understanding of intestinal LVs in patients with cirrhosis might lead us to novel therapeutic strategies. To this end, we thoroughly investigated duodenal (D2) LVs in patients with liver cirrhosis and studied their association with disease severity, inflammatory markers, and survival. Along with this, we prepared animal model of liver cirrhosis and modulated the gut lymphatic vessels with vascular endothelial growth factor-C (VEGF-C), a key pro-lymphangiogenic factor, that activates lymphangiogenesis by binding to tyrosine kinase receptor VEGF receptor-3 (VEGFR3) (Nurmi et al. 2015). VEGF-C is known to be effective in improving lymphatic drainage and ameliorating inflammation diseases such as IBD, rheumatoid arthritis, skin inflammation, and hepatic encephalopathy (Bouta et al. 2018; Güç et al. 2017; Hagura et al. 2014; Hsu et al. 2021). We hypothesized a therapeutic role of VEGF-C for restoring gut lymphatic drainage and immune response in cirrhosis models. VEGF-C, a hydrophilic molecule, is highly unstable and has a positive charge at physiological pH (Claaßen et al. 2017; Geng et al. 2011). To minimize its side effects of systemic delivery and increase its gut bioavailability, we fabricated an engineered VEGF-C protein using human recombinant VEGF-C protein (Cys156Ser), which specifically binds to VEGFR3 homodimers, present on lymphatic endothelial cells (LyECs) lining the LVs. VEGF-C protein was encapsulated within nanoscale reverse micelle (RM)-based lipo-carriers and delivered via oral route to ensure its uptake in mLVs *in vivo*.

Objectives:

1) **To characterize gut lymphatic vessels in liver cirrhotic patients and in experimental animal models of liver disease.**
2) **To modulate gut lymphatic vessels in experimental animal models of liver cirrhosis.**
3) **To study the effect of lymphatic vessels modulation on lymphatic drainage, hepatic hemodynamic parameters, and gut immune surveillance in experimental animal models.**

Materials and Methods:

Study design and patients

We performed a prospective, observational, single-center cohort study in about six months in patients with histologically confirmed cirrhosis (viral, alcoholic, or other aetiology) and clinical parameters from the Department of Hepatology of Institute of Liver and Biliary Sciences, listed in **Table 1**. We excluded patients with active bacterial infection at evaluation, hepatocellular carcinoma, active alcohol abuse, variceal bleed (4 weeks), recent previous transjugular intrahepatic portosystemic shunt insertion, occlusive portal vein thrombosis, liver transplantation (LT), chronic kidney disease, gastrointestinal mucosal diseases (e.g., celiac disease, inflammatory bowel disease) or intestinal surgery. Patients with cirrhosis were subsequently divided into two groups: decompensated (n=19) or compensated (n=12) cirrhosis based on the presence or history of at least one decompensating event, i.e., ascites, jaundice, variceal bleeding, and overt hepatic encephalopathy (HE). The control group (n=9) comprised individuals

without any liver disease matched for age and sex which underwent an upper gastrointestinal tract endoscopy for non-cirrhotic causes, e.g., functional dyspepsia. All subjects enrolled in study underwent an upper gastrointestinal tract endoscopy for clinical indications, during which biopsies were obtained from second portion of duodenum, distal to the ampulla of Vater, after their consent before the study. The study was conducted according to the principles of the Declaration of Helsinki and was approved by the institutional ethics committee (IEC/2019/68/NA05). All patients gave written informed consent for participation.

In addition, routine laboratory tests for liver dysfunction severity were measured, e.g., *Model for End-Stage Liver Disease* (MELD) and Child-Pugh scores (CTP). Along with this, *TNF-α* and *IL-6* mRNA and their serum levels were assessed as biomarkers of gut and systemic inflammation by ELISA following the manufacturer's instructions. Commercial ELISA kits were used to measure the biomarker levels (E-lab-sciences, Unites States). All the clinical and biochemical investigations were done at the time of biopsy collection. After collecting the D2-biopsy, patients were followed for three months to record mortality.

RNA Isolation and RT-PCRs

D2-biopsies were snap-frozen in liquid nitrogen, and total RNA was isolated from the TRIzol reagent (Thermofisher, India). RNA was quantified with Thermo Scientific Nanodrop 2000 Spectrophotometer. According to the manufacturer's instructions, cDNA was synthesized using 1 µg of RNA with reverse transcriptase (Thermo Revert aid cDNA synthesis kit). qRT-PCR was performed with SYBR green PCR master mix (Fermentas Life Sciences) on the ViiA7 PCR system (Applied Biosystems, United States). The cycling parameters used were as follows: start at 95°C for 5 min, denaturing at 95°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s, and final 5 min extra extension at the end of the reaction and repeated for 40 amplification cycles. After normalization, relative quantification of the expression was done using the ΔΔCt method to the expression of the housekeeping gene, GAPDH. The genes and primer pairs are mentioned in **Supplementary Table 1**.

Immunohistochemistry

D2-biopsies were fixed in 10% buffered formalin and processed. Sections of 4-µm-thick paraffin-embedded tissues were heat-fixed, deparaffinized at 45 C, and rehydrated in descending ethanol series. Following antigen retrieval by heating for 8 minutes in a microwave with citrate buffer, pH 6, sections were incubated for 20 minutes with peroxidase-1 solution to quench endogenous peroxidase. Protein blocking was done with 3% BSA. Slides were then incubated overnight at 4C in the humid chamber with primary antibodies **(Supplementary Table 2)**. Staining was completed using the HRP-conjugated mouse/rat/human detection kit and DAB chromogen as a substrate, according to the manufacturer's instructions. Lastly, sections were counterstained with hematoxylin. Slides were mounted with a coverslip using DPX (Dibutylphthalate Polystyrene Xylene). LVs were semi-quantitatively quantified based on the expression of podoplanin (PDPN), a surface marker present on lymphatic endothelium. The total PDPN score was the sum of (1) the intensity of stained area measured in percentage using ImageJ [0- none; 1 (1-25%); 2 (25-50%); 3 (50-75%); 4 (75-100%)] and (2) the percentage proportion of PDPN+ stained area/field [0 (0-5%); 1 (6–25%); 2 (26–50%), and 3 (51–75%) and 4 (76-100%)] (Klein et al. 2001) **(Supplementary Figure 1, Supplementary Table 3**). Scores between 0–4 were considered to indicate low PDPN expression, while scores of 5–8 represented high expression. Pathologists, blinded to clinicopathological details of patients and identity of slides, conducted IHC analysis. PDPN scores in each sample were based on analysis of six randomly selected fields per slide. The diameter of LVs was quantified using ImageJ. Each image was calibrated as per the scale bar, and the diameter was measured in PDPN-stained LVs. Per slide, six random fields were selected for analysis. For scoring intraepithelial lymphocytes (IELs) and macrophages, D2-biopsy sections were stained with ready-to-use CD3 and CD68 anti-human antibodies (PathnSitu Biotechnologies), respectively. According to the manufacturer's instructions, the staining was completed using the PathnSitu HRP-conjugated detection kit and DAB chromogen as a substrate and counterstained with hematoxylin. For the quantification of CD3+ and CD68+ cells, six fields per slide were selected. CD3+ IELs were counted per 100 epithelial cells, and less than 30 IELs were considered normal. CD68+ cells were quantified per field. For VEGF-C expression, slides were stained with VEGF-C (VEGF-C antibody, MA5-26494, Invitrogen, Unites States, 1:100 dilution). Six random fields per slide were selected. Mean staining density was determined using ImageJ (ImageJ, NIH, USA), and pathologists interpreted staining results.

Preparation of E-VEGF-C

To prepare RMs, high-pressure homogenization/micro-fluidization techniques were adapted as described by *Bai et al.* (Bai et al. 2019), followed by slight modification. Recombinant human VEGF-C (rhVEGF-C (Cys156Ser), R&D system, 752-VC-025) was incorporated in prepared RMs to obtain nanosized RMs containing VEGF-C protein termed

as engineered VEGF-C (E-VEGF-C) **(Supp-Figure 1).** E-VEGF-C was characterized and its uptake was studied in mesenteric LyECs in vitro, as given in supplementary materials.

Study Groups and treatment

All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The Animal Ethics Committee of ILBS, New Delhi, approved the study according to standard guidelines (Ethics Protocol No: IAEC/ILBS/18/01). Rats were housed in a room at 22 ± 3^0C for 12h. light-dark cycle and were given food and water ad libitum. Studies were performed on 8-week-old male Sprague Dawley (SD) rats weighing 250–300g. Three cirrhotic models of portal hypertension and one non-cirrhotic portal hypertension model were prepared. Cirrhosis was induced by (1) intraperitoneal (i.p.) injection of 1.0 ml/kg body weight of CCl4:olive oil in 1:1 ratio, two times a week for 12-14 weeks until ascites formation (2) i.p injection of 250mg/kg body weight of thioacetamide (TAA), two times a week for 12 weeks (3) Bile duct ligation (BDL) model were developed by ligating the common bile duct as previously described (Tripathi et al. 2018). Non-cirrhotic animal models of portal hypertension were developed using partial portal vein ligation (PPVL), as previously described (Tripathi et al. 2018). To inhibit LVs, a selective VEGFR-3 inhibitor, SAR131675, was orally administered in TAA cirrhotic rats. For *in vivo* studies, animals were randomized to study the efficacy of E-VEGF-C. In cirrhotic models of portal hypertension, these groups were prepared: healthy control, vehicle (CCl4-V, TAA-V, and BDL-V), and treated E-VEGF-C (E-VEGF-C, TAA+E-VEGF-C, and BDL+E-VEGF-C). After hemodynamics, these rats were sacrificed 48h after the last dose of vehicle, or E-VEGF-C. In the non-cirrhotic model of portal hypertension, PPVL rats were prepared and randomized into two groups: (1) vehicle (PPVL-V) and (2) treated (PPVL+E-VEGF-C). The preparation of animal models and treatments are described in detail in supplementary methods.

Statistical Analysis:

Patient Studies: Categorical variables were reported as absolute frequencies (n) and relative frequencies (%); continuous variables as mean ± SD. Normal distribution was assessed by Kolmogorov-Smirnov tests. Categorical data were analyzed with the Chi-square or Fisher's exact test wherever appropriate. Continuous variables were compared with an independent sample t-test or Mann-Whitney U test for two groups and by One-way ANOVA or Kruskal-Wallis's test for more than two groups depending on the distribution (normal or skewed). For the assessment of prognostic factors in decompensated cirrhosis, clinical predictors were analyzed by binary logistic regression as the time of decompensation was not available. For analysis of mortality predictors, all clinical variables were entered into the Cox hazard regression model to assess the effects of factors on 3-month mortality from the date of biopsy collection. We used unadjusted linear regression models and multivariate models adjusted for clinical covariates. The receiver operating characteristic (ROC) curves were plotted to explore the area under the curve (AUC). Cutoff points to discriminate the survivors from the non-survivors were calculated by obtaining the best Youden index (sensitivity% + specificity% − 100). The cutoff values were taken where sensitivity and specificity were optimal.

AUCs were expressed with their 95% confidence interval (CI). The study participants were divided into two groups according to the optimal cutoff value of 3-month mortality/LT probability. Then the mortality rates of the groups were compared using Kaplan–Meier curve analysis. A log-rank test was conducted to compare the survival curves of the groups. Correlation analysis between the variables was performed by using Pearson's Correlation analysis. All statistical analyses were performed using IBM SPSS Statistics (version 20.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, CA, USA). Statistical analysis was done using GraphPad Prism (version Prism 8.4.3; GraphPad Software, San Diego, CA, USA) and SPSS. The level of significance was set at a 2-sided p-value <0.05.

Animal studies: Continuous variables are expressed as either mean ± standard deviation for normal distribution or as median values for skewed distribution. Continuous variables were compared between the two groups using an unpaired two-tailed Student's t-test or Mann-Whitney U test. Variables greater than two were compared using one-way ANOVA followed by a post hoc Tukey's test. Bar diagrams with various data points, dot plots, and box whisker plots were plotted using GraphPad Prism (version 8.0.1.GraphPad Software, San Diego, CA, USA), and statistical analysis was performed using GraphPad Prism. Statistical significance was set at $p<0.05$.

Results

Demographic Features of the Study Groups

Characteristics of the patient groups are summarized in **Table 1**. The mean age was comparable between the study groups, with 48 ± 11.9 years for controls, 52 ± 10.5, and 52 ± 9.49 for compensated and decompensated cirrhotic patients. The control group was significantly different from the cirrhotic patients with respect to the clinical variables. Decompensated cirrhotic had lower platelet and serum albumin levels and higher serum bilirubin, AST, INR, and creatinine compared to compensated cirrhosis ($p<0.05$, **Table 1**). Non-alcoholic fatty liver disease (45.1%) and alcohol-related liver disease (38.7%) were the most common etiologies for liver cirrhosis in the study cohort. Among patients with decompensated cirrhosis, 19 (100%) had ascites, 8 (42.1%) had variceal bleeding, and 9 (47.3%) had hepatic encephalopathy. The CTP and MELD scores were significantly higher in decompensated versus compensated cirrhosis ($p<0.0001$ for both, **Table 1**).

Enhanced PDPN+ dilated lymphatic vessels density in decompensated cirrhosis

An impaired gut lymphatic system in terms of increased density, dilation, leakage, and reduced drainage has been reported previously in experimental liver cirrhosis with ascites (Ribera et al., 2013; Juneja et al., 2022). In cirrhotic rats, high levels of pro-lymphangiogenic factors in the liver, such as VEGF-C and VEGF-D, have also been found to be positively associated with disease progression (Tugues et al., 2005). The density and diameter of gut LVs in human cirrhosis, however, remain elusive. Therefore, to characterize intestinal LVs in cirrhotic patients, we collected D2-biopsies from control and cirrhotic patients and immunostained with PDPN, a widely accepted marker of LVs presents on the surface of lymphatic endothelium (**Figure 1A**). PDPN stained sections were semi-quantitatively accessed and scored from 0-8. The diameter of LVs was also measured for dilation. Histologically, there was a weak and minimal PDPN positivity in the control biopsies. Patients with compensated and decompensated cirrhosis displayed a large number of PDPN+ dilated lymphatic channels, consisting of a single layer of endothelial cells in the mucosal and submucosal regions of D2-biopsies. Detailed calculation of PDPN scores in different patients is given in **Supplementary Table 4**. The mean PDPN scores in decompensated cirrhosis (6.91 ± 1.26) were significantly higher than in patients with compensated cirrhosis (3.25 ± 1.60, $p<0.0001$, **Figure 1B**). We observed significantly dilated LVs in decompensated (193.1 ± 28.8 µm) and compensated (158 ± 11.1 µm) patients as compared to controls (138.5 ± 9.8 µm) ($p<0.05$ each, **Figure 1C**). We have also checked the expression of key pro-lymphangiogenic factor VEGF-C protein in the D2-biopsies of control and cirrhotic patients and found significantly increased levels in decompensated (1.7 arbitrary unit (a.u.)) and compensated (0.9 a.u.) cirrhotic patients, compared to controls (0.6 a.u.) ($p<0.0043$, **Supplementary Figure 2A-B**). Along with this, we have also quantified the mRNA levels of some pro-lymphangiogenic factors, *FLT4, LYVE1*, and *PDPN*, in the D2-biopsies of control and cirrhotic patients. *LYVE1* (4-fold) and *PDPN* (9-fold) mRNA levels were elevated in patients with cirrhosis compared to controls ($p<0.0001$, **Supplementary Figure 2C**). We also compared PDPN scores in cirrhotic patients with and without different complications of decompensated cirrhosis. PDPN scores were significantly increased in patients with ascites and variceal bleeding versus those without ascites and bleeding ($p<0.05$, **Figure 1D, E**). We did not find any significant difference in the PDPN scores of patients with and without HE ($p=0.13$, **Figure 1F**).

PDPN Score Correlated with Intestinal Permeability and Systemic Inflammation

We next investigated if PDPN scores are associated with intestinal inflammation and permeability in cirrhotic patients. Intestinal inflammation was characterized by the numbers of CD3+ duodenal intraepithelial lymphocytes (IELs), CD68+ macrophages, and villi structures in different study groups. The IELs were not significantly different between compensated and decompensated cirrhotic and also were not significantly correlated with PDPN scores ((**Supplementary Figure 3A-B**, **Figure 2A**, $r = 0.33$, $p=0.06$). CD68+ macrophages per field were found to be non-significantly increased in compensated (4.1 ± 1.16) patients compared to controls (2.4 ± 0.9, $p=0.09$) but significantly elevated in decompensated cirrhotic patients (5.8 ± 2.0) compared to compensated or controls (**Supplementary Figure 3C-D**, $p<0.05$ each). Also, the villi: crypt ratio was 1:1 in the duodenum of decompensated cirrhotic with patchy mild blunting of villi as compared to 2:1 in control and compensated cirrhotic patients with no blunting of villi. In decompensated cirrhotic patients, there was also a depletion of goblet cells on the surface epithelium. (**Supplementary Figure 3E**). For intestinal permeability and inflammation, we examined the mRNA level of tight junction protein, ZO-1 (*TJP1*), Occludin (*OCLN*), *TNF-α,* and *IL-6* in some of the biopsy samples (**Supplementary Figure 4A-D**). The expression of *TJP1* gene was significantly reduced in patients with decompensated cirrhosis compared to compensated cirrhosis ($p<0.05$). A correlation analysis between *TJP1* gene expression and PDPN score revealed a significant negative correlation in the patients ($r=-0.46$, $p=0.05$, **Figure 2B**). *OCLN* mRNA levels were significantly reduced in decompensated cirrhotic patients compared to controls ($p<0.05$). Both *TNF-α* and *IL-6* mRNA levels were significantly increased in cirrhotic patients compared to control ($p<0.05$ each). Next, we measured serum TNF-α and IL-6 levels to investigate the association of PDPN score with systemic inflammation. Serum TNF-α levels did not

significantly differ between compensated and decompensated cirrhotic patients (**Supplementary Figure 5A**). IL-6 levels, on the other hand, were significantly higher in patients with decompensated cirrhosis (**Supplementary Figure 5B**, p=0.02). High systemic levels of TNF-α (r=0.38, p= 0.03) and IL-6 (r=0.48, p= 0.006) significantly correlated with high PDPN scores in cirrhotic patients (**Figure 2C, D**).

PDPN Score Correlates with MELD and CTP Scores of Patients with Cirrhosis

Given a significant increase in the PDPN+ lymphatic channels in decompensated patients, we evaluated if the PDPN score correlates with liver disease severity. We performed a binary logistic regression analysis along with other clinical factors of decompensation. Cox regression was not performed as we did not have the data for the time of decompensation. The analysis showed that high levels of bilirubin and PDPN scores were relevant risk factors, and albumin was a protective factor (**Supplementary Table 5**). Multivariate logistic regression analysis excluded all these factors, and none of them emerged as a significant prognostic factor, suggesting a high correlation between these factors. The PDPN scores were significantly correlated with the Albumin (r=-0.62, p<0.0001) bilirubin (r=0.69, p<0001) and creatinine (r= 0.48, p=0.005) in the cirrhotic patients. PDPN score also showed a correlation with both MELD (r=0.64, p<0.0001) and CTP scores (r=0.39, p= 0.06) (**Figure 3A, B**). Although a definite relationship between the existing scores and PDPN was observed, variability was observed in certain patients. For example, patients with MELD scores of 18-20 had PDPN scores ranging from 4 to 8, and similarly for patients with CTP scores of 7 had PDPN scores from 2 to 8.

PDPN score associates with 3-month mortality in decompensated cirrhotic patients

We next studied if PDPN scores varied between the survivors and non-survivors. There was no mortality in patients with compensated cirrhosis within three months. Among patients with decompensated cirrhosis, 36.8% mortality was observed. PDPN scores were significantly different between the survivors and the non-survivors [Median values: 5 (1-8) vs. 7 (7-8), respectively, p= 0.001, **Figure 4A**]. Next, we studied whether PDPN scores also predicted 3-month mortality in cirrhotic patients by Cox regression. Along with the laboratory parameters, we included the presence of complications such as bleeding, varices, ascites, and HE in this analysis. The univariate analysis showed several laboratory parameters and complications as significant predictors of mortality, including high bilirubin, creatinine, INR, presence of variceal bleeding, jaundice, HE, and oesophageal varices. High CTP, MELD, and PDPN scores also emerged as significant predictive factors of mortality in the univariate analysis (**Table 2**). In multivariate analysis, we excluded the clinical parameters that were a part of the severity scores and included the presence of variceal bleeding, CTP, MELD, and PDPN score for analysis. PDPN score emerged as a significant and independent mortality predictor (**Table 2**). The ROC curve for PDPN score showed an AUC of 84.2 (95% CI:70.6-97.8, p<0.007, **Figure 4B**). The cutoff value of PDPN scores for predicting significant 3-month mortality was above 6.5, with a sensitivity of 100% and a specificity of 75%. With the obtained cutoff values of PDPN, we also constructed survival curves in cirrhotic patients with high and low PDPN scores in liver cirrhosis using the Cox regression. The log-rank analysis showed that mortality in patients with a PDPN score less than equal to 6.5 was significantly lower at three months than in patients with a PDPN score greater than 6.5 (**Figure 4C**, Chi-Square: 20.5, p<0.0001). There was no death in cirrhotic patients with a PDPN score less than 6.5, while 54% of patients (all decompensated) died within three months, which had a PDPN score greater than 6.5. The cause of death in all patients was septic shock and multiple organ failure.

Mesenteric lymphatic vessels are dilated in experimental liver cirrhosis

To first evaluate mesenteric LVs (mLVs) in liver cirrhosis, we performed immunohistochemical staining of mesentery sections in control rats and CCl4 experimental models of liver cirrhosis with antibodies recognizing Podoplanin (Pdpn), a widely accepted marker of lymphatic vasculature. Control rats showed sporadic, thin LVs near blood vessels, whereas cirrhotic rat's mesentery contained numerous, readily detectable, and dilated LVs compared to control (**Figure 5A**). Quantitative analysis revealed that the total number of Pdpn+ LVs per field in CCl4 cirrhotic rats was significantly higher with increased diameter than control (**Figure 5B and C,** p<0.05 each).

The mRNA level of LV markers, Prox1, and LyVE1, were also enhanced in the mesentery of CCl4 rats compared to control rats (**Figure 5D,** p<0.05 each). Also, mRNA levels of CCL21, COX2, and eNOS were upregulated, reflecting an inflamed gut (p<0.05 each). VEGF-C expression was enhanced, suggesting inflammation-induced lymphangiogenesis (p<0.05). VEGF-C/VEGFR3 axis is pronounced in intestinal lymphangiogenesis and inflammation-induced lymphangiogenesis (Hagura et al. 2014). Therefore, we checked the expression of VEGFR-3

by immunofluorescence staining in mesentery sections. The protein expression of VEGFR-3 was significantly increased in CCl4 cirrhotic rats compared to that seen in controls (**Figure 5E, F** $p<0.01$).

Development of Nanoengineered VEGF-C for targeted delivery to Mesenteric Lymphatic Vessels

Dilation and lymph transport failure of the mLVs in experimental cirrhosis with ascites have been previously reported (Ribera et al. 2013); therefore, we hypothesized that enhancing the number of new functional mLVs by treatment with VEGF-C may improve lymphatic drainage in cirrhotic rats. To this end, we developed an engineered VEGF-C (E-VEGF-C) nanoformulation to enhance its uptake by gut LVs after oral delivery (**Supp-Figure 6**). Human recombinant VEGF-C protein (rhVEGF-C- Cys156Ser), specifically binds to VEGFR-3 homodimer present on LyECs, was encapsulated inside lipo-carriers prepared with distearoyl-rac-glycerol-PEG2K (**Figure 6A**). In dynamic light scattering (DLS) analysis, the z-average/mean particle size (MPS) of E-VEGF-C was found to be 134.8 ± 0.47 nm with a polydispersity index (PDI) value of 0.126 ± 0.01 (**Figure 6B**). The zeta potential (ZP) and pH value of the E-VEGF-C were -21.9 ± 1.24 mV and 6.369 ± 0.004, respectively (**Figure 6C**). A stability study of up to one month indicated no drastic change in the physicochemical properties of E-VEGF-C nanoformulation **(Supp-Table 6).** The surface morphology and size of E-VEGF-C were studied using field emission scanning electron microscope (FE-SEM), and field emission-transmission electron microscopy (FE-TEM) indicated that particles were spherical, smooth in appearance and uniformly distributed (**Figure 6D, E**). Atomic force microscopy (AFM) micrographs provided two-dimensional and three-dimensional images of E-VEGF-C surface morphology and showed that E-VEGF-C was compact, smooth, and spherical (**Figure 6F**). The average diameter of individual particles was 16 nm with an encapsulation efficiency of 93.28±1.85%. The release profile of E-VEGF-C showed an initial burst release of 31.95±1.52% VEGF-C observed at 2h post mixing, followed by an increase in VEGF-C release of 84.66±1.82% at 4h, which then gradually decreased at other post-mixing time points (**Figure 6G).** After the *in vitro* release study, R^2 values obtained from different kinetic models listed in **Supp-Table 7** suggested the VEGF-C release from RMs showed zero-order kinetic and, therefore, independent of concentration. To investigate the internalization of E-VEGF-C *in vitro*, we isolated LyECs from rat mesentery and mesenteric lymph nodes (MLN) using fluorescence-activated cell sorting (FACS) **(Supp-Figure 7A, B)** and incubated them with coumarin-6-labeled E-VEGF-C. Fluorescence microscopy revealed the efficient internalization of E-VEGF-C by LyECs at 4h **(Figure 6H, I).** Next, to ensure the specificity of E-VEGF-C delivery *in vivo*, tissue biodistribution studies of coumarin-tagged E-VEGF-C were performed 2h after oral administration using spectrofluorimetry and fluorescence microscopy **(Figure 6J).** A weak fluorescence signal was observed in all tissues in control and CCl4 rats **(Supp-Figure 7C),** with an intense signal observed in the mesentery of both **(Figure 6K, L).** CCl4 rats also showed increased human VEGF-C levels in mesenteric and duodenal tissues compared to the control **(Figure 6M, P< 0.05).** In the serum of CCl4 animals, levels of VEGF-C exhibited a biphasic peak; the first peak appeared at about 10 min, and the second peak appeared at approximately 5h **(Figure 6N).** There were no adverse effects or mortality after E-VEGF-C treatment in either control or CCl4 rats.

Mesenteric Lymphatic Vessel proliferation by E-VEGF-C

Next, we studied the therapeutic effects of E-VEGF-C in different animal models of liver cirrhosis. Rats were injected intraperitoneally with CCl4 and TAA for 14 and 12 weeks respectively. All CCl4 rats had ascites at 14week, while ascites were absent in cirrhotic TAA rats. E-VEGF-C was administered orally, 600µg/kg, on alternate days for up to the next two weeks in CCl4 and TAA rats (**Figure 7A**). SAR, a VEGFR-3 selective inhibitor, was orally administered to ablate LVs in TAA cirrhotic rats from 8week up till 14 week. Rats were sacrificed 48h after the last dose of E-VEGF-C or SAR. Immunohistochemical analysis of mesentery showed increased numbers of Pdpn+ mLVs ($p>0.05$) with a significantly reduced diameter ($p<0.05$) in E-VEGF-C treated rats compared to CCl4-V rats (**Figure 7B-D**). In the mesentery of TAA-V and TAA+SAR rats, we observed dilated mLVs. Treatment with E-VEGF-C increased the number of mLVs and reduced their dilation in TAA rats (**p<0.05, Figure 7E-G**). There was a significant increase in hVEGF-C and VEGFR-3 protein in the mesentery of E-VEGF-C treated rats vs. CCl4-V ($p<0.01$ each, **Figure 7H-J**). Along with mesentery, marked LVs proliferation was observed in MLNs and duodenum of E-VEGF-C rats compared to CCl4-V and controls ($p<0.05$ each, **Figure 7K-N**). Mesenteric tissues also revealed reduced inflammation in E-VEGF-C rats compared to CCl4-V rats. There was a reduction in the expression of inflammatory markers eNOS, iNOS, IL-6, and IFN-gamma and increased TGF-β in mesentery tissues of E-VEGF-C rats vs. CCl4-V rats (**Supp-Figure 8A, B**). Since VEGF-C also participates in angiogenesis, we evaluated whether E-VEGF-C affected angiogenesis in the mesentery. We observed no significant difference in the number of CD31+ blood vessels between CCl4-V and E-VEGF-C treated rats ($p>0.05$, **Supp-Figure 8C, D**).

Improved Drainage of Mesenteric Lymphatic Vessels by E-VEGF-C

To investigate the effect of E-VEGF-C on LVs patterning, whole-mount immunostaining of mesentery was performed using pdpn antibody. In control rats, thin LVs of diameter around 50µm were observed, which were increased to 100-150µm in CCl4-V rats. In E-VEGF-C treated CCl4 rats, sprouting of new LVs from the existing one was marked with reduced diameter ranging from 60-100µm compared to the CCl4-V rats (**Figure 8A, B**). Also, branching points of mLVs close to the intestine increased in E-VEGF-C rats compared to CCL4-V (**Figure 8C**, **D**). To assess the functionality of LVs, we gavaged BODIPY FL-C16 in all study groups and analyzed drainage and leakage of mLVs after 2h (**Figure 8E-L, Supp Figure 9A, B).** CCl4-V, TAA-V, and TAA+SAR rats displayed increased BODIPY fluorescence inside mLVs with an increased diameter compared to control rats, representing incomplete drainage ($p<0.05$ each). On the other hand, E-VEGF-C-treated CCl4 and TAA rats had significantly reduced fluorescence inside the mLVs with decreased diameter ($p<0.05$ each). Since dilated LVs would cause leakage of lymph in tissue spaces, we assessed leakage from mLVs by estimating fluorescence outside the mLVs. We observed increased fluorescence outside the LVs in both CCl4 and TAA rats compared to controls and reduced fluorescence in both the E-VEGF-C treated rats ($p<0.05$ each).

E-VEGF-C reduces Ascites and ameliorates Portal Pressure in Cirrhotic and Non-cirrhotic Portal Hypertensive Rats

We next probed whether an improvement in mesenteric lymphatic drainage by E-VEGF-C has any effect on ascitic fluid volume in cirrhotic rats. No ascites was observed in the control group, whereas all CCl4-V rats displayed severe ascites **(Figure 9A).** E-VEGF-C rats showed a marked reduction in ascites volume compared to CCl4-V ($p< 0.05$, **Figure 9A, B, Supp-Table 8**). Along with ascites reduction, Evans blue staining showed a significant increase in plasma volume in the E-VEGF-C group compared with CCl4-V ($p< 0.05$, **Figure 9C**). To assess whether E-VEGF-C could also prevent formation of ascites, we developed bile duct ligation (BDL) animal model of liver cirrhosis (**Supp Figure 10A).** One week after surgery, a prophylactic dose of 600ug/kg E-VEGF-C was administered orally twice a week for next 3 weeks. BDL rats were sacrificed at the end of 4^{th} week. In the BDL-V group, five out of six rats showed the presence of ascites, whereas only one animal developed mild ascites in BDL+E-VEGF-C rats (**Supp Figure 10B**). Next, we analyzed whether this reduction in ascitic fluid volume after E-VEGF-C treatment was also associated with hepatic hemodynamic changes. In CCl4-V rats, portal pressure (PP) was markedly increased as compared to that in controls ($p<0.001$, **Figure 9D**). However, E-VEGF-C treatment significantly attenuated PP compared to the vehicle ($p<0.001$). We also monitored these parameters to ascertain whether the observed reduction in PP was due to the change in portal blood flow (PBF) or intrahepatic resistance (IHR). E-VEGF-C treatment significantly decreased PBF, in turn increasing the mean arterial pressure (MAP) as compared to CCl4-V ($p<0.05$, **Figure 9E-F**). However, IHR in E-VEGF-C-treated rats was similar to that in CCl4-V ($p>0.05$, **Figure 9G**). Compared to the control, liver weights in E-VEGF-C and CCl4-V rats were similar (**Supp-Figure 11**). In TAA-V and TAA+SAR rats, there was increased PP and PBF compared to controls and which significantly reduced in E-VEGF-C treated rats ($p<0.05$, **Supp Figure 12**). Also, MAP was reduced in TAA+E-VEGF-C rats compared to TAA-V and TAA+SAR rats ($p<0.05$). In BDL+E-VEGF-C treated rats, PP was decreased significantly ($p<0.05$, **Supp Figure 13**), and MAP was increased compared to vehicle ($p>0.05$). We next evaluated changes in histological and biochemical parameters of the liver in cirrhotic rats, including CCl4, TAA, and BDL after E-VEGF-C. Masson's trichrome staining of liver tissues revealed that in CCl4 cirrhotic rats, there was no improvement in liver fibrosis after E-VEGF-C treatment. However, BDL+E-VEGF-C and TAA+E-VEGF-C rats showed a slight reduction in liver fibrosis compared to BDL-V and TAA-V respectively (**Figure 9H, Supp Figure 14**). CCl4 significantly increased serum ALT levels and decreased albumin levels compared to the control (**Supp-Table 8**). There was no significant improvement in serum albumin and ALT levels in E-VEGF-C-treated CCl4 rats compared to CCl4-V. Kidney functions were normal in CCl4-V as well as in E-VEGF-C CCl4 rats.

We also investigated whether E-VEGF-C treatment improved PP irrespective of liver cirrhosis by measuring hepatic hemodynamics in non-cirrhotic portal hypertensive (PPVL) rats (**Supp Figure 15A**). Hemodynamic analysis revealed a significant reduction in PP and PBF of E-VEGF-C treated PPVL rats compared with vehicle ($p<0.01$, **Figure 9I-K**). Histology of these animals displayed portal inflammation in the liver but no significant fibrosis (**Supp-Figure 15B**). PPVL+E-VEGF-C rats also showed increased Pdpn+ mLVs number and VEGF-C protein expression with reduced inflammation in mesentery compared to PPVL-vehicle. E-VEGF-C-treatment also reduces the mRNA expression of iNOS and eNOS in mesentery compared to the vehicle ($p<0.05$, **Supp-Figure 15C-G**).

E-VEGF-C increases the trafficking of immune cells and clearance of bacteria load in mesenteric lymph node

Cirrhosis increases gut bacterial translocation to MLNs and other organs (Wiest, Lawson, and Geuking 2014). Therefore, to test the effect of E-VEGF-C on endogenous bacterial load/clearance in MLN and other organs, 100 mg of each tissue was extracted from cirrhotic rats in sterile conditions and plated on LB agar (**Figure 10A**). In CCl4 and TAA rats treated with E-VEGF-C, we observed a decrease in bacterial load in all MLNs and other organs studied (**Figure 10B-C, p<0.05)**. In TAA+SAR rats, there was a significantly increased bacterial load in Peyer's patches and liver compared to TAA-V ($p<0.001$ each). To ascertain the role of E-VEGF-C in the reduction/clearance of bacterial load from MLNs, we next looked at the immune cell trafficking in MLNs (Hsu et al. 2021; JM et al. 2018). Cells were isolated from MLNs of controls, CCl4-V, and E-VEGF-C rats and labeled with antibodies for T cell subsets and DCs for quantification using flow cytometry (**Supp Figure 16A, B**). Total T cell, Th, and Tc cell populations in MLNs did not change significantly in control and CCl4-V as previously reported ($p>0.05$, **Figure 10D-E**) (Úbeda et al. 2010). Treatment with E-VEGF-C also led to no change in the populations mentioned above. We further quantified recently activated T cells with CD134 and regulatory T cells with CD25 expression. No significant change was observed in CD134 expression in varied groups ($p>0.05$, **Figure 10D, E),** but we observed a significant decrease in CD8 Treg cells after E-VEGF-C treatment in comparison to CCl4-V ($p<0.05$, **Figure 10F-G**). DCs, along with the expression of T cell activation coreceptor CD80, were increased after treatment with E-VEGF-C compared to that observed in the vehicle ($p<0.05$, **Figure 10H**).

As we observed no significant changes in T cell subsets in MLNs of either CCl4-V or E-VEGF-C, therefore we investigated changes in the priming of immune responses in the presence of antigen challenge. To analyze the effect of activated T cells on bacterial clearance in MLNs after E-VEGF-C treatment, we gavaged rats with GFP-labelled *Salmonella typhimurium* in CCL4 cirrhotic and PPVL non-cirrhotic model of portal hypertension. (**Figure 11A, Supp Figure 16C**) (Wiest et al. 2014). After 48 h of gavaging, GFP-labelled bacteria were found only in the MLNs of control. In contrast, in CCl4-V and PPVL-V, GFP-labelled bacteria were present in all the collected tissues, including blood in CCl4-V (**Figure 11B**, **Supp-Figure 16D-E**). Interestingly, in E-VEGF-C treated CCl4 rats, bacterial translocation was confined only to MLNs with reduced live bacteria than CCl4-V ($p<0.001$, **Figure 11B, C**), suggesting clearance of bacterial load. In PPVL+E-VEGF-C rats, bacterial translocation was reduced in MLN and other organs (**Supp Figure 16F**). After the bacterial challenge, we found a significant increase in recently activated Th cells in MLNs of E-VEGF-C treated rats compared to CCl4-V, which indicates an active immune response in MLNs in the presence of antigen (**Figure 11D-G**). We also assessed the levels of endotoxins and inflammatory cytokines in serum and ascites. Endotoxin levels were reduced in ascites of E-VEGF-C rats ($p<0.05$); however, no change was observed in serum endotoxins (**Supp figure 17A, B**). Serum IL-6 levels were significantly reduced in E-VEGF-C rats compared to CCL4-V ($p<0.05$); however, no change was observed in serum TNF-α levels in the E-VEGF-C treated rats compared to CCl4-V **(Supp-Figure 1C, D).**

E-VEGF-C treatment modulates gene expression in lymphatic endothelial cells of mesentery and lymph nodes

To gain mechanistic insights into VEGF-C-induced lymphangiogenesis in mLVs, LyECs from mesentery and MLNs were isolated using FACS, and the expression of relevant genes was examined (**Figure 12A**). The mRNA level of differentiation and proliferation markers of LyECs, i.e., Prox1 and LyVE1, were upregulated in LyECs of E-VEGF-C treated rats compared to vehicle ($p<0.05$, **Figure 12B**). mRNA levels of VCAM1 were decreased in LyECs of CCL4-V rats, indicating reduced adhesion in LyECs of mLVs. No significant change was observed in VE-cadherin in CCl4-V rats compared to the control. Treatment with E-VEGF-C led to increased expression of both VCAM1 and VE-cadherin in LyECs ($p<0.05$). LyECs also act as APCs, presenting Ag to T cells for tolerance or induction of immune responses. The costimulatory marker, CD86, was significantly upregulated in LyECs of treated rats. In contrast, no significant change was observed in MHC-II levels in the vehicle vs. treated group ($p>0.05$). The inflammatory marker COX2 was markedly increased in LyECs of CCl4-V rats suggesting inflamed lymphatic endothelium, which was further reduced in LyECs of the treated group ($p<0.05$). CCL21 and CCL19 chemokine mRNA levels were increased in LyECs of E-VEGF-C treated rats compared to the vehicle resulting in increased immune cell trafficking to MLNs ($p<0.05$). PD-L1 was increased in LyECs of CCl4-V rats, which was further reduced in the treated group ($p<0.01$). We next asked if these transcriptional changes in LyECs specifically VE-cadherin could lead to differences in the ability to allow passage of BODIPY (labeled fatty acid) through their cell-cell junctions (i.e., permeability). To answer this question, we utilized a barrier transwell assay in which a confluent monolayer of mesenteric LyECs, isolated from controls, CCl4-V and E-VEGF-C treated rats and plated on the upper chamber of transwell. From initial time points

onwards (4-24h), there was increased permeability in LyECs of CCl4-V rats compared to controls, as measured by an increase in the amount of BODIPY fluorescence in the lower chamber ($p<0.001$, **Figure 12C**). In LyECs of E-VEGF-C rats, BODIPY could not pass through the monolayer; therefore, its reduced amount was observed in the lower chamber compared to CCl4-V rats ($p<0.001$).

Discussion

Inflammation-induced remodeling of the lymphatic network occurs by vascular endothelial growth factor (VEGF)-A/C/D signaling through VEGF receptor 2/3 (VEGFR-3). Besides these VEGFs, PDPN has also been reported to have significant effects on the proliferation, migration, and tube formation of lymphatic endothelial cells (Navarro et al. 2008, 2011). Our study reports that patients with cirrhosis have a significantly increased number of lymphatic channels characterized by PDPN and VEGF-C immunostaining and *PDPN* and *LYVE1* expression in the D2-biopsy lysates. Immunostaining with PDPN demonstrated enlarged and dilated lymphatic channels in decompensated cirrhosis patients compared with compensated ones. Patients with and without HE did not differ significantly for PDPN score, possibly because all decompensated patients had ascites, to begin with, and HE was a second decompensation event present only in some patients.

An increased number of dilated PDPN+ intestinal LVs in cirrhotic patients signifies impaired lymph drainage and compensatory lymphangiogenesis. Compared with the lymphatic flow in healthy subjects (1L/day), studies have demonstrated a significant increase in abdominal lymph production (30-fold) and lymph flow in the thoracic duct of cirrhotic patients with ascites (8–9 L/day) (Vollmar et al. 1997). The most common cause of ascites in cirrhosis is elevated pressure in the portal circulation, causing hypertension. A positive correlation exists between lymph production in the gut and lymph flow with increasing portal pressures (Megevand R, 1969). Gut LVs are capable of draining a moderate amount of lymph and returning it to the systemic circulation, preventing the accumulation of fluid in the abdominal cavity. But as cirrhosis and portal hypertension progresses, the vascular dysfunction or hyperdynamic systemic and splanchnic circulation increases the mesenteric blood flow and portal pressure, which in turn, causes more fluid to escape from the vessels. This excess fluid greatly enhances the production and flow of interstitial hepato-intestinal lymph leading to fluid loss in a significant amount from the peritoneal lining and serosal surfaces of the bowel (Aller et al. 2010; Olkawa et al. 1998). Concerning intrahepatic LVs, it is known that there is an increase in the number of dilated D2-40/podoplanin liver LVs and lymphangiogenesis in patients with cirrhosis and portal hypertension, regardless of disease etiology (Olkawa et al., 1998; Yokomori et al., 2010; Ma et al., 2021). Hence, it might be deduced that there is an increased production of intestinal and hepatic lymph due to ongoing inflammation in the gut-liver axis and vascular blood flow changes. The overwhelmed and defective lymphatic flow and drainage mechanism leads to the accumulation of ascites.

PDPN scores significantly correlated with many clinical parameters of cirrhosis, including the severity scores, MELD, and CTP in our patients, suggesting the utility of PDPN scores in assessing disease severity. However, it is to be noted that many patients with a particular value of MELD or CTP score showed a range of values for the PDPN score, indicating that the variables are different. We also observed an inverse correlation between the expression of *TJP1*, an intestinal permeability gene, and PDPN scores in our patients, underscoring the contribution of increased intestinal permeability in LVs remodeling. Gut dysbiosis with increased intestinal permeability is a characteristic feature of cirrhosis (Ponziani et al., 2018). Intestinal permeability has been reported to be significantly higher in decompensated cirrhosis patients with ascites and encephalopathy compared with compensated cirrhosis (Pascual et al. 2003). Patients with decompensated cirrhosis also have significantly reduced expression of tight junction proteins in the D2-biopsies as compared to compensated (Assimakopoulos et al. 2012). With increased intestinal epithelial permeability, gut LVs are exposed to increased fluid and pathogen load in cirrhosis that might be causing LVs remodeling, dilation, and lymphangiogenesis, as observed in intestinal inflammation (Stephens, Liao, and Von Der Weid 2019).

In our study, a higher PDPN score was identified as an independent predictor of 3-month mortality when MELD, CTP, and the presence of variceal bleeding were included in the multivariate analysis. This signifies the importance of dilated intestinal lymphatic channels in predicting mortality in cirrhotic patients along with the MELD and CTP scores. This also calls for further prospective studies to determine the use of PDPN scores for organ allocation to reduce mortality in patients on the liver transplantation waiting list. Increased mortality due to lymphatic abnormality may be attributed to its role in inducing systemic inflammation. Intestinal hyperpermeability is associated with an increase in pathological bacterial translocation in cirrhosis. Gut bacteria and other endotoxins use the mesenteric collecting lymph vessels and drain into the mesenteric lymph nodes that serve as key points to handle the bacterial load in the gut and prevent their transfer in the systemic circulation (Macpherson and Smith 2006). Impaired lymph drainage and persistent lymph stasis in the intestine could contribute to chronic inflammation by reducing the drainage of bacteria and bacterial products to the lymph nodes. Gut inflammation in cirrhosis might also produce profound changes in the mesenteric lymph composition. Elevated endotoxins and inflammatory cytokines are found in the

mesenteric lymph and thoracic duct before they appear in the portal vein (Deitch 2002). In fact, mesenteric lymph can be termed as a splanchnic vehicle for systemic proinflammatory responses. Disruption of intestinal lymphatics and lymph nodes has earlier been shown to cause systemic infection, massive inflammatory responses, and mortality (Jang et al., 2013). A pathogenic role of mesenteric lymph has also been suggested in multiple organ dysfunction. It has been reported that although there were no live bacteria in the thoracic duct lymph, the levels of lymph inflammatory cytokines were higher in ICU patients with multi-organ dysfunction as compared to those without organ dysfunction (Deitch 2002; Lemaire et al. 1999). It has been postulated that during gut inflammation and infection, mesenteric lymph with toxic factors potentiate distant organ failure, especially lung failure, by exaggerating the systemic inflammatory responses, even when these translocating bacteria do not reach the systemic circulation (Ma et al. 2021). High PDPN scores in our patients were significantly associated with both systemic TNF-α and IL-6 levels and also IELs in the D2-biopsies, suggestive of both gut and systemic inflammatory immune responses in patients with dilated lymphatic channels. Systemic inflammation, as reflected by plasma IL-6 levels, is a valuable biomarker of advanced chronic liver disease progression. IL-6 predicts the risk of the first decompensation in patients with compensated cirrhosis and also one-year liver-related mortality predictor or the need for liver transplantation in those with decompensated cirrhosis (Costa et al. 2021). It would be worthwhile to study the levels of IL-6 and other inflammatory cytokines in mesenteric lymph of patients with cirrhosis with varying severity and correlate them with duodenal PDPN scores and mortality.

In experimental animal models of liver cirrhosis, we investigated morphological and molecular alterations of mLVs and MLNs in experimental cirrhosis and portal hypertension. Significantly increased number of dilated and leaky mLVs with reduced drainage were observed in cirrhotic portal hypertensive rats implying gut lymphatic dysfunction similar to that observed in intestinal lymphangiectasia and IBD (Rossi et al. 2011; Zhang et al. 2021a). Increased dysfunctional mLVs in cirrhosis may be attributed to compensatory lymphangiogenesis response to lymphatic occlusion (Zhang et al. 2021b). Therefore, we focused on increasing the number of new functional lymphatic channels via VEGF-C treatment.

Due to short half-life and systemic effects of VEGF-C (Lohela et al. 2003), we constructed recombinant VEGF-C molecules using RM-based nano-lipo-carriers, which are nanoparticle-sized water-in-oil microemulsions with controlled size. A recent report illustrated the therapeutic potential of a human fusion protein F8-VEGF-C for targeted delivery of VEGF-C in mouse models of chronic inflammatory skin disease (Schwager et al. 2018). However, the study did not report sustained release of VEGF-C. In another study, VEGF-C mRNA was encapsulated in lipid nanoparticles for sustained release in experimental lymphedema (Szőke et al. 2021). In our study, RM-based lipid nanocarriers allowed VEGF-C to be carried in chylomicron-sized particles in LVs. Encapsulation also ensured their sustained and programmable release (Banerjee and Pillai 2019). *In vitro,* release studies indicated more than 80% VEGF-C release in 4h. *In vivo,* the release profile showed a biphasic peak, first at 10 min, and then at 5h. Biphasic drug release is a characteristic feature of drugs encapsulated in nano-lipo-carriers (Anon n.d.). Also, cirrhotic rats showed maximum expression of VEGF-C in the mesentery, indicating efficacy of our delivery vehicle. We did not follow any conjugation chemistry to prepare the E-VEGF-C molecule focused on preparing simple solid lipid nanoparticles with an aqueous template using a modified multiple emulsification technique (Banerjee et al. 2020).

In both CCl4 and TAA-induced cirrhosis, treatment with E-VEGF-C enhanced expression of VEGF-C protein in the mesentery, along with concomitant increase in the sprouting of mLVs, both in the mesentery and MLNs, suggestive of VEGF-C driven proliferation of LyECs (Deng, Zhang, and Simons 2015). Dilations in mLVs of cirrhotic rats were also attenuated with E-VEGF-C treatment. Specific genes, such as Prox1 and LyVE1, were significantly increased in mesenteric LyECs of E-VEGF-C rats, indicating differentiation and proliferation of LyECs. Expression of inflammatory genes, such as Cox2 was decreased while VCAM-1 and VE-cadherin, which govern cell adhesion and vessel permeability, increased in LyECs of E-VEGF-C rats as compared to cirrhotic rats. The functional implications of enhanced gene expression were evident in terms of reduced permeability, lymph leakage and improved drainage in treated rats. Also, there was a conspicuous decrease in mesenteric tissue inflammation in treated rats. This is in accordance to the previously reported drainage-promoting function of VEGF-C (D'Alessio et al. 2014; Visuri et al. 2015). We did not detect any significant changes in CD31+blood-vessels with E-VEGF-C treatment, indicating that LV-specific VEGF-C(Cys156Ser) did not affect mesenteric blood vessels (K et al. 2009).

An improvement in lymphatic drainage and functionality of the mLVs in cirrhotic rats was also associated with a reduction in ascites in CCl4 rats. The decrease in ascites was accompanied by an increase in the plasma volume of treated rats (Chung and Iwakiri 2013). Compared to the 4-week BDL vehicle, E-VEGF-C treatment also prevented

ascites formation in BDL models when given as a prophylactic treatment. We, however, did not follow these models beyond four weeks. Along with a reduction in ascites, there was also a decrease in PP in CCl4 and TAA-induced portal hypertensive rats treated with E-VEGF-C, which was associated with attenuated PBF and increased MAP. However, there was no change in IHR, indicating that improvement in PBF and not hepatic resistance (fibrosis) led to improved portal pressure after treatment. It has been postulated that removal of ascites plays a role in post-paracentesis systemic hemodynamic changes through mechanical decompression of the splanchnic vascular bed (Cabrera et al. 2001). However, reduction in PP due to increased LVs and decreased interstitial fluid pressure may also have led to decreased ascites (Chung and Iwakiri 2013). This decrease in PP was also observed in the non-cirrhotic PPVL animals after E-VEGF-C treatment, validating the favorable effects of VEGF-C on PBF. An improvement in PP was not associated with an improvement in liver pathology in the treated CCl4 and TAA rats, indicating no protective effects of E-VEGF-C treatment on hepatic compartment per se. In these models, we administered therapeutic E-VEGF-C treatment when liver cirrhosis had already been established. A slight reduction in liver fibrosis was however seen in E-VEGF-C treated BDL compared to BDL vehicle, in which an early treatment was given.

A reduction in permeability and improved drainage of lymphatic vessels was associated with reduced endogenous bacterial translocation in cirrhotic animals treated with E-VEGF-C. When we challenged these cirrhotic animals with live bacteria, we observed live bacteria in MLNs and other organs and blood, indicating impaired gut immune responses in the MLNs. In cirrhotic animals treated with E-VEGF-C, bacteria remained confined to MLNs only. Translocation of live bacteria was reduced in MLNs of non-cirrhotic PPVL animals treated with E-VEGF-C. This decrease in bacterial translocation could explain the observed reduction in PP of PPVL animals (Wiest et al. 2014).

VEGF-C increases immune cell trafficking to the draining lymph node (Güç et al. 2017). We did not observe any change in T cell recruitment in MLNs of E-VEGF-C treated animals compared to CCl4 rats. There was however, an increase in costimulatory markers, such as CD86 in the LyECs of E-VEGF-C treated CCl4 rats, suggesting active antigen presentation by these cells (Macpherson and Smith 2006; Santambrogio, Berendam, and Engelhard 2019). In MLNs of CCl4 animals, VEGF-C treatment increased recently activated CD4 T cells, indicative of an appropriate immune response. An earlier study documented that stimulation of cardiac lymphangiogenesis with VEGF-C improved trafficking of immune cells to draining lymph nodes after myocardial infarction resolving inflammation (Vieira et al. 2018). Along with improved gut immune responses, we also observed a decrease in systemic endotoxins and inflammatory cytokines after E-VEGF-C treatment (Dieterich, Seidel, and Detmar 2014).

To summarize, we report here that an increased number of dilated LVs, characterized by PDPN expression in the D2-biopsies, are a characteristic feature of patients with decompensated cirrhosis and ascites and PDPN score serves as a valuable predictor of 3-month mortality. Future studies with a larger sample size to confirm the prognostic value of PDPN score across stages of cirrhosis are warranted. Further understanding of whether gut lymphangiogenesis is a pathological or protective mechanism in cirrhosis would help us to design specific therapeutic interventions for such patients. In experimental animal models, our study underscores the use of nano-lipo-carriers incorporating LV-specific VEGF-C as a novel therapy for improving lymphatic drainage, gut immunity, and portal hypertension by providing an efficient exit route for ascites. Gut LVs-targeted delivery of E-VEGF-C may open new and exciting avenues for treating and preventing decompensation in cirrhosis.

Impact of the research in the advancement of knowledge or benefit to mankind: Literature reference

The impact of this study on mankind could be significant. The study found that dilated and dysfunctional gut lymphatic vessels (LVs) are a characteristic feature of patients with decompensated cirrhosis. This suggests that LV dysfunction may play a role in the development of complications of cirrhosis, such as ascites and portal hypertension.
The study also found that PDPN score, a marker of LV dysfunction, is an independent predictor of 3-month mortality in patients with cirrhosis. This means that PDPN score can be used to identify patients who are at high risk of death.
These findings could lead to the development of new treatments for cirrhosis. For example, drugs that improve LV function (such as VEGF-C mediated lymphangiogenesis) could be used to prevent the development of complications of cirrhosis and improve survival.

The study on experimental animal models of liver cirrhosis found that E-VEGF-C treatment can improve the function of mesenteric lymphatic vessels (mLVs) in cirrhotic rats. This could lead to the development of new treatments for cirrhosis, such as drugs to improve functional lymphatic vessels. The study also found that E-VEGF-C treatment can

reduce ascites and portal pressure in cirrhotic rats. This is important because ascites and portal hypertension are two of the most serious complications of cirrhosis. In addition, the study found that E-VEGF-C treatment can strengthen cytotoxic T-cell immunity in mesenteric lymph nodes (MLNs) in cirrhotic rats.

Overall, the findings of this study could help to improve the diagnosis of cirrhosis and suggested that PDPN score could be used as a diagnostic tool for cirrhosis. E-VEGF-C treatment could be a promising therapy for cirrhosis and management of its complications. It could help reduce ascites and portal hypertension, and could also help strengthen the immune system. E-VEGF-C therapy thus holds the potential to manage ascites and portal pressure and reduce gut bacterial translocation in patients with cirrhosis. Further research is needed to confirm these findings and to determine the optimal dose and route of administration of E-VEGF-C for the treatment of cirrhosis.

Table 1: Demographic and clinical characteristics of the patients (N=40)

Parameters	**Controls (N=9)**	**Compensated (N=12)**	**Decompensated (N=19)**	**P value (all 3 groups)**	**P value Comp Vs Decomp**
Age (years)	48 ± 11.9	52 ± 10.5	52 ± 9.49	NS	NS
Male N (%)	6 (60)	8 (66.6)	17 (89.4)	NS	NS
Platelet count (x 10^9/L)	254.1 ± 84.43	172.5 ± 57.11	117.4 ± 48.2	< 0.0001	0.02
Bilirubin (mg/dL)	0.6±0.27	1.4 ±0.95	5.1 ± 6.71	0.03	0.07
Aspartate transaminases (AST*, U/L)	27.5 (20.3-44)	39 (28-72)	60 (24-221)	0.01	0.04
Alanine transaminase (ALT*, U/L)	28.85 (19-47)	30.50 (21-80)	33 (20-144)	NS	NS
Albumin (g/dL)	4.2 ± 0.64	3.7 ± 0.79	3.1 ± 0.56	0.0006	0.01
Serum Sodium (mEq/L)	138.2 ± 3.06	137.8 ± 3.72	131.7 ± 4.30	NS	NS
INR	1.1 ± 0.12	1.3 ± 0.22	1.6 ± 0.59	0.006	0.04
Creatinine (mg/dL)	0.9 ± 0.16	0.7 ± 0.19	1.3 ± 0.74	0.01	0.01
Etiology of liver diseases n (%)					
Alcohol N (%)		3 (25)	9 (47.3)		NS
HBV N (%)		1 (8.3)	2 (10.5)		NS
HCV N (%)		0	0		
NASH N (%)		7 (58.3)	7 (36.8)		NS
Others N (%)		1 (8.3)	1 (5.2)		NS
Ascites N (%)		0	19 (100)		-
Hepatic encephalopathy N (%)		0	9 (47.3)		-

Variceal Bleeding N (%)		0	8 (42.1)		-
Esophageal Varices N (%)		1 (8.3)	14 (73.6)		0.0004
Liver-Related 3-month mortality N (%)		0	7 (36.8)		-
CTP Score	-	5.67 ± 2.8	9.1 ± 2.06	<0.0001	<0.0001
MELD score	-	8.3 ± 5.4	18.6 ± 6.41	<0.0001	<0.0001

For continuous variables, values are given as mean (SD) or Median (min-max), and p values have been calculated by Student's 't' test or Mann-Whitney U test. Categorical variables are given as N (%), and p values for categorical variables have been calculated using Fisher exact test. HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; NASH: Non-alcoholic steatohepatitis; CTP: Child-Turcotte-Pugh; MELD: a model for end-stage liver disease.

Table 2: Predictive Factors for 3-month Mortality in Patients with Cirrhosis (N=31)

	Univariate Analysis	
Risk Factor	**OR (95% CI)**	**p value**
Age	0.99 (0.91-1.06)	0.79
Sex	2.02 (0.24-16.88)	0.51
Globulin	0.58 (0.22-1.49)	0.26
TLC	1.04 (0.98-1.10)	0.17
Albumin	0.51 (0.21-1.25)	0.15
Bilirubin	1.12 (1.03-1.22)	0.006*
Sodium	0.88 (0.76-1.01)	0.08
AST[#]	3.1 (0.86-11.2)	0.08
ALT[#]	1.54 (0.39-6.10)	0.53
INR	3.24 (1.19-8.80)	0.05*
Creatinine	18.67 (2.42-144.10)	0.005*
Platelet	0.99 (0.98-1.01)	0.81
Variceal Bleeding	5.09 (1.12-23.02)	0.03*
Esophageal Varices	4.39 (0.96-19.99)	0.06*
Ascites	386.1 (0.22-654354)	0.116
HE	9.43 (1.61-55.1)	0.01*
CTP	1.59 (1.13-2.23)	0.007*
MELD	1.16 (1.04-1.29)	0.006*
PDPN Score	6.01 (1.17-30.74)	0.03*
Multivariate Analysis (with scores and presence of variceal bleeding)		
PDPN Score	5.61 (1.08-29.109)	0.04*

'#' Log values of these parameters were taken. '' denotes significant p values (Cox regression). Significance was taken as p<0.05. OR: Odd ratio; CI: Confidence Interval. TLC: Total lymphocyte count; HE: Hepatic Encephalopathy; MELD: Model for end-stage liver disease; CTP: Child-Turcotte-Pugh.*

Figure Legends

Figure 1: PDPN score in different stages of cirrhosis and associated complications. **(A)** Representative PDPN immunostained sections (40x) of D2-biopsies in control, patient with compensated and decompensated cirrhosis. Scale bar: 100µM. Bar graph showing **(B)** PDPN score and **(C)** Diameter of LVs in duodenum biopsy of control (n=9), compensated (n=12), and decompensated (n=19) cirrhotic patients. Bar graph showing PDPN score in patients with compensated and decompensated cirrhosis **(D)** with (n=19) and without (n=12) ascites, **(E)** with (n=8) and without (n=23) variceal bleeding, and **(F)** with (n=10) and without (n=21) hepatic encephalopathy (HE). Data represent mean ± standard deviation. Differences between groups were calculated by students' unpaired 't-test. LVs: Lymphatic vessels; D2: Duodenal

Figure 2: (A) Pearson's Correlation between PDPN score and number of IELs in liver cirrhosis patients (R=0.33, P=0.06, n=31). **(B)** Pearson's Correlation between PDPN score and relative mRNA expression of *TJP1* (R=-0.46, P=0.05, n=20*).* **(C, D)** Pearson's Correlation between PDPN score and plasma level of TNF-α (R=0.38, P=0.03) and IL-6 (R=0.48, P=0.006) in patients with liver cirrhosis (n=31 each). Differences between groups were calculated by students' unpaired t-test. Data represents mean±standard deviation. The dotted lines represent 95% Confidence Interval (CI). R is correlation co-efficient. IEL: Intraepithelial lymphocytes**.**

Figure 3: Pearson's Correlation between PDPN score and **(A)** MELD Score (R=0.64, P=0.0001), **(B)** CTP Score (R=0.39, P=0.06) in liver cirrhosis patients (n=24-30). Dotted lines represent 95% CI. R is correlation co-efficient. MELD: Model for end-stage liver disease; CTP: Child-Turcotte-Pugh.

Figure 4: PDPN score as a mortality predictor in liver cirrhosis patients. **(A)** Dot plots showing PDPN scores in survivors (n=24) and non-survivors (n=7) in patients with cirrhosis (P=0.001). **(B)** ROC curve of PDPN score discriminating the survivors from the non-survivors (AUC=84.2, P=0.007). **(C)** Kaplan–Meier curve of survival with low and high PDPN score (cut-off value: 6.5), P<0.0001 (log-rank analysis). Differences between groups were calculated by Mann-Whitney 'U' test. ROC: Receiver operating characteristics; AUC: Area under the curve.

Figure 5: Mesenteric Lymphatic vessels (mLVs) density increased in liver cirrhosis. (A) IHC with podoplanin (pdpn) antibody was performed in mesentery tissue of control and CCl4 model of liver cirrhosis. Scale bar:500µm. **(B)** Number of the pdpn+ mLVs was quantified using ImageJ. *P*=0.0240. **(C)** Diameter of mLVs in control and CCl4 rats was measured using ImageJ's diameter plugin. n=5 each. *P*=0.0119 (**D)** mRNA levels for Prox1, LyVE1, VEGF-C, CCL21, eNOS, and COX2 were quantified in mesentery tissue. n=5 each, *P*=0.05-0.01, the dotted line represents control. **(E)** Immunofluorescence staining for VEGFR-3 and DAPI was performed in mesentery tissue of control and CCl4 rats. Scale bar:186.2µm**. (F)** VEGFR-3 expression was quantified using ImageJ. n=5 each. *P*=0.0147. Data expressed as mean±SD. Two-tailed, unpaired T-test.'*' represents p< 0.05.

Figure 6: Formation, characterization, uptake, and biodistribution of E-VEGF-C. (A) Schematic representation of VEGF-C-engineered stealth nano-lipo-carriers (E-VEGF-C). Histograms showing **(B)** Mean Particle Size (MPS), Polydispersity Index (PDI), and **(C)** zeta potential of E-VEGF-C. **(D)** Field emission-scanning electron microscope (FE-SEM) and of E-VEGF-C. Surface morphology and size of E-VEGF-C were visualized using FE-SEM at 4.70KX and 50.67KX magnification. **(E)** Field emission-transmission electron microscopy (FE-TEM) analysis of E-VEGF-C The morphology and size of E-VEGF-C were examined using FE-TEM, indicating spherical shape. **(F)** Atomic force micrographs (AFM)- 2D and 3D images of E-VEGF-C surface morphology. (**G)** *In vitro* release profile of E-VEGF-C in PBS at pH 7.4. **(H)**Primary lymphatic endothelial cells (LyECs) were cultured and incubated with coumarin-6 labeled E-VEGF-C and observed at different time points. Scale bars:100µm. **(I)** Line graph of coumarin-positive LyECs depicting percentage of E-VEGF-C uptake at different time points. **(J)** Schema of *in vivo* biodistribution studies. n=6 each. Tissues were collected after 2h and measured in a fluorimeter. **(K)** Representative fluorescence images of the control and CCl4-V rats' mesentery section showed localization of coumarin-tagged E-VEGF-C in the

gut. Scale bars:200μm. **(L)** Quantitative fluorescence analysis in different tissue of control and CCl4 rats using fluorimeter. n=6 for each group. **(M)** ELISA of human VEGF-C (pg/mg of total protein) in different tissue of control and CCl4 rats. n=3 each. *P*=0.01-0.001. **(N)** Levels of human VEGF-C (pg/ml) in control and CCl4-V rat plasma samples at indicated time points were measured using ELISA. n=3 each. Data expressed as mean±SD. '*'represents p< 0.05 and '**'represents p< 0.01

Figure 7: Effect of E-VEGF-C treatment on mesenteric lymphatic vessels (mLVs) in a cirrhotic rat model of portal hypertension (PHT).
(**A)** Schema of *in vivo* studies. CCl4 and TAA cirrhotic rats were treated with E-VEGF-C 600ug/kg on alternate days for 2wk. n=8 for each group. TAA rats treated with SAR, 30mg/kg, twice a week for 7wk. n=6. **(B)** IHC staining of mesentery sections of CCl4-V and E-VEGF-C rat. Scale bars:200μm. Arrow indicated at pdpn+ LVs. (**C)** Number of mLVs quantified using ImageJ. *P* > 0.05. (**D)** Diameter of pdpn+ mLVs was measured using ImageJ. *P*=0.0073. **(E)** IHC staining of mesentery sections of TAA-V, TAA+SAR and TAA+E-VEGF-C rats. Scale bars:200μm. Arrow indicated at LyVE1+ LVs. (**F)** Number of LyVE1+ mLVs quantified using ImageJ. P<0.0001. (**G)** Diameter of LyVE1+ mLVs was measured using ImageJ. P<0.0001. (**H)** Expression of human VEGF-C and VEGFR-3 protein in mesentery was measured using western blotting in control, CCl4-V, and E-VEGF-C groups. (**I-J)** Quantitative analysis of VEGF-C (P=0.0076) and VEGFR-3 (P=0.0263) protein is represented in bar graph. The dotted line represents control. n=4 each. **(K)** IHC of MLNs tissue in control, CCl4-V and E-VEGF-C treated rats. Scale bars:500μm **(L)** Stained area mean intensity was quantified as IHC scores using ImageJ . *P*=0.0197 for control vs CCl4-V and *P*=0.0346 for CCl4-V vs E-VEGF-C. **(M)** IHC staining of duodenum sections of control, CCl4-V and E-VEGF-C treated rats. Scale bars:200μm. **(N)** Stained area mean intensity was quantified as IHC scores using ImageJ. *P*=0.0002 for control vs. CCl4-V and P=0.0017 for CCl4-V vs E-VEGF-C. Data expressed as mean±SD. One-way ANOVA with Tukey's post hoc test was performed. '*'represents p< 0.05, '**'represents p< 0.01, and '***'represents p< 0.001.

Figure 8: Effect of E-VEGF-C on proliferation and drainage of mLVs. (**A)** Whole-mount immunostaining of mesentery for visualization of mLVs in control, CCl4-V, and E-VEGF-C study groups. Upper two panels represent collecting mLVs, and the lower two represent lymphatic capillaries. Scale bar:309.4μm. (**B)** Diameter of collecting mLVs was measured using the ImageJ diameter plugin. n=3 or 4 rats in each group. *P*<0.0001 for control vs. CCl4-V and *P*=0.0183 for CCl4-V vs. E-VEGF-C (**C)** Mesentery tissue of CCl4-V and E-VEGF-C rats. Arrowheads indicated proliferation and branching of mLVs in the mesentery of E-VEGF-C treated rats. (**D)** Quantitative analysis of branching points in mesentery in control, CCl4-V, and E-VEGF-C. n=3-4 rats in each group. *P*>0.05 for each comparison. (**E)** Whole mount images of mLVs 2h after BODIPY FL-C16 administration in control, CCl4-V, and E-VEGF-C rats. Scale bar:309.1μm Representative graphs for characterization of functional mLVs. Mean of three points from each field was taken. **(F)** Diameter of LVs was measured using ImageJ diameter-plugins. (**G)** Drainage of LVs was measured by quantifying fluorescence intensity inside the vessels using ImageJ. (**H)** Leakage from mLVs was quantified by measuring fluorescence intensity in the extraluminal space of mLVs using ImageJ. n=4 or 5 rats in each group. *Represent comparison with CCl4-V. (**I)** Whole mount images of mLVs 2h after BODIPY FL-C16 administration in TAA-V, TAA+SAR, and TAA+E-VEGF-C rats. Scale bar:200μm Representative graphs for characterization of functional mLVs. A mean of three points from each field was taken. **(J)** Diameter of LVs was measured in μm using ImageJ diameter-plugins. (**K)** Drainage of LVs was measured by quantifying fluorescence intensity inside the vessels using ImageJ. (**L)** Leakage from mLVs was quantified by measuring fluorescence intensity in the extraluminal space of mLVs using ImageJ. n=4 or five rats in each group. Data expressed as mean±SD. One-way ANOVA with Tukey's post hoc test was performed. '***'represents p<0.001, and '*'represents p<0.05. *Represent comparison with TAA-V

Figure 9: Effect of E-VEGF-C treatment on ascitic fluid volume and hemodynamic parameters in cirrhotic and non cirrhotic portal hypertensive rats. (A) Representative CT scan slices of control, CCl4-V, and E-VEGF-C rat showing abdominal cavity. Regions of interest are marked with red dotted outline and correspond with fluid accumulation. **(B)** Dot plots showing ascitic fluid volume(ml) in CCl4-V and E-VEGF-C treated rats, n=6 for each group. *P*=0.0168. (**C)** Histograms showing plasma volumes(ml) using the Evans Blue dye dilution technique. n=4 each. Bar Diagrams showing hepatic hemodynamic parameters, **(D)** Portal pressure(PP), **(E)** Portal Blood Flow(PBF),

and **(F)** Intrahepatic resistance (IHR) and **(G)** Mean arterial pressure (MAP) in study groups n=6 each. *P*<0.05 **(H)** Masson Trichrome(MT) stained images of liver tissues in different animal groups. Liver fibrosis was assessed using the Laennec fibrosis scoring system. Bar Diagrams showing hepatic hemodynamic parameters **(I)** PP, **(J)** PBF, and **(K)** IHR in control, PPVL vehicle, and PPVL+E-VEGF-C rats. n=5 each Data expressed as mean±SD. Unpaired two-tailed t-tests were performed. One-way ANOVA with Tukey's post hoc test was performed. '*'represents p< 0.05 and '**'represents p< 0.01, and '***'represents p< 0.001. *Represent comparison with CCl4-V.

Figure 10: Effect of E-VEGF-C on clearance of bacterial load in different organs. (A) Bacterial load in different organs extract from CCl4-V, E-VEGF-C, TAA-V, TAA+SAR, and TAA+E-VEGF-C. **(B)** Quantitative analysis of CFU/gm in MLNs, Peyer's patches, Liver, Spleen, and Lung of CCl4-V and E-VEGF-C. n=3 each. *P*<0.05 **(C)** Quantitative analysis of CFU/gm in MLNs, Peyer's patches, Liver, Spleen, and Lung of TAA-V, TAA+SAR, and TAA+E-VEGF-C. n=3 each. **(D)** Immune cell quantifications from MLNs of control, CCl4-V, and E-VEGF-C rats. Dot Plots of T cell subsets in respective study groups. **(E)** Percentage-population of CD3 T cells, CD4 helper T cells, CD8 cytotoxic T cells, CD134+ recently activated helper and cytotoxic T cells. **(F)** Dot plots of CD25+ regulatory helper T cells in respective study groups. **(G)** Percentage-population of T regulatory cells positive for CD3, CD4/CD8, and CD25. (**H)** Percentage-population of dendritic cells (DCs) positive for CD11c, CD103, and CD80.. *P*<0.05. Data expressed as mean±SD. One-Way ANOVA with post hoc Tukey's test was performed '#' represents comparison with control.'*' represent a comparison with CCl4-V.'***'represents p<0.001,'**'represents p<0.01, and '*'represents p<0.05.'**#**'represents p<0.05.

Figure 11: Effect of E-VEGF-C on priming of immune cells after bacterial challenge. (A) Schema of workflow, 10^9 GFP+ bacteria were given to rats orally, and tissue was collected in sterile condition after 48h of gavage. 100mg of MLN tissue was used for bacterial load quantification. Cells were isolated from MLNs for quantification using flow cytometry. (**B)** GFP+ *S. typhimurium* colonies in 100mg of MLN tissue extract of each group were visualized using UV transilluminator. (**C)** Quantitative analysis of CFU/gm of MLN tissue for GFP+ *S. typhimurium.* n=3 each. *P*<0.001. **(D)** Dot Plots of T cells subsets in cells isolated from MLNs of all study groups after bacterial challenge. **(E)** Percentage-population of CD4 helper T cells, CD8 cytotoxic T cells, CD134+ recently activated helper and cytotoxic T cells. **(F)** Dot plots of CD25+ regulatory helper T cells. **(G)** Percentage-population of T regulatory cells positive for CD3, CD4/CD8, and CD25. n=4 each. Data expressed as mean±SD. One-Way ANOVA with post hoc Tukey's test was performed.'#' represents comparison with control.'*' represent a comparison with CCl4-V.'***'represents p<0.001,'**'represents p<0.01, and '*'represents p<0.05.'**#**'represents p<0.05.

Figure 12: Effect of E-VEGF-C on gene expression profiling and permeability of lymphatic endothelial cells (LyECs). (A) Schema of workflow. LyECs were isolated from mesentery and MLNs using FACS. RNA isolation and qRT-PCR analysis were done for different marker genes of LyECs. **(B)** Relative gene expression of LyVE1, Prox1, VCAM1, VE-Cad, MHCII, CD86, CCL21, and COX2 genes in CCl4-V and E-VEGF-C treated rats were plotted. **(C)** Transwell permeability assay using BODIPY in cultured mesenteric LyECs from control, CCL4-V, and E-VEGF-C treated rats. The concentration of BODIPY in collected media after 4, 6, 10, and 24h. n= 3 each. Data expressed as mean±SD. One-Way ANOVA with post hoc Tukey's test was performed. '**'represents p<0.01,'*'represents p<0.05. *Represent comparison with CCl4-V.

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