

ORIGINAL RESEARCH

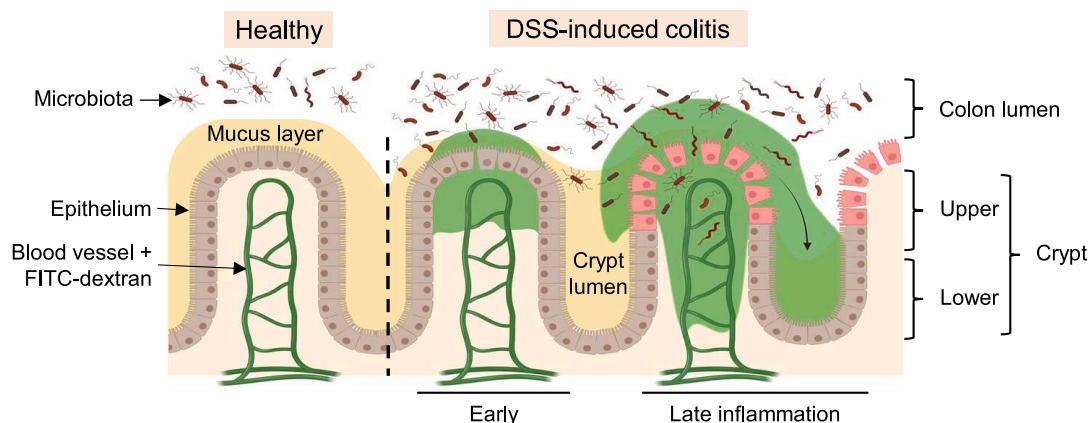
Early Gut–Vascular Barrier Breakdown Precedes Colitis Onset in Murine Models



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Early gut–vascular barrier breakdown precedes colitis onset in murine models



SUMMARY

Early disruption of the gut–vascular barrier and increased vascular permeability precede classical inflammation

markers in colitis. Vascular changes may serve as sensitive indicators for early inflammatory bowel disease detection and flare prediction, offering a promising target for diagnostic and monitoring strategies.

WHAT YOU NEED TO KNOW

Background: Previously, we reported that increased permeability of colon blood vessels is closely associated with and functionally contributes to the pathogenesis of inflammatory bowel disease.

Impact: Using a novel multiphoton endomicroscopy-based approach, we show that disruption of the gut-vascular barrier and increased vascular permeability precede classical inflammation markers in colitis.

Future Directions: Vascular permeability may serve as a sensitive indicator for early inflammatory bowel disease detection and flare prediction, offering a promising target for diagnostic and monitoring strategies.

BACKGROUND & AIMS: The gut-vascular barrier plays a pivotal role in inflammatory bowel disease pathogenesis. We introduce a novel 3-dimensional multiphoton endomicroscopy approach for real-time and sensitive detection of vascular permeability in the colon to identify colitis-associated vascular changes in the early stages.

METHODS: Using fluorescence-based multiphoton endomicroscopy, we visualized dynamic changes in vascular permeability in vivo during longitudinal observations in different experimental colitis models (dextran sodium sulfate- and T cell transfer-induced colitis). Vascular permeability changes were systematically compared with conventional inflammatory markers, including weight loss, endoscopic scoring, colon length, histopathology, and immune cell infiltration. To assess molecular regulation of barrier functions, the expression of key molecules of vascular (plasmalemma vesicle-associated protein, vascular endothelial cadherin) and epithelial (epithelial cadherin) barriers was investigated during development of colitis at the single-cell level.

RESULTS: Multiphoton endomicroscopy provided unprecedented 3-dimensional visualization of vascular permeability dynamics and showed that vascular dysfunction occurs prior to epithelial barrier breakdown and the detection of traditional inflammatory markers across all colitis models. Distinct spatial vascular permeability patterns strongly correlated with mucosal damage severity, further supporting that early gut-vascular barrier disruption precedes mucosal barrier breakdown. Additionally, this sequence was confirmed at the molecular level, with the vascular upregulation of the trans-endothelial permeability channel plasmalemma vesicle-associated protein occurring before downregulation of the epithelial barrier molecule E-cadherin.

CONCLUSIONS: Multiphoton endomicroscopy with 3-dimensional imaging demonstrated gut-vascular barrier dysfunction in the very early stages of experimental colitis. Further development of multiphoton endomicroscopy-based vascular permeability analysis for use in routine clinical monitoring of patients may provide new perspectives to improve diagnosis and clinical decision-making in inflammatory bowel disease. (*Cell Mol Gastroenterol Hepatol* 2026;20:101795; <https://doi.org/10.1016/j.jcmgh.2026.101795>)

Keywords: In Vivo Microscopy; Multiphoton Endomicroscopy; Vascular Permeability.

Inflammatory bowel diseases (IBDs) include chronic inflammatory disorders of the intestine, such as ulcerative colitis (UC) and Crohn's disease.¹ Although treatment outcomes are unpredictable,² various therapies and lifestyle changes can mitigate disease impact. The rising incidence of IBD³ and its intermittent nature, characterized by remission and flare-ups,⁴ highlight the need for sensitive and noninvasive inflammatory disease monitoring tools. Moreover, early detection of inflammation is crucial for timely intervention, enabling therapy adjustments and preventing disease progression.² For developing sensitive diagnostic and predictive tools, the understanding of underlying mechanisms of IBD pathogenesis is an essential prerequisite.

At the cellular level, IBD is presently considered to be primarily driven by a disrupted intestinal epithelial barrier and an excessive inflammatory response.⁵ Previous research on gut homeostasis and defense against microbial invasion has largely focused on the epithelial barrier and innate immune system,^{6–8} overlooking the potential role of the gut-vascular barrier (GVB), despite the intestine's highly vascularized nature.⁹ Only recently has the GVB gained recognition as a crucial secondary intestinal barrier.¹⁰

Blood vessels in the intestine serve as access points for immune cells, with endothelial cells regulating their recruitment, adhesion, and extravasation into the tissue.¹¹ At steady state, the GVB is permeable to small molecules up to 4 kDa.¹² Proinflammatory cytokines, such as interferon- γ (IFN- γ), increase GVB permeability, allowing large molecules of approximately 70 kDa to extravasate^{10,13,14} and pass from the gut lumen to the blood during inflammation.¹² Increased vascular permeability (VP) has recently been linked to inflammation in human IBD.^{13,15} Probe-based confocal laser endomicroscopy (pCLE) studies have

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Abbreviations used in this paper: 3D, 3-dimensional; DSS, dextran sodium sulfate; E-cadherin, epithelial cadherin; FFPE, formalin-fixed paraffin-embedded; FITC, fluorescein isothiocyanate; GVB, gut-vascular barrier; IBD, inflammatory bowel disease; IFN- γ , interferon- γ ; i.v., intravenous; IVM, intravital microscopy; MPEM, multiphoton endomicroscopy; MPM, multiphoton microscopy; mRNA, messenger RNA; NK, natural killer; pCLE, probe-based confocal laser endomicroscopy; PMT, photomultiplier tube; PV1, plasmalemma vesicle-associated protein; Rag1^{-/-}, recombination activating gene 1 knockout; ROI, region of interest; SEPIA, Shared Experimental Inflammation Data; TNF- α , tumor necrosis factor- α ; TRITC, tetramethylrhodamine isothiocyanate; UC, ulcerative colitis; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VP, vascular permeability.



Most current article

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shown that fluorescein leakage, following intravenous (i.v.) injection, correlates with active inflammation and decreases during remission,^{13,16,17} offering superior predictive precision in comparison with other diagnostic techniques such as endoscopy and histology.¹⁸

To date, no in vivo study has focused on early VP changes in IBD. To fill this gap, we used multiphoton microscopy (MPM),¹⁹ which provides high optical resolution with significantly deeper tissue penetration than single-photon confocal microscopy.^{20,21} In preclinical ex vivo studies, MPM enabled the 3-dimensional (3D) reconstruction of the colon, which revealed inflammation-induced changes in the colonic mucosa.²² This technology was further advanced into multiphoton endomicroscopy (MPEM) for in vivo use, which allows label-free visualization of inflammatory changes in colon morphology with a sensitivity and resolution comparable to ex vivo MPM.^{23,24} In this study, we introduce fluorescence marker-based MPEM to evaluate the kinetics, severity, and 3D distribution of vascular barrier defects in a noninvasive manner in different preclinical models of colitis.

Our findings demonstrate that VP increases during the very early stages of the disease. MPEM detected early inflammatory responses, even before visible mucosal damage. At the molecular level, upregulation of plasmalemma vesicle-associated protein (PV1) occurred before downregulation of vascular endothelial (VE) cadherin in endothelial cells and epithelial (E) cadherin in epithelial cells and, therefore, is likely responsible for the early breakdown of the vascular barrier. Further development of MPEM-based VP analysis for the use in routine clinical monitoring of patients may provide new perspectives to improve diagnosis and clinical decision-making in IBD.

Results

Multiphoton Endomicroscopy Enables In Vivo Characterization of Vascular Permeability in Preclinical Murine Colitis Models

Increased VP is associated with inflammation in murine colitis models and patients with IBD.¹³ The goal of the present study was to establish a new methodology to determine the earliest onset and location of VP in the course of different experimentally induced colitis models in mice. The established methodology offers sensitive, noninvasive, and repeated observations of VP in the same animal.

To determine the onset of VP, we performed MPEM imaging after i.v. injection of fluorescently labeled dextrans to monitor the colonic vasculature in the same mouse over time. Imaging of the colonic vasculature in healthy mice revealed immediate leakage of the 10-kDa dextran into the colon tissue within 5 minutes post-injection (Figure 1A; asterisk). In contrast, the 70- and 500-kDa dextrans remained in the blood vessels (Figure 1A; arrows). Analysis of the serum concentrations revealed that, unlike the 10-kDa dextran, these higher molecular weight dextrans

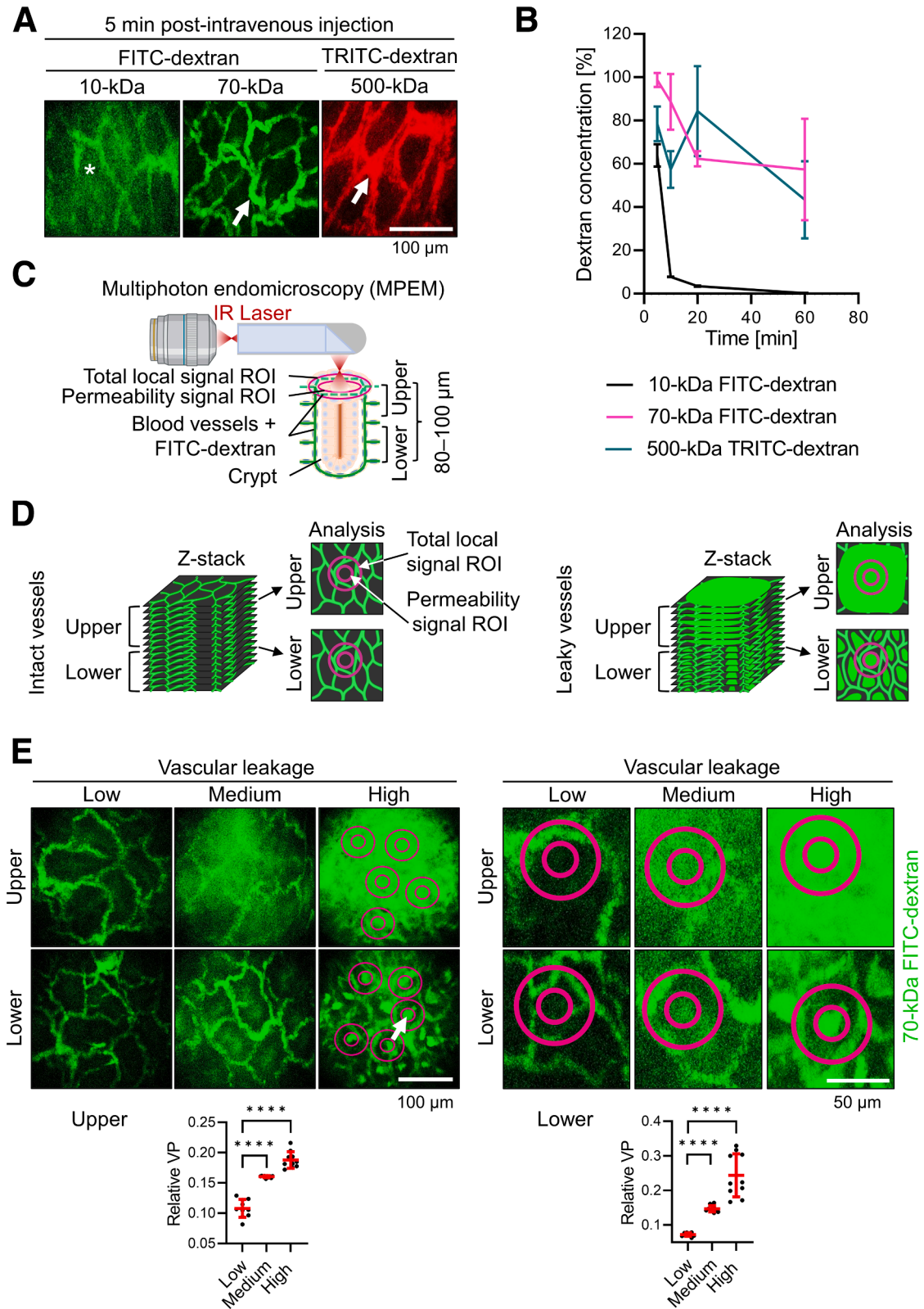
remained stable over long time intervals (Figure 1B). Increased circulation times and lacking capability to penetrate healthy vessels directed the use of only the 70- and 500-kDa dextrans for subsequent experiments to ensure sensitive discrimination of VP between healthy and inflamed colons.

Exploiting the acquisition speed and penetration depth of MPEM, multiple images of the colonic vasculature were captured at several positions along the distal colons at depths of 80 to 100 μm in the mucosa (Figure 1C and D). Notably, within a stack of images captured from the same position, we observed differences in dye extravasation and accumulation patterns in dextran sodium sulfate (DSS)-treated animals between the upper areas and lower regions of the crypts, located near the colonic lumen and within the crypts, respectively (Figure 1D and E). Extravasation and increased VP appeared as diffuse signals in the upper regions (Figure 1E; upper panels). The lower regions exhibited focal dye accumulation in the crypts (Figure 1E; lower panels, arrow). Therefore, for the remainder of the study, the stacks were divided into upper and lower regions, with 1 representative plane per region chosen for VP analysis.

To analyze VP, we developed a semiautomated quantification tool to calculate the relative VP as a ratio of the extravasated dextran molecules (Figure 1C and D) (permeability signal regions of interest [ROIs]) to the total local signal captured (Figure 1C and D) (total local signal ROI). Validation with representative images of low, medium, and high permeability confirmed the tool's accuracy in reflecting visually determined leakage (Figure 1E).

Vascular Disruption and Leakage Are Early Events in Dextran Sodium Sulfate-Induced Colitis

To investigate the kinetics of VP, we performed MPEM analyses through the course of DSS-induced colitis. As an important aspect of our experimental design was to reduce the number of animals, the scores of each mouse on day 0 were used as controls throughout treatment and analyses. In addition to MPEM-based detection of VP after injection of 70-kDa fluorescein isothiocyanate (FITC)-dextran, we assessed body weight and endoscopic score in the same animals on days 0, 2, 4, 7, and 9 after the beginning of DSS treatment (Figure 2A). Significant changes in body weight were observed from day 6 onward (Figure 2B), and in endoscopic scores from day 7 onward (Figure 2B). Notably, MPEM-based analysis revealed a significant increase in VP in the upper colonic region as early as 2 days after DSS administration (Figure 2B; arrows indicate intact vessels, asterisks denote areas with vascular leakage). In the lower region, significant extravascular dye leakage and accumulation within the crypt lumen were observed only from day 7 onward (Figure 2B; arrowheads) and coincided with severe mucosal damage (Figure 2B; endoscopy, damages framed in red).



To further assess the severity of vascular barrier defects, the body weight and endoscopic scores were compared with extravasation of the 500-kDa dextran on days 0, 2, 4, 7, and 10 post-DSS administration (Figure 2A and C). MPEM measurements revealed a significant increase in VP to 500-kDa dextran starting on day 4 in the upper region (Figure 2C; arrows indicate intact vessels, asterisks denote areas with vascular leakage). In the lower region, the accumulation of extravascular 500-kDa dextran in the crypt lumen and a significant increase in VP were observed on days 7 and 10 (Figure 2C; arrowheads).

Collectively, these results demonstrate that an increase in VP occurs early in DSS-induced colitis, with leakage of 70- and 500-kDa dextrans occurring as early as days 2 and 4 of disease induction, respectively, before visible mucosal damage is observed. These findings highlight the MPEM's potential to detect VP as an early predictor and sensitive indicator of inflammation.

Early Vascular Leakage Precedes Established Macro- and Microscopic Markers of Inflammation in Dextran Sodium Sulfate-Induced Colitis

Next, we investigated the potential of VP as an early marker for colitis by comparing, in the same animals, the sensitivity of MPEM for detecting VP dynamics with conventional approaches used to monitor disease severity and inflammation at the cellular level. First, VP, body weight changes, and endoscopic scores were measured in vivo on days 0, 2, 4, and 7 post-DSS administration, whereas colon length, histopathologic scoring, and immune cell infiltration were assessed at each timepoint ex vivo (Figure 3A).

Consistent with the above findings, VP increased early, with significant changes in the upper and lower crypt regions starting on days 2 and 4 post-DSS administration,

respectively (Figure 3B). In contrast, a decrease in body weight and colon length as well as an increase of the endoscopic score, were first observed on day 7 (Figure 3C and D). Histologic colitis score (Methods and Table 1) began to rise on day 4, with significant differences observed on day 7 (Figure 3E–G). Innate immune cells, including natural killer (NK) cells (NKp46+), neutrophils (CD45+ Ly6Ghigh), and macrophages (CD45+ MHCII+ F4/80+), assessed by immunohistochemical staining (NK cells) and by flow cytometry (neutrophils and macrophages) were increased from day 4 onward (Figure 4A and B). Total CD45+ immune cells and adaptive immune populations such as T cells (CD45+ CD3+) and B cells (CD45+ CD19+) were only increased at day 7 (Figure 4B). These results show that the MPEM-based detection of increased VP identifies colitis-associated changes in the gut earlier than other commonly used disease activity monitoring tools, thereby confirming that VP is an early marker of inflammation in the DSS-colitis model.

Multiphoton Endomicroscopy Detects Ethanol-Induced Vascular Permeability With High Sensitivity

DSS induces colitis by damaging epithelial cells and compromising barrier integrity, allowing luminal antigens to penetrate the mucosa and trigger rapid inflammation.²⁸ However, because DSS is administered via the drinking water, the exact timepoint and frequency of application, as well as the dosage, cannot be controlled or monitored accurately for each animal. This makes it challenging to precisely determine when changes in VP first appear and to assess whether VP also increases in case of mild mucosal damage, even in the complete absence of visible inflammation. Therefore, we used a single dose of 50% ethanol enema to assess whether a direct and controlled mild colon damage increases VP.²⁹

Figure 1. (See previous page). MPEM imaging enables in vivo detection of colonic VP in experimental colitis. (A) MPEM imaging was performed on healthy mice 5 minutes after intravenous injection of 10-, 70-, and 500-kDa dextrans conjugated with FITC or TRITC. Representative images are shown. Scale bar, 100 μ m. Perivascular leakage is indicated with an asterisk and nonleaky vessels with an arrow. (B) Circulation times of the different fluorescently labeled dextrans in healthy mice were investigated following an i.v. injection. After different timepoints (5, 10, 20, and 60 minutes), the mice sera were harvested, and dextran concentrations were determined photometrically. Individual mice were used for each dextran, and 2 mice were used for each timepoint. Data are presented as mean $\pm$ SD. (C) MPEM schematic overview showing an external microscope objective delivering an infrared laser beam through an endoscope containing a prism positioned in the distal colon of the mouse. Imaging typically covered 80- to 100- μ m depth. Magenta circles indicate ROIs to measure the permeability signal (inner ROI) and total local signal (outer ROI). Diagram partly created with BioRender.com. (D) After injection of fluorescent dextran, Z-stack images of the distal colon at 7 different positions were acquired. Z-stack and single-plane images representing upper (near-lumen) and lower (crypt lumen and base) regions are shown for healthy (left) and inflamed (right) colon. Magenta circles denote ROIs for permeability signal and total local signal determination. Diagram created with BioRender.com. (E) To validate the semi-automated quantification, MPEM images of untreated or DSS-treated animals injected with 70-kDa dextran, and previously visually divided by the experimenter into low, medium, and high leakage groups, were analyzed (n = 10/group). Representative pictures illustrating quantification using the custom semiautomated analysis tool are shown as full images (left panel) and enlarged crypt regions (right panel). Paired concentric ROIs were placed on selected vessels in the upper and lower crypt layers. The inner ROI is positioned in the perivascular region to capture the extravascular signal (vascular leakage), whereas the outer ROI encompasses the vessel to measure the total local signal for normalization. VP was calculated as the ratio of extravasated fluorescence (inner circle) to local background fluorescence (outer circle) across 5 ROIs per image and is presented in graphs where each dot represents the mean permeability for 5 ROIs per image. Data correspond to means $\pm$ SDs, with P values determined using 2-tailed unpaired t tests and Mann-Whitney tests (****P < .0001). SD, standard deviation.

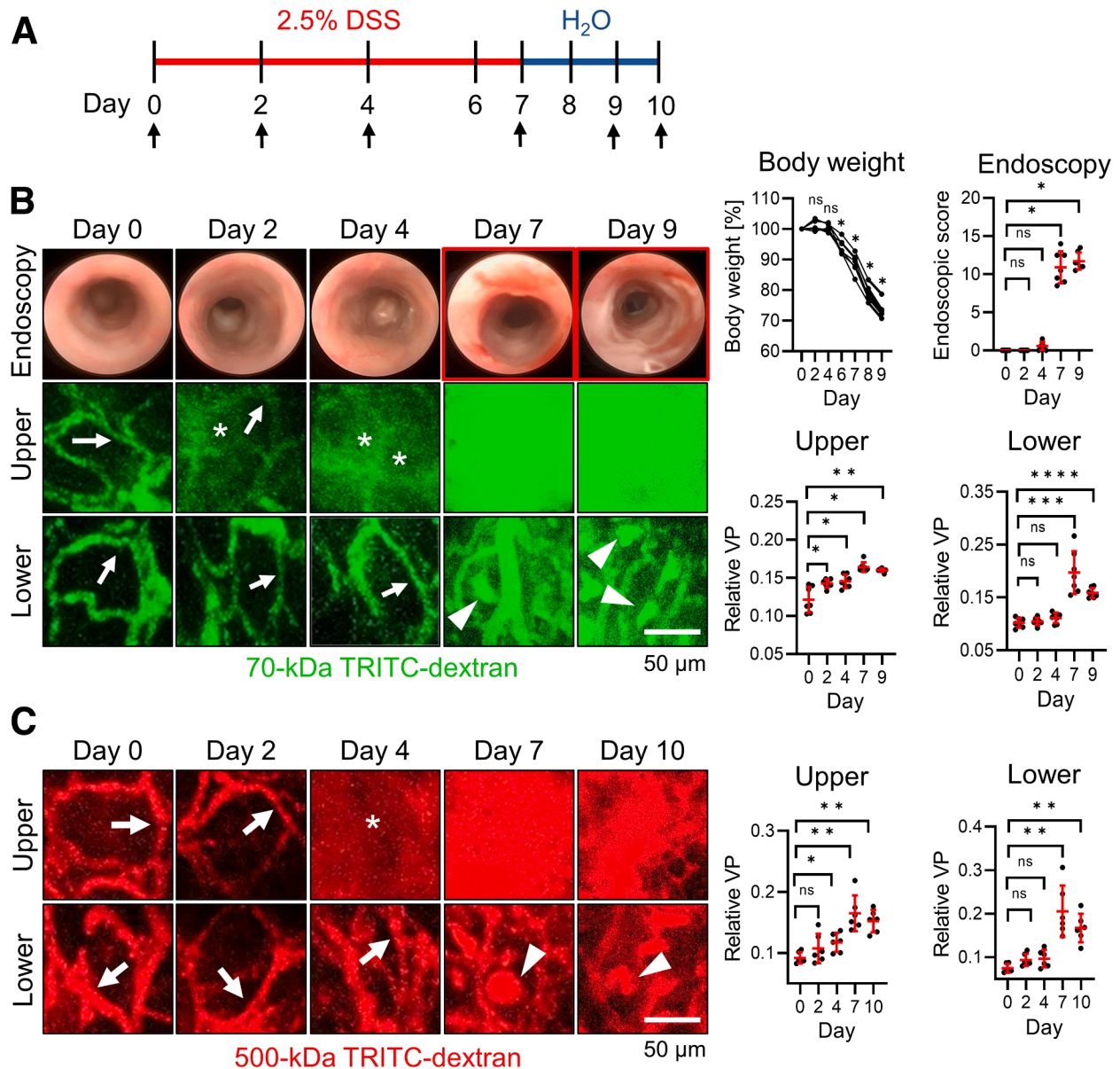
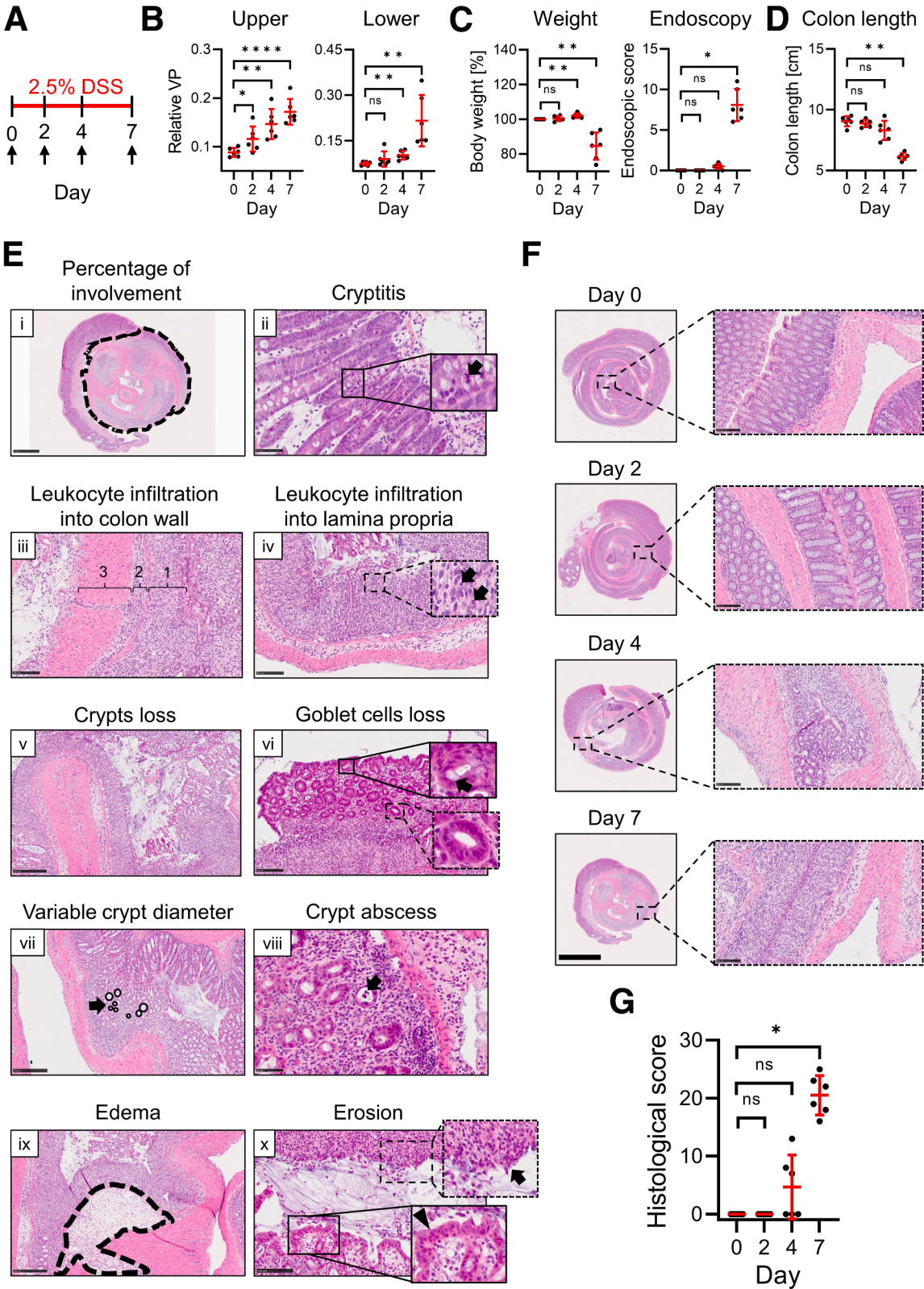


Figure 2. Vascular leakage is an early event in DSS-induced colitis preceding epithelial barrier disruption. (A) Experimental workflow of DSS treatment. Mice were treated with 2.5% DSS for 7 days and subjected to longitudinal assessments, including body weight monitoring, endoscopic evaluation of inflammation, and MPEM-based VP analysis on days 0, 2, 4, 7, and 9 or 10 (black arrows). Two groups were analyzed using 70-kDa FITC-dextran ($n = 7$) and 500-kDa TRITC-dextran ($n = 6$). (B) Left panel, top row: endoscopy images. Red frames highlight the timepoints with visible mucosal inflammation. Middle and bottom rows: MPEM images after 70-kDa dextran injection from upper and lower crypt regions. Arrows indicate intact vessels, asterisks denote perivascular leakage, and arrowheads point to dye accumulation in crypt lumina. Scale bar, 50 μ m. Right panel: plots depict body weight as a percentage of initial weight, endoscopic scores, and relative VP in upper and lower crypt regions. (C) Left panel: MPEM images after 500-kDa dextran injection of the upper and lower crypt regions. Arrows indicate intact vessels, asterisks denote vascular leakage, and arrowheads highlight dye accumulation in crypt lumina. Scale bar, 50 μ m. Right panel: plots show relative VP quantifications for each region. In the dot plots of (B and C), each dot represents an individual mouse. In the MPEM analyses, the mean relative VP across 7 distal colon positions per animal was determined. Results are shown as means $\pm$ SDs. P values were calculated using 2-tailed paired t tests and Wilcoxon matched-pairs signed rank tests, comparing each timepoint to the baseline (day 0) (* $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$). Results in (B) were pooled from 2 independent experiments, whereas (C) represents results from 1 experiment. SD, standard deviation.



Our data revealed no visible mucosal damage during endoscopy 12, 24, or 72 hours post-enema (Figure 5A and B). In contrast, a rapid and significant increase in VP was detected in the upper and lower crypt regions within 12 hours, although this effect was transient (Figure 5C; upper; D, lower). These results indicate that VP can be induced by epithelial penetrating substances in the absence of endoscopically evident epithelial barrier defects.

Vascular Leakage Precedes the Onset of Macroscopic Signs of Inflammation in T Cell Transfer-Induced Colitis

The murine DSS and ethanol models used in our study involved chemical triggers acting from the luminal side. To further explore whether VP can be detected in colitis models based on alternative mechanisms of initiation, we used a T cell transfer-induced colitis model. T cells isolated from wild-type mice were transferred into recipient recombination activating gene 1 knockout (Rag1^{-/-}) mice lacking mature T cells, disrupting T cell homeostasis and resulting in colonic inflammation usually 5 to 8 weeks later.^{30,31}

Endoscopic score and VP were assessed 18, 21, 24, 28, and 31 days after T cell transfer, with sham-treated littermates used as controls (Figure 6A). Endoscopic scoring revealed a minor increase in inflammation 24 and 28 days post-transfer, although not statistically significant (Figure 6B and C; red frames indicate pathologic alterations). In contrast, increased VP, as detected using MPEM, was apparent on day 21 and became significant in the upper crypt region on days 24 and 28 (Figure 6B and C). These findings demonstrate that MPEM can also detect early inflammatory changes in immune-driven colitis models that are not based on epithelial layer injury.

Vascular Leakage Patterns in 3-Dimensional Multiphoton Endomicroscopy Imaging Indicate Mucosal Damage Severity and Inflammation

In our kinetics experiments, diffuse vascular leakage was observed in the upper regions during early or mild inflammation across all 3 murine models. As inflammation progressed, leakage intensified, particularly during severe inflammation accompanied by macroscopic mucosal damage. This was marked by an increased, diffusely distributed signal in the upper areas and dye accumulation in the crypt centers. The latter was most prominent in DSS-induced colitis and less evident in the T cell transfer colitis model (compare Figures 2B and 6B).

To investigate the development of these signal patterns in more detail, we employed Z-stack images from MPEM at days 0, 4, and 7 of 2.5% DSS treatment to assess the 3D distribution of VP in the colonic mucosa (Figure 7A). On day 0, before the induction of inflammation, VP was absent or very low without any differences between upper and lower layers (Figure 7A; day 0). On day 4 of DSS treatment, leakage was apparent in the upper region (Figure 7A; asterisks), whereas vessels in the lower region were still intact (Figure 7A; lower). Notably, no dye accumulation was detected in the lower crypt lumina (Figure 7A; lower). By day 7, FITC-dextran was leaking diffusely in the upper regions (Figure 7A; asterisks) and accumulated in the lower crypt centers (Figure 7A; arrowheads). Lateral Z-stack views revealed cone-shaped crypts with distinct dark borders, indicative of intact epithelium at these levels (Figure 7A; arrowheads). Of note, no significant dye leakage from neighboring vessels was observed in the lower crypt regions.

For validation of MPEM results, intravital microscopy (IVM) was used on day 7 in a 3% DSS-treated mouse and compared with a healthy control (Figure 7B). IVM is an

Figure 3. (See previous page). Vascular leakage precedes macroscopic and microscopic indices of inflammation in DSS-induced colitis. (A) Experimental design. On days 0, 2, 4, and 7 of 2.5% DSS treatment (black arrows), separate sets of mice (n = 6 per timepoint) underwent MPEM imaging (in vivo) and evaluations for colitis-related inflammation (in vivo), before being sacrificed for further ex vivo analyses. (B) Relative VP was quantified in the upper and lower colonic crypts after intravenous injection of 70-kDa FITC-dextran, capturing in vivo microscopic changes. (C) Body weight changes and endoscopic mucosal damage scores were recorded. (D) Colon length shortening was evaluated at each timepoint. (E) Histologic scoring was performed using 10 criteria illustrated by images taken from inflamed colons harvested after a 7-day cycle of 2.5% DSS treatment. Criteria included (i) percentage of involvement by colitis based on the assessment of entire colon (scale bar, 1 mm); (ii) cryptitis as indicated as neutrophils (arrow) found between crypt epithelial cells (scale bar, 50 μ m); (iii) leukocyte infiltration into the colon wall including the mucosal (1), submucosal (2), and transmural (3) layers (scale bar, 100 μ m); (iv) leukocyte infiltration into the lamina propria (arrows; scale bar, 100 μ m); (v) crypt loss (scale bar, 250 μ m); (vi) goblet cell loss in crypts (lower enlarged image to be compared with crypts containing goblet cells indicated by an arrow in the upper enlarged image; scale bar, 100 μ m); (vii) variable crypt diameters (arrow, circled; scale bar, 250 μ m); (viii) crypt abscess with neutrophils (arrow) in crypt lumen (scale bar, 25 μ m); (ix) edema (area indicated by a dashed line; scale bar, 250 μ m); (x) erosion with loss of epithelium (arrow, upper enlarged image) to be compared with intact epithelium (arrowhead, lower enlarged image); scale bar, 100 μ m. (F) Exemplary images of the H&E-stained colonic tissues harvested for histological scoring of colitis on day 0, 2, 4, and 7 of 2.5% DSS treatment. Images were taken at 1.25 $\times$ magnification, with an enlarged region acquired at 20 $\times$ magnification for better visualization. Scale bars are 2.5 mm and 100 μ m, respectively. (G) Histologic score was evaluated using colons harvested from 6 mice per timepoint on day 0, 2, 4, and 7 of 2.5% DSS treatment. (B–G) Results are shown as means $\pm$ SDs. Statistical comparisons were made using 2-tailed unpaired *t*-tests and Mann-Whitney tests for all, except endoscopic and histologic scores, where Wilcoxon signed rank tests were applied (**P* < .05, ***P* < .01, and *****P* < .0001). Each timepoint was compared with healthy controls (day 0). Each dot represents an individual mouse, and relative VP reflects mean VP across 7 distal colon positions per animal. Data were pooled from 2 independent experiments. H&E, hematoxylin and eosin; SD, standard deviation.

Table 1. Histologic Scoring of Colitis

Category	Criterion	Definition	Score value
Involvement	Percentage of involvement	Percentage of inflamed areas within the whole colon:	0–3
		None: 0%	0
		1%–25%	1
		26%–50%	2
		>50%	3
Within the involved areas, check:			
I: Inflammatory cell infiltrate	Severity	Leukocyte density infiltrated to the lamina propria area:	0–4
		None: 0%	0
		Minimal: <10%	1
		Mild: 10%–25%; scattered neutrophils	2
		Moderate: 26%–50%	3
	Extent	Marked: >51%; dense infiltrate	4
		Expansion of leukocyte infiltration:	0–3
		None: 0	0
		Mucosal	1
		Mucosal and submucosal	2
II: Epithelial changes	Goblet cell loss	Mucosal, submucosal and transmural	3
		Reduction of goblet cell numbers relative to baseline goblet cell numbers per crypt:	0–4
		None: 0%	0
		Minimal: <20%	1
		Mild: 21%–35%	2
	Cryptitis	Moderate: 36%–50%	3
		Marked: >50%	4
		Neutrophils between crypt epithelial cells	0 or 1
		None	0
		Present	1
	Crypt abscesses	Neutrophils in crypt lumen	0 or 1
		None	0
		Present	1
	Erosion	Loss of surface epithelium:	0–4
		None: 0%	0
Minimal: 1%–20%		1	
Mild: 21%–35%		2	
Moderate: 36%–50%		3	
III: Mucosal architecture	Irregular crypts	Marked: >50%	4
		Variable crypt diameters	0 or 1
		None	0
	Crypt loss	Present	1
		Mucosa devoid of crypts	0 or 1
None		0	
IV: Edema	Severity	Present	1
		The space between the muscularis mucosa and the muscularis propria compared with the mucosal thickness	0–3
		None: 0%	0
		Mild: 1%–30%	1
		Moderate: 31%–99%	2
Marked: ≥100%	3		
			Maximum score = 25

NOTE. Paraffin-fixed colons were stained with hematoxylin and eosin and assessed for the degree of colitis using the following categories: percentage of involved areas (inflamed areas) in the overall colon tissue, and within these involved areas, the degree of immune cell infiltration, epithelial changes, mucosal architecture, and the extent of edema were analyzed. Each category was given a score, as shown in the Table, and the summation of the scores was given as the histologic score of colitis with a maximum score of 25. The scoring system was developed by adapting criteria from.^{25–27}

end-point experiment and therefore prevents following the evolution of VP in the same animal over time. The advantages of IVM are its higher resolution due to reduced motion and the possibility of coinjecting lectin as a vascular marker alongside the 70-kDa FITC-dextran. In agreement with the results obtained with MPEM, a healthy control showed no vessel permeability with lectin (*magenta*) and FITC-dextran (*green*) signals overlapping (*Figure 7B; left panel*). In DSS-treated mice, FITC-dextran significantly accumulated within the crypts (*Figure 7B; right panel*). In agreement with MPEM, Z-stack lateral views revealed cone-

shaped crypts with defined dark borders indicative of an intact epithelium (*Figure 7B; right panel, lateral views, arrowhead*). No detectable dye leakage from neighboring vessels was observed in the lower crypt regions. In contrast, in the upper regions, the crypts appeared connected by the diffuse flow of the FITC-dextran signals (*Figure 7B; asterisk*). Thus, IVM convincingly confirmed the MPEM results.

This VP marker distribution is highly similar to the pooling of contrast agents in the crypt regions observed in patients with UC when injected with fluorescein, as

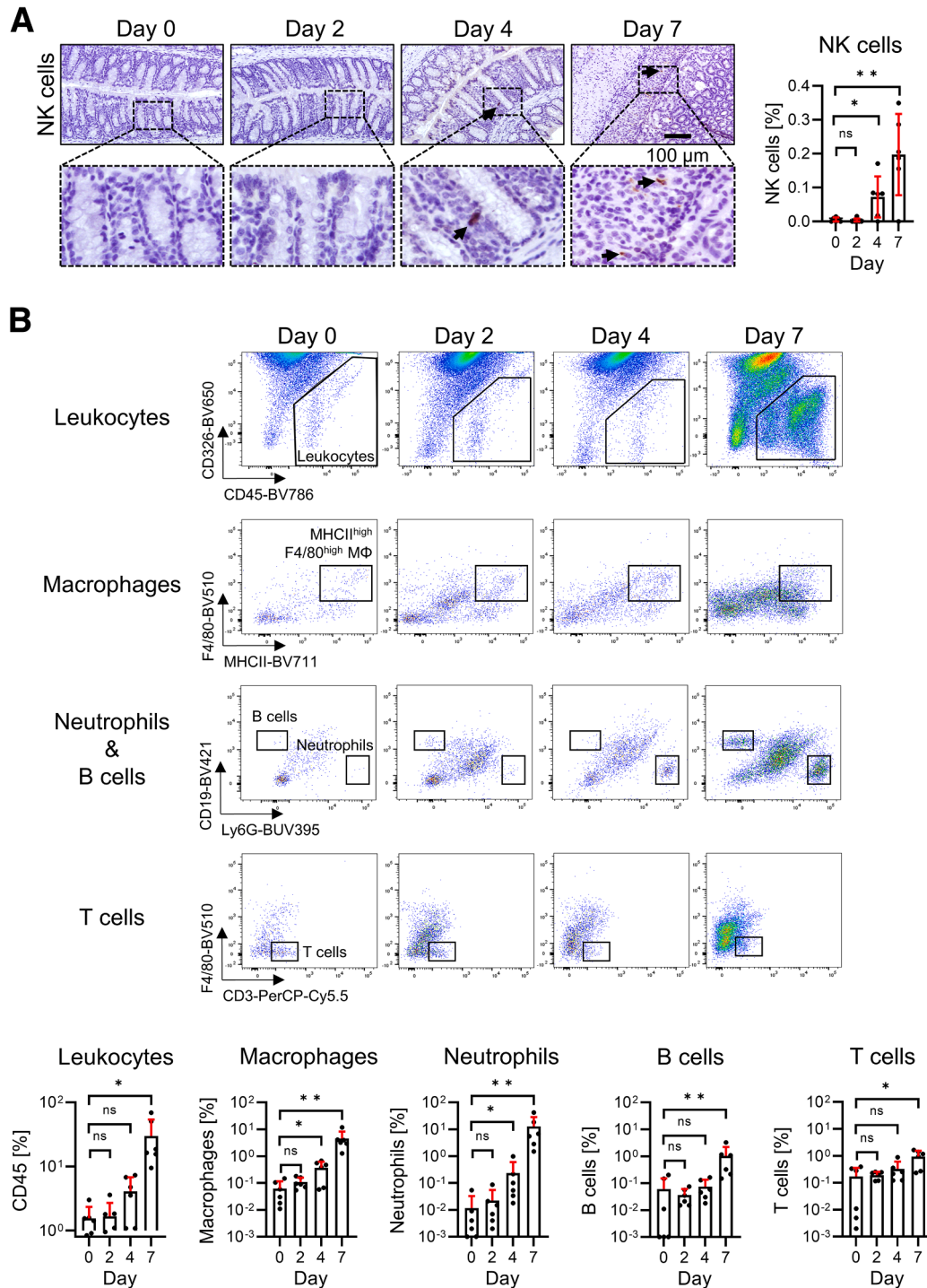


Figure 4. Vascular leakage precedes immune cell recruitment during DSS-induced colitis. (A) NK cell populations (NKp46+) were determined in colon tissues from the same mice used in Figure 3 using immunohistochemical staining. *Black arrows* point at the NK cells. Enlarged views (*dashed black outlines*). Scale bar, 100 μ m. The plot shows the percentage of NK cells relative to total cells at each timepoint. Results are shown as means $\pm$ SDs. (B) Immune cell populations were determined by flow cytometry analysis of freshly harvested colon tissues from the same mice used in Figure 3. Representative dot plots show leukocytes (CD45+), macrophages (CD45+ MHCII+ F4/80+), neutrophils (CD45+ Ly6G^{high}), B cells (CD45+ CD19+ Ly6G-), and T cells (CD45+ CD3+ F4/80-) at all timepoints. Immune cell populations were quantified as percentages of total cells at each timepoint. Results are shown as means $\pm$ SDs. (A and B) Statistical comparisons were made using 2-tailed unpaired *t* tests and Mann-Whitney tests for all (**P* < .05, ***P* < .01). Each timepoint was compared with healthy controls (day 0). Each *dot* represents an individual mouse. Data were pooled from 2 independent experiments. SD, standard deviation.

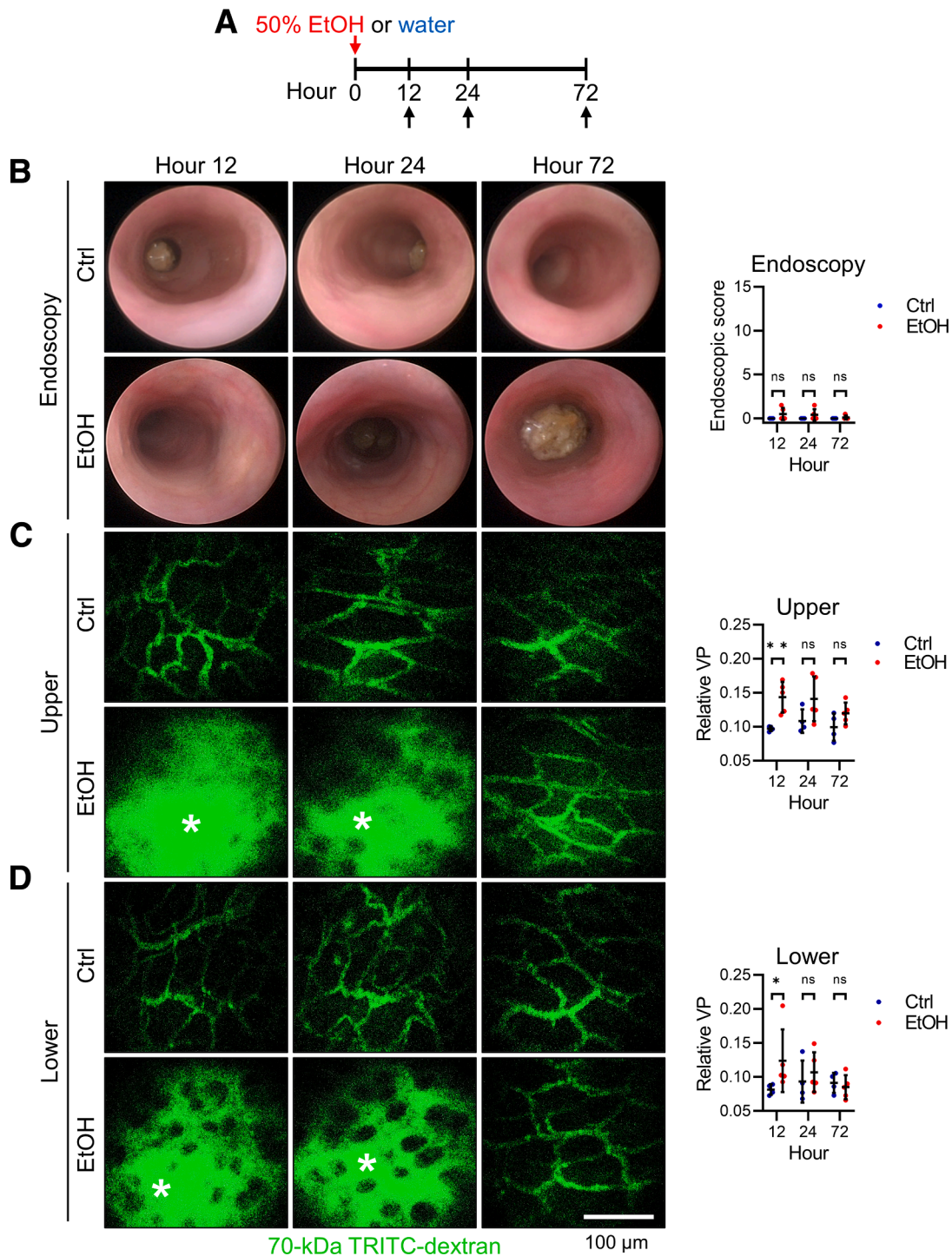
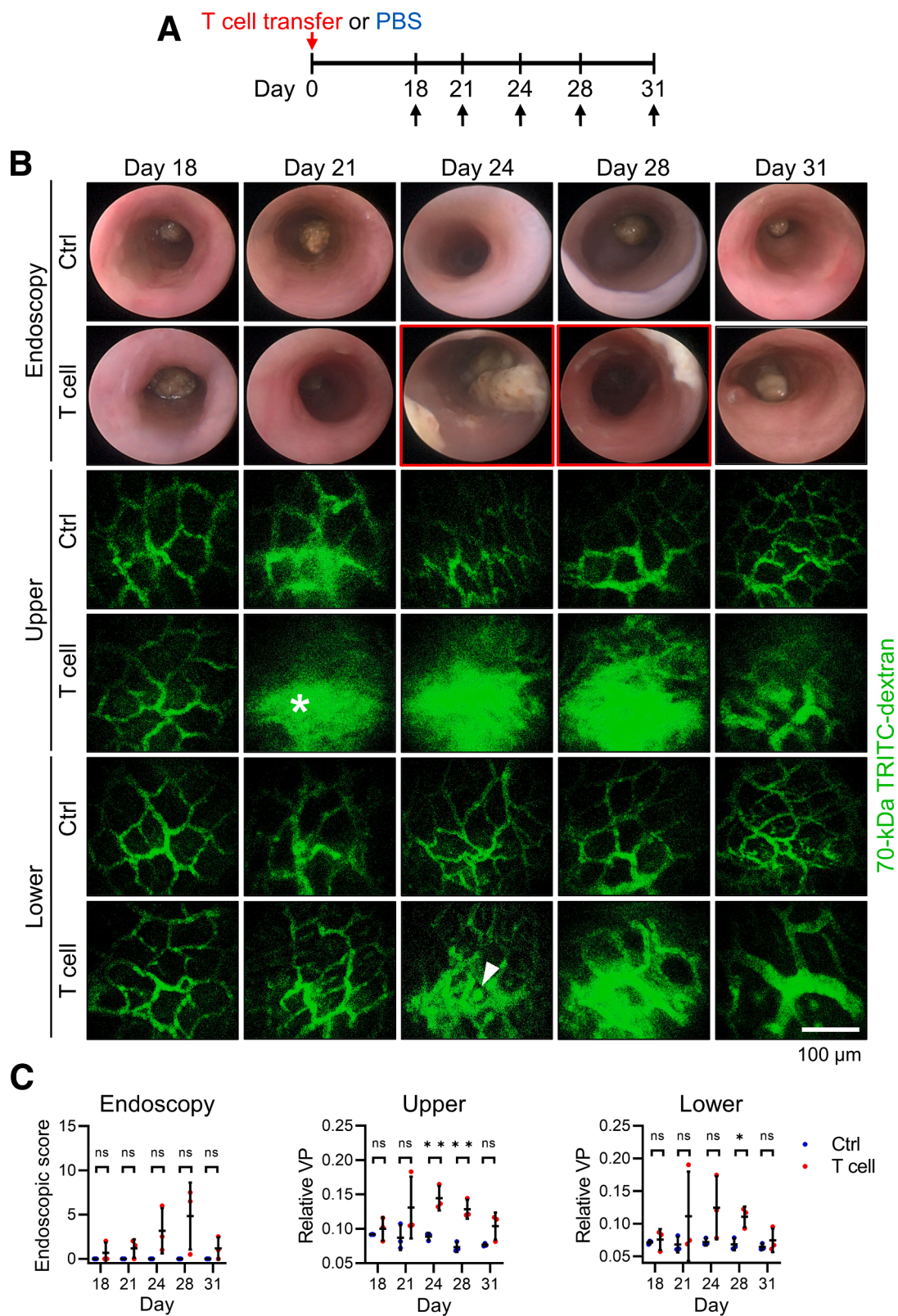


Figure 5. MPEM detects ethanol-induced VP with high sensitivity. (A) Experimental design. The mice were treated intrarectally with 50% ethanol ($n = 5$) or water as controls ($n = 4$) and assessed at 12, 24, and 72 hours post-treatment with endoscopy and MPEM (red arrow indicates the treatment timepoint; black arrows indicate the assessment timepoints). (B) Representative endoscopy images (left) and endoscopic scores (right), with each dot representing a mouse. (C and D) Exemplary MPEM images of colonic vasculature (left panels) from the upper (C) and lower (D) crypt regions post i.v. injection of 70-kDa FITC-dextran. Asterisks denote areas with vascular leakage. Scale bar, 100 μ m. Quantitative analysis (right panels) shows mean relative VP across 7 positions per mouse. Comparisons are shown as means $\pm$ SDs. P values were calculated using the Wilcoxon signed rank test (B), 2-tailed unpaired t test (C), or Mann-Whitney test (D) ($*P < .05$ and $**P < .01$). Comparisons were made between ethanol-treated and controls at each timepoint. Results were pooled from 2 independent experiments. EtOH, ethanol; SD, standard deviation.



observed with pCLE (Figure 7C; arrows), which is absent in patients in remission (Figure 7C).

Altogether, these findings show that early DSS-induced inflammation is reliably characterized by diffuse leakage patterns in the upper regions of the colonic mucosa (Figure 7D). In the course of disease progression, severe mucosal damage and epithelial barrier disruption occur, allowing fluorochromes from the upper vessels to enter the crypt lumen through a disrupted adjacent epithelial barrier and leading to characteristic accumulation patterns in the crypt centers, indicating extensive epithelial barrier breach (Figure 7D).

Expression of Vascular and Epithelial Barrier Proteins Confirms Early Onset of Vascular Permeability in Patients With Inflammatory Bowel Disease and Experimental Colitis

GVB disruption during full-blown inflammation is demonstrated at the molecular level by the loss of VE-cadherin, a key endothelial junction protein. In this study, we confirmed a marked VE-cadherin disruption in patients with UC (Figure 8A), consistent with previous findings by Langer et al, in both patients with IBD and DSS-treated mice.¹³ Another hallmark of GVB dysfunction is the upregulation of PV1, a transendothelial channel protein. At the RNA level, we observed increased PV1 expression in patients with UC (Figure 8B) and in DSS-treated mice (Figure 8C), in agreement with earlier studies by Carloni et al.³²

To follow colitis-associated disintegration of the epithelial and vascular barriers, we investigated the expression of major regulatory molecules of both barriers (vascular: PV1, VE-cadherin; epithelial: E-cadherin) in the course of colitis at the single-cell level (Figure 8D) with quantitative evaluation (Figure 8D; bottom row). PV1 was significantly increased already after 2 days (Figure 8D; upper 2 rows, yellow arrows), whereas a significant downregulation of VE-cadherin in CD31-positive vessel endothelial cells was only observed after day 4 (Figure 8D; middle 2 rows, yellow arrows). This suggested that PV1 may be the primary regulator of early increase in permeability, whereas decreased VE-cadherin contributes to increased permeability at later stages. Notably, alterations in the epithelial barrier, observed both in the histologic hematoxylin and eosin stain and by downregulation of E-cadherin, were only observed after 4 days (Figure 8D; lower 2

rows). These results confirmed, at the molecular level, that GVB dysfunction precedes epithelial breakdown during early inflammation and may serve as a sensitive and early marker of IBD initiation.

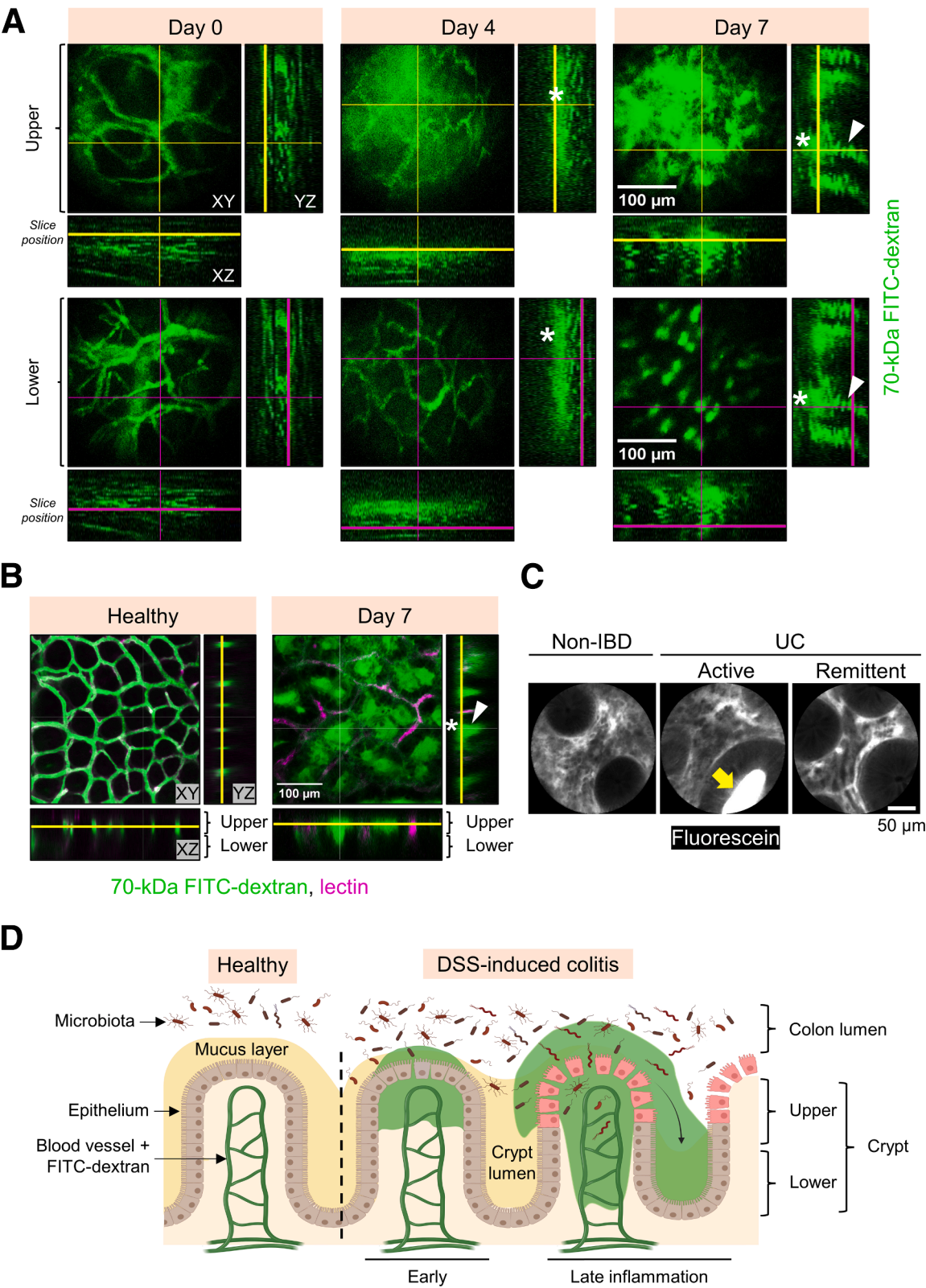
To assess early cytokine dynamics, we examined publicly available data on bulk RNA sequencing analysis from DSS colitis reported by Patankar et al.³³ The expression of several proinflammatory cytokines (IFN- γ , tumor necrosis factor- α [TNF- α], interleukin-1 β , and interleukin-17A)^{13,34–36} and chemokines (C-X-C motif chemokine ligand 1 and C-X-C motif chemokine ligand 10)^{34,35} known to activate endothelial cells was significantly increased at day 3, rising further until day 8 (peak inflammation), and decreased during recovery (day 12) (Figure 9A). To extend this analysis to the T cell transfer model, we utilized the Shared Experimental Inflammation Data (SEPIA) platform from Gonzalez-Acera et al.³⁷ A comparison of inflamed vs healthy tissue revealed increases in the same cytokines and chemokines in this model (Figure 9B). Unfortunately, early timepoint data required to assess initial inflammatory events were not available for the T cell transfer model. Altogether, these findings indicate that several cytokines exerting proinflammatory activation of the endothelium, are present early in the inflammatory microenvironment and may contribute to the appearance of VP.

Discussion

In this study, we present a novel MPEM-based technique for longitudinal monitoring of VP in preclinical colitis models. By tracking the extravasation of fluorescently labeled dextrans with different molecular weights, we successfully visualized VP and its progression over time in vivo within the same animal.

Our findings identified increased MPEM-detected VP as an early marker of colitis, present by day 2 of DSS treatment. This shows that alterations in VP detect colitis-associated changes in the gut earlier than other diagnostic methods. For example, traditional indicators, including immune cell infiltration, histologic scoring, and epithelial barrier disruption (via E-cadherin staining) only detected inflammation on days 4 and 7. Similarly, macroscopic signs such as body weight loss, elevated endoscopic scores, and colon shortening became significant only after 7 days. Consistent with our findings, Tolstanova et al³⁸ reported that Evans blue extravasation in colon biopsies from DSS-treated rodents preceded epithelial barrier permeability changes. As reported by Carloni et al,³² we found

Figure 6. (See previous page). Vascular leakage precedes the onset of macroscopic signs of inflammation in T cell transfer-induced colitis. (A) Experimental design. On day 0, Rag-deficient mice received naïve T cells ($n = 3$) for an adoptive T cell transfer-induced colitis model or PBS ($n = 3$) as controls (red arrow). On days 18, 21, 24, 28, and 31, post-transfer (black arrows), colitis score using endoscopy and VP were determined using MPEM. (B) Representative images of endoscopic scoring of inflammation (framed in red are timepoints with visible inflammation) and MPEM images of colon vasculature at upper (asterisk denoting area with leakage) and lower crypt regions (arrowheads indicating the dye accumulation in the lumina of crypts) post-i.v. injection of 70-kDa FITC-dextran. Scale bar, 100 μ m. (C) Plots show endoscopic scores and the relative VP. Each dot represents an individual mouse, and MPEM values reflect mean VP across 7 distal colon positions per animal. Comparisons are shown as means $\pm$ SDs. P values were calculated using the Wilcoxon signed rank test for the endoscopic score or 2-tailed unpaired t test for relative VP plots (* $P < .05$ and ** $P < .01$). Results are from 1 experiment. PBS, phosphate-buffered saline; SD, standard deviation.



that PV1 expression in DSS-treated mice was significantly upregulated as early as day 2. Collectively, our *in vivo* imaging study is consistent with findings of previous *ex vivo* studies and establishes VP as an early marker of colitis. It is a specific advantage of the MPEM method that it adheres to the 3R principle (refinement, replacement, and reduction) by minimizing animal usage to monitor microscopic changes during inflammation.

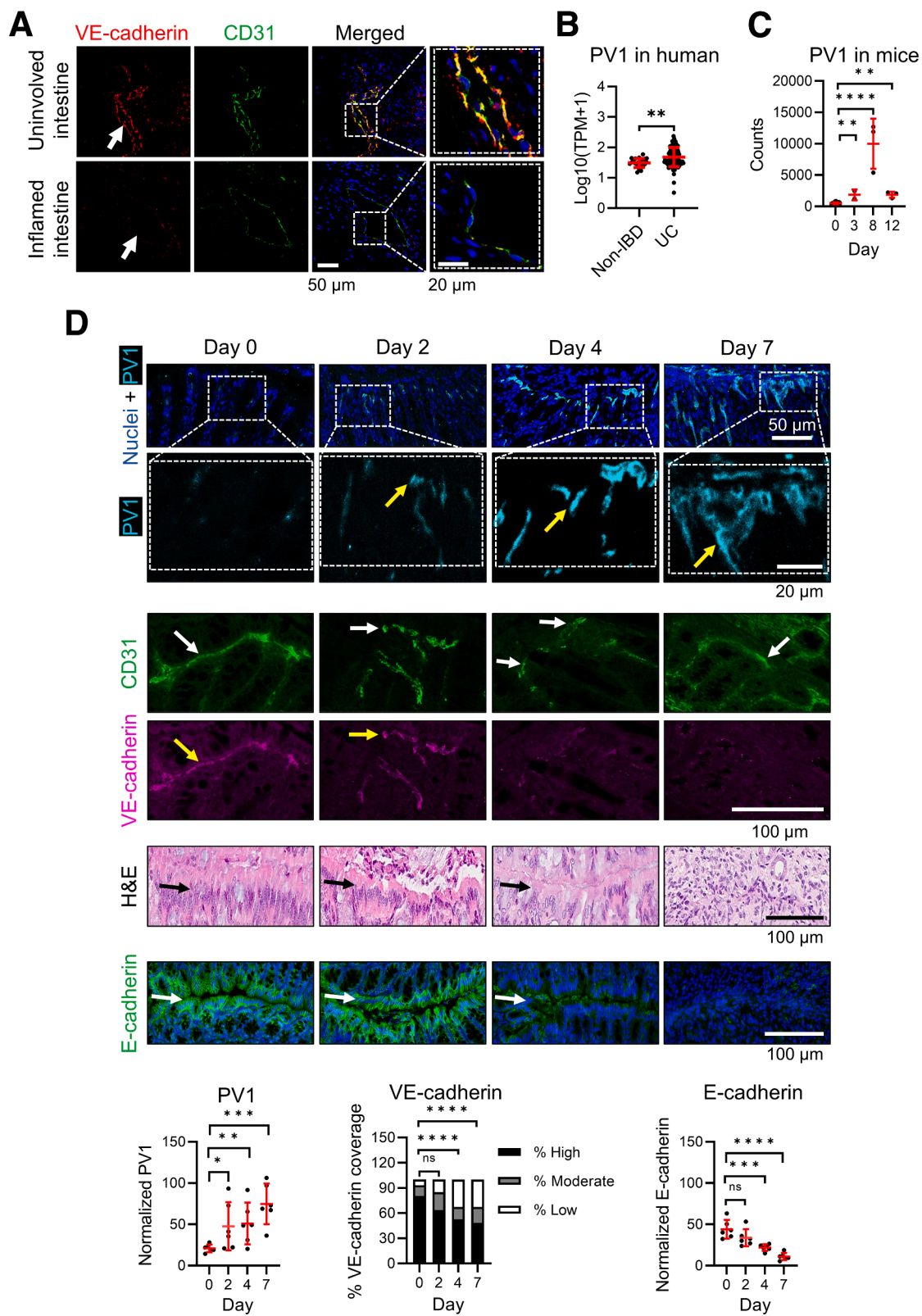
The sensitivity of MPEM to detect VP changes was successfully validated following a single enema of 50% ethanol. Despite the absence of visible epithelial damage, robust VP changes were detected. Ethanol can activate endothelial cells leading to increased monocyte adhesion and cytokine production, as previously shown *in vitro*.³⁹ Accordingly, the activated endothelial cells may independently initiate a proinflammatory response upon stimulation via substances that can permeate the epithelial cell barrier, further highlighting the role of the GVB in the pathogenesis of IBD. The early increase in VP in the T cell transfer-induced colitis model revealed that mucosal VP is not merely a response to chemical and luminal triggers but also a consequence of disrupted T cell homeostasis elicited by the implantation of naïve T cells into T cell-deficient recipients.³⁰ Although inflammation in the T cell transfer model is thought to originate in the lamina propria, early infiltration of transferred T cells also occurs in upper subepithelial regions along the crypt axis, where they form clusters with dendritic cells prior to overt colitis.⁴⁰ Local immune activation, for example, by microbiota-derived signals, in these superficial compartments likely promotes Th1 cytokine production (eg, IFN- γ and TNF- α), macrophage recruitment, and endothelial activation, thereby contributing to the earlier and more pronounced VP observed in the upper crypt layer.^{31,41–43} Taken together, VP may establish a precise measure of inflammation, regardless of the cause, even in clinical settings where mucosal damage may not be apparent and flares might otherwise escape detection.

The high tissue-penetration depth of MPEM enables 3D tissue visualization, which allows optical dissection of the

mucosal layers and the identification of specific leakage sites. By assessing leakage patterns in 3 murine models, we established a correlation between dye leakage, dye deposition patterns, and the degree of inflammation. Diffuse dextran signals restricted to the luminal mucosa were associated with early or mild inflammation. In contrast, a more intense and diffusely distributed signal, accompanied by dye accumulation within the crypts, indicated increased epithelial barrier disruption. Interestingly, these changes in the VP pattern were closely related to PV1 expression, which increases early in the course of the disease, initially in the upper part of the mucosal tissue, and subsequently spreads to deeper tissue areas as the disease progresses. This and the observations that PV1 upregulation occurred at day 2, before the downregulation of VE-cadherin in endothelial and of E-cadherin in epithelial cells (day 4) indicated that VP in colitis may be initiated by PV1 and amplified by the combined effects of PV1-mediated transcellular and VE-cadherin breakdown-associated pericellular permeability. At the molecular level, both PV1 upregulation and VE-cadherin alterations are induced by vascular endothelial growth factor (VEGF), a well-known mediator of vessel permeability, which is expressed in colitis.¹⁴ VEGF-mediated activation of VEGF receptor 2 in endothelial cells causes phosphorylation of VE-cadherin, resulting in its proteolytic cleavage and clathrin-mediated endocytosis.^{44–48} Moreover, VEGF induces the expression of caveolin.^{49,50} Caveolin is essential for PV1 stability and diaphragm formation and regulates VE-cadherin junction stability through modulation of VE-cadherin interaction with catenins.⁵¹ Accordingly, caveolin induction by VEGF may represent a molecular link between both processes.⁵² Monitoring 3D dye permeation patterns through MPEM includes both processes and allows for a detailed real-time assessment of mucosal inflammation and damage *in vivo*.

In clinical studies, an increased VP has also been observed during acute IBD, with a decrease during remission.¹³ Conventionally, permeability is assessed in the clinics by measuring the leakage of intravenously

Figure 7. (See previous page). Three-dimensional MPEM colonic vascular imaging as an *in vivo* indicator of mucosal damage severity and inflammation. (A) MPEM imaging was performed on days 0, 4, and 7 of 2.5% DSS treatment following intravenous injection of 70-kDa FITC-dextran. A 3D image stack of the distal colon was reconstructed to visualize spatial patterns of vascular leakage across different mucosal layers. Representative images show orthogonal views of the Z-stacks (XY, YZ, and XZ perspectives) for upper and lower crypt regions. The slice positions within the stack are indicated (*upper, yellow; lower, magenta*). Asterisks indicate diffusely distributed dextran in areas with perivascular leakage. Arrowheads indicate dextran accumulation in the crypt lumen. Scale bars, 100 μ m. (B) IVM imaging of vascular leakage in healthy mice and mice treated with 3% DSS for 7 days after i.v. coinjection of 70-kDa FITC-dextran (*green*, permeability marker) and lectin (*magenta*, vessel marker). Representative orthogonal images are shown. Asterisks indicate diffusely distributed dextran in areas with perivascular leakage. Arrowheads indicate dextran accumulation in the crypt lumen. Scale bars, 100 μ m. (C) Representative pictures of VP in non-IBD individuals ($n = 6$) and patients with UC with active ($n = 6$) or remittent colitis ($n = 6$), visualized using pCLE after intravenous fluorescein injection. Scale bar, 50 μ m. Arrows indicate dye accumulation in colon crypts. (D) Model of vascular leakage progression: A lateral schematic of the colon wall represents vascular and epithelial barrier integrity in healthy and inflamed states. In healthy colons, mucus, epithelial, and endothelial barriers maintain intestinal barrier function. DSS-induced inflammation disrupts epithelial integrity and crypt microarchitecture while impairing mucus production. Early-stage inflammation initiates leakage from vessels just below the mucosal surface (see A; day 4), likely driven by endothelial barrier activation due to bacterial invasion and local cytokine production. Epithelial integrity is only very slightly compromised in the early phase. At later stages, epithelial integrity is severely compromised, and vascular leakage increases. Dye accumulation in crypt lumina (A; day 7) suggests that the epithelial barrier remains intact at the crypt base but is breached apically, allowing dye to enter crypt spaces. Scale bars, 100 μ m. Diagram created with [BioRender.com](https://www.biorender.com).



injected fluorescein via pCLE.^{13,16,17} This tracer has a low molecular weight of only 0.332 kDa, and Spadoni et al¹⁰ reported that molecules up to 4 kDa diffuse freely through the GVB even in the healthy state. Our current work extends this finding to 10 kDa (Figure 1A and B). During inflammation, we and others observed permeability to 70-kDa molecules,^{10,13–15} and this study showed that molecules up to 500 kDa also leaked as a consequence of disease induction, much prior to when macroscopic signs of inflammation became visible. Optimizing dye size may further improve the sensitivity and specificity of clinical monitoring in patients with IBD.

The limited imaging depth is a clear disadvantage of pCLE, specifically under consideration of the thicker mucosal layer in humans compared with that in mice. The use of MPEM may overcome this shortcoming, but the translation of the MPEM imaging method from rodent models to human patients presents challenges, including the availability of 2-photon excitation endoscopes and flexible imaging probes. Nevertheless, these challenges can be overcome through continued innovation in medical imaging technology, similar to MPEM, which, according to our findings, may evolve to be an important tool for the clinical detection and monitoring of IBD.

Overall, our results suggest that MPEM-based VP scoring may be a sensitive and precise marker for early diagnosis and flare prediction in patients with IBD. Although quantitative analysis of individual cytokines or chemokines may appear more cost-effective, such approaches typically require tissue biopsies and repeated sampling, making them poorly suited for minimally invasive, repeated, longitudinal monitoring. Moreover, detection of individual cytokines or chemokines does not fully recapitulate the complexity of the inflammatory process or reliably reflect their functional activity within different tissue microenvironments. In contrast, MPEM-based VP imaging directly reports the functional vascular response

in vivo, providing a real-time, minimally invasive readout that can be repeatedly assessed over time.

Importantly, the observation that VP increases at very early stages of disease, together with our previous findings that endothelial cell-specific knockout of the IFN- γ receptor or pharmacologic treatment with imatinib or an anti-VEGF-antibody (BV13) reduces VP and ameliorates colitis in murine models,¹³ suggests that early detection of VP may define a therapeutic window before the onset of overt inflammation. This concept is further supported by the increasing number of pharmacologic agents targeting pathologic VP, including razuprotafib and PMC-403, which are currently under clinical or preclinical evaluation.^{53–55}

Overall, our results suggest that MPEM-based VP scoring holds great promise as a sensitive and precise marker for early diagnosis and flare prediction in patients with IBD. When integrated into routine clinical testing, this approach could significantly improve the accuracy of inflammation assessment, refine disease monitoring and treatment strategies, and reduce the need for invasive biopsies in the clinical management of IBD.

Materials and Methods

Study Approval and Participants

The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and received approval from the Ethics Committee of Friedrich-Alexander-Universität Erlangen-Nürnberg and the Medical Faculty Institutional Review Board. All patients gave their written informed consent prior to the pCLE examination.

Probe-Based Confocal Laser Endomicroscopy in Patients

Adult patients with active UC (n = 6), remittent UC (n = 6), and without IBD (n = 6) underwent colonoscopy. A standardized pCLE system was used for endomicroscopy,

Figure 8. (See previous page). Upregulation of VP channel protein PV1 precedes epithelial barrier disruption. (A) Human IBD and matched noninvolved intestinal tissues (n = 11) were stained for VE-cadherin (red, arrows) and CD31 (green). Clinical characteristics are listed in Table 2. Scale bar, 50 μ m. (B) Transcriptomic analysis of colon biopsies from non-IBD individuals (n = 22) and patients with UC (n = 96), obtained from the TRR241 IBDome cohort (Table 3), showed significantly elevated PV1 expression in UC samples. *P* values were calculated using the Mann-Whitney test (***P* < .01). Each dot represents an individual patient. Data are presented as means $\pm$ SDs. (C) PV1 expression was assessed using bulk RNA sequencing of colon tissue from healthy control mice and 3% DSS-treated mice at various timepoints (n = 3 for days 0, 8, and 12; n = 2 for day 3). PV1 read counts are shown. Each dot represents an individual mouse. Data are shown as means $\pm$ SDs. *P* values were adjusted using the Benjamini-Hochberg method and are reported as *Padj* (***Padj* < .01, *****Padj* < .0001). (D) Immunofluorescence staining of PV1 (cyan, first and second rows), costaining of CD31 (green, third row) and VE-cadherin (magenta, fourth row), as well as H&E (fifth row) and E-cadherin (green, sixth row) staining were performed on consecutive colonic sections from 2.5% DSS-treated mice on days 0, 2, 4, and 7, (n = 6 per timepoint). PV1-expressing vessels are marked with yellow arrows. Enlarged views (dashed white outlines) highlight vascular staining within the colonic mucosa. CD31-positive vessels are marked with white arrows, whereas those coexpressing VE-cadherin are indicated by yellow arrows in the fourth row. Black and white arrows in the fifth and sixth rows mark the epithelial barrier. Scale bar, 100 μ m. Normalized PV1 (left) and E-cadherin (right) signals are shown; each dot represents the average signal per mouse. *P* values were calculated using a 1-tailed unpaired *t* test (**P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001). The middle plot shows the percentage of VE-cadherin coverage in CD31-positive vessels, categorized as high (black), moderate (gray), or low (white). *P* values were analyzed using the χ^2 test comparing each timepoint with day 0, with Bonferroni correction and are reported as *Padj* (*****Padj* < .0001). H&E, hematoxylin and eosin; SD, standard deviation.

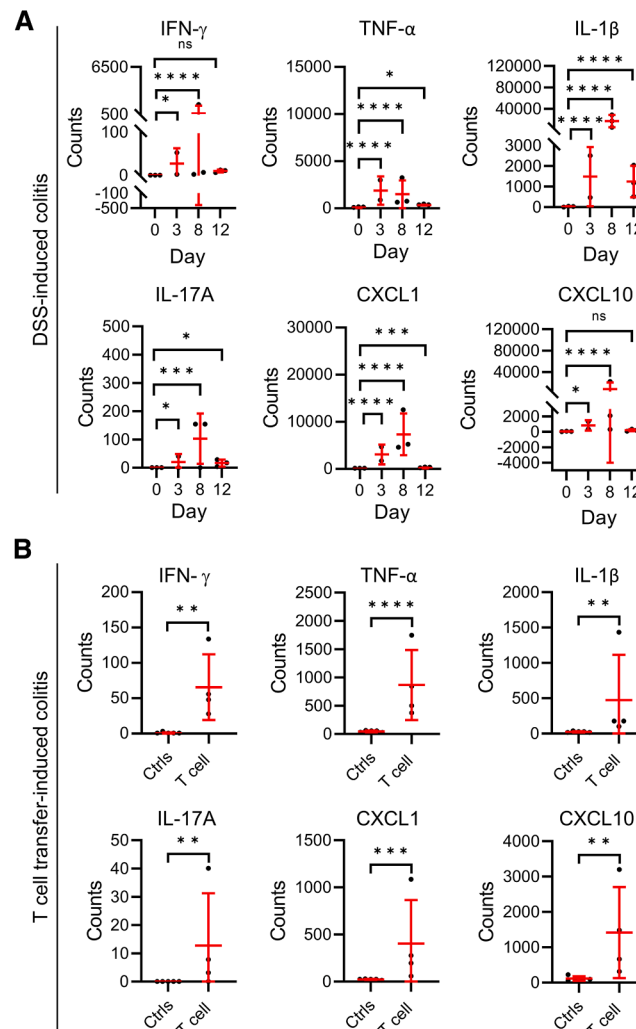


Figure 9. Pro-inflammatory vasoactive cytokines and chemokines are upregulated in DSS and T cell transfer colitis murine models. The expression of endothelial cell activating cytokines (IFN- γ , TNF- α , IL-1 β , and IL-17A) and chemokines (CXCL1 and CXCL10) were assessed using bulk RNA sequencing of (A) colonic tissues from healthy control mice (day 0, $n = 3$) and DSS-treated (3%) mice at various timepoints (days 3, 8, and 12, all $n = 3$ except day 3 with $n = 2$) or (B) colonic tissues from healthy control (Ctrl) mice ($n = 5$) and T cell transfer colitis mice ($n = 4$). (A) Gene read counts are shown across different timepoints for the DSS colitis model; (B) healthy and diseased tissues are compared in the T cell transfer colitis model. Each dot represents an individual mouse. Data are shown as means $\pm$ SDs. P values were adjusted using the Benjamini-Hochberg method and are reported as P_{adj} (* $P_{adj} < .05$, ** $P_{adj} < .01$, *** $P_{adj} < .001$, and **** $P_{adj} < .0001$). CXCL1, C-X-C motif chemokine ligand 1; CXCL10, C-X-C motif chemokine ligand 10; IL-1 β , interleukin-1 β ; IL-17A, interleukin-17A.

consisting of a portable laser station (Cellvizio) and confocal miniprobes (ColoFlex UHD, Mauna Kea Technologies). After reaching the cecum, 5 mL of 10% fluorescein solution was i.v. injected as a contrast agent, and confocal imaging was performed at the cecum and rectosigmoid junction. At each site, a CLE video of at least 2 minutes in duration was recorded with an image acquisition rate of 8 frames per second.

Mice

Experiments were conducted according to German law and approved by the Institutional Animal Care and Use Committee of the University of Erlangen and the Animal

Experiment Committee of the State Government of Lower Franconia, Würzburg, Germany.

Age- and sex-matched 8- to 12-week-old littermate C57BL/6 mice were used. Wild-type mice were either purchased from Charles River Laboratories (#C57BL/6NCRL) and acclimated for approximately 12 days or bred locally. B6.129S7-Rag1^{tm1Mom}/J (Rag1^{-/-}) mice, bred in-house, were used for T cell transfer experiments. Experimental and control groups were allocated blindly. Mice were housed separately for DSS experiments and cohoused for T cell transfer and ethanol experiments, with a maximum of 5 animals per cage. All mice had ad libitum access to a standard diet and water, were maintained under specific pathogen-free conditions, and were screened per

Federation of European Laboratory Animal Science Associations guidelines.

Murine Models of Colitis

Dextran sodium sulfate-induced colitis. Acute colitis was induced by administering DSS (MP Biomedicals, #0216011090) concentrations of 2.5% (MPEM experiments) or 3% (IVM experiments) in the drinking water for 7 days, followed by normal drinking water for 2 to 3 days.²⁸ Control mice received normal drinking water.

Ethanol-induced vascular permeability. Sedated mice were intrarectally administered 100 μ L of 50% ethanol (1:2, ethanol:water for injection). Control mice received water only.

T cell transfer-induced colitis. Naïve CD4⁺ splenic T cells were isolated from C57BL/6J splenocytes using a naïve CD4⁺ T cell isolation kit (Miltenyi Biotec, #130-104-453). Experimental Rag1^{-/-} mice were injected intraperitoneally with 1×10^6 cells in 100 μ L phosphate-buffered saline, whereas controls received phosphate-buffered saline only.

In Vivo Multiphoton Microscopy Imaging of Colonic Vasculature

We used a compact, rack-mounted, side-view MPEM equipped with a 780-nm femtosecond fiber laser, galvanometric scanners, and an electrically tunable lens to rapidly shift the focal plane in z-axis. The endoscope head is a graded index-based needle objective with a reflective prism at the tip; during colonoscopy, the prism is placed in direct contact with the mucosa, enabling noninvasive optical sectioning of the colon wall in vivo. With this configuration, we routinely acquire 3D image stacks (field of view, $290 \times 290 \mu\text{m}$; axial depth, ~ 100 – $120 \mu\text{m}$; spacing, $3 \mu\text{m}$) (Figure 1C–E).

The system was originally established for label-free autofluorescence/second-harmonic generation imaging.^{23,24} Here, we optimized it for longitudinal in vivo quantification of VP after i.v. injection of fluorescently labeled dextran (FITC or tetramethylrhodamine isothiocyanate [TRITC]; 10, 70, or 500 kDa at 0.5, 0.75–1.5, and 3.75 mg per mouse, respectively; Sigma-Aldrich #FD10S, #90718, #52194) (Figure 1A). Anesthesia was induced with 3% isoflurane (2 L/min; CP-Pharma) and maintained at 2% to 2.5%. Before MPEM, colons were flushed and inspected using endoscopy. Eye ointment was applied during imaging. Image stacks were acquired at 5 and/or 40 to 45 minutes postinjection at 7 distal colonic sites.

Acquisition Standardization

To ensure comparability across imaging sessions, laser power and all microscope settings were kept identical throughout each experiment. The same acquisition parameters were used for all mice and days: excitation at 780 nm; emission filters of 525/20 (FITC) and 571/72 (TRITC); image size of 512×512 pixels; photomultiplier tube (PMT) gain of 0.8 to 0.87 for both channels; and a z-spacing of 3

μm between planes. Laser power and PMT gain were determined on day 0 and maintained for all subsequent timepoints. The 3D image stacks were acquired at 5 minutes and/or 40 to 45 minutes after dextran injection at 7 distal colonic sites. Details of the fluorescence quantitation workflow and the derivation of the relative VP value are provided in the following subsection. The full image-analysis code is publicly available for transparency and reuse (see Data Availability).

Quantification of Vascular Permeability in Multiphoton Endomicroscopy-Acquired Images

MPEM-acquired images from mice injected i.v. with fluorescently labeled dextrans were quantified to derive a relative VP metric. For this purpose, we developed a custom semiautomated Fiji/ImageJ⁵⁶ (<https://fiji.sc/>, v2.3.0) macro (see Data Availability). For each image, the user selects representative “upper” and “lower” planes from the Z-stack and places 5 predefined ROI pairs (outer ROI, intravascular; inner ROI, perivascular) on vessels (Figure 1D and E). The inner ROI captures the signal in the extravascular space to measure vascular leakage, whereas the outer ROI captures the total local signal (including both extravascular and intravascular signals) for normalization. Due to biological variability in vessel architecture and diameter, a single ROI size cannot precisely match all vessels. Users were therefore instructed to place the ROI pairs such that the inner ROI did not overlap with the vessel lumen, whereas the outer ROI included the intravascular signal and surrounding background. After ROI placement by the experimenter, the macro automatically measures intensities (all channels if multiple dyes were coinjected), calculates relative VP (ratios of inner ROI to outer ROI values) and averages from 5 ROI pairs at each position, exports the results to an Excel file, and proceeds automatically to the next position until all files in the selected folder are processed. Quantification requires approximately 30 seconds per position. For each mouse, relative VP was calculated as the average of measurements from 7 positions and plotted for the upper or lower crypts separately (Figure 1E). In cases of extensive leakage, particularly in the upper crypt layer, as illustrated in Figure 1E, leaked tracer may saturate the surrounding tissue, making complete separation of intra- and extravascular signals difficult. Under these conditions, the measured relative VP reflects a high-permeability state rather than precise compartmental separation.

Intravital Microscopy

IVM was performed on day 9 of DSS treatment, as previously described by Langer et al.¹³ In brief, sedated mice were intravenously injected with 70 kDa FITC-dextran (0.75 mg; Sigma-Aldrich, #90718) and DyLight 649-lectin (100 mg; Invitrogen, #L32472) as VP and blood vessel markers. The distal colon was excised, longitudinally incised, and in vivo imaged using confocal laser-scanning microscopy (TCS SPE, Leica Microsystems) using 488- and 650-nm channels.

Table 2. Cohort of Patients With IBD Subjected to VE-Cadherin Staining (Figure 8A)

Patient	Sex	Age, y	Disease	Location
1	F	23	Crohn's disease	Ileocecal
2	F	29	Crohn's disease	Sigmoid colon
3	F	46	Crohn's disease	Ileocecal; ileoascendostomy
4	M	25	UC	Colon
5	M	29	Crohn's disease	Ileocecal
6	M	22	Crohn's disease	Ileocecal; ileoascendostomy
7	M	23	Crohn's disease	Jejunum; vermiform appendix
8	F	22	Crohn's disease	Ileocecal; terminal ileum
9	M	36	Crohn's disease	Ileocecal; ileum
10	M	33	Crohn's disease	Jejunum; ileum; sigmoid colon
11	M	60	Crohn's disease	Sigmoid colon; ileotransversostomy

NOTE. Classification of inflammation status (inflamed vs uninvolved) was based on macroscopic tissue evaluation performed by a pathologist after surgery.

F, female; IBD, inflammatory bowel disease; M, male; UC, ulcerative colitis; VE, vascular endothelial.

Endoscopic Score

Gross mucosal damage and inflammation in the distal colon were assessed using a high-resolution mini-endoscope (Karl-Storz) in all 3 murine models at various time-points, scored using a grading system adapted from Becker et al,⁵⁷ and plotted as endoscopic score.

Colon Harvesting in Mice

Colon lengths were measured from below the cecum to 3 mm above the anus and plotted in centimeters. The most distal 1 cm was used for flow cytometry; the remaining tissue was Swiss-rolled, fixed in 10% formalin for 18 hours at 4 °C, embedded in paraffin to prepare formalin-fixed paraffin-embedded (FFPE) blocks, and sectioned (4 µm) for staining.

Histologic Scoring of Colitis

Hematoxylin (hematoxylin solution modified according to Gill III, Sigma-Aldrich, #1.05174) and eosin (Eosin Y Solution Alcoholic, Sigma-Aldrich, #HT110132)-stained colon sections were scanned using a Hamamatsu Nano-Zoomer S60 Digital slide scanner and evaluated by a blinded pathologist using NDP.view software (v2.9.29, Hamamatsu Photonics). Our histologic scoring of colitis was developed by combining different criteria^{25–27} (Table 1) as follows: assessing the degree of colitis involvement at 1.25× to 5× magnification (Figure 3E). Involved areas were then analyzed at 10× to 80× magnifications to evaluate inflammatory cell infiltration severity and extent, epithelial changes (goblet cell loss, cryptitis, crypt abscess, erosion), mucosal architecture, and edema extent (Figure 3E). Scores (Table 1) were summed and plotted to assess colitis progression at days 0, 2, 4, and 7 post-DSS administration.

Immunostaining

Immunofluorescence staining. FFPE intestinal tissue sections of patients with IBD (n = 11) (Table 2) and

murine colon sections were used for immunofluorescence staining. For VE-cadherin, CD31, and E-cadherin staining, antigen retrieval solution at pH 9 (Agilent Dako, #S2367) was used, whereas pH 6 (Agilent Dako, #S1699) was used for PV1 staining. Sections were incubated with the following primary antibodies: rabbit anti-human VE-cadherin (1:100, Abcam, #ab33168) and mouse anti-human CD31 (20.5 µg/mL, Dako, #M0823) for 1 hour at room temperature, or overnight at 4 °C for goat anti-mouse VE-cadherin (1:100, R&D Systems, #AF1002), rat anti-mouse CD31 (1:100, Dianova, #DIA-310), rabbit anti-mouse E-cadherin (1:1000, Cell Signaling, #96743), and rat anti-mouse PV1 (1:500, BD Pharmingen, #550563). Secondary antibodies were AF546-conjugated donkey anti-rabbit (1:500, Thermo Fisher Scientific, #A10040), AF488-conjugated donkey anti-mouse (1:500, Thermo Fisher Scientific, #A21202), cyanine3 F(ab')₂ fragment rabbit anti-goat (1:500, Jackson ImmunoResearch, #305-166-047), rabbit

Table 3. IBDome Cohort of Patients with UC and Non-IBD controls (Transcriptome Analysis Figure 8B)

Disease	Inflammation status	No.
Non-IBD	Inflamed	22
	Noninflamed	0
UC	Noninflamed	22
	Inflamed	96
	Noninflamed	66
	NA	28
		2
Total		118

NOTE. PV1 expression was screened using the IBDome (v0.6.4) online tool. Colonic samples from patients with UC (n = 96) and non-IBD individuals (n = 22) were included in our study.

IBD, inflammatory bowel disease; NA, not available; PV1, plasmalemma vesicle-associated protein; UC, ulcerative colitis.

anti-rat AF488 (1:500, Invitrogen, #A21210), AF488-conjugated donkey anti-rabbit (1:500, Thermo Fisher Scientific, #A21206), or AF546-conjugated donkey anti-rat (1:500, Thermo Fisher Scientific, #A48270) and incubated for 1 to 2 h at room temperature. Nuclei were counterstained with either 4',6-diamidino-2-phenylindole (1:5000, Molecular Probes, #D-21490) or DRAQ5 (1:800, Cell Signaling, #4084). Images were acquired using a confocal laser-scanning microscopy system (TCS SPE, Leica Microsystems) or Leica DM6000B (Leica Microsystems) epifluorescence microscope.

Plasmalemma vesicle-associated protein and epithelial cadherin quantification. PV1 and E-cadherin immunofluorescence stainings were performed on consecutive colon tissue sections. Signal intensities were quantified in Fiji/ImageJ by measuring the mean gray value within defined ROIs in the PV1 or E-cadherin channels. These values were normalized to the corresponding 4',6-diamidino-2-phenylindole-positive nuclei counts within the same ROIs. For each colon, a minimum of 5 images were analyzed, and the average signal per mouse is shown in Figure 7D.

Vascular endothelial cadherin quantification. Tissue sections were costained for CD31 and VE-cadherin. Blood vessels were identified based on CD31 positivity, and their total number was recorded. Only the blood vessels located in the mucosal layers, in particular the sub-epithelial ones, were considered. Each vessel was then assessed for VE-cadherin coverage and categorized as high (70%–100%), moderate (40%–70%), or low (0%–40%). Results were expressed as the percentage of vessels falling into each category relative to the total vessel count per optical field. For each mouse, 10 optical fields were analyzed per colon section, yielding approximately 30 vessels per sample ($n = 6$ mice per timepoint for days 0, 2, 4, and 7).

Immunohistochemical staining. FFPE murine colon sections were used for immunohistochemical permanent staining. For NK cell (Nkp46) staining, antigen retrieval was performed using a pH 9 retrieval solution (Agilent Dako, #S2367). Blocking steps included endogenous peroxidase blocking with 7.5% H_2O_2 for 10 minutes, followed by endogenous biotin blocking using an avidin/biotin blocking kit (Biozol, #SP-2001). Sections were incubated overnight at 4 °C with the primary antibody goat anti-mouse Nkp46/NCR1 (1:500, R&D Systems, #AF2225), diluted in antibody diluent solution (Zytomed, #ZUC025-500). Subsequently, VECTASTAIN Elite ABC-HRP Kit, Peroxidase (Goat IgG) (Vector Laboratories, #PK-6105) was used. Sections were incubated with the biotinylated anti-goat secondary antibody for 30 minutes, followed by incubation with the ABC reagent for 30 minutes, both at room temperature. Signal detection was performed using NovaRed substrate kit (Vector Laboratories, #SK-4800) with a 20-minute incubation at room temperature. Nuclei were counterstained with hematoxylin. Images were acquired using Leica DM6000 light microscope (Leica Microsystems).

Natural killer cell quantification. NK cell populations were quantified in each image by counting Nkp46+ cells and reporting them as a percentage of the total number of cells (hematoxylin-stained nuclei) present in the same image. For each colon, a minimum of 5 images were analyzed, and the average value per mouse is shown in Figure 3E.

Flow Cytometry

Single-cell suspensions were prepared from the most distal 1-cm colon segments. Cells were stained with the following antibodies (dilution 1:200) and incubated at room temperature for 20 minutes: anti-CD326-BV650 (BD Biosciences, #740559), anti-Ly6C-FITC (BD Biosciences, #553104), anti-CD11b-BUV737 (BD Biosciences, #612800), anti-I-A/I-E-BV711 (BD Biosciences, #563414), anti-F4/80-BV510 (BD Biosciences, #743280), anti-Ly6G-BUV395 (BD Biosciences, #563978), anti-CD19-BV421 (BD Biosciences, #562701), anti-CD45.2-BV786 (BD Biosciences, #563686), and anti-CD3-PerCP-Cy5.5 (BD Biosciences, #560527). Cells were characterized as epithelial cells (CD326+), leukocytes (CD45+), macrophages (CD45+ MHCII+ F4/80+), neutrophils (CD45+ Ly6Ghigh), T cells (CD45+ CD3+ F4/80-), or B cells (CD45+ CD19+ Ly6G-). Data were acquired using a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (v10.8.1, FlowJo LLC).

Transcriptome Analysis

PV1 messenger RNA (mRNA) expression in humans was obtained from the IBDome⁵⁸ cohort (v0.6.4) using colon samples from patients with UC ($n = 96$) and non-IBD controls ($n = 22$) (Table 3).

For mice, PV1, IFN- γ , TNF- α , interleukin-1 β , interleukin-17A, C-X-C motif chemokine ligand 1, and C-X-C motif chemokine ligand 10 mRNA expression was initially screened using SEPIA transcriptomic database,³⁷ followed by downloading the publicly available original data of the DSS experiment from Patankar et al³³ and from Gonzalez-Acera et al³⁷ for the T cell transfer experiment. As described by Patankar et al, for the DSS experiment, 10-week-old C57BL/6 mice received 3% DSS in drinking water for 8 days, followed by normal water. Colon tissues were collected at days 0, 3, 8, and 12 ($n = 3$ per timepoint; $n = 2$ for day 3). For the T cell transfer experiment, as described by Gonzalez-Acera et al, 8- to 12-week-old Rag1 knockout mice were used. An amount of 1×10^6 naïve T cells was injected intraperitoneally, and colon tissues were collected 3 weeks post-transfer (healthy controls, $n = 5$; T cell-injected mice, $n = 4$). Colon tissues from both experiments were processed for bulk RNA sequencing.

Dextran Preparations

The 10- and 70-kDa FITC-dextran (Sigma-Aldrich, #FD10S and #90718, respectively) were dissolved in normal saline solution at concentrations of 10 and 15 mg/mL, respectively, whereas the 500-kDa TRITC-dextran

(Sigma-Aldrich, #52194) was prepared at a concentration of 75 mg/mL by dissolving it in a saline solution with the pH adjusted to 8 to 8.2. The solution was kept in a water bath for 10 minutes at 45 °C to increase its solubility.

Dextran Concentration Measurements in Mice Sera

FITC- and TRITC-dextran concentrations in mice sera were measured at 5, 10, 20, and 60 minutes following i.v. injection in healthy C57BL/6 mice. Individual mice were used to test each dye concentration separately at each timepoint ($n = 2$ per timepoint per dye). Mice were euthanized by cervical dislocation at the above timepoints, and a volume of 500 μ L of blood was collected by cardiac puncture, which was subsequently centrifuged at 3000 $\times g$ for 15 minutes to obtain the serum fraction. Serum fluorescence was measured using a fluorescence plate reader with excitation and emission wavelengths of 490 nm and 525 nm for FITC-labeled dextrans and 550 nm and 575 nm for TRITC-labelled dextran. Subsequently, dextran concentrations were determined from a standard curve generated with known concentrations of the respectively labeled dextrans diluted in human serum.

Single-Cell Isolation From Murine Colon Tissues

Single-cell suspensions were obtained as follows: the most distal 1-cm segments of freshly harvested colons were excised and subjected to enzymatic digestion with 450 U/mL collagenase I (Sigma-Aldrich, #C0130), 60 U/mL collagenase XI (Sigma-Aldrich, #C7657), 60 U/mL hyaluronidase (Sigma-Aldrich, #H1115000), 60 U/mL DNase I (Sigma-Aldrich, #D5319), and 20 mmol/L Hepes (Thermo Fisher Scientific, #15630080) for 1 hour at 37 °C while shaking. Digested colon tissues were homogenized through a 40- μ m cell strainer.

Statistical Analyses

Data normality was assessed using the Shapiro-Wilk test. For comparisons across timepoints in the same mice, either a 1- or 2-tailed paired t test (for normally distributed data) or the Wilcoxon matched-pairs signed-rank test (for non-normal data) was used, as specified in figure legends. For comparisons between different groups of mice, 2-tailed unpaired t tests or Mann-Whitney tests were applied, depending on data normality. When all control values were 0, the Wilcoxon signed-rank test was used. For multiple comparisons involving non-normally distributed data, the Kruskal-Wallis test was applied. Correlations were assessed using Spearman's rank correlation. Statistical significance was defined as $P < .05$, with the following thresholds: $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$. For differential expression analyses, P values were adjusted using the Benjamini-Hochberg method and reported as $Padj$, with significance defined as $Padj < .05$ ($*Padj < .05$, $**Padj < .01$, $***Padj < .001$, and $****Padj < .0001$). For contingency analysis, χ^2 tests were used to compare each timepoint with day 0, applying Bonferroni correction, and the results are reported as $Padj$. All analyses were performed

using GraphPad Prism (v9.5.1), except for data processed via IBDome and SEPIA web tools.

Declaration of Generative AI in Scientific Writing

During the preparation of this work, the authors used Grammarly and ChatGPT only to improve the language and readability of the manuscript. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the publication's content.

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Conflicts of interest

The authors disclose no conflicts.

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Data Availability

Study materials will be made available to other researchers on request. Human transcriptomic data was obtained from the IBDome cohort (v0.6.4), available at <https://ibdome.org>. Mouse transcriptomic messenger RNA expression levels

were derived from the Shared Experimental Inflammation Data webtool (v0.3.6), available at <http://trr241.hosting.rze.uni-erlangen.de/SEPIA/>. The original sequencing data of the DSS experiment was obtained from Patankar et al and from Gonzalez-Acera et al for the T cell transfer model and finally downloaded from the European Nucleotide Archive through ArrayExpress (<https://ebi.ac.uk/biostudies/arrayexpress>), with accession numbers E-MTAB-9850 and E-MTAB-14306, respectively. Quantification of vascular permeability in multiphoton endomicroscopy images was performed using the following macro (<https://github.com/LucasKreiss/VascularPermeability>).