Interferon Gamma Counteracts the Angiogenic Switch and Induces Vascular Permeability in Dextran Sulfate Sodium Colitis in Mice

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Background: Interferon (IFN)-γ is a central pathogenesis factor in inflammatory bowel disease (IBD) with pleiotropic effects on many different cell types. However, as yet, the immune modulatory functions of IFN-γ in IBD have been predominantly investigated. Based on previous studies showing that IFN-γ acts antiangiogenic in colorectal carcinoma, we investigated the effects of IFN-γ on the vascular system in IBD.

Methods: Colon tissues of patients with IBD and dextran sulfate sodium–induced colitis in mice were subjected to immunohistochemistry, quantitative real-time polymerase chain reactions, and in situ hybridization to quantify cell activation, angiogenesis, and immune responses. Vascular structure and permeability in mice were analyzed by ultramicroscopy and in vivo confocal laser endomicroscopy.

Results: We showed a significantly increased blood vessel density in IBD and dextran sulfate sodium colitis. In mice, this was associated with a disorganized blood vessel structure and profound vascular leakage. As compared with genes associated with angiogenesis, genes associated with inflammatory cell activation including IFN-γ were more strongly upregulated in colitis tissues. IFN-γ exerted direct effects on endothelial cells in IBD tissues in vivo, as indicated by the expression of IFN-γ–induced guanylate binding protein 1 (GBP-1). Neutralization of IFN-γ in the acute dextran sulfate sodium colitis model demonstrated that this cytokine exerts endogenous angiostatic activity in IBD and contributes to increased vascular permeability.

Conclusions: The dissection of the pleiotropic activities of IFN-γ in IBD provides new insights to the pathological functions of this cytokine and may be of high relevance for the optimization of combination therapy approaches.

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Key Words: interferon-γ, IBD, angiogenesis

Inflammatory bowel disease (IBD) is an intermittent chronic inflammatory disorder of the gastrointestinal tract affecting roughly 2.2 million patients in Europe and about 1 million patients in the United States.[1] The 2 major clinically relevant forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Histologic hallmarks of IBD include leukocyte infiltration, mucosal damage, ulcerations, edema, and an increase in the blood vessel density.[2–4] Increased angiogenesis has been shown to directly correlate with disease severity in patients with CD and UC as well as in experimental colitis.[2,5] Nevertheless, the pathophysiologic function of

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angiogenesis in IBD is still poorly understood. For instance, genetic and pharmacological approaches targeting angiogenic growth factors in experimental colitis revealed conflicting results on the association of angiogenesis with disease progression. Neutralization of the vascular endothelial growth factor A in dextran sulfate sodium (DSS)–induced and 2,4,6-trinitrobenzenesulfonic acid (TNBS)–induced colitis resulted in a decreased vessel density and improvement of the disease.[6,7] Deficiency of placental growth factor was also associated with reduced angiogenic activity but, in contrast, failed to ameliorate colitis in the same experimental models.[8] Overall, these results suggest that not only the vessel number is of relevance for the outcome of the disease but also the quality of the vessels formed in diseased tissues. In fact, evidence has been provided that IBD vessels are immature and/or functionally defective, as indicated by a lack of pericytes, leakiness, and the formation of stenosis and hyperthrombic edema.[2] Accordingly, analyses of the phenotypes and functions of blood vessels within the complex network of angiogenic and inflammatory factors in the IBD microenvironment may provide new insights for the understanding of blood vessel functions in IBD pathogenesis.

Interferon (IFN)-γ is a multifunctional cytokine known to be highly upregulated in mucosal tissues of CD and UC and different murine model systems.[3,9,10] Due to its immune regulatory function in the differentiation and activation of macrophages and effector T cells, as well as its proapoptotic effects on epithelial cells, IFN-γ is supposed to perpetuate inflammation in the setting of IBD.[9,11]

Interestingly, IFN-γ has also profound effects on blood vessels. It has been shown that this cytokine inhibits the proliferation, migration, and invasion of human microvascular and macrovascular endothelial cells.[12–14] The inhibitory effects of IFN-γ have been confirmed by the identification of the large GTPase guanylate binding protein 1 (GBP-1) as the intracellular mediator of the angiostatic activities of IFN-γ.[12–14] Moreover, GBP-1 expression has been shown to indicate the activation of blood vessel endothelial cells by IFN-γ both in vitro and in vivo in different carcinomas and autoimmune disorders.[15–18] In patients with colorectal carcinoma, an IFN-γ–dominated micromilieu, characterized by prominent GBP-1 expression, was associated with a highly significantly decreased angiogenic activity and increased cancer-related 5-year survival of the patients.[12,17] In contrast, increased vessel numbers and high IFN-γ expression coincide in patients with IBD.

These clinical findings suggest that IFN-γ may either act differently on the vascular system in IBD and colorectal carcinoma or that the angiostatic effects of IFN-γ may be compensated by alternative effects of the specific disease-associated microenvironment. Both considerations would be of high relevance for the understanding of the pathogenesis mechanisms of IBD and for the development of specific therapy approaches. Therefore, we investigated in this study the effect of IFN-γ on the vascular system in IBD both in humans and mouse models.

MATERIAL AND METHODS

Patients

Formalin-fixed paraffin-embedded endoscopic biopsy specimens from patients with active or moderately active CD (n = 28) and UC (n = 23) and healthy control specimens (n = 14) were obtained from the Institute of Pathology (Bayreuth, Germany). All specimens were taken for routine purposes. The retrospective use of biopsies was approved by the Ethics Committee and the Institutional Review Board of the University of Erlangen-Nuremberg.

Animals and Treatment

Pathogen-free C57BL/6 mice (6–12 weeks old) were obtained from the central animal facility of Erlangen or The Jackson Laboratory (Bar Harbor, Maine). Acute and chronic DSS colitis was induced as previously described.[19] For neutralization of endogenous IFN-γ in the acute DSS colitis, 100 μg of a monoclonal rat anti-mouse IFN-γ antibody (αIFN-γ mAb; clone R4 6A2; BioXcell, Lebanon, New Hampshire) were injected intraperitoneally. Injections were performed daily for 5 days during the induction of colitis and every other day thereafter. Control groups received 100 μg of a rat IgG1 isotype control antibody directed against horseradish peroxidase (control mAb; clone HRPN; BioXcell). All animal experiments were reproduced at least twice with similar results. The animal studies were conducted at the University of Erlangen-Nuremberg, Germany, and approved by the State Government of Middle Franconia, Germany.

In Vivo Confocal Laser Endomicroscopy

Fluorescein isothiocyanate (FITC)–dextran (75 kDa) was injected intravenously, and vessel morphology and permeability were imaged with a confocal microscope (FIVE1; Optiscan, Victoria, Australia) as previously described.[20] Vascular permeability was quantified by measuring the total FITC-signal using the software ImageJ v1.45 (U.S. National Institutes of Health, Bethesda, MD).

Ultramicroscopy

Ultramicroscopic analysis of vessel perfusion and permeability was performed as previously described (for details, see Text, Supplemental Digital Content 1, http://links.lww.com/IBD/A975).[21]

Cell Proliferation Assay

C57BL/6 Mouse Small Intestinal Endothelial Cells (Cell Biologics, Chicago, IL) were seeded in triplicates in 24-well plates (Nunc; Thermo Fisher Scientific, Bonn, Germany) at a density of 4×10^3 cells per well (cell culture details, see Text, Supplemental Digital Content 1, http://links.lww.com/IBD/A975). Cells were treated with angiogenic growth factors ([10 ng/mL vascular endothelial growth factor A and 10 ng/mL basic fibroblast growth factor], Biolegend, San Diego, CA) and IFN-γ (100 U/mL, Biolegend) with or without mAbs as indicated. Medium including additives was renewed every 2 days. Five days

after stimulation, total cell numbers were determined using an automatic cell counter (Z2 Coulter Particle Count and Size Analyzer; Beckmann Coulter GmbH, Krefeld, Germany).

Western Blot Analysis

Western blotting was performed as described previously[22] using 10 μg of cell lysate per lane. Detection of murine GBP-1 was performed using a monoclonal rat anti-mouse GBP-1 antibody (clone 2C4, 1:10; see Text, Supplemental Digital Content 1, http://links.lww.com/IBD/A975 and Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/A976) as a primary antibody and a sheep anti-rat horseradish peroxidase–coupled secondary antibody (1:5,000, Dako, Hamburg, Germany).

Immunocytochemistry

Cells were seeded in gelatin-coated chamber slides (Nunc) at a density of 2×10^4 cells per well and 24 hours after treatment fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% triton-X100 for 30 minutes at room temperature. Nonspecific binding sites were blocked using 10% rabbit-normal serum (Dianova, Hamburg, Germany). A monoclonal rat anti-mouse GBP-1 antibody (clone 2C4; see Text, Supplemental Digital Content 1, http://links.lww.com/IBD/A975 and Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/A976 1:50, 1 h) was used as a primary antibody. Afterward, cells were incubated for 30 minutes with a biotinylated mouse absorbed rabbit anti-rat antibody, followed by 30 minutes of incubation with an avidin–biotin complex (ABC kit, Vector Laboratories, Burlingame, CA). The reaction was developed using the NovaRED Substrate Kit (Vector Laboratories). Slides were counterstained with Mayer's hemalaun (VWR International, Ismaning, Germany), dehydrated, and mounted in DPX mounting medium (Sigma-Aldrich, Taufkirchen, Germany).

Immunohistochemistry and Determination of the Vessel Density

Formalin-fixed paraffin-embedded tissue sections (4 μm) of human and mouse tissues were deparaffinized in xylol (Merck Chemicals, Darmstadt, Germany) and rehydrated in a descending ethanol series. After antigen retrieval in target retrieval solution pH 9.0 (TRS9; Dako), slides were treated with 7.5% hydrogen peroxide for 10 minutes at room temperature to block endogenous peroxidases. Endogenous biotin was blocked with the Vector biotin blocking kit (Vector Laboratories). The following primary antibodies were used: a monoclonal mouse anti-human CD31 antibody (5 μg/mL; Dako), a monoclonal rat anti-human GBP-1 antibody (Clone 1B1[16]; 1:300), a monoclonal rat anti-mouse CD31 antibody (clone SZ31; 0.5 μg/mL; Dianova), and a monoclonal rat anti-mouse GBP-1 antibody (clone 2C4; 1:50). Incubations were for 1 hour at room temperature. Subsequently, sections were incubated for 30 minutes with a mouse absorbed biotinylated rabbit anti-rat or a biotinylated horse anti-mouse IgG antibody and further processed as described for the immunocytochemistry. For CD31 immunofluorescence, an additional protein block (Dako) was performed before incubation with the primary antibody. Slides were incubated overnight at 4°C and further processed using the TSA Cyanine 3 System (Perkin Elmer, Shelton, CT). Nuclei were stained with DAPI, and slides were mounted with fluorescence mounting medium (Dako). The vessel density in murine colitis was quantified by determining the total pixel density of CD31 and DAPI staining in each image using the software ImageJ. The ratio of CD31 pixel density to DAPI pixel density for each image was used to calculate relative vessel densities. In human samples, the number of CD31-stained vessels per image was counted and normalized to the tissue area.

Isolation of mRNA and Quantitative Real-time Polymerase Chain Reaction

For gene expression analyses in mouse tissues, 2-step quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the SensiFAST SYBR No-ROX Kit (Bioline, Taunton, MA) in combination with QuantiTect Primer Assays (Qiagen, Hilden, Germany). Samples were normalized using the expression of hypoxanthine guanine phosphoribosyltransferase. For mRNA expression analyses in human specimens, total RNA was isolated from 10-μm formalin-fixed paraffin-embedded sections as previously described.[23] Primers and 5′-FAM-3′-TAMRA–labeled probes (see Table, Supplemental Digital Content 3, http://links.lww.com/IBD/A977) were designed using Primer Express 3.0 (Applied Biosystems, Life Technologies, Carlsbad, CA) or PrimerBlast (NCBI) and purchased at Eurogentec (Serain, Belgium). All primers/probes were checked for specificity using BLAST (NCBI). DNA contamination was excluded with a primer/probe mix for the progestagen-associated endometrial protein (PAEP) gene (gene ID 5047). Samples were normalized to the housekeeping gene ribosomal protein L37a (RPL37a) (NM_000998.4). qRT-PCR reactions were performed with the SuperScript III Platinum One-Step qRT-PCR kit with ROX (Invitrogen, Darmstadt, Germany). For all qRT-PCR analyses, the ΔΔCT-method was used for further calculations (for details, see Text, Supplemental Digital Content 1, http://links.lww.com/IBD/A975).

In Situ Hybridization

Specimens were processed as described previously.[24] The plasmid for probe synthesis consisted of a full-length cDNA of human IFN-γ (NM_000619.2) inserted into the pcDNA4/myc-His expression vector as previously described.[25] Transcription of ^{35}S-radioactively labeled RNA hybridization probes was performed with a T7 polymerase (Agilent Technologies, Böglingen, Germany). After autoradiography, sections were stained with Mayer's hemalaun (VWR International), dehydrated, and mounted in DPX mounting medium (Sigma-Aldrich). Signals were analyzed using a Leica DM6000 (Leica Microsystems, Wetzlar, Germany).

Statistical Analyses

Pairwise comparisons were performed using the Student's *t* test for parametric or Mann–Whitney test for nonparametric

analyses. Correlations were analyzed according to Spearman's correlation test. All statistical analyses were conducted using the GraphPad Prism software version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Blood Vessel Density Is Increased in Human IBD and DSS Colitis in Mice

Blood vessel content in colonic tissue samples of patients with IBD was measured using immunohistochemical detection of CD31 (Fig. 1A). Quantitative evaluation confirmed that the vessel density was significantly ($P \leq 0.001$) increased in IBD tissues as compared with normal control tissues (Fig. 1B). As an additional marker for blood vessel content and angiogenic endothelial cell activation,[26–28] the mRNA expression of the endothelial marker von Willebrand factor (vWF) was measured in consecutive sections of patients with IBD by qRT-PCR (Fig. 1C). Expression of vWF was significantly ($P \leq 0.001$) increased in CD and UC as compared with healthy colon tissues, indicating higher vascularization and/or angiogenic activity in these tissues.

The DSS-induced colitis in mice is commonly used as an animal model to investigate different aspects of human IBD, including angiogenesis.[5,7] To mirror acute and chronic inflammation, age- and gender-matched C57BL/6J mice were treated with 2% DSS for 1 (acute) or 3 (chronic) cycles (Fig. 1E). All DSS-treated mice developed intestinal inflammation (see Text, Supplemental Digital Content 1, http://links.lww.com/IBD/A975 and Fig., Supplemental Digital Content 4, http://links.lww.com/IBD/A978). In accordance with the observations in human IBD, the vessel density as determined by CD31 immunohistochemical staining was higher in acute and chronic DSS colitis as compared with untreated controls (Fig. 1D). Quantitative evaluation confirmed that the vessel density was significantly ($P \leq 0.001$) increased in diseased tissues (Fig. 1F).

Blood Vessels in DSS Colitis Exhibit a Disorganized Structure and Increased Permeability

Blood vessels in inflammatory tissues exhibit alterations.[2] Therefore, we characterized structure, perfusion, and permeability of the vascular network in acute and chronic DSS colitis. Perfused vessels were detected by intravenous injection of a fluorescence-coupled lectin, which binds to carbohydrate residues exposed on the surface of blood vessel endothelial cells (Fig. 2A). Although quantification of lectin deposition in defined tissue areas demonstrated comparable perfusion in DSS-treated mice and healthy controls (Fig. 2B), visualization of the 3-dimensional vascular network depicted massive structural alterations in colitis tissues (Fig. 2A). Although blood vessels in untreated mice were organized in a size-dependent manner with large vessels at the periphery (Fig. 2A, upper panel, white arrow) and a highly regular network of small capillaries in the center (Fig. 2A, upper panel, asterisk), vascular networks in the colonic mucosa of acute and chronic DSS colitis models showed a highly irregular structure which partially did not allow sharp resolution of smaller vessels (Fig. 2A, middle, lower panel, asterisks). Quantification of colonic vascular leakage by measuring the extravascular accumulation of a non-binding fluorescence-labeled IgG antibody demonstrated a significantly increased permeability in acute ($P < 0.01$) and chronic ($P < 0.05$) DSS colitis as compared with colon tissues of healthy control animals (Fig. 2C).

Genes Associated with Inflammatory Vessel Activation Are More Strongly Upregulated in DSS Colitis as Compared with Angiogenesis-associated Genes

The microenvironment of IBD is characterized by the presence of many different inflammatory and angiogenic factors acting simultaneously on vessel endothelial cells during disease progression.[11,29] Angiogenic factors activate endothelial cell proliferation and increase permeability, whereas inflammatory factors are predominantly involved in the regulation of the extravasation of immune cells into the tissues.[2,30] Interestingly, certain inflammation-associated cytokines and chemokines such as IFN-γ and CXCL10 have been shown to exert angiostatic activity.[12,13,17] To investigate which group of factors dominates vessel activation in IBD, the expression of inflammation-associated cytokines/chemokines (Fig. 3A, dark gray bars) and of angiogenesis-associated factors (Fig. 3A, light gray bars) was investigated with qRT-PCR in mice with acute DSS colitis and compared with control mice. Interestingly, all of the selected inflammation-associated genes were more strongly upregulated in colitis tissues as compared with the angiogenesis-related factors (Fig. 3A). This suggested that an inflammatory vessel phenotype predominates in colitis tissues. Among the highest induced genes in acute DSS colitis was IFN-γ, which is regarded as an important pathogenesis factor in IBD and experimental colitis[9,11,31] and also exerts angiostatic activity.[12,13,17] The latter is in clear contrast to the increased vascular density observed in IBD and experimental colitis (Fig. 1). To investigate whether this cytokine is acting on vascular endothelial cells in colitis tissues, we used the expression of GBP-1 as a marker of an active IFN-γ response in IBD vessel endothelial cells, as previously described.[15,17,32] In accordance with the results observed in human endothelial cells, the expression of murine GBP-1 was highly upregulated in cultivated primary mouse intestinal endothelial cells after treatment with IFN-γ, but not to detectable levels by TNF-α (Fig. 3B). Using qRT-PCR, the expression of GBP-1 was found to be significantly ($P < 0.01$) increased in acute ($n = 5$) and chronic ($n = 4$) DSS colitis tissues as compared with healthy controls ($n = 7$, Fig. 3C), indicating an active IFN-γ signaling in vivo. Immunohistochemical staining of GBP-1 at the single-cell level showed expression in many different cell types in colitis tissues including endothelial cells (Fig. 3D; arrows). In contrast, GBP-1 expression was low or undetectable in colon tissues of untreated mice (Fig. 3D). Endothelial staining of GBP-1 was

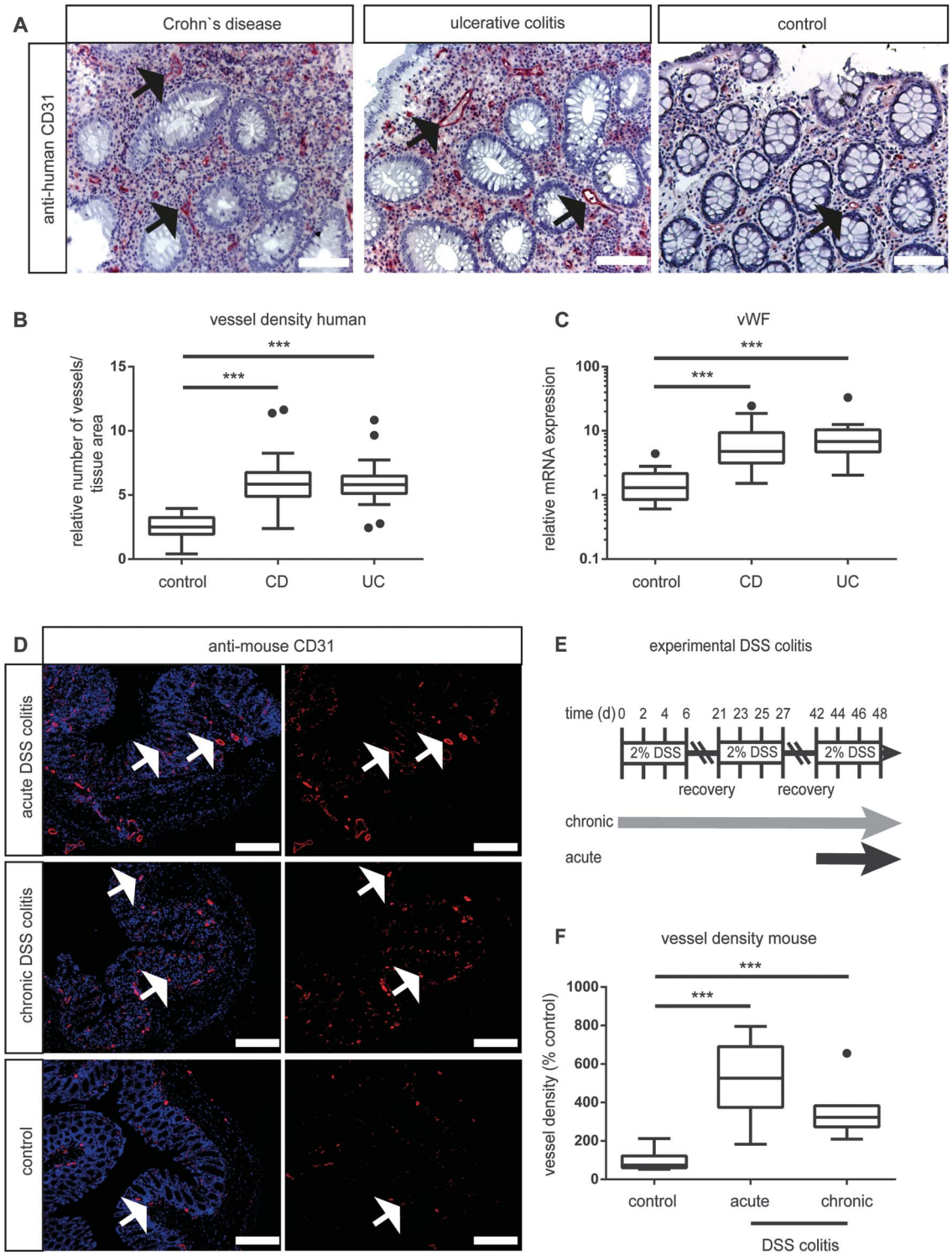

FIGURE 1. Blood vessel density is increased in tissues of IBD and DSS colitis. A and B, The vessel density is significantly increased in colonic tissues of patients with IBD (n = 28 [CD]; n = 23 [UC]) in comparison with healthy controls (n = 14) as indicated by an increased staining for CD31 (scale bars = 100 μm; positive vessels are indicated by arrows). B, Quantitative analysis of CD31 staining results. C, The significantly increased expression of vWF mRNA in patients with IBD indicates higher vessel content and/or angiogenic activity. D, Vascular density in murine colitis tissues was measured by staining for CD31 (red; scale bars = 100 μm; positive vessels are indicated by arrows). Nuclei were stained with DAPI (blue). E, Acute and chronic colitis were induced by administration of 2% DSS in the drinking water in 1 (acute) or 3 (chronic) cycles. F, Quantification of the vessel density in DSS colitis (n = 15 [control]; n = 14 [acute]; n = 7 [chronic]). Tukey boxplots; • = outliers (< first or > third quartile ± 1.5 interquartile range; ***$P \leq 0.001$; Mann–Whitney test.

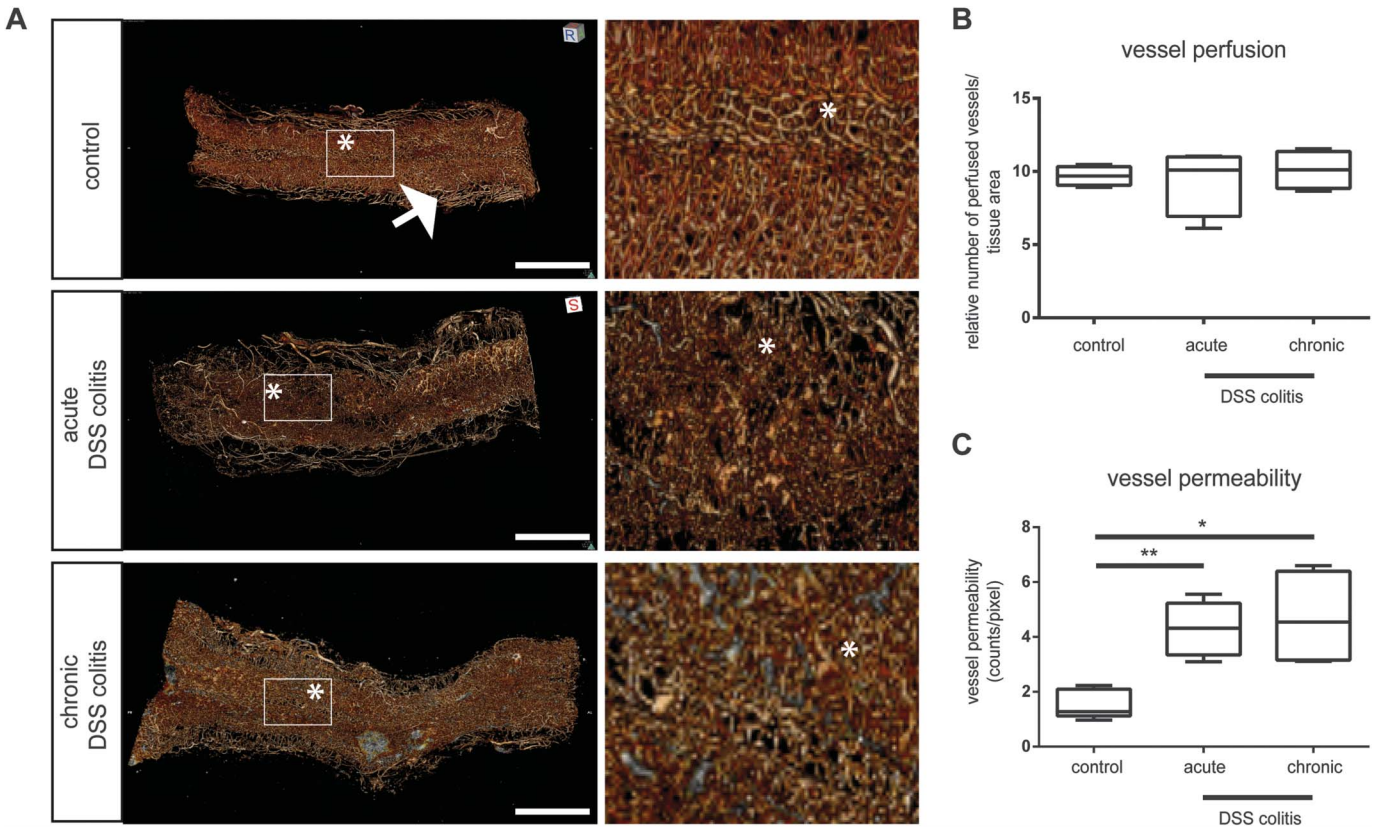

FIGURE 2. Blood vessels in DSS colitis show a disorganized structure and increased permeability. A, Vessels were stained by intravenous injection of an Alexa Fluor 750-coupled lectin and visualized by ultramicroscopy. Arrow indicates large vessels in the periphery, and asterisks indicate small capillaries in the mucosa. Framed areas (left panel) are depicted as a higher magnification in the right panels. B, Quantification of the lectin signal per tissue area. C, Vessel permeability was significantly increased in the acute and chronic DSS colitis (*$P < 0.05$; **$P < 0.01$; Tukey boxplots; Mann–Whitney test; n = 4).

further confirmed by identifying endothelial cells with CD31 staining on consecutive tissue sections (Fig. 3E). These results implied that blood vessel endothelial cells are exposed to and activated by IFN-γ in DSS colitis in mice.

IFN-γ Is Expressed in CD and UC and Is Acting on Blood Vessel Endothelial Cells

IFN-γ expression was investigated in human IBD by in situ hybridization with strand specific S^{35}-labeled RNA hybridization probes. Although robust IFN-γ expression was detected in tissues of CD and UC, expression was low or absent in healthy colon tissues (Fig. 4A). qRT-PCR confirmed a significantly increased IFN-γ expression ($P \leq 0.001$) in tissues from CD (n = 28) and UC (n = 23) as compared with healthy controls (n = 14, Fig. 3B). Furthermore, GBP-1 expression was upregulated in blood vessel endothelial cells of these patients, indicating that these cells are activated by IFN-γ (Fig. 4C, inset, arrow). In contrast, GBP-1 was not expressed in healthy colon tissues (Fig. 4C). A statistically significant ($P \leq 0.001$) increase of GBP-1 expression in CD and UC as compared with healthy colon tissues could also be confirmed by qRT-PCR (Fig. 4D). Moreover, the mRNA expression of GBP-1 highly correlated (r = 0.70; $P \leq 0.001$) with the expression of IFN-γ in patients with IBD (Fig. 4E).

IFN-γ Inhibits Proliferation of Primary Mouse Intestinal Endothelial Cells

IFN-γ exhibits angiostatic activity on human microvascular and macrovascular endothelial cells.[12,13,17] However, species-specific differences may result in different activity of murine IFN-γ on murine primary intestinal endothelial cells. Proliferation experiments showed that angiogenic growth factor significantly ($P \leq 0.001$) induced proliferation of endothelial cells, which could be significantly ($P \leq 0.001$) inhibited by simultaneous application of murine IFN-γ (Fig. 5A). The addition of a neutralizing antibody against IFN-γ (αIFN-γ mAb) reduced the inhibitory effect of IFN-γ on cell proliferation in a concentration-dependent manner, whereas a control antibody applied in the highest concentration had no effect (Fig. 5A). The neutralizing activity of the αIFN-γ mAb could be confirmed by investigating the expression of GBP-1 at the single-cell level (Fig. 5B).

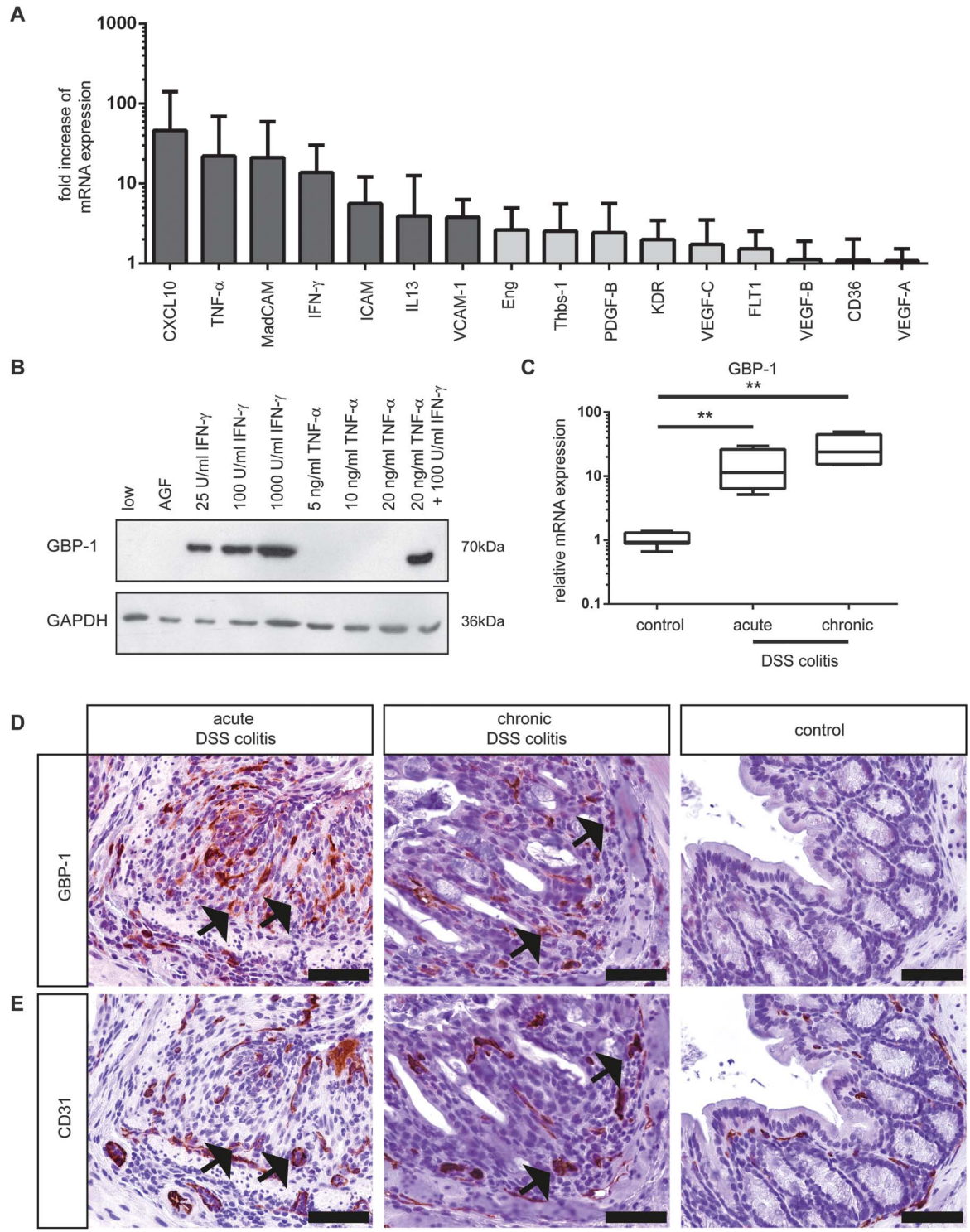

FIGURE 3. GBP-1 expression in DSS colitis indicates endothelial cell activation by IFN-γ. A, Expression profile of genes associated with inflammatory (dark gray) and angiogenic (light gray) blood vessel activation in acute DSS colitis (n = 19). Expression was analyzed by qRT-PCR and ranked according to the fold change expression of the respective gene in colitis tissues as compared with healthy control tissues (n = 15). B, Western blot shows that IFN-γ induces the expression of GBP-1 in cultures of mouse intestinal endothelial cells (24-h stimulation). C, qRT-PCR shows that GBP-1 expression is significantly increased in acute (n = 5) and chronic (n = 4) DSS colitis (**$P < 0.01$; Tukey boxplot; Mann–Whitney test). D, GBP-1 expression is detected in blood vessel endothelial cells (arrows) of DSS colitis as detected by immunohistochemistry. E, Endothelial cells were identified by CD31 staining of consecutive sections [corresponding cells in (D and E) are indicated by arrows; scale bars = 50 μm].

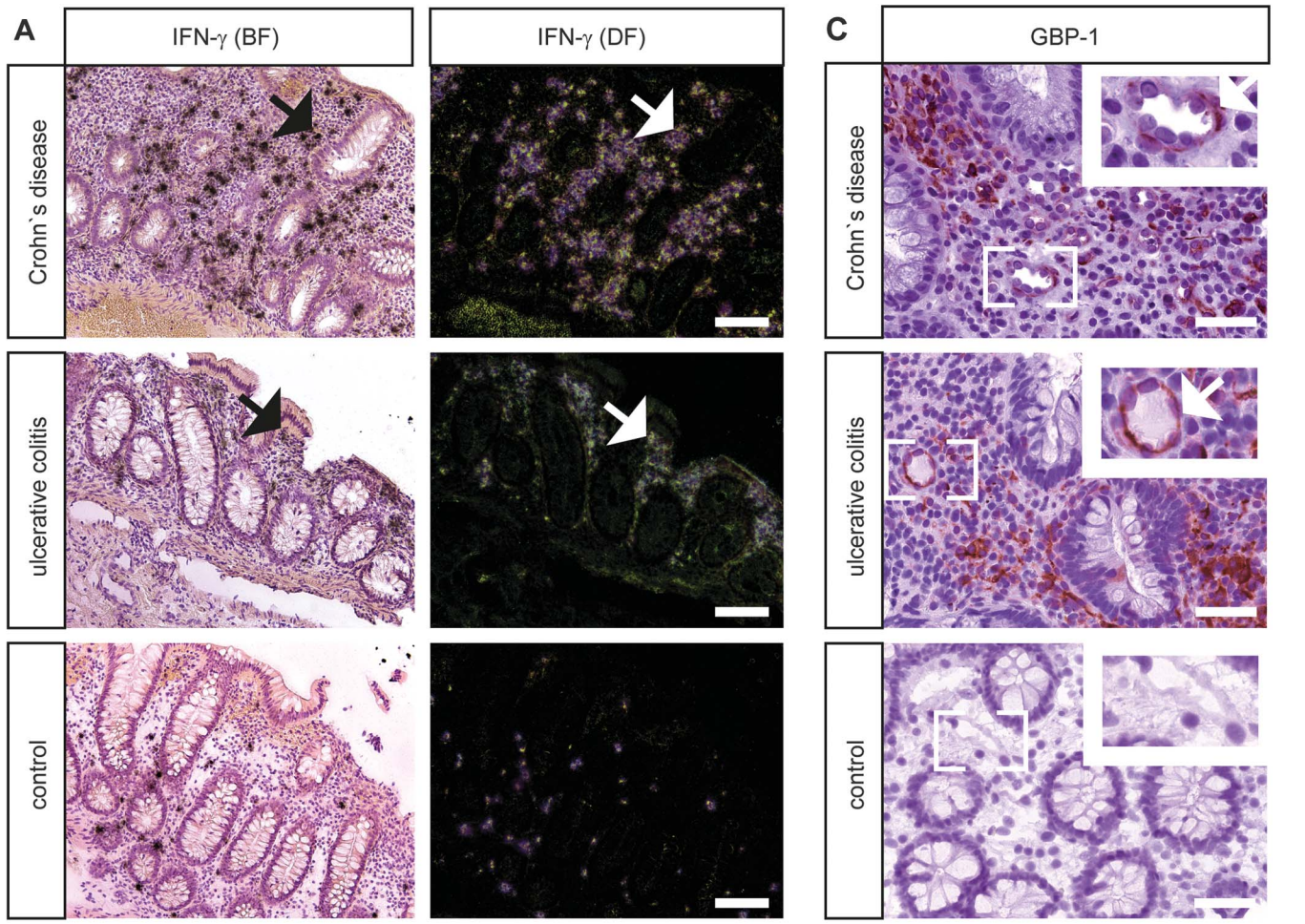

FIGURE 4. Blood vessel endothelial cells in IBD are exposed to activation by IFN-γ. A, In situ hybridization with strand specific S^{35}-labeled RNA hybridization probes detects robust IFN-γ expression in CD and UC. IFN-γ signals (arrows) are shown in bright field (BF, black silver grains) and corresponding dark field (DF, gray to pink signals). Scale bars = 100 μm. B, qRT-PCR of IFN-γ expression in tissues of CD (n = 28) and UC (n = 23) as compared with healthy controls (n = 14; Tukey boxplot; • = outliers (< first or > third quartile ± 1.5 interquartile range; ***$P \leq 0.001$; Mann–Whitney test). C, Immunohistochemical detection of GBP-1 expression in vascular endothelial cells of CD and UC. An increased magnification of the framed area is shown in the inset. Morphologically characterized vessels expressing GBP-1 are indicated by arrows. Scale bars = 50 μm. D, qRT-PCR of GBP-1 expression in CD (n = 28) and UC (n = 23; • = outliers (< first or > third quartile ± 1.5 interquartile range; Tukey boxplot; ***$P \leq 0.001$; Mann–Whitney test). E, The mRNA expression of GBP-1 highly correlates with the expression of IFN-γ in patients with CD (n = 28) and UC (n = 23) (r = 0.7; ***$P \leq 0.001$; Spearman's correlation).

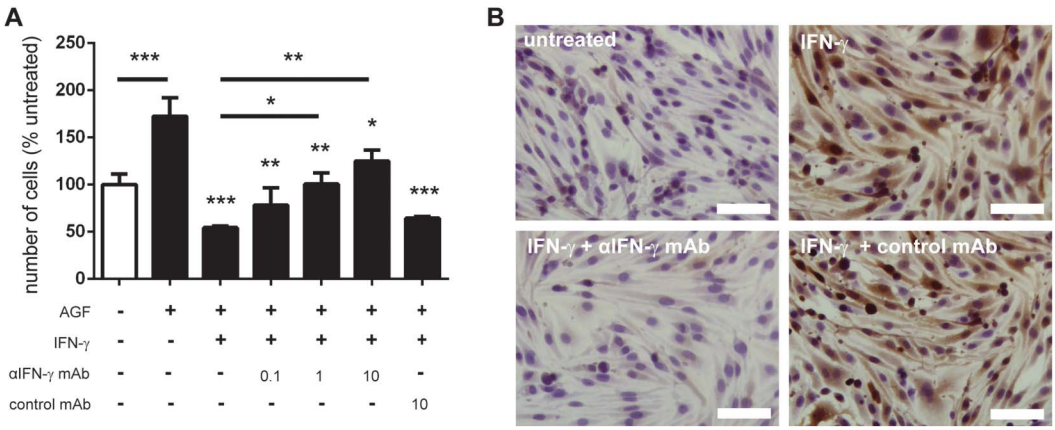

FIGURE 5. Neutralization of IFN-γ inhibits the antiproliferative effects of IFN-γ and reduces GBP-1 expression. A, Cell count after 5-day stimulation with angiogenic growth factor (vascular endothelial growth factor 10 ng/mL; basic fibroblast growth factor 10 ng/mL), IFN-γ (100 U/mL) or mAbs (αIFN-γ mAb [0.1, 1, 10 μg/mL]; control mAb [10 μg/mL]). *$P < 0.05$; **$P < 0.01$; ***$P \leq 0.001$; Student's t test. B, Immunocytochemical staining shows GBP-1 expression after stimulation with IFN-γ (100 U/mL, 24 h) in mouse intestinal endothelial cells. This was inhibited by the αIFN-γ mAb (10 μg/mL) but not by the control mAb (10 μg/mL). Scale bar = 10 μm. Experiments were reproduced at least 3 times with identical outcomes.

Neutralization of IFN-γ Increases Vessel Density and Reduces Vascular Permeability in Acute DSS Colitis

In the final step, we investigated the effects of endogenous IFN-γ on the vasculature in colitis tissues in vivo. Therefore, we blocked IFN-γ effects in the acute DSS colitis by administration of the above-tested neutralizing αIFN-γ mAb and subsequently analyzed blood vessel density and permeability. In line with our previous observations, CD31 immunohistochemistry showed that the vessel density was significantly increased after DSS-treatment as compared with healthy control animals (Fig. 6A). Interestingly, this effect was further enhanced after neutralization of endogenous IFN-γ, indicating that IFN-γ acts in an angiostatic manner in IBD tissues (Fig. 6A). Quantitative evaluation confirmed that the vessel density was significantly increased ($P < 0.05$) in DSS colitis in the presence of the αIFN-γ mAb (n = 5) as compared with colitis tissues treated with control antibody (n = 6) or healthy colon tissues (n = 3, Fig. 6C).

To investigate whether IFN-γ is involved in colitis-associated vascular permeability, we performed confocal laser endomicroscopy after intravenous injection of FITC–dextran (Fig. 6B). In agreement with our previous results, permeability was highly increased in DSS-treated mice as compared with healthy control animals (Fig. 6B). Although vessels in healthy mice were characterized by well-defined borders (Fig. 6B, upper panel), vessel borders in acute DSS colitis were blurry and indistinct (Fig. 6B, middle panel). Furthermore, accumulation of FITC–dextran in the central area of the crypts indicated vascular leakage (Fig. 6B, middle panel; Fig. 6D). Of note, mice injected with the neutralizing αIFN-γ mAb (Fig. 6B, lower panel) demonstrated a less severe phenotype as compared with those treated with a control antibody (Fig. 6B, middle panel). Quantitative evaluation confirmed that application of the IFN-γ–neutralizing antibody significantly ($P < 0.05$) ameliorated blood vessel permeability in DSS colitis (Fig. 6E). Altogether, these results clearly demonstrated that IFN-γ exerts angiostatic activity in vivo and is involved in vascular leakage during IBD.

DISCUSSION

IFN-γ is a pleiotropic cytokine involved in multiple regulatory pathways and acting on many different cell types. Concerning its role in angiogenesis, we have previously shown that IFN-γ exhibits potent antiangiogenic activity on human microvascular and macrovascular endothelial cells. Here, we could confirm that IFN-γ also inhibits the proliferation of mouse intestinal endothelial cells in vitro.[12–14] However, in the setting of IBD, IFN-γ expression was highly upregulated and coincided with an increased vessel density. To investigate whether IFN-γ may directly act on blood vessels in intestinal inflammation in vivo, we used the expression of GBP-1 as a marker for an active IFN-γ response as previously described.[12,13,15,17] Consistent with observations in human endothelial cells, the expression of murine GBP-1 was highly increased in cultivated primary mouse intestinal endothelial cells after stimulation with IFN-γ. Immunohistochemical detection of GBP-1 expression in endothelial cells in colonic tissues further implied that IFN-γ acts on intestinal endothelial cells in the acute and chronic DSS colitis in mice and human IBD.

It has recently been reported that endogeneous inhibitors of angiogenesis are active in intestinal inflammation.[5] Therefore, IFN-γ and IFN-γ-inducible antiangiogenic chemokines like CXCL10 may contribute to the angiostatic forces active on the angiogenic balance in IBD. In line with this, we found that antibody-mediated neutralization of IFN-γ increased the vessel density in experimental DSS colitis. This demonstrated for the first time that IFN-γ acts as an endogenous antiangiogenic factor in the endothelial microenvironment of colitis tissues in vivo. The specific activity of the neutralizing antibody was confirmed by

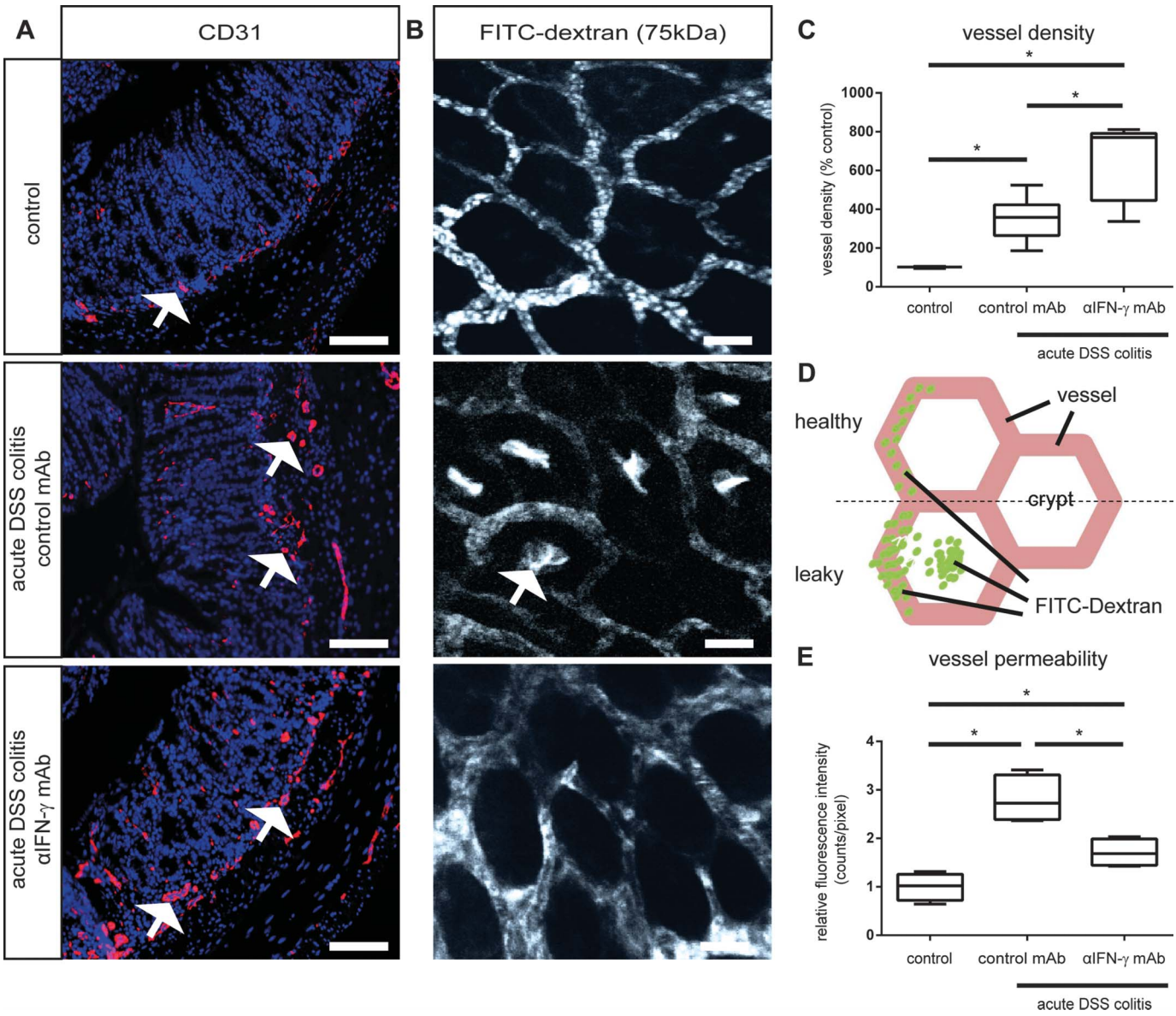

FIGURE 6. Neutralization of IFN-γ increases the vessel density and decreases vascular permeability in acute DSS colitis. A, Colonic tissue sections of untreated or DSS-treated animals who received a neutralizing anti-IFN-γ mAb (αIFN-γ mAb) or control mAb were stained for CD31 (red) to determine blood vessel density (positive blood vessels are indicated by arrows; nuclei were stained with DAPI [blue]; scale bars = 100 μm). B, Vessel permeability was measured by confocal laser endomicroscopy in vivo after intravenous injection of FITC–dextran (scale bar = 50 μm). C, Quantification of the relative blood vessel density in mice treated with the αIFN-γ mAb (n = 5) or the mAb control (n = 6) as compared with untreated controls (n = 4). D, Scheme of dextran deposition in colitis tissues. E, Quantification of vessel permeability (Tukey boxplots; *$P < 0.05$; Mann–Whitney test).

in vitro experiments showing that the inhibition of cell proliferation and activation of GBP-1 expression by ectopically added IFN-γ can be efficiently blocked by this antibody.

Because it is known that blood vessels in IBD are structurally and functionally altered,[2] we further investigated vessel structure, perfusion, and permeability. We found that the overall perfusion into the tissues was similar in normal colon and DSS colitis tissues. However, the highly increased vessel numbers detected in the mucosal tissues of DSS-treated mice indicated that the performances of a given length unit of the vessels may be lower in colitis as compared with vessels in the healthy colon. This was supported at the structural level where vessels in DSS colitis were found to be highly disorganized as compared with the vascular network in healthy colon tissue. Moreover, vessels in DSS colitis exhibited a significantly increased permeability for a nonbinding antibody and FITC–dextran.

Vascular barrier dysfunctions are common in IBD and perpetuate disease by various mechanisms, most importantly by the regulation of leukocyte infiltration, altered perfusion and blood flow velocity, and the development of edema and hyperthrombic vessels.[2] Furthermore, increased vascular leakage has been linked to epithelial cell damage in experimental colitis and was therefore suggested to directly contribute to disease establishment.[33] In this study, we showed that neutralization of endogenous IFN-γ significantly reduced macromolecular leakage in the acute DSS colitis in vivo. Therefore, IFN-γ might be causatively involved in vascular permeability barrier defects observed in IBD. Because IFN-γ has been described to downregulate or relocate the junctional adhesion molecule-A, as well as the platelet endothelial cell adhesion molecule-1 and occludin in vitro,[10,34,35] it is likely that disturbance of these proteins may be responsible for the increased permeability of the vascular system in IBD.

As yet, inhibition of IFN-γ activity for the treatment of IBD did not provide any clear results in clinical studies.[36,37] In line with this, genetic or pharmacologic interference with the IFN-γ signaling pathway in experimental colitis revealed different outcomes concerning disease establishment and progression based on the experimental design. For instance, IFN-γ–deficient mice did not develop DSS colitis, indicating an essential role of IFN-γ in IBD.[9] However, TNBS colitis could be induced independent of IFN-γ signaling.[38] Overall, these studies clearly demonstrate the need to carefully dissect the different effects of this pleiotropic cytokine on different cell types in IBD.

Here, we provided evidence that besides its well-known roles in the regulation of immune cell functions and epithelial barrier defects; IFN-γ also strongly affects vascular functions in IBD which might be of high clinical relevance. It has been recently shown that the endothelial microenvironment in experimental colitis is highly diverse, based on the model system and the genetic background of the mice.[5,39] This might reflect the heterogeneity in patients and provide an explanation about varying therapeutic success of neutralizing antibodies in the treatment of IBD.[40] Based on the expression levels of other vascular modulating factors within the mucosal microenvironment, pharmacological inhibition of IFN-γ signaling might either rescue or foster disease progression. Therefore, individualized therapy according to the unique mucosal cytokine profile and the involved immune cells might be a future prospective in the treatment of IBD. In this framework, a combinational therapy of fontolizumab with the anti–vascular endothelial growth factor antibody bevacizumab or other currently available drugs addressing angiogenic pathways might be a future solution. Moreover, the specific activity of IFN-γ on different cell types may open new strategies to predict therapy responses and outcomes. For this purpose, the use of GBP-1 as a marker for an active IFN-γ response might be a suitable approach.

Altogether, our results indicate that the inhibition of IFN-γ activity as a treatment option for IBD should be revised by more specifically dissecting the complex activity of this cytokine on different cell types and by taking into account the specific composition of the microenvironment in IBD tissues.

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REFERENCES

1. Loftus EV Jr. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology*. 2004; 126:1504–1517.
2. Cromer WE, Mathis JM, Granger DN, et al. Role of the endothelium in inflammatory bowel diseases. *World J Gastroenterol.* 2011;17:578–593.
3. Funderburg NT, Stubblefield Park SR, Sung HC, et al. Circulating CD4+ and CD8+ T cells are activated in IBD and are associated with plasma markers of inflammation. *Immunology*. 2013;140:87–97.
4. Zhang YZ, Li YY. Inflammatory bowel disease: pathogenesis. *World J Gastroenterol.* 2014;20:91–99.
5. Chidlow JH Jr, Langston W, Greer JJ, et al. Differential angiogenic regulation of experimental colitis. *Am J Pathol.* 2006;169:2014–2030.
6. Cromer WE, Ganta CV, Patel M, et al. VEGF-A isoform modulation in an preclinical TNBS model of ulcerative colitis: protective effects of a VEGF164b therapy. *J Transl Med.* 2013;11:207.
7. Scaldaferri F, Vetrano S, Sans M, et al. VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. *Gastroenterology*. 2009;136:585–595.e585.
8. Hindryckx P, Waeytens A, Laukens D, et al. Absence of placental growth factor blocks dextran sodium sulfate-induced colonic mucosal angiogenesis, increases mucosal hypoxia and aggravates acute colonic injury. *Lab Invest.* 2010;90:566–576.
9. Ito R, Shin-Ya M, Kishida T, et al. Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin Exp Immunol.* 2006;146:330–338.
10. Oshima T, Laroux FS, Coe LL, et al. Interferon-gamma and interleukin-10 reciprocally regulate endothelial junction integrity and barrier function. *Microvasc Res.* 2001;61:130–143.
11. Neurath MF. Cytokines in inflammatory bowel disease. *Nat Rev Immunol.* 2014;14:329–342.
12. Guenzi E, Topolt K, Cornali E, et al. The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *EMBO J.* 2001;20:5568–5577.
13. Guenzi E, Topolt K, Lubeseder-Martellato C, et al. The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. *EMBO J.* 2003;22: 3772–3782.
14. Weinlander K, Naschberger E, Lehmann MH, et al. Guanylate binding protein-1 inhibits spreading and migration of endothelial cells through induction of integrin alpha4 expression. *FASEB J.* 2008;22:4168–4178.
15. Grenz S, Naschberger E, Merkel S, et al. IFN-gamma-driven intratumoral microenvironment exhibits superior prognostic effect compared with an IFN-alpha-driven microenvironment in patients with colon carcinoma. *Am J Pathol.* 2013;183:1897–1909.
16. Lubeseder-Martellato C, Guenzi E, Jorg A, et al. Guanylate-binding protein-1 expression is selectively induced by inflammatory cytokines

and is an activation marker of endothelial cells during inflammatory diseases. *Am J Pathol.* 2002;161:1749–1759.
17. Naschberger E, Croner RS, Merkel S, et al. Angiostatic immune reaction in colorectal carcinoma: impact on survival and perspectives for antiangiogenic therapy. *Int J Cancer.* 2008;123:2120–2129.
18. Naschberger E, Wenzel J, Kretz CC, et al. Increased expression of guanylate binding protein-1 in lesional skin of patients with cutaneous lupus erythematosus. *Exp Dermatol.* 2011;20:102–106.
19. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. *Nat Protoc.* 2007;2:541–546.
20. Waldner MJ, Wirtz S, Neufert C, et al. Confocal laser endomicroscopy and narrow-band imaging-aided endoscopy for in vivo imaging of colitis and colon cancer in mice. *Nat Protoc.* 2011;6:1471–1481.
21. Poschinger T, Renner A, Eisa F, et al. Dynamic contrast-enhanced microcomputed tomography correlates with 3-dimensional fluorescence ultramicroscopy in antiangiogenic therapy of breast cancer xenografts. *Invest Radiol.* 2014;49:445–456.
22. Britzen-Laurent N, Lipnik K, Ocker M, et al. GBP-1 acts as a tumor suppressor in colorectal cancer cells. *Carcinogenesis*. 2013;34:153–162.
23. Ostalecki C, Konrad A, Thurau E, et al. Combined multi-gene analysis at the RNA and protein levels in single FFPE tissue sections. *Exp Mol Pathol.* 2013;95:1–6.
24. Sturzl M, Roth WK, Brockmeyer NH, et al. Expression of platelet-derived growth factor and its receptor in AIDS-related Kaposi sarcoma in vivo suggests paracrine and autocrine mechanisms of tumor maintenance. *Proc Natl Acad Sci U S A.* 1992;89:7046–7050.
25. Kuhn E, Naschberger E, Konrad A, et al. A novel chip-based parallel transfection assay to evaluate paracrine cell interactions. *Lab Chip.* 2012;12:1363–1372.
26. Garlanda C, Dejana E. Heterogeneity of endothelial cells. Specific markers. *Arterioscler Thromb Vasc Biol.* 1997;17:1193–1202.
27. Lienau J, Schmidt-Bleek K, Peters A, et al. Differential regulation of blood vessel formation between standard and delayed bone healing. *J Orthop Res.* 2009;27:1133–1140.
28. Zanetta L, Marcus SG, Vasile J, et al. Expression of Von Willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: a potential method for objective assessment of tumor angiogenesis. *Int J Cancer.* 2000;85:281–288.
29. Kanazawa S, Tsunoda T, Onuma E, et al. VEGF, basic-FGF, and TGF-beta in Crohn's disease and ulcerative colitis: a novel mechanism of chronic intestinal inflammation. *Am J Gastroenterol.* 2001;96:822–828.
30. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature*. 2011;473:298–307.
31. Rauch I, Muller M, Decker T. The regulation of inflammation by interferons and their STATs. *JAKSTAT*. 2013;2:e23820.
32. Naschberger E, Werner T, Vicente AB, et al. Nuclear factor-kappaB motif and interferon-alpha-stimulated response element co-operate in the activation of guanylate-binding protein-1 expression by inflammatory cytokines in endothelial cells. *Biochem J.* 2004;379:409–420.
33. Tolstanova G, Deng X, French SW, et al. Early endothelial damage and increased colonic vascular permeability in the development of experimental ulcerative colitis in rats and mice. *Lab Invest.* 2012;92:9–21.
34. Jaczewska J, Abdulreda MH, Yau CY, et al. TNF-alpha and IFN-gamma promote lymphocyte adhesion to endothelial junctional regions facilitating transendothelial migration. *J Leukoc Biol.* 2014;95:265–274.
35. Bruewer M, Luegering A, Kucharzik T, et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *J Immunol.* 2003;171:6164–6172.
36. Hommes DW, Mikhajlova TL, Stoinov S, et al. Fontolizumab, a humanised anti-interferon gamma antibody, demonstrates safety and clinical activity in patients with moderate to severe Crohn's disease. *Gut*. 2006;55:1131–1137.
37. Reinisch W, Hommes DW, Van Assche G, et al. A dose escalating, placebo controlled, double blind, single dose and multidose, safety and tolerability study of fontolizumab, a humanised anti-interferon gamma antibody, in patients with moderate to severe Crohn's disease. *Gut*. 2006;55:1138–1144.
38. Camoglio L, te Velde AA, de Boer A, et al. Hapten-induced colitis associated with maintained Th1 and inflammatory responses in IFN-gamma receptor-deficient mice. *Eur J Immunol.* 2000;30:1486–1495.
39. Knod JL, Crawford K, Dusing M, et al. Mouse strain influences angiogenic response to dextran sodium sulfate-induced colitis. *J Surg Res.* 2014;190:47–54.
40. Ben-Horin S, Kopylov U, Chowers Y. Optimizing anti-TNF treatments in inflammatory bowel disease. *Autoimmun Rev.* 2014;13:24–30.