



Published in final edited form as:

*Inflamm Bowel Dis.* 2011 July ; 17(7): 1501–1512. doi:10.1002/ibd.21525.

## VEGF<sub>164</sub> Isoform Specific Regulation of T-cell-dependent Experimental Colitis in Mice

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### Abstract

**Background**—Inflammatory bowel disease (IBD) consists of Crohn's disease (CD) and ulcerative colitis (UC), two widespread diseases of unknown, multifactorial etiology. Colitis pathology involves a pathological angiogenic response where increases in vascular density participate in colitis histopathology. Vascular endothelial growth factor-A (VEGF-A) is a potent angiogenesis stimulator known to be involved in pathological angiogenesis in several diseases including colitis. However, the pathogenic importance of specific VEGF-A isoforms during T-cell-mediated experimental colitis remains largely unknown.

**Methods**—The CD4<sup>+</sup>CD45RB<sup>high</sup> T-cell transfer model of experimental colitis was used for these studies. The VEGF lac-Z trans-genic reporter mouse was used to examine specific cellular sources of VEGF-A production. The VEGF<sub>164</sub> aptamer (Macu-gen), adenoviral VEGF<sub>164</sub>, and the VEGF Trap were used to evaluate pathological importance.

**Results**—VEGF lac-Z reporter mice experiments showed that both infiltrating T cells and local tissue cells produce VEGF-A in the colon during disease. Inhibition of VEGF<sub>164</sub> using a highly selective RNA aptamer significantly attenuated CD4<sup>+</sup>CD45RB<sup>high</sup> T-cell-dependent experimental colitis by reducing pathological angiogenesis and inflammatory pathology. Conversely, broad-spectrum VEGF inhibition with VEGF Trap did not attenuate disease, nor did adenoviral VEGF<sub>164</sub> overexpression significantly alter colitis pathology.

**Conclusions**—VEGF<sub>164</sub> is actively produced by multiple cell types during T-cell-mediated colitis. Importantly, specific inhibition of the VEGF<sub>164</sub> isoform during T-cell-mediated colitis dose-dependently attenuated disease progression, while broad-scale inhibition of all VEGF-A isoforms was not therapeutically beneficial.

### Keywords

IBD; colitis; VEGF; inflammation; angiogenesis; T cell

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Inflammatory bowel disease (IBD) occurs in people of all ages and is a life-long debilitating condition. It consists of two diseases, Crohn's disease (CD) and ulcerative colitis (UC), both of which have unknown, multifactorial etiologies. Dietary, immune, and genetic factors are all implicated in the onset of these diseases; however, the contribution of each remains unknown. Clinical data has long shown increased vascular density to be a hallmark characteristic of IBD.<sup>1-4</sup> Recently, we and others have examined and quantified this angiogenic response.<sup>5,6</sup> There is now considerable evidence that angiogenesis contributes to the establishment and maintenance of this chronic disease.<sup>4-7</sup> Angiogenesis is a critical component of many chronic inflammatory diseases, including IBD, rheumatoid arthritis, atherosclerosis, and age-related macular degeneration (AMD), making modulation of pathological angiogenesis therapeutically relevant for these diseases. Recent studies from our laboratory and others provide strong evidence that a pathological angiogenic response is necessary for establishing chronic inflammation in IBD and experimental colitis, and that attenuation of this angiogenic response ameliorates disease in animal models.<sup>5,8,9</sup> Our data shows that, not only do increases in vascular density correlate highly with tissue pathology, but inhibition of angiogenesis reduces disease activity in the CD4<sup>+</sup>CD45RB<sup>high</sup> and dextran sulfate sodium (DSS) mouse models of experimental colitis.<sup>5</sup> Specifically, the use of antiangiogenic agents such as thalidomide have been shown to induce remission in patients with active disease and protect against experimental colitis.<sup>5,10,11</sup>

Vascular endothelial growth factor-A (VEGF-A) production is significantly increased during disease in human IBD and in models of experimental colitis.<sup>5,12-14</sup> VEGF-A is a potent angiogenic growth factor that stimulates endothelial cell growth, multiplication, and motility. VEGF-A also activates endothelial signaling pathways, resulting in transient microvasculature dilation and permeability increases, and upregulation of endothelial adhesion molecule expression, factors that all contribute to pathological angiogenic responses and when not properly regulated potentially contribute to chronic inflammatory states. Production of VEGF-A is known to occur in many cell types including endothelial cells, leukocytes, epithelial cells, and others.<sup>15-18</sup> The production of VEGF by different tissues in the body has been elegantly documented during development and postnatally with use of VEGF lac-Z transgenic mice.<sup>19-21</sup> However, the cellular sources of VEGF-A production during T-cell-mediated experimental colitis have not been examined.

VEGF-A involvement in pathological processes of both chronic inflammatory and cancer settings has been documented, and new therapeutic approaches targeting it are being developed and used for the treatment of these and other diseases.<sup>22-28</sup> The overproduction of VEGF-A and other proangiogenic mediators during pathological states can cause a dysregulated angiogenic response characterized by the development of immature, tortuous, and leaky neovascular structures that aid edema formation and ease leukocyte entry into inflamed tissues.<sup>5,29</sup> This occurrence allows inflammation to continue unchecked and likely is key to establishing a chronic inflammatory state. Importantly, VEGF-A has four major isoform splice variants of the VEGF gene: VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>205</sub>, each of which exist as a one amino acid shorter protein in mice. These isoforms play various roles in developmental, wound healing, and pathological angiogenesis as dictated by their size, solubility, and binding properties. It has been proposed that VEGF<sub>164</sub> may be responsible for many of the pathological VEGF-A responses seen during disease, due to its

intermediate biological characteristics and strong affinity for VEGFR2 and neuropilin-1 (Nrp1), a coreceptor that amplifies VEGFR2 signaling in endothelium.

Here we examine the hypothesis that the VEGF<sub>164</sub> isoform plays a key role in the linking of angiogenesis and chronic inflammation during T-cell-mediated colitis, and is therefore the pathogenic isoform necessary for the establishment of chronic disease. Herein we show, using VEGF lac-Z transgenic mice, T cell and local tissue production of VEGF-A during colitis, verifying that VEGF-A is produced in large quantities by multiple cell types during colitis. We also demonstrate that broad inhibition of multiple VEGF-A isoforms with the VEGF Trap did not attenuate disease, while VEGF<sub>164</sub> isoform neutralization significantly decreased T-cell colitis pathology and associated angiogenesis. Importantly, these data suggest that other VEGF family members may play protective or regulatory roles during T-cell-dependent colitis, indicating that selective targeting of specific VEGF isoforms may be more beneficial than wide-scale inhibition of multiple isoforms across different protein families. These findings contribute new insight into how chronic inflammation during IBD is regulated by VEGF-A, and delineates VEGF<sub>164</sub> as a specific target for the treatment of colitis through unlinking angiogenesis and inflammation to prevent the development of a chronic inflammatory state.

## Materials and Methods

### Mice

Animals used in these studies were bred and housed at the Association for Assessment and Accreditation of Laboratory Animal Care, international-accredited Louisiana State University Health Sciences Center-Shreveport (LSUHSC) animal resource facility and maintained according to the National Research Council's Guide for Care and Use of Laboratory Animals. C57B6/J and Rag-1<sup>-/-</sup>(tm1Mom) mice used in these experiments were ordered from Jax Mice and housed at LSUHSC. VEGF Lac-Z mice were bred in-house and backcrossed onto the Rag-1<sup>-/-</sup> background for eight generations.

### CD4<sup>+</sup>CD45RB<sup>High</sup> T-cell Transfer Model of Colitis

T-cell-dependent CD4<sup>+</sup>CD45RB<sup>High</sup> experimental colitis was induced in 10-12-week-old male Rag-1<sup>-/-</sup> and VEGF lac-Z Rag-1<sup>-/-</sup> mice as previously reported.<sup>5</sup> Animals were monitored throughout the study for weight loss as a determinant of disease progression.

### VEGF Antagonist Dosing Regimens

One day after T-cell transfer, animals were randomized to either vehicle or experimental treatment groups. VEGF Trap or human Fc placebo was administered at a dose of 20 mg/kg by intraperitoneal (i.p.) injection three times a week (M,W,F) throughout the 6-week study period. VEGF<sub>164</sub> aptamer was administered at a range of doses including 40 mg/kg, 80 mg/kg, and 160 mg/kg by i.p. injection every 2 days throughout the 6-week study period.

### Histopathological Scoring of the CD4<sup>+</sup>CD45RB<sup>High</sup> Model of Colitis

Histopathological scoring of the colons from mice used in these experiments was done on paraffin-embedded hematoxylin and eosin (H&E)-stained sections in a double-blinded

manner as previously reported.<sup>5</sup> Briefly, eight parameters were examined, including: 1) the degree of inflammation in the lamina propria (scored 1–3); 2) goblet cell loss indicative of mucin depletion (scored 0–2); 3) reactive epithelial hyperplasia/atypia with nuclear changes (scored 0–3); 4) number of intraepithelial lymphocytes in the epithelial crypt (scored 0–3); 5) abnormal crypt architecture (scored 0–3); 6) number of crypt abscesses (scored 0–2); 7) mucosal erosion to frank ulceration (scored 0–2); and 8) submucosal spread to transmural involvement (scored 0–2). Final histopathological scores for each mouse were based on the sum of these scores for a maximal score of 20.

### **Histological Staining for Lac-Z**

Staining for the lac-Z reporter gene was done using an anti- $\beta$ -gal primary antibody (AbCam, Cambridge, MA) at a concentration of 1:200, incubated overnight at 4°C in conjunction with the Vector ABC Elite Kit (Vector Laboratories, Burlingame, CA), Nova Red (Vector Laboratories), and hematoxylin nuclear counterstain (Vector Laboratories).

### **Tissue Angiogenic Index**

Cross-sections of colon tissue from each animal were analyzed as previously reported.<sup>5</sup> Slides were fixed and stained for platelet-endothelial cellular adhesion molecule-1 (PECAM-1) (CD31) and a Nikon Eclipse TE2000-S epifluorescent scope was used to capture images using Simple PCI software (C-Imaging Systems; Compix, Lake Oswego, OR) as previously described.<sup>5</sup> Total pixel area stained for PECAM-1 was divided by DAPI pixel area staining to calculate an angiogenic index (AI).

### **Enzyme-linked Immunosorbent Assays (ELISAs)**

VEGF<sub>164</sub> ELISAs were obtained from Calbiochem (La Jolla, CA) and used according to their protocols with tissue lysates from the colons of control and experimental mice.

### **In Vitro Transwell Assay of VEGF164 Chemotaxis**

Transwell assays were performed as previously described.<sup>30</sup> Briefly, cells were seeded at 400,000 cells per insert and allowed to sit for 2 hours. Fifty ng/mL VEGF<sub>164</sub> was then added to the abluminal chamber and cells were allowed to chemotax for 6 hours, then fixed. Cells were cleared from the top of each insert and fluorescence readings were taken.

### **Statistical Analyses**

Statistical analyses were done using Student's *t*-test or analysis of variance (ANOVA) with a Bonferroni's posttest among all groups and are presented as mean  $\pm$  standard error of the mean (SEM). *P* values <0.05, <0.01, or <0.0001 are significant as indicated in the figures. All experiments were replicated at least twice and experimental *P* values are reported in the text and *N* values are reported in the figure legends.

## Results

### Cellular Production of VEGF-A During CD4<sup>+</sup> Colitis

VEGF-A is known to play a role in pathological angiogenic settings where its overexpression has been documented, including rheumatoid arthritis, IBD, atherosclerosis, diabetic retinopathy, and psoriasis.<sup>5,29</sup> Here we examine VEGF-A production during CD4<sup>+</sup> T-cell-induced experimental colitis in order to determine the cellular sources of VEGF-A during disease. Using CD4<sup>+</sup>CD45RB<sup>High</sup> T cells isolated from VEGF lac-Z transgenic mice we induced colitis in Rag-1 mice, and T cells isolated from C57BL/6J mice were used to induce colitis in VEGF lac-Z Rag-1<sup>-/-</sup> mice. These mice were followed for 6 weeks and weight loss was documented to measure disease activity. Figure 1A shows the percent change in weight and colon lengths of the control and experimental groups, these data were not significantly different between groups, and all mice lost significant weight indicative of disease (10%-20% total body weight). Figure 1B shows the survival data for these groups over the 6-week experimental time course. Figure 1C (lac-Z T cells into Rag-1<sup>-/-</sup> mice) and 1D (wildtype [WT] T cells into VEGF lac-Z Rag-1<sup>-/-</sup> mice) show sample pictomicrographs of the histopathology from both experimental groups verifying the full development of colitis; for comparison, sample images of normal colon tissue are shown in Figure 3A and Supporting Information Figure 2. Figure 1E shows  $\beta$ -gal staining of colon tissue from Rag-1<sup>-/-</sup> mice where colitis was induced with VEGF lac-Z T cells; this sample image shows the nuclei of T cells producing VEGF-A (brown) with a hematoxylin counterstain (blue); black arrows indicate positive staining of T-cell nuclei. Figure 1F is a sample image of  $\beta$ -gal staining in VEGF lac-Z Rag-1<sup>-/-</sup> mice; here the nuclei of all tissue cells producing VEGF-A stain positive for  $\beta$ -gal (brown) and hematoxylin counterstain was used (blue); black arrows indicate positive staining of nuclei for  $\beta$ -gal. These data clearly show that multiple cell types (e.g., infiltrating leukocytes and mucosal epithelial cells) actively produce VEGF-A during disease. These findings are significant as they clearly demonstrate VEGF-A cytokine expression is involved in multiple tissue niches during the development of chronic disease.

### Isoform-specific Dose-dependent Attenuation of CD4<sup>+</sup> Experimental Colitis by the VEGF<sup>164</sup> aptamer

Having shown previously that VEGF-A is upregulated during CD4<sup>+</sup> T-cell-mediated colitis we next examined the effect of VEGF<sub>164</sub> isoform-specific inhibition in our model using a VEGF<sub>164</sub> aptamer. These studies make use of three increasing doses of the aptamer (40 mg/kg/day, low dose; 80 mg/kg/day, med dose; and 160 mg/kg/day, high dose) to examine the utility of VEGF<sub>164</sub> inhibition and dose-dependent responses to the same. Table 1 depicts the aptamer doses used, number and frequency of dosing, and total amount of the aptamer given to each group as compared to the dose given to patients for treatment of AMD. Figure 2A shows the weight loss data from all groups revealing that the medium and high dose groups of aptamer significantly attenuated weight loss during the experimental time course beginning at experimental week 4. Survival data for all four groups is reported in Figure 2B. Measurements of colonic shortening as an indication of disease progression are reported in Figure 2C, revealing increased colon lengths with the aptamer treatment (low, 83.50  $\pm$  3.423,  $P = 0.0160$ ; med, 88.33  $\pm$  1.252,  $P < 0.0001$ ; high, 87.78  $\pm$  1.553,  $P < 0.0001$ ) compared to control (73.45  $\pm$  2.033). Figure 2D reports the gross scores for the combined

control group ( $2.375 \pm 0.1451$ ), and low ( $1.625 \pm 0.1825$ ,  $P = 0.0103$ ), med ( $1.385 \pm 0.1804$ ,  $P = 0.0002$ ), and high ( $1.100 \pm 0.2333$ ,  $P < 0.0001$ ) dose aptamer treated groups. Here we saw a significant, dose-dependent reduction in gross score for all groups with increasing significant differences for each increase in dose. ELISAs of colon tissue lysates from these groups revealed decreasing concentrations of VEGF<sub>164</sub> levels concomitant with increasing doses of the aptamer (control,  $1329 \pm 57.96$  pg/ $\mu$ L; low dose,  $1082 \pm 156.7$  pg/ $\mu$ L,  $P = 0.0973$ ; med dose,  $755.2 \pm 53.08$  pg/ $\mu$ L,  $P < 0.0001$ ; and high dose,  $605 \pm 40.00$  pg/ $\mu$ L,  $P < 0.0001$ ) (Fig. 2E).

We next examined the effects of the aptamer on disease histopathology. Figure 3 contains sample histopathology pictomicrographs of normal (A), control (B), low dose (C), medium dose (D), and high dose (E) aptamer treated colons. Graphical analysis of combined histopathology scores for the same groups is reported in Figure 3F. The dose-dependent response is evident with decreased pathology compared to controls ( $14.41 \pm 0.6138$ ) in the low ( $11.50 \pm 0.4564$ ,  $P = 0.0019$ ) and medium ( $7.412 \pm 1.536$ ,  $P < 0.0001$ ) groups, and a near absence of pathological indicators in the high ( $2.000 \pm 0.000$ ,  $P < 0.0001$ ) dose group. The attenuation of tissue histopathology provides clear evidence in a highly relevant disease model that the targeting of VEGF<sub>164</sub> is cytoprotective during T-cell-mediated colitis.

We then examined changes in pathological angiogenesis and vascular density in the aptamer treated groups using the angiogenic index (AI) analysis. Figure 4 shows sample images from untreated (A), low dose (B), medium dose (C), and high dose (D), VEGF<sub>164</sub> aptamer treatment cohorts of CD4<sup>+</sup> colitis stained for PECAM-1 (red) and DAPI nuclear counterstain. PECAM-1 staining showed a trend for reduced vascular density in the low-dose group ( $0.2043 \pm 0.2080$ ,  $P = 0.1355$ ), and significantly attenuated vascular density in the medium ( $0.1700 \pm 0.03215$ ,  $P = 0.0012$ ) and high ( $0.06067 \pm 0.006434$ ,  $P < 0.0001$ ) dose aptamer-treated groups versus control ( $0.2445 \pm 0.01507$ ) (Fig. 4E). These data clearly demonstrate that the isoform-specific inhibition of VEGF<sub>164</sub> results in attenuation of CD4<sup>+</sup> experimental colitis, and that high doses of the aptamer largely inhibit development of disease.

### Heat-denatured Aptamer Does Not Inhibit T-cell-mediated Colitis

Having shown that the VEGF<sub>164</sub> aptamer is able to attenuate CD4<sup>+</sup> colitis in mice, we next heat-denatured the aptamer (HD aptamer) and administered it at the medium dose (80 mg/kg/day) as a control experiment. Figure 5A,B show weight loss and survival data for control and HD aptamer-treated groups, respectively. Histopathology scores ( $15.30 \pm 0.3958$ , control;  $16.12 \pm 0.2241$ , HD aptamer;  $P = 0.0703$ ) and AI analysis ( $0.2400 \pm 0.01281$ , control;  $0.2287 \pm 0.02451$ , HD aptamer;  $P = 0.6636$ ), are shown in Figure 5C,D, respectively. Figure 5E shows VEGF<sub>164</sub> ELISA data for the control ( $1366 \pm 50.45$  pg/ $\mu$ L) and HD aptamer ( $1307 \pm 91.21$  pg/ $\mu$ L) treated groups ( $P = 0.3666$ ). These data confirm the efficacy of the aptamer as a specific VEGF<sub>164</sub> inhibitor and effective therapy for colitis.

As a further control we performed an in vitro Transwell cell migration assay to verify that the aptamer was efficiently binding and inhibiting VEGF<sub>164</sub>. Mouse recombinant VEGF<sub>164</sub> treatment (50 ng/ $\mu$ L) resulted in significantly increased transmigration of colonic endothelial cells as measured by relative fluorescence intensity ( $40,024 \pm 2708$ ), 6.27-fold increase

versus control (nonlabeled) ( $6379 \pm 210.6$ ,  $P = 0.0005$ ) and 2.61-fold increase versus untreated (CellTracker green labeled, nonstimulated) ( $15,312 \pm 205.5$ ,  $P = 0.0119$ ) mouse colonic endothelial cells (Supporting Information Fig. 1). Stimulation with VEGF<sub>164</sub> and coincubation with the aptamer at doses used in AMD therapy (patient dose 0.3 mM) ( $26,483 \pm 5134$ ,  $P = 0.1613$ , 1.5-fold decrease), and our low (1 mM) ( $24,700 \pm 1608$ ;  $P = 0.0132$ , 1.6-fold decrease), medium, 2 mM ( $21,503 \pm 1928$ ;  $P = 0.0051$ , 1.86-fold decrease), and high, 4 mM ( $18,179 \pm 2406$ ;  $P = 0.0053$ , 2.20-fold decrease) doses resulted in a stepwise reduction in endothelial transmigration versus the VEGF<sub>164</sub>-treated group (ANOVA,  $P = 0.0003$ ) (Supporting Information Fig. 1). Together, these data verify the activity of the aptamer used in these experiments and highlight that the VEGF<sub>164</sub> aptamer dose-dependently attenuates CD4<sup>+</sup> colitis in mice.

### Overexpression of VEGF-A in CD4<sup>+</sup> Colitis

Having shown that pharmacologic inhibition of all VEGF-A isoforms was not beneficial in the previous experiment, we next tested whether or not VEGF<sub>164</sub> over-expression had an effect on disease. Here we used VEGF<sub>164</sub> adenovirus or  $\beta$ -gal control adenovirus treatment in the CD4<sup>+</sup> model of colitis. Figure 6A reports the observed weight change during the experimental period; a transient separation was observed at week 5 with greater weight loss in the Ad. $\beta$ -gal ( $86.88 \pm 1.945$ ) than the Ad.VEGF-treated group ( $94.90 \pm 1.672$ ) ( $P = 0.0198$ ), but no difference was present at the end of the experimental period. Survival data for both groups is reported in Figure 6B. Gross scores from the colons of both groups are shown in Figure 6C; no difference was observed between these groups ( $2.400 \pm 0.2449$ , Ad. $\beta$ -gal versus  $2.400 \pm 0.4000$ , Ad.VEGF;  $P = 1.000$ ). Supporting Information Figure 2A–C illustrates sample H&E-stained images of normal, Ad. $\beta$ -gal, and Ad.VEGF-treated colon cross-sections, respectively. Histopathological analysis of colon sections showed no difference in score between groups ( $13.30 \pm 1.100$ , Ad. $\beta$ -gal versus  $12.70 \pm 0.9000$ , Ad.VEGF;  $P = 0.7140$ ) (Fig. 6D). We next examined the colons of these mice for differences in vascular density. Supporting Information Figure 2 contains sample images of normal (D), Ad. $\beta$ -gal (E), and Ad.VEGF (F) treated colons, respectively, stained for PECAM-1 (red) and DAPI nuclear counterstain (blue). AI analysis showed that both groups have increased vascular density compared to normal colons ( $0.2375 \pm 0.03259$ , Ad. $\beta$ -gal, and  $0.2258 \pm 0.01877$ , Ad.VEGF). Importantly, VEGF<sub>164</sub> ELISA revealed increased levels of VEGF<sub>164</sub> in the Ad. $\beta$ -gal group ( $3628 \pm 549.8$  pg/ $\mu$ L) and an even larger increase in VEGF<sub>164</sub> protein in Ad.VEGF-treated colon tissue ( $8540 \pm 321.3$  pg/ $\mu$ L), indicating the effectiveness of the adenovirus ( $P = 0.0007$ ) (Fig. 6F). These data suggest that induction of VEGF<sub>164</sub> normally observed during the development of CD4<sup>+</sup> colitis is sufficient to develop robust tissue damage and vascular density, and that further increasing the VEGF<sub>164</sub> concentration has little additional effect on T-cell-mediated colitis.

### Inhibition of All VEGF-A Isoforms in CD4<sup>+</sup> Colitis

As noted previously, the CD4<sup>+</sup> model exhibited rampant upregulation of proangiogenic factors, including VEGF-A, similar to what is seen in human disease, with some downregulation of antiangiogenic mediators.<sup>5</sup> Lastly, we examined whether inhibition of all VEGF-A isoforms using the VEGF Trap altered the development of CD4<sup>+</sup> T-cell-dependent colitis. VEGF Trap and human Fc (control) treated groups were used in this experiment.

Importantly, neither VEGF Trap nor Fc control significantly altered weight loss; however, a significant decrease in survival occurred in the VEGF Trap-treated group (Supporting Information Fig. 3A,B, respectively). Gross scores of the colons from the human Fc ( $3.000 \pm 0.5164$ ) and VEGF Trap ( $2.429 \pm 0.4266$ ) ( $P = 0.4085$ ) were not significantly different (Supporting Information Fig. 3C). Moreover, histo-pathological scores of diseased colons between the human Fc ( $11.76 \pm 2.240$ ) and VEGF Trap ( $15.94 \pm 2.399$ ) treated groups showed no significant difference ( $P = 0.2114$ ), with both groups receiving high scores (Supporting Information Fig. 3D). Sample images of H&E-stained colon cross-sections from human Fc and VEGF Trap-treated experimental groups revealing significant pathology in both groups are shown in Supporting Information Figure 3E,F, respectively. Surprisingly, angiogenic index analysis of vascular density showed that both groups have similar vascular density levels ( $0.2325 \pm 0.04502$ , human Fc versus  $0.1988 \pm 0.01794$ , VEGF Trap;  $P = 0.7593$ ) (Supporting Information Fig. 4A). Sample images of human Fc and VEGF Trap-treated colons, stained for PECAM-1 (red) and DAPI nuclear counterstain (blue), are shown in Supporting Information Figure 4B,C, respectively. Conversely, VEGF-A ELISA data revealed a reduced level of VEGF-A protein in tissues from VEGF Trap-treated mice ( $704.0 \pm 152.0$  pg/ $\mu$ L) versus human Fc treated mice ( $2874 \pm 539.8$  pg/ $\mu$ L), verifying the potency of the VEGF Trap ( $P = 0.0083$ ) (Supporting Information Fig. 4D). Both groups in this experiment exhibited significant development of disease, suggesting that inhibition of all VEGF-A isoforms may not be beneficial during experimental colitis. These data combined with the VEGF<sub>164</sub> aptamer data support the hypothesis that VEGF<sub>120</sub> and 188 may act in a physiological/wound healing manner during disease, while VEGF<sub>164</sub> drives pathological angiogenesis.

## Discussion

While the etiology of IBD remains largely unknown, the pathological basis of disease progression is widely studied and involves both innate mucosal and acquired immune responses. However, work over the past decade has revealed that the colon microvasculature plays an important role in controlling disease progression and severity through angiogenesis and leukocyte recruitment processes.<sup>5,6,29,31</sup> As such, several clinical and experimental studies have shown increased VEGF-A levels in the serum and colon tissue during colitis.<sup>12–14,29,32</sup> Increased VEGF-A expression could emanate from immune cells including neutrophils, monocytes, T cells, and platelets, as well as produced locally by endothelial cells, epithelial cells, and other cell types. Our findings are the first to unambiguously document specific tissue niches of VEGF-A expression during T-cell colitis implicating infiltrating leukocytes as well as local colon tissue production of angiogenic cytokines during disease. These data also provide additional explanation for the abundant amount of VEGF-A protein expression in situ during the T-cell transfer model,<sup>5</sup> indicating that VEGF-A expression must occur from multiple cell sources to result in high tissue levels seen during T-cell-mediated colitis.

Our current findings clearly show that VEGF<sub>164</sub> plays an important role in the process of pathological angiogenesis occurring during colitis. We have previously shown that this isoform drives inflammatory responses of colonic endothelium that contribute to colitis.<sup>33</sup> Importantly, VEGF<sub>164</sub> has been repeatedly indicated as the pathological isoform of VEGF-A



due to its role in multiple disease pathologies.<sup>34</sup> Moreover, inhibition of angiogenesis attenuates inflammation and disease development in different models of colitis, and the reduced inflammatory capacity of CD18<sup>-/-</sup> mice results in decreased vascular density and attenuated disease.<sup>5</sup> Gene array analysis of both the CD4<sup>+</sup> T cell and DSS models of experimental colitis, and data from clinical studies shows that during disease there is a prominent upregulation of multiple proangiogenic factors such as VEGF, bFGF, TGF- $\beta$ , TNF- $\alpha$ , and others which could outweigh the biological effects of antiangiogenic factors which are found at lower levels during disease.<sup>5</sup> However, the fact that selective inhibition of VEGF<sub>164</sub> conferred protection against disease in a dose-dependent manner surprisingly demonstrates that this angiogenic factor serves as an important intermediary between angiogenesis and inflammation despite upregulation of other cytokines that could engage in similar processes. This relationship between angiogenesis and inflammation suggests that the ideal targets for therapy may be those factors that are closely tied to both events, such as VEGF<sub>164</sub>.<sup>35,36</sup>

In the VEGF<sub>164</sub> adenovirus experiments, we showed that overexpression of VEGF<sub>164</sub> did not affect the disease process. This is important because were VEGF<sub>164</sub> to act in a protective and wound healing manner, its overexpression should protect against disease; however, this was not the case. Moreover, these data suggest that during T-cell-mediated colitis, VEGF<sub>164</sub> is produced at elevated levels that are above maximal pathologic VEGF<sub>164</sub>-dependent effects, as tissue histopathology does not go beyond previously documented severity levels, even with a greater overexpression of VEGF-A above disease levels. Another possible explanation for our findings could be that such significant overexpression of VEGF<sub>164</sub> downregulates VEGFRs or VEGF coreceptors (Nrp-1), which would diminish further induction of angiogenesis and inflammation. Whichever the explanation may be, our data demonstrate that VEGF<sub>164</sub> overexpression does not significantly alter chronic T-cell-mediated colitis.

However, pharmacological inhibition of the VEGF<sub>164</sub> isoform using a specific RNA aptamer (Macugen) did confer significant protection against T-cell-mediated colitis in a dose-dependent manner. We found that a low dose (40 mg/kg/day) of the aptamer was unable to drastically alter the disease phenotype. This was not entirely unexpected, since Macugen is used for the treatment of AMD, where it is injected and retained in a localized intraocular environment, thereby necessitating lesser amounts of the drug for efficacy. However, when treating a large and less confined organ with greater surface area and ability to produce VEGF<sub>164</sub> (e.g., the colon), it seems logical that larger drug doses may be necessary to effectively neutralize elevated tissue VEGF<sub>164</sub> protein levels. As we increased the dose of the aptamer we observed a clear, dose-dependent therapeutic response, resulting in near total attenuation of disease and significant reduction of tissue VEGF<sub>164</sub> protein levels at the highest dose. These data support the hypothesis that VEGF<sub>164</sub> is the dominant pathological isoform of VEGF-A during T-cell-mediated colitis.<sup>34</sup> It is likely that the loss of VEGF<sub>164</sub> affects the progression of inflammation through altered regulation of immune cell recruitment, as previously reported.<sup>33</sup> Moreover, the known roles of the different VEGF family members (i.e., VEGF-B) during physiological angiogenesis and other functions of

VEGF-A isoforms (120 and 188) are consistent with the hypothesis that specific inhibition of VEGF<sub>164</sub> could be very beneficial during disease.

Data from our VEGF Trap (Aflibercept) studies, which binds all isoforms of VEGF-A, VEGF-B, and PlGF, showed no protection against disease, with a surprising increase in mortality. While at first this may seem contrary to potential pathological roles of VEGF-A in disease, upon further consideration this result is not unexpected. VEGF-B, has been shown to have a “decoy effect” when interacting with VEGFR1 to diminish VEGF-A binding to and signaling by VEGFR2, the major ligand for VEGF<sub>164</sub>, which may partly explain the effects of VEGF Trap therapy in the T-cell colitis model.<sup>17</sup> If, as the literature suggests, various VEGF-A isoforms are important for tissue healing, then broad-scale isoform inhibition would not be protective, which agrees with our data.<sup>37,38</sup> It has also recently been reported that genetic deficiency of PlGF expression exacerbates DSS-induced mucosal hypoxia and aggravates colon injury, which could be due to a possible protective role in regulating vascular growth which could also occur in the T-cell colitis model.<sup>39</sup>

Results from our current study significantly advance our understanding of VEGF-A during experimental colitis as initially reported by Scaldaferrri et al<sup>36</sup> showing that VEGF-A inhibition with soluble Flt-1 (VEGFR1) adenovirus attenuates DSS-mediated colitis. Several important differences between our studies include: 1) soluble Flt-1 confers limited VEGF protein binding (i.e., all VEGF-A isoforms), whereas the VEGF<sub>164</sub> aptamer only binds this isoform and the VEGF Trap (containing binding regions of VEGFR1 and VEGFR2) binds VEGF-A, VEGF-B, and PlGF; 2) different disease mechanisms are involved between the DSS and CD4<sup>+</sup> T-cell transfer models of colitis that could influence VEGF isoform function and biology; and 3) our study employed approved pharmacological agents to antagonize VEGF function in the Rag-1<sup>-/-</sup> C57BL/6J mouse strain versus adenovirus-based approaches in C57BL/6N mouse strain which may involve strain-dependent responses. It is important to emphasize that inhibition of multiple VEGF family proteins may not be protective due to the broad range of actions they are able to regulate. Numerous clinical side effects have been noted with broad-scale anti-VEGF therapy including disturbances of cardiovascular (thrombosis, hemorrhage, and hypertension) and renal (proteinuria and renal dysfunction) systems, and in wound healing.<sup>40,41</sup> Nonetheless, it is clear that VEGF<sub>164</sub> isoform specific inhibition is therapeutically beneficial, whereas VEGF Trap therapy does not confer protection against T-cell-mediated colitis and actually increases disease mortality. These results demonstrate that proper consideration and thought must be given to any potential antiangiogenic therapy for colitis.

The profound effect of VEGF<sub>164</sub> inhibition is of critical importance in defining key physiological links between pathological angiogenesis and chronic inflammation during T-cell-mediated colitis.<sup>5,33</sup> The complexity of angiogenic regulation during colitis is only beginning to be elucidated and we report that VEGF-A expression occurs from several different cell types and that VEGF<sub>164</sub> plays a prominent pathophysiological role during T-cell-mediated colitis. While the VEGF<sub>164</sub> aptamer doses required to treat experimental colitis are clearly cost-limiting in human disease, our findings represent a major step forward in understanding potential antiangiogenic targets for novel IBD therapies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Supported by NIH grant P01 DK043785-18 Project 4, and Animal models and Histopathology cores.

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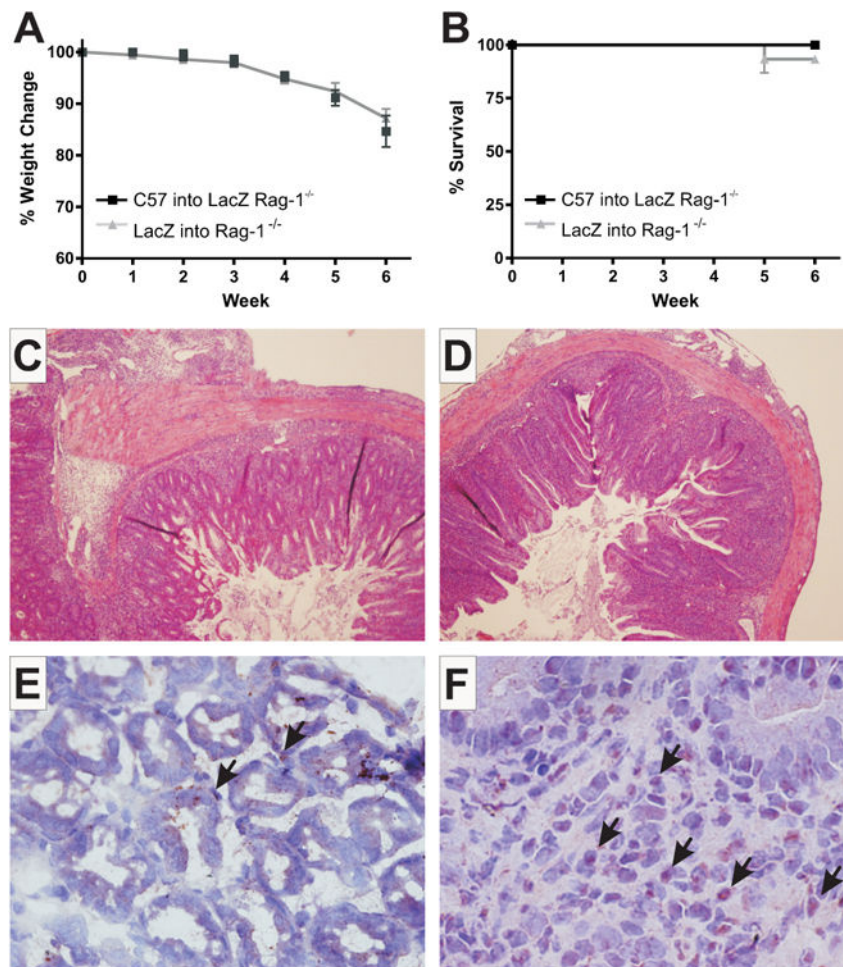
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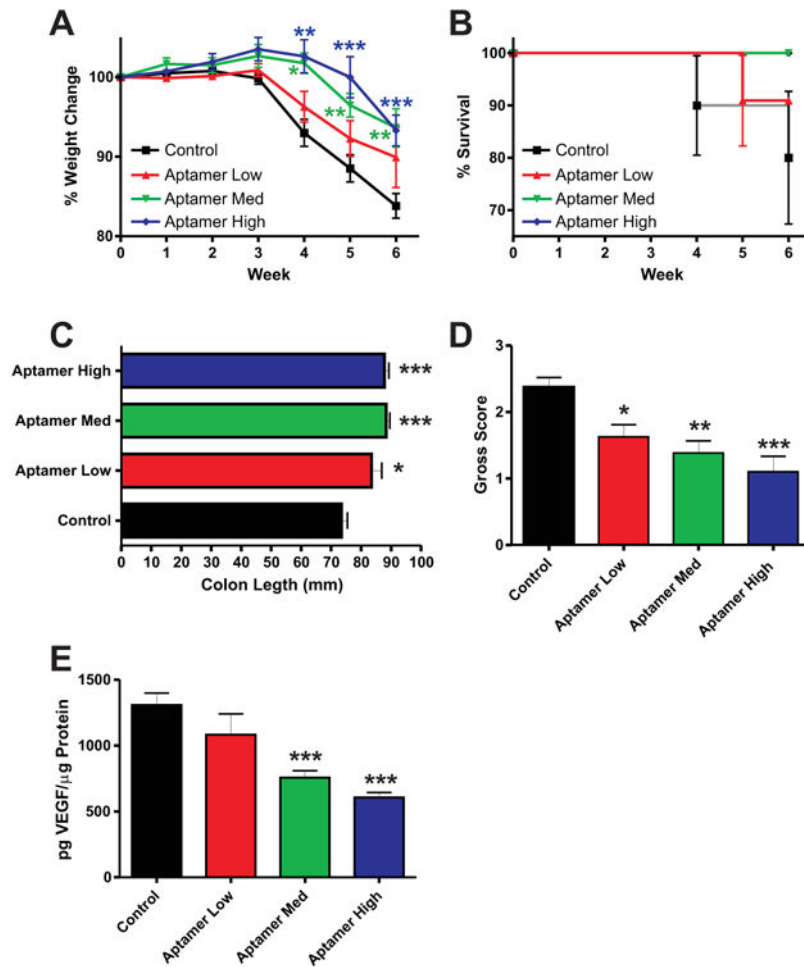
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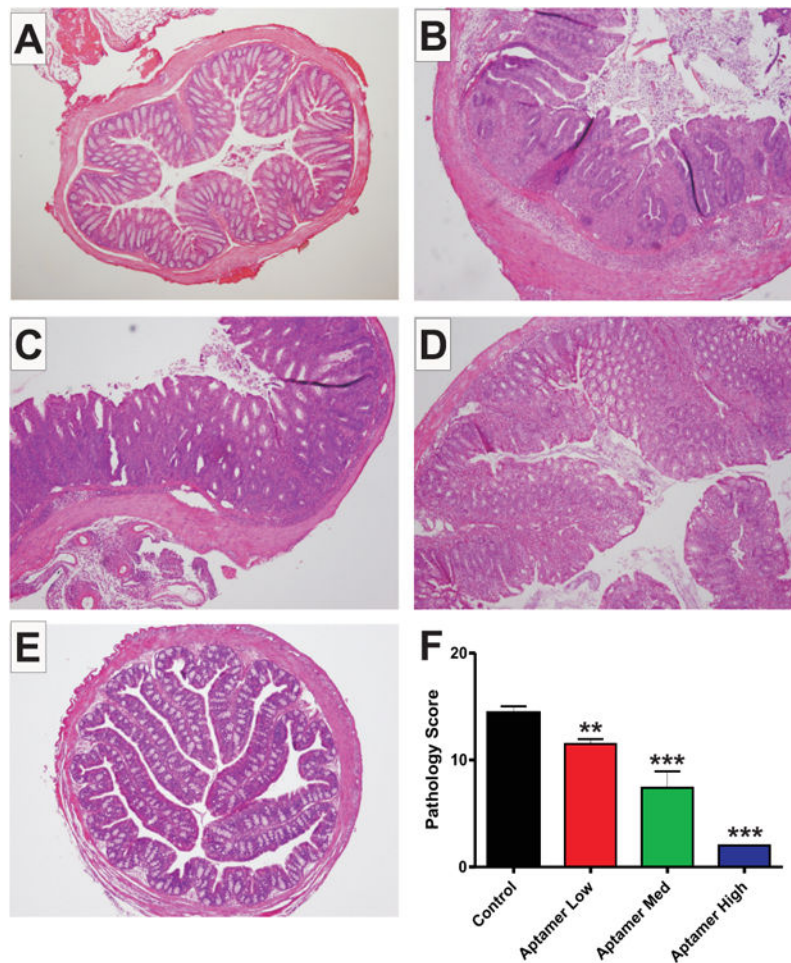
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**Figure 1.** VEGF lac-Z reporter mice reveal VEGF-A production by infiltrating immune and local tissue cells. Weight loss data for VEGF Lac-Z T cells transferred into Rag-1<sup>-/-</sup> (gray) and C57 T cells transferred into VEGF Lac-Z Rag-1<sup>-/-</sup> (black) mice is shown in (A). (B) Survival data for the same groups. Sample H&E stained histopathology images are shown in (C,D) for Rag-1<sup>-/-</sup> and VEGF Lac-Z Rag-1<sup>-/-</sup> groups, respectively. (E) Lac-Z staining (brown) with hematoxylin counterstain (blue) of T cells producing VEGF-A during colitis. (F) The same for leukocytes other than T cells and local tissue cells.  $N = 14$  for Lac-Z into Rag-1<sup>-/-</sup>,  $N = 10$  for C57 into Lac-Z Rag-1<sup>-/-</sup> groups.



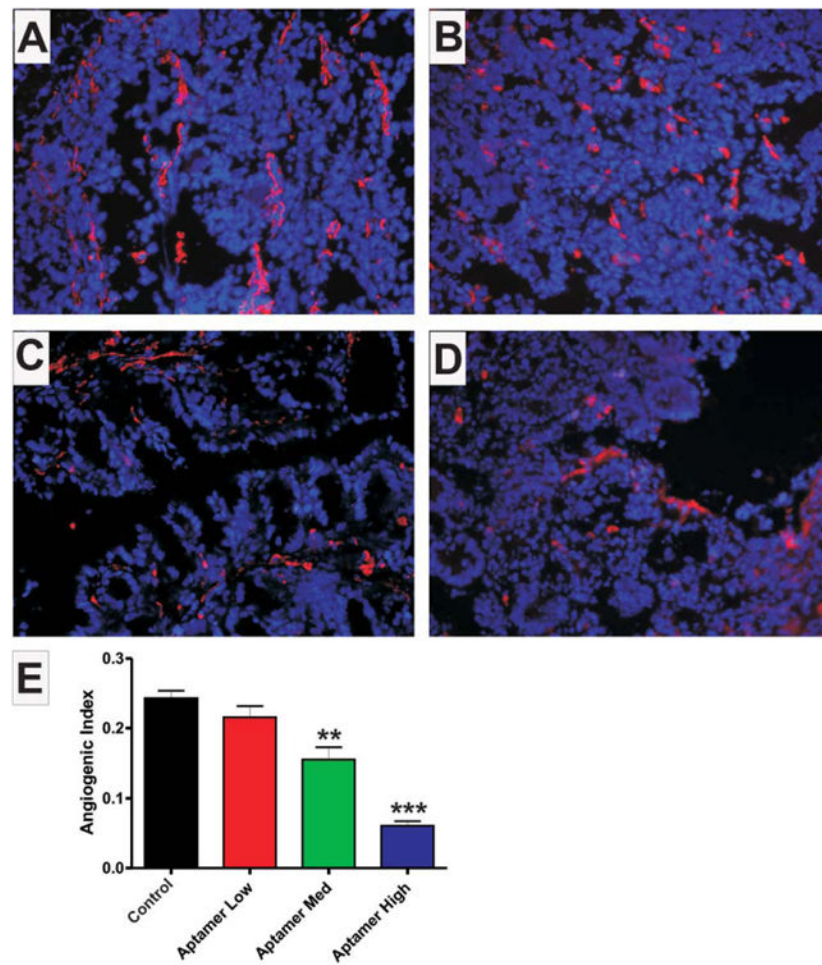
**Figure 2.** Disease activity and progression in the VEGF<sub>164</sub> aptamer treatment groups. (A) Weight loss data for the aptamer-dosed groups; control (black), Aptamer Low (red), Aptamer Med (green), and Aptamer High (blue). (B) Survival data for the same. (C,D) Colon lengths and gross scores, respectively, upon takedown for all groups. (E) VEGF<sub>164</sub> ELISA data for the same.  $N = 28$  for Control,  $N = 14$  for Aptamer Low,  $N = 18$  for Aptamer Med,  $N = 18$  for Aptamer High,  $N = 5$  for ELISA data.



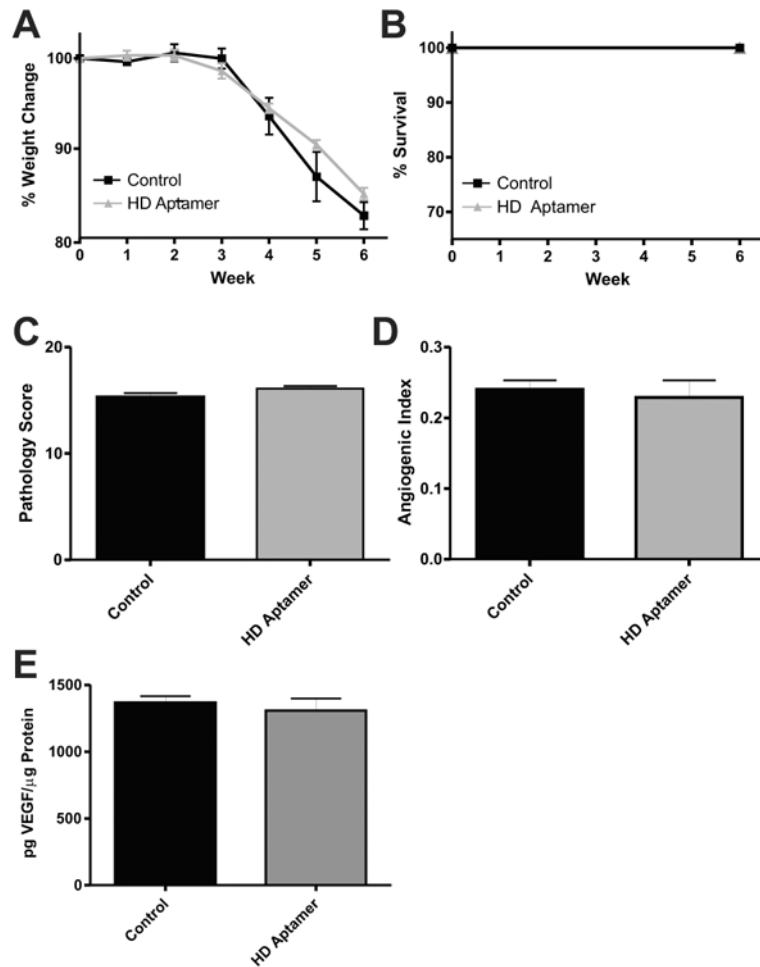
**Figure 3.**

The VEGF<sub>164</sub> aptamer dose-dependently inhibits CD4<sup>+</sup> experimental colitis. This figure shows sample images of H&E staining of colonic cross-sections from Control noncolitic (A), Control colitic (B), Aptamer Low (C), Aptamer Med (D), and Aptamer High (E) dose groups. (F) The histopathology scores for these groups, Control colitic (black), Aptamer Low (red), Aptamer Med (green), and Aptamer High (blue). *N* = 28 for Control, *N* = 14 for Aptamer Low, *N* = 18 for Aptamer Med, *N* = 18 for Aptamer High.

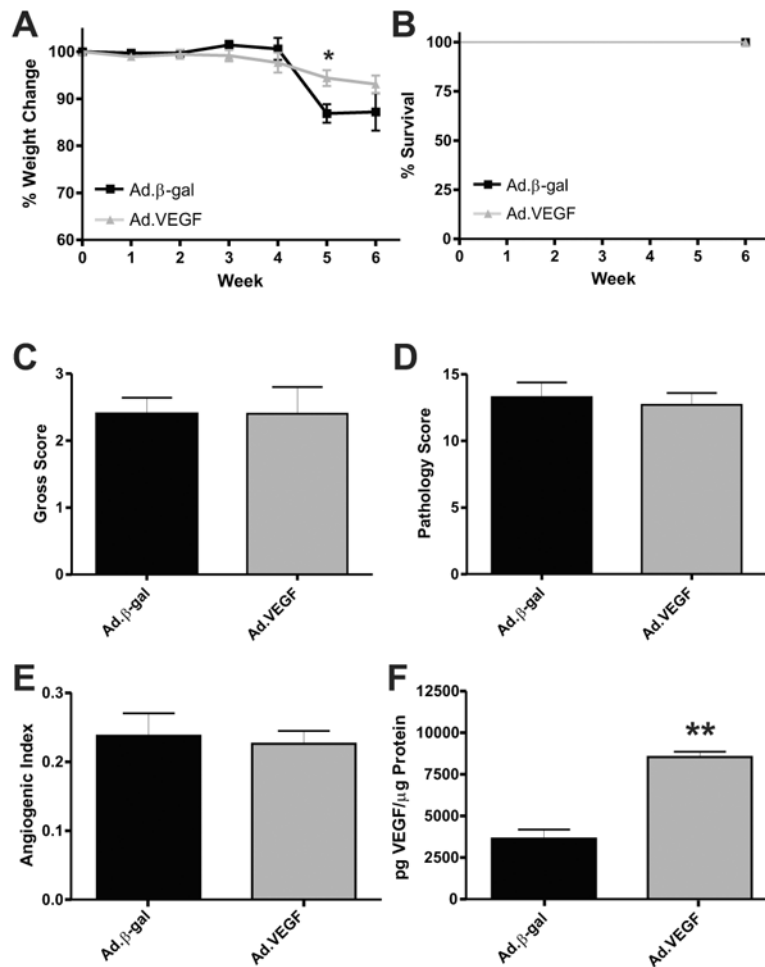




**Figure 4.** The VEGF<sub>164</sub> aptamer dose-dependently prevents increases in vascular density associated with CD4<sup>+</sup> experimental colitis. This figure shows sample images of PECAM-1 (red) staining with DAPI nuclear counterstain (blue) of colonic cross-sections from Control colitic (A), Aptamer Low (B), Aptamer Med (C), and Aptamer High (D) dose groups. (E) The angiogenic indexes for these groups, Control colitic (black), Aptamer Low (red), Aptamer Med (green), and Aptamer High (blue).  $N = 28$  for Control,  $N = 14$  for Aptamer Low,  $N = 18$  for Aptamer Med,  $N = 18$  for Aptamer High.



**Figure 5.** Heat-denatured aptamer fails to prevent disease pathology and increases in vascular density. (A) Weight loss data for control (black) and HD aptamer (gray) treated groups. (B) Survival data for the same. The histopathology scores and angiogenic indexes for these groups are shown in (C,D), respectively. VEGF<sub>164</sub> ELISA data is shown in (E).  $N = 8$  for control and HD aptamer groups,  $N = 5$  for ELISA data.



**Figure 6.** Adenoviral overexpression of VEGF<sub>164</sub> results in maximal development of CD4<sup>+</sup> experimental colitis. (A) Weight loss data for Ad.β-gal (black) and Ad.VEGF<sub>164</sub> (gray) treated mice. (B) Survival data for the same. The gross scores and histopathological scores for these groups are reported in (C,D), respectively. Angiogenic Index data for the same are shown in (E). (F) The VEGF<sub>164</sub> ELISA data for these groups. *N* = 8 for Ad.β-gal, *N* = 9 for Ad.VEGF, *N* = 5 for ELISA data.

**Table 1**  
**Various Dosing Regimens for VEGF<sub>164</sub> Aptamer during 6 Week T cell Colitis Model**

Dose Group	Total Amount	Time between injections	Total Amount Given
AMD Patient	0.3 mg	6 weeks	0.3 mg
Low	40 mg/kg	2 days	18 mg
Medium	80 mg/kg	2 days	36 mg
High	160 mg/kg	2 days	72 mg

The amount of the VEGF<sub>164</sub> aptamer (Macugen) given in AMD treatment, and the amount given in our low, medium, and high doses per day, the frequency of dosing, and the total dose given over a 6- week period.

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