Supplementary Information

Supplemental Methods

Production of anti-DLL4 F(ab')$_2$

For all efficacy and safety studies (with the exception of the definitive rat and monkey F(ab')$_2$ studies designed to enable first-in-human dosing; Fig. 5 and 6), anti-DLL4 F(ab')$_2$ material was made as follows, using good scientific practices. Previously purified anti-DLL4 antibody (YW152F, Ridgway et al 2006) was diluted to 5mg/mL in 50mM sodium citrate buffer, pH 3.0, and porcine gastric mucosa pepsin (#P7000; Sigma-Aldrich, St. Louis, MO) was added at a ratio of 1:100 (w/w). The antibody/enzyme solution was incubated at 37ºC for 4 hours without agitation. The expected proteolytic site for pepsin on the anti-DLL4 molecule is between leucine positions 235 and 236 of the heavy chain portion of the antibody. The enzymatic digest was halted with the addition of a 5% volume of 1.5M TRIS buffer at pH 8.6, resulting in a final pH 8.0. The antibody digestion solution was then filtered using a 0.22µm filter (#567-0020; Nalgene, Waltham, MA) and dialyzed into 20mM sodium acetate buffer, pH 5.0. The F(ab')$_2$ solution was then bound to SP Sepharose high performance resin (#17-1087-05; GE Healthcare, Uppsala, Sweden), washed with 4 column volumes of 20mM sodium acetate, 0.1% Triton X-114, and washed with 4 column volumes of 20mM sodium acetate. The F(ab')$_2$ was eluted from the resin using a 20 column volume 0%-30% gradient of 1M sodium chloride in 20mM sodium acetate and formulated into 10mM histidine sulfate buffer, pH 5.8, 6% sucrose and 0.02% Tween-20 (#28320; Thermo Scientific, Rockford, IL). The anti-DLL4 F(ab')$_2$ was correctly identified by mass spectrometry at 96208.07 Da confirming the predicted cleavage site.

For the definitive safety evaluations of anti-DLL4 F(ab')$_2$ in rat and monkey (Figs. 7, 8), F(ab')$_2$ dosing material was made as follows, using Good Manufacturing Practices (GMP). Anti-DLL4 IgG1 was expressed by CHO cells grown in a non–serum containing chemically defined culture medium, secreted

into the cell culture fluid, and isolated from cellular debris by centrifugation. The IgG-containing harvested cell culture (HCCF) is purified through a series of chromatographic steps, including immobilized pepsin to generate purified anti-DLL4 $F(ab')_2$. The purified protein was formulated by ultrafiltration and diafiltration. Anti-DLL4 $F(ab')_2$ was provided as a single-use liquid formulation containing 200 mg of anti-DLL4 $F(ab')_2$ per glass vial with a protein formulation buffered at pH 5.5 with stabilizers.

HUVEC fibrin gel bead assay

Details of the assay have been described previously (*16, 28*). In the current study, conditioned medium from human skin fibroblast (SF) culture was used instead of the co-cultured SF cells on top of the fibrin gels. HUVEC sprouts were visualized by staining with AlexaFluor488-phalloidin (Invitrogen) and 4', 6-diamidino-2-phenylindole (DAPI, Sigma).

Mouse neonatal retina assay

Details of the assay have been described previously (*16*). In brief, P2 CD1 mice were injected IP with PBS, anti-DLL4 IgG1, or anti-DLL4 $F(ab')_2$ at a dose of 30 mg/kg daily. Eyes were collected on P5, and dissected retinas were subjected to immunostaining procedure.

Development of xenograft tumor models

$5X10^6$ HM7 cells were inoculated SC into 8- to 10-week-old beige nude female mice. When the average tumor size reached 150 mm^3 animals were sorted into groups consisting of 8-10 mice/group and subjected to affinity variants of anti-DLL4 (LM1 and HM6) administration for anti-tumor efficacy evaluations.

SW620 colon carcinoma tumors were maintained by serial SC transplantation in athymic nude mice (nu/nu, 10-11 week old female mice, Harlan). Each test mouse received a tumor fragment (1 mm^3) implanted SC in the right flank.

For Calu-6 model, $10X10^6$ tumor cells were inoculated SC into 8- to 10-week-old beige nude female mice.

Histological analysis of mouse liver

Adult CD1 mice were used to assess the liver effect of various anti-DLL4 antibodies. Following completion of the dosing period, formalin-preserved liver tissues were evaluated via microscopic anatomic pathology using hematoxylin and eosin (H&E) staining by a board-certified veterinary pathologist.

Gene expression analysis of mouse livers after antibody treatment

Following antibody treatment, mouse livers were snap frozen for RNA analysis. RNA was extracted using RNeasy Mini kit (Qiagen), and 2 μg total RNA was used to make cDNA using the Taqman-RT kit. Gene expression was analyzed using 20 ng cDNA, Taqman Gene Expression Master Mix (Applied Biosystems), and gene specific Taqman Gene Expression Assays (Applied Biosystems) on an Applied Biosystems 7500 RT-PCR system. Probe sets used for this analysis were GAPDH, CD93, Egfl7, and Lgals1. Results were normalized to GAPDH.

PK studies in mice, rats and monkeys

PK studies of anti-DLL4 $F(ab')_2$ and IgG1 in each species were conducted in separate studies. The PK study in athymic nude mice was conducted at Genentech, Inc, while the toxicokinetic studies in Sprague-Dawley rats and cynomolgus monkeys were conducted at Covance Laboratories, Inc. (Madison, WI).

PK Assay Method

For rat and cyno monkey PK study samples, recombinant human DLL4 extracellular domain (ECD) was diluted to 1 µg/mL in carbonate buffer pH 9.6 and added to a 96-well high binding polystyrene ELISA plate (Nunc 439454), 100 µL per well, and incubated overnight at 4°C. The plate was washed with wash buffer (PBS/0.05% Tween 20), and then 200 µL of assay buffer (PBS, 0.5% BSA, 0.05% polysorbate 20, 0.05% ProClin 300, pH 7.4) was added to each well. After incubation and washing, 100 µL of diluted serum sample was added and incubated for 2 hours at room temperature. The plate was washed, and captured antibodies were detected by adding 100 µL of monoclonal 10C4 anti-IgG (GNE IgG1 framework-specific (*50*) HRP conjugate. After incubation and washing, signal was generated by adding tetramethyl benzidine (TMB; KPL) and the reaction was terminated with 1M phosphoric acid. Absorbance was measured at 450 nm using 620 nm reference on a Tecan ELISA plate reader.

For mouse PK study samples, recombinant human DLL4 ECD was diluted in coating buffer and 25 µL per well was added to a 384-well ELISA plate (Nunc, Rochester, NY; cat # 464718), and incubated overnight at 4°C. The plate was washed with wash buffer, and then 50 µL of blocking buffer (PBS, 0.5% BSA, 0.05% Proclin 300, pH 7.4) was added to each well. Serum samples were diluted in sample buffer (PBS pH 7.4 + 0.5% BSA + 10 ppm Proclin + 0.05% Tween 20 + 0.25% CHAPS + 5 mM EDTA + 0.35M NaCl). After plate was washed, 25 µL of diluted serum sample was added onto the plate and incubated for 2 hours at room temperature with gentle agitation. The plate was washed, and captured analytes were detected by 25 µL of HRP- conjugated goat anti-human IgG $F(ab')_2$ fragment specific or goat anti-human IgG, Fcg fragment specific reagents (Jackson ImmunoResearch, West Grove, PA; cat # 109-036-097 for anti-DLL4 $F(ab')_2$, and cat # 109-036-098 for intact huIgG1). After incubation at room temperature for 1 hour with gentle agitation followed by washing, signal was generated by adding 25 µL of 3,3',5,5' tetramethylbenzidine (TMB-E); (Moss, Inc; Pasadena, MD, cat TMBE-100) and the reaction was

terminated with 25mL of 1M phosphoric acid. Absorbance was measured at 450 nm using 620 nm as reference on a MultiSkan Ascent (Thermo, Waltham, MA) plate reader. The absorbance values were then analyzed with 4-p fit equations to calculate the concentration.

Bridging Anti-Therapeutic Antibody (ATA) Assay Method

Anti-DLL4 $F(ab')_2$ was conjugated to biotin (Pierce Sulfo-NHS-LC-biotin, Cat No. 21327) or digoxigenin (Invitrogen 3-amino-3-deoxydigoxigenin hemisuccinamide, succinimidyl ester, Catalog No. A2952, DIG). The two conjugates were incubated at a concentration of 2 µg/mL in a 1:1 stoichiometric ratio, with serum sample or control, overnight, at room temperature on an orbital mixer. 70 µL of combined $F(ab')_2$-DIG solution in assay buffer was added to 96-well polypropylene plate (Corning Catalog No. 3365) with 70 µL of diluted serum sample. The reaction mixture was then transferred to a washed Streptavidin High Bind plate (Roche Catalogue No. 11989685001) and incubated for two hours at RT with shaking. Bound antibodies were detected by adding 100 µL of mouse monoclonal anti-DIG HRP conjugate (125 ng/mL, Jackson Catalog No. 200-032-156) to the streptavidin plate. Signal was generated by adding tetramethyl benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD), then the reaction was terminated with 1M phosphoric acid. Absorbance was measured at 450 nm/630 nm on a spectrophotometer (Tecan Sunrise). Assay Buffer was used as a negative control due to the pre-existing anti-$F(ab')_2$ antibodies observed in cynomolgus monkey serum. Hyperimmunized goat antiserum, which had been affinity purified to enrich for anti-drug CDR reactivity (Genentech, South San Francisco, CA), was used as a positive control. The anti-DLL4 CDR antibodies were added to the assay buffer at 750 ng/mL (low control) and at 125 µg/mL (high or titer control).

Table S1: Pharmacokinetic parameters of anti-DLL4 IgG1 and F(ab')2 in mouse.

Parameter	Anti-DLL4 IgG	Anti-DLL4 F(ab')2	
Dose	20 mg/kg	10 mg/kg	50 mg/kg
Cmax (nM)	3493	1600	9160
AUC_{0-7} (day*nM)	13427	276	1500
CL (mL/day/kg)	8	362	333

C_{max}: maximum concentration; AUC_{0-7}: area under the concentration-time curve during the week following first dose administration (Day 0-Day 7); CL: clearance.

Table S2: Pharmacokinetic parameters for anti-DLL4 F(ab')2 and IgG1 in rat and monkey.

Compound	Rat			Monkey		
	Dose (mg/kg)	**C_{max}** (nM)	**$AUC_{(0-7)}$** (day*nM)	**Dose** (mg/kg)	**C_{max}** (nM)	**$AUC_{(0-7)}$** (day*nM)
Anti-DLL4 $F(ab')_2$	**3**	310 ± 120	252	**5**	1270 ± 80	800 ± 110
	10	1480 ± 200	891	**15**	3620 ± 310	2040 ± 190
	30	4090 ± 270	2710	**50**	11730± 940	6400 ± 640
	100	14100 ± 1460	9290	**na**	na	na
Anti-DLL4 IgG1	**1**	280 ± 220	400	**0.2**	33 ± 7	60 ± 13
	3	387 ± 167	907	**0.8**	147 ± 13	420 ± 47
	10	1593 ± 167	4007	**3**	560 ± 53	1680 ± 187
	30	3073 ± 2193	10367	**12**	2227 ± 287	7540 ± 947

C_{max}= maximum concentration; AUC_{0-7}= area under the concentration-time curve during the week following the first dose administration (Day 0-Day 7).

Table S3: Anti-DLL4 F(ab') 2 mitigates the occurrence of vascular neoplasm in rats.

AUC 0-7 (day*nM)	Dose level (mg/kg/wk)	Incidence of vascular neoplasms in skin (%)	AUC 0-7 (day*nM)	Dose level (mg/kg/wk)	Incidence of vascular neoplasms in skin (%)
Anti-DLL4 IgG1			Anti-DLL4 $F(ab')_2$		
0	0	0	0	0	0
400	1	0	NT	NT	NT
907	3	7	891	10	0
4007	10	27	2710	30	0
10367	30	33	9290	100	0

A

	CDR-L1						CDR-L2						CDR-L3					
Antibody	28	29	30	31	32	33	50	51	52	53	54	55	91	92	93	94	95	96
YW152F	D	V	S	T	A	V	S	A	S	F	L	Y	S	Y	T	G	T	V
LM1	D	V	S	T	A	V	S	A	S	F	L	Y	S	Y	T	A	T	V
HM6	D	V	S	T	A	V	S	A	S	F	L	Y	S	Y	T	G	T	V

	CDR-H1								CDR-H2										CDR-H3								
Antibody	28	29	30	31	32	33	34	35	50	51	52	A	53	54	55	56	57	58	95	96	97	98	99	100	101	102	103
YW152F	T	F	T	D	N	W	I	S	Y	I	S	P	N	S	G	F	T	Y	D	N	F	G	G	Y	F	D	Y
LM1	T	F	T	D	N	W	I	S	Y	I	S	P	P	S	G	F	T	Y	D	N	F	G	G	Y	F	D	Y
HM6	T	F	T	D	N	W	I	S	Y	I	S	P	N	S	G	F	T	Y	D	N	F	G	G	A	F	D	Y

B

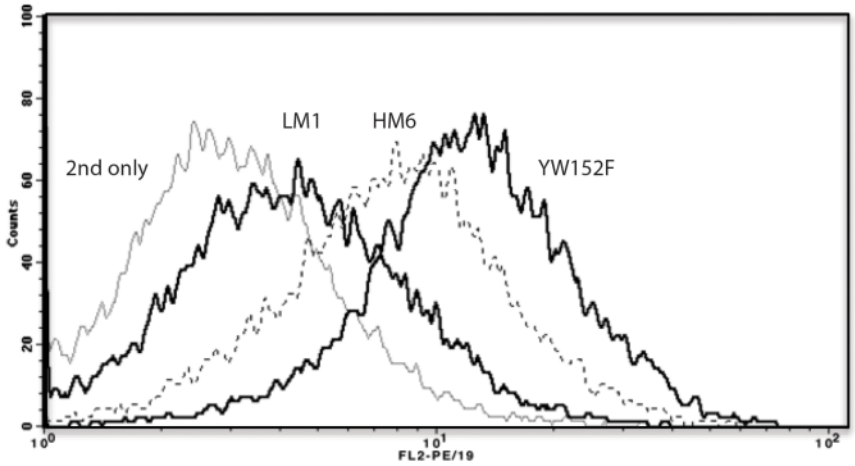

Figure S1. Generating and characterizing low affinity anti-DLL4 antibodies. (A) Sequence alignment of the original anti-DLL4 IgG1 molecule (clone YW152F) with mutant antibodies (LM1 and HM6, shown in Fig. 1). (B) FACS analysis of antibody binding to HUVECs. 2nd only= secondary only control.

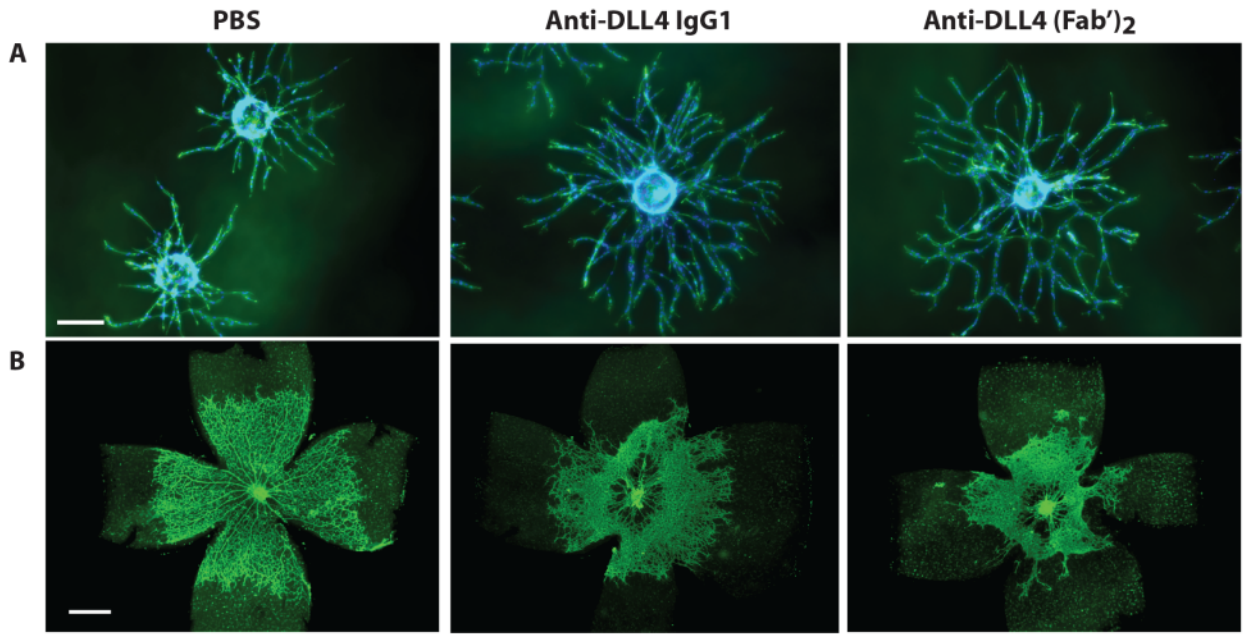

Figure S2. Characterization of anti-DLL4 F(ab')2 (A) Anti-DLL4 F(ab')2 caused a marked increase of angiogenic sprouting and branching of HUVECs in fibrin gels with a similar potency relative to anti-DLL4 IgG1, indicating effective DLL4 pathway blockade with the F(ab')2 molecule. HUVEC sprouts were visualized by staining with DAPI and AlexaFluor488-phalloidin; Scale bar = 175 mm. (B) Anti-DLL4 F(ab')2 also caused defective vascular development of neonatal mouse retina. Whole-mount retinas from P5 mice were stained with isolectin B4. Anti-DLL4 F(ab')2 treatment resulted a marked increase of retinal vascular density (isolectin B4), similar to that observed with anti-DLL4 IgG1. Scale bar = 500 mm.

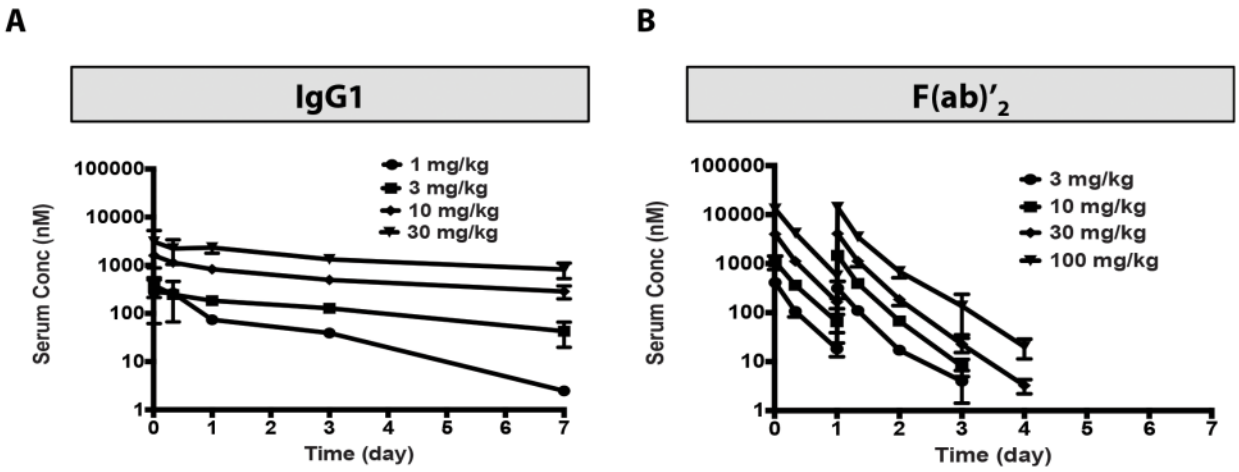

Figure S3. Pharmacokinetic profile of anti-DLL4 IgG1 and F(ab')2 in rat toxicity studies. Serum-concentration time data in Sprague–Dawley rats for (A) anti-DLL4 IgG1 using a weekly dosing schedule and (B) anti-DLL4 F(ab')2 using a 2 on/5 off dosing schedule.

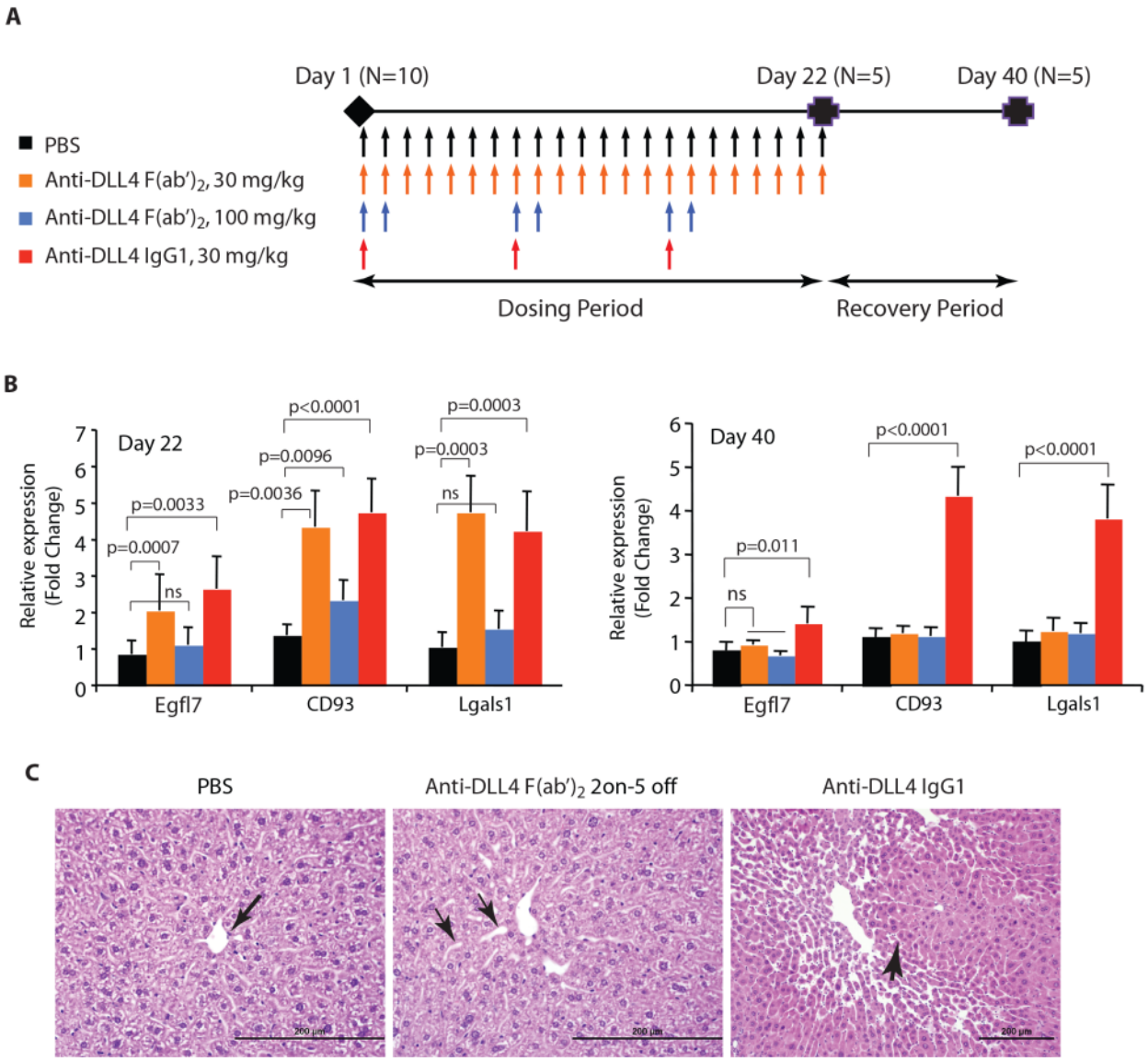

Figure S4. Mitigation of liver toxicity in mice with anti-DLL4 F(ab')2. (A) Depiction of the experimental design/dosing regimens used to compare the impact of anti-DLL4 F(ab')2 and IgG1 on mouse liver. (B) Liver gene expression changes previously shown to be associated with anti-DLL4 treatment were evaluated as a direct readout of DLL4 pathway inhibition. Results are represented as fold change in mRNA levels (mean ± SEM), n=5/group. Two-tailed unpaired t test was used to calculate P values. (C) Liver histopathology was evaluated on Day 40 to assess relative toxicity of anti-DLL4 F(ab)'2 versus IgG1 following intermittent or continuous exposure at the same cumulative weekly dose. Scale bar= 200 uM. Left panel, normal mouse liver, arrow indicates the central vein. Middle panel, minimally dilated hepatic sinusoids (arrows) observed following F(ab)'2 treatment. Right panel, IgG1 induced a more severely dilated central vein and hepatic sinusoids as well as a hyperplastic nodule (arrow).

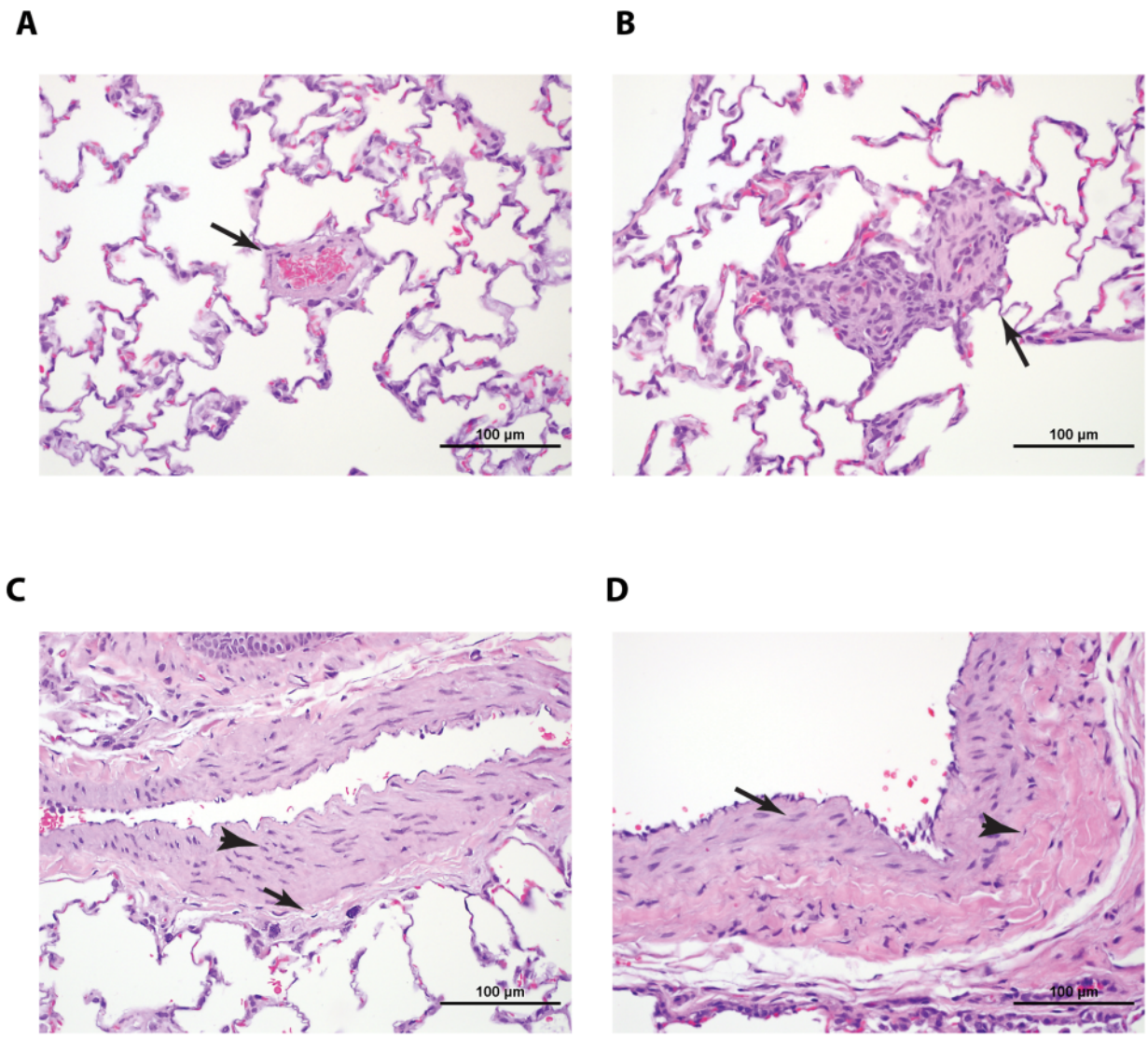

Figure S5. Novel toxicities identified with anti-DLL4 F(ab')2 in rats. (A) Normal lung in a vehicle-treated rat; pulmonary small artery (arrow). (B) Hypercellularity and hypertrophy of muscularis layer of small pulmonary artery (arrow) in the lung of a rat treated with anti-DLL4 F(ab')2 (15 mg/kg/wk; 2 on/5 off schedule). (C) Normal lung and pulmonary artery in a vehicle-treated rat after an 8-week recovery period, including the arterial muscularis layer (arrowhead) and arterial adventitial layer (arrow). (D) Reversibility of lung and pulmonary artery lesions (arterial muscularis layer, arrow) observed in a rat treated with anti-DLL4 F(ab')2 (50 mg/kg/wk; 2 on/5 off schedule) after an 8-week recovery period, with the development of a fibrotic arterial adventitial layer (arrowhead). All panels are representative H&E sections from the indicated dose group.