Supporting Information

Al-Jamal et al. 10.1073/pnas.0908401107

SI Text

Experimental Procedures. Advanced RPMI, FBS, penicillin/streptomycin, PBS were bought from Invitrogen; Murine vascular endothelial cells SVEC 4-10 was a kind gift from AstraZeneca; human umbilical vein endothelial cells (HUVECs) were a kind gift from Hammersmith Hospital; and [^{3}H] Lysine.HCl (5 mCi) was purchased from Amersham. Optiphase® scintillation cocktail was purchased from Wallac and BTS450® tissue solubilizer was purchased from Beckman instruments. Isoflurane was purchased from Abott.

Cultrex® BME without phenol red was purchased from R&D; unfractionated heparin, 30% Brij-35, Drabkin's reagent, hemoglobin standard, Giemsa stain, MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), Anti-β-tubulin monoclonal, (clone TUB.2.1) were purchased from sigma; DeadEnd™ Fluorometric TUNEL System was purchased from Promega; Dulbecco's modified eagle medium (DMEM) and bFGF were purchased from Invitrogen; and Clonetics EGM-2 BulletKit (CC-3162) was purchased from Cambrex. Murine melanoma B16F10 (CRL-6475) was purchased from ATCC; Annexin-V-Fluos staining kit was purchased from Roche Diagnostics GmbH. EDTA containing tubes (0.5 mL) were purchased from Teklab. Texas Red-conjugated phalloidin was purchased from Molecular Probes, Invitrogen. Biotinylated secondary antimouse immunoglobulin G, fluorescein isothiocyanate (FITC)-labeled avidin D and Vectashield with DAPI were purchased from Vector Laboratories. Synthesis of PLL dendrimer and [^{3}H] PLL-dendrimer synthesis is described in supplementary material.

PLL dendrimer and [^{3}H]-PLL-dendrimer synthesis. The synthesis of [^{3}H] labelled Boc-Lys(Boc)-OH was first carried out. To a 100 mL round bottom flask containing 12 mL of 1 M NaOH, cold lysine.HCl (2 g, 10.87 mmol), [^{3}H] lysine.HCl (5 mCi) were added and stirred at ambient temperature and then diluted with tert-butyl alcohol (9 mL). To a well-stirred, clear solution di-tert-butyl dicarbonate (4.9 g, 21.97 mmol) was added dropwise within 1 h and kept stirring for 24 h. A white precipitate appears during addition of di-tert-butyl dicarbonate. The reaction mixture was extracted with hexane (2×3 mL), and then the hexane layer was extracted with saturated $NaHCO_3$ solution (3×20 mL). The combined aqueous layers were acidified to pH 1-1.5 by careful addition of solution of 1.5 g of $KHSO_4$ in 10 mL water. The turbid reaction mixture is then extracted with ethyl acetate (4×20 mL). The combined organic layers were then washed with water (2×40 mL), dried over anhydrous $MgSO_4$ and filtered. The solvent is removed under reduced pressure using a rotary evaporator. Radio-labelled Boc-Lys(Boc)-OH.HCl was yielded at 31% in the form of oil and dissolved in DMF for the dendrimer synthesis.

The synthesis and purification of the water soluble, glycine cored, PLL-dendrimer bearing 64-surface amino groups used in this study (MW 8149 Da) has been described in detail (1). Briefly, The dendrimer 6th generation PPL dendrimer was prepared by stepwise solid-phase peptide synthesis on a 4-Methylbenzhydralamine (MBHA) resin with a loading capacity of 0.67 mmol/gm using tert-butoxycarbonyl (Boc) methodology. In brief, Boc-Gly-OH (4 equivalents relative to resin loading), HBTU (4 equivalents), HOBt (4 equivalents) were dissolved in the minimum volume of DMF necessary to dissolve all the components and finally DIEA (8 equivalents) was added to the solution to make the first coupling. The efficiency of coupling was checked by the Kaiser test during the first 30–240 min. Double couplings were sometimes used to ensure the completion of the coupling reactions before the subsequent deprotection. *N*-termini deprotection was carried out with 100% TFA (2×1 min). Six successive couplings/deprotections used four fold excess HBTU activated Boc-Lys(Boc)-OH.DCHA, HOBt, and DIEA were performed. The compound was cleaved from the resin by HF (1 g resin peptide, 10 mL HF, 1.5 h) in a salted ice bath at -5 °C. After cleavage, the PPl-dendrimer was precipitated with ether to remove fluoride salt. The precipitated PLL dendrimer and resin were filtered, and washed with 20% glacial acetic acid or water and freeze-dried, dried over P_2O_5 for three days and stored over silica gel.

For the synthesis of [^{3}H]-PLL dendrimer, the same method described in ref. 18 was used except that the reaction started with 0.25 g MBHA resin using one fold excess HBTU-activated [^{3}H]-Boc-Lys (Boc)-OH.HCl (8.5×10^7 dpm/58 mg or equivalent), and followed by a coupling using four fold excess HBTU-activated cold Boc-Lys (Boc)-OH.DCHA. The labelling was performed at first, second, third, and fourth lysine coupling, leading to incorporation of 35% of total [^{3}H]-Boc-Lys(Boc)-OH.HCl added to the reaction. The yielded PPL dendrimer has a specific radioactivity of 0.28 mCi/mg.

Tubule formation assay. In vitro endothelial tubular formation assay was performed as described previously with minor modifications (2). Briefly, 200 μL of the basement membrane extract Cultrex® BME was added to each well of 48-well plates and allowed to polymerize for 1 h at 37° °C. SVEC4-10 (1×10^5 cells/well) were plated on the Cultrex® BME and cultured in a final volume of 0.5 mL of DMEM medium alone or with various concentrations of PLL dendrimer (60 or 100 μg/mL). After 3, 5, and 7 h of incubation in 5% CO_2 at 37 °C, cell growth and 3-dimensional organization were observed through an Olympus inverted photomicroscope and photographed with 10X lens, with and without Giemsa counterstaining. Percentage of inhibition of tubular formation was measured by counting and averaging the branch points from 4 different fields (x100). Data were analyzed by Student's *t* test (one-tailed) on Excel program and $P \leq 0.05$ was considered significant.

Chick chorioallantoic membrane (CAM) angiogenesis assay. Antiangiogenic activity in CAM was assayed as described in ref. 3. The CAM of day 4 chick-fertilized embryos was exposed by making a window in the egg shell, and a silicone disc was then placed directly on the CAM. Ten microliter of dendrimer or protamine sulphate solution in water, containing 10–100 μg per CAM, was mixed with 10 μL of 1% methyl cellulose in 0.9% NaCl, and pipetted into the silicone ring, the window was sealed, eggs were reincubated, and the grafts were recovered after 24 h. Angiogenic inhibition was indicated by the formation of an avascular zone around the ring of 3 mm diameter. The results were expressed as the percentage of embryos showing inhibition ($n = 7 - 10$) from three different experiments.

Animal-handling procedures. Six to eight week old female C57/BL6 mice (Harlan Laboratories) were caged in groups of 4–7 with free access to food and water. A temperature of 19–22 °C was maintained, with a relative humidity of 45–65%, and a 12 h light/dark cycle. Animals were acclimatized for 7 days before each experiment. All animal experiments were performed in compliance with the UK Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989).

PLL dendrimer tissue distribution and pharmacokinetics in vivo. Mice were inoculated subcutaneously with 1×10^6 B16F10 Murine melanoma cells in 100 μL PBS on the left flank. When the tumor reached 100 mm^2 in diameter, mice were anesthetized using isofluorane and injected via tail vein with 25 mg/kg in 200 μL PBS as a single dose (approximately 0.14 mCi per animal). Animals were placed in metabolic cages for 24 h for urine and feces collection at 24, 48, and 72 h post-dosing. Blood was collected by bleeding 50 μL from superficial tail vein at 1 min, 3 min, 5 min, 10 min, 30 min, 6 h, 24 h, 72 h, and 168 h. Each animal was bled only twice during the whole experiment. At the scheduled time points (0 h, 0.5 h, 6 h, 24 h, 72 h, and 168 h post dosing), animals were culled by cervical dislocation. Skin, heart, lung, liver, spleen, kidney, thigh muscle, femur bone, brain, and right and left tumors were removed before radioactivity counting; an aliquot of 0.1 mL of blood or 200 μg of each organ was solubilized with 1.0 mL of BTS450® tissue solubilizer shaken overnight at 55 °C, combined with 17 mL of the acidified Optiphase® scintillation cocktail, and kept in a dark cold room at 4 °C for 24 h before counting in a LS6500 Multi-Purpose Scintillation Counter (Beckman). Colored samples were decolorized with 200–400 μL of 30% hydrogen peroxide, and with 200–400 μL of isoamyl alcohol added to stop foaming. Mouse plasma volume was calculated based on the formula that blood constitutes 8.5% of total body weight. Results were expressed as the percent of the injected dose per organ, or the percent of the injected dose per gram tissue ± S.D. ($n = 4$).

Matrigel plug in vivo assay. The assay was performed as described in details by Passaniti (4). Briefly, 500 μL of Cultrex® BME containing bFGF (100 ng/mL) and heparin (40 units/mL) was injected subcutaneously into C57BL/6 mice at 4 °C ($n = 4$). After injection, matrigel polymerized to form a plug. Twenty-four and forty-eight h later, mice were administered with dendrimer (50 mg/kg) or PBS via tail injection. After 7 days, animals were sacrificed and the matrigel plug was removed and cut into halves. One half was homogenized in 0.5 mL of 0.1% Brij-35 lysis buffer), and centrifuged for 5 min at 13,000 rpm. To quantify the formation of functional vasculature in the plug, hemoglobin contents were measured in duplicate in the supernatant using Drabkin's reagent (40); 200 μL of the supernatant was transferred into a different tube containing 3 mL of Drabkin's reagent. The absorbance was read at 540 nm after 20 min incubation using Drabkin's reagent solution as a blank. The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. Data were analyzed by Student's t test (one-tailed) on Excel program and $P < 0.05$ was considered significant. For histological analysis, other halves of matrigel plugs were fixed in 10% buffered formalin and processed for routine histology with hematoxylin and eosin stain as described in Heamatoxylin/Eosin (H&E) Tissue Histology.

Window chamber assay and tumor implantation. Female severe combined immunodeficiency (SCID) mice (12–16 weeks old, 28–32 g) were anesthetized using fentanyl-fluanisone and midazolam i.p., as described previously (5). Briefly, an aluminum window chamber (total weight approximately 2 g), holding two parallel glass windows, was implanted into a dorsal skin flap. A tumor fragment (approximately 0.5 mm in diameter) from a donor animal was implanted onto the exposed panniculus muscle before closing the chamber, allowing a depth of approximately 200 μm for tumor growth. Animals were given a s.c. injection of dextrose saline (1 mL) and an i.p. injection of buprenorphine (0.1 mL, Vetergesic) to aid recovery and then kept in a warm room (28–30 °C), until the day of the experiment.

Intravital microscopy. SCID mice received 200 μL of dendrimer in PBS (50 mg/kg/day/i.v.) once daily on day 4 and 7 post-tumor implantation or the same volume of PBS. Tumors were approximately 3–5 mm in diameter when used. For the assessment of tumor vascularization in treated and untreated tumors, transmitted light images were captured at various magnifications once per day for 10–12 days after tumor transplantation.

Tumor growth delay study. Mice were inoculated subcutaneously with 1×10^6 B16F10 Murine melanoma cells in 100 μL PBS on the left flank. The tumor volume was estimated by bilateral Vernier caliper measurement once daily and calculated using the formula (width × width) × (length) × ($\pi/6$), where length was taken to be the longest diameter across the tumor. For systemic administration of the PLL dendrimer, mice were anesthetized using isofluorane and injected via tail vein with (*i*) 50 mg/kg i.v., in 200 μL PBS once a day on days 1 and 2 post-tumor implantation, (*ii*) 60 mg/kg s.c., in 200 μL PBS twice daily for 10 days starting on days 1 post-tumor implantation, and (*iii*) PBS. Mice were sacrificed by cervical dislocation on day 10 when tumors of the naïve group reached 800 – 1,000 mm^3. Growth delay was calculated by subtracting the average time for control tumors to grow 4-fold in volume from the time required for treated tumors to increase in volume by the same amount from the day of PLL dendrimer first PLL-dendrimer injection. Tumors were cut into two halves and fixed in formalin or zinc fixative for TUNEL or CD31 staining, respectively.

Tumor necrosis and apoptosis assays. Tissue sections were deparaffinised in Histoclear and rehydrated through graded ethanol. The DeadEnd™ Fluorometric TUNEL System (Promega) was used to label nicked DNA through incorporation of fluorescein-12-dUTP. Samples were incubated with recombinant Terminal Deoxynucleotidyl Transferase (rTdT) as per manufacturer's instructions and fluorescein labelling was visualised using confocal microscopy (LSM 510, Zeiss). Propidium iodide was used to counterstain nuclei. Areas of necrosis and apoptosis were assessed qualitatively by examination of H&E and TUNEL processed sections respectively.

Immunohistochemistry staining for CD31. Paraffin-embedded zinc fixed sections were kept at 60 °C in incubator overnight, dewaxed in 2 sequential Histo Clear II baths, and then rehydrated in a series of descending concentrations of 3 alcohol baths. Antigen retrieval was done by incubating the sections with 20 μg/mL Proteinase K at 37 °C for 10 min. Endogenous peroxidise activity was blocked by 0.3% solution of hydrogen peroxide in methanol. Sections were then incubated with diluted blocking serum (rat IgG ABC kit, Vectastain) to prevent non-specific bindings. The rat polyclonal anti-mouse CD31 antibody (SantaCruz Biotechnology) was used at 1 : 100 dilution as a primary antibody. Sections were covered with the primary antibody and left in a humid chamber for 1 h. After washing, sections were incubated with biotinylated anti-rat IgG secondary antibody (IgG ABC kit, Vectastain) for 45 min. Preformed ABC reagent was then applied onto the sections which was then followed by DAB (Vector) treatment to visualize peroxidise activity. Counterstaining was performed by hematoxylin treatment. After dehydration in a series of ascending concentrations of ethanol baths and refixation in Histo Clear II, sections were mounted with mounting medium. Twelve random light microscopy images were captured (X10 magnification) from each tumor section, CD31 positive cells were then counted using Image J software.

Cell cultures. HUVECs cells were maintained in endothelial cell basal medium-2 supplemented with hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, Heparin, FBS, hEGF, and GA-1000 at 37 °C at 5% CO_2. Cells were passaged when they reached 80% confluence and used up to passage 5.

PLL dendrimer dynamic light scattering. The hydrodynamic Z-average diameter and zeta potential of the PLL dendrimer were measured using a Zetasizer Nano ZS (Malvern Instruments) at dendrimer concentration of 100 μM (1 mg/mL) in PBS at pH 7.4. The average of three measurements provided results expressed as Z-average diameter (nm) ±S.D. and zeta potential (mV) ±S.D.

Cell viability assays. HUVECs cells (50,000 cells/well) were subcultured into 24-well plates. Three days later, cells were allowed to interact with the PLL dendrimer in complete media for 15 or 60 min at 37 °C in a humidified atmosphere (5% CO_2) then allowed to recover for another 24 h. Final concentrations reached were 10–1,000 μg/mL. The live-death cell assay was performed with a PI/annexin-V-FITC staining kit according to the instructions of the manufacturer. In brief, monolayers were trypsinised into single-cell suspensions and stained according to the manufacturer's instructions. Samples were analyzed on a flow cytometer using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a filter 615 nm for PI detection. Electronic compensation of the instrument was performed to exclude overlapping of the two emission spectra. Cell death was expressed as percentage cell population stained with Annexin V or with PI staining. A CyAn ADP flow cytometer (DakoCytomation) was used to analyze 20,000 cells per sample. All conditions were tested in triplicate.

SVEC4-10 (50,000 cells/well) were subcultured into 24-well plates. After 24 h, , cells were allowed to interact with the dendrimer in complete media for 2, 12, or 24 h at 37 °C in a humidified atmosphere (5% CO_2). Final concentrations reached were 1–1,000 μg/mL. Cell death was assayed by MTT assay. Cells were incubated with MTT solution at 0.5 mg/mL MTT final concentration for up to 4 h. Media was then removed and the formazan produced was dissolved in 200 μL DMSO and absorbance was read in a plate reader at 560 nm. All conditions were tested in triplicate.

HUVECs cytoskeleton assay. Analysis of the cytoskeleton was performed in HUVECs which were plated on fibronectin-coated Permanox slides at a density of 5×10^3 cells/cm^2. Cells were treated 48 h after plating with PLL dendrimer (0–600 μg/mL diluted in OptiMem) for 1 h and then fixed with 3.7% formaldehyde and permeabilized in 0.1% Triton X-100. Cells were then incubated with anti-β-tubulin for 2 h followed by sequential incubations with biotin-labeled antimouse IgG and FITC-labeled avidin D, the latter added together with 5 U/mL of Texas Red-conjugated phalloidin. Slides were mounted in Vectashield with DAPI and fluorecence images were taken with a Leica DMI4000B fluorescence microscope and using LASAF control and analysis software package.

Toxicity of PLL dendrimer in vivo. Non-tumor bearing mice were used in this study. Mice were anesthetized using isofluorane and injected via tail vein with 25 mg/kg in 200 μL PBS as a single dose. The mice were placed individually into metabolic cages (Tecniplast) and monitored for 24 h. Animal weight, water consumption, urine, and feces productions were monitored at time points 24, 48, and 72 h post-dosing. Whole blood was collected from the inferior vena cava of the mice under terminal dose of anaesthesia, using 21 G x 38 mm BD needles in 0.5 mL EDTA containing tubes, immediately were placed on ice, transferred to the Royal Veterinary College Laboratory where automated complete blood count (CBC) and differential WBC counts were performed. Urine collected from mice over a 24 h period was analyzed using Multistix 10 SG reagent strips for the following parameters: glucose, protein, RBCs, and leukocytes. After confirmation of animal death, liver, spleen, and kidneys were removed and fixed in 10% buffered formalin for routine histology and hematoxylin and eosin stain by the Laboratory Diagnostic Service of the Royal Veterinary College (London). Microscopic observation of tissues was carried out with Nikon Microphot-FXA microscope coupled with Infinity 2 digital camera.

1. Al-Jamal KT, Ruenraroengsak P, Hartell N, Florence AT (2006) An intrinsically fluorescent dendrimer as a nanoprobe of cell transport. *J Drug Target*, 14:405–412.
2. Medhora M, et al. (2002) Epoxygenase-driven angiogenesis in human lung microvascular endothelial cells. *Am J Physiol Heart Circ Physiol*, 284:H215–H224.
3. Kasai S, et al. (2002) Design and synthesis of antiangiogenic/heparin-binding arginine dendrimer mimicking the surface of endostatin. *Bioorg Med Chem Lett*, 12:951–954.
4. Passaniti A, et al. (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest*, 67:519–528.
5. Tozer GM, et al. (2005) Intravital imaging of tumour vascular networks using multiphoton fluorescence microscopy. *Adv Drug Deliver Rev*, 57:135–152.

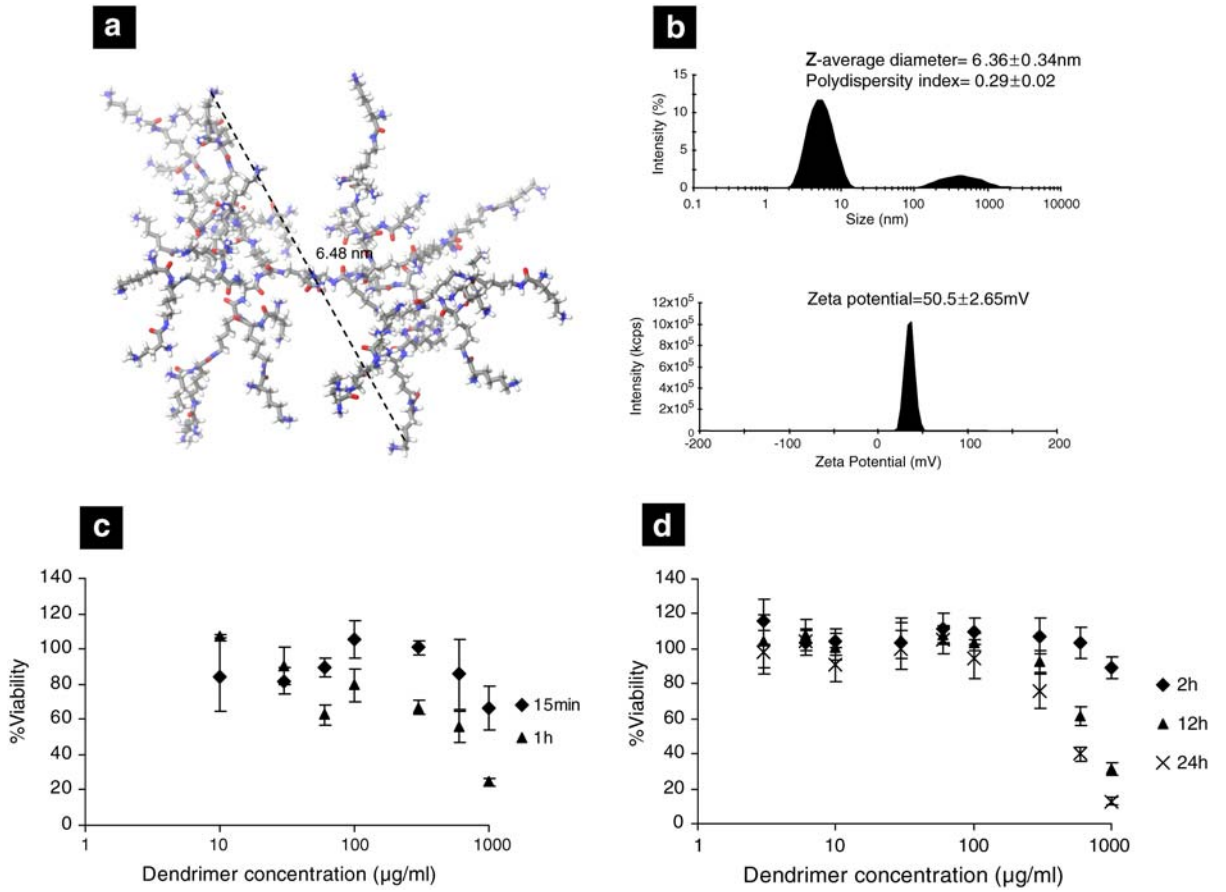

Fig. S1. PLL-dendrimer effect on endothelial cell viability. (*A*) Molecular modeling and (*B*) physicochemical properties of the 6th generation PLL dendrimer used in this study. (*C*) % viability of HUVECs assessed by Annexin V-FITC/PI staining. (*D*) % viability of SVEC4-10 assessed by MTT assay, after incubation with the dendrimer for various time points.

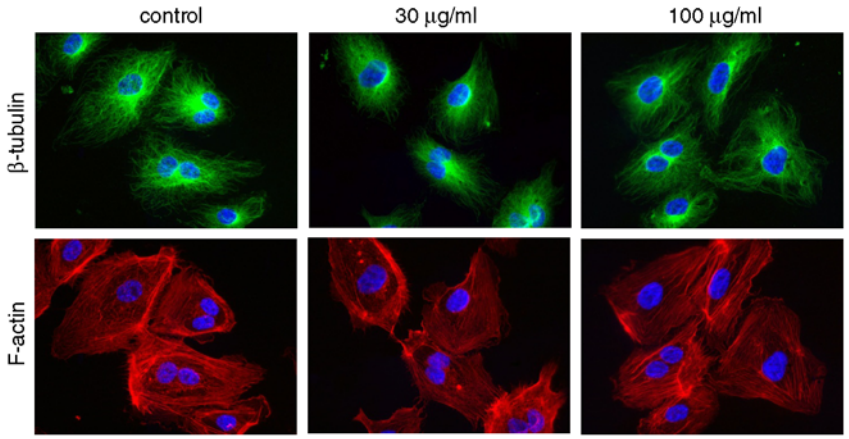

Fig. S2. PLL-dendrimer effect on endothelial cytoskeleton. Staining of microtubule and actin filaments of HUVECs incubated with the PLL dendrimer for 1 h, microtubules stained with a β-tubulin antibody (green) and Texas-red phalloidin for actin filaments (red). Microtubules appeared thinner and partially disrupted in cells exposed to ≥30 μg/mL dendrimer. Actin structures remodeled into stress fibers in cells exposed to ≥100 μg/mL dendrimer.

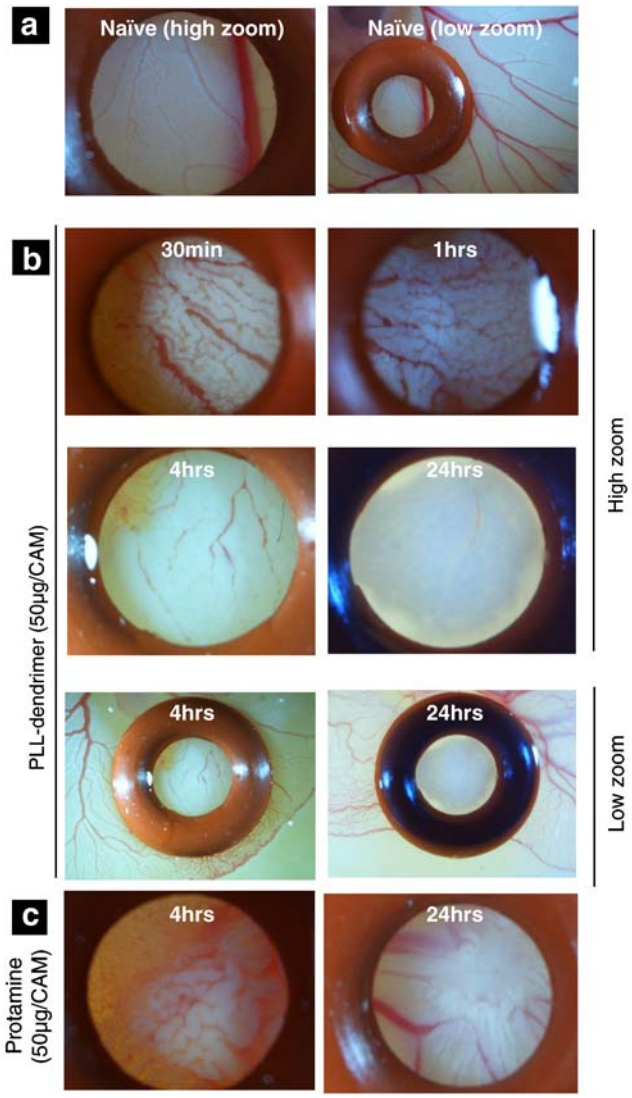

Fig. S3. Inhibitory effect of dendrimer on in vivo angiogenesis in CAM. (*A*) 50 μg/CAM PLL dendrimer was added to the CAM surfaces of 4 day old fertilized eggs, and then the eggs were incubated and photographed after 30 min, 1 h, 4 h, and 24 h. Low zoom images show the retarded growth of the whole CAM on the region where PLL dendrimer was applied indicating its antiangiogenic activity. (*B*) 50 μg/CAM protamine sulphate was added, as a positive control, to the CAM surfaces of 4 day old fertilized eggs, and then the eggs were incubated and photographed after 4 h and 24 h.

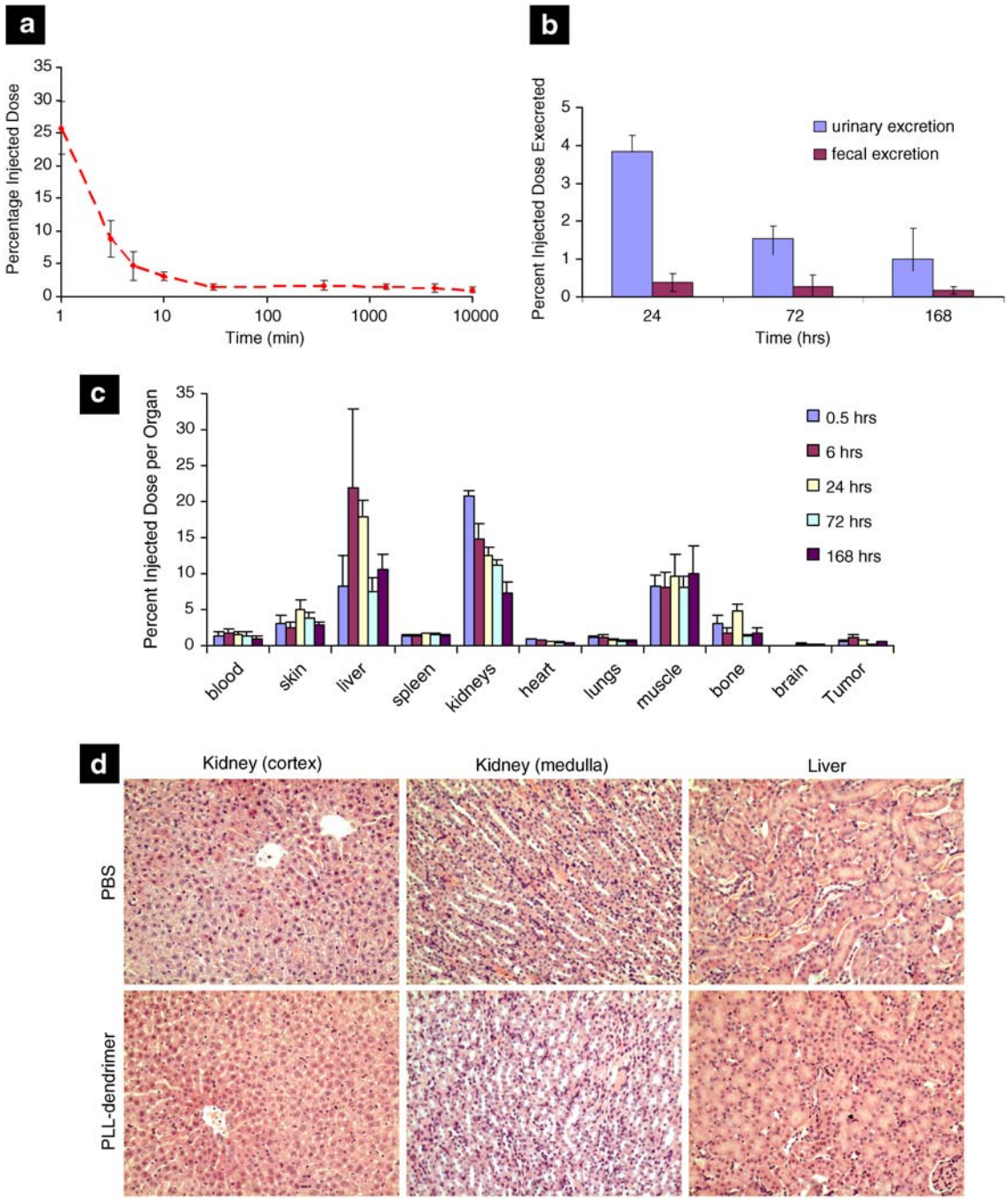

Fig. S4. In vivo biodistribution of PLL dendrimer after i.v. administration of 25 mg/kg as a single dose in tumor-bearing C57Bl/6 mice. (*A*) Blood clearance profile; (*B*) % injected dose excreted in urine or faeces. Data expressed as the mean ± SD ($n = 4$) using tumor-bearing mice. (*C*) The percentage injected dose per collected organ at 0.5, 6, 24, 72, and 168 h. Data were expressed as means ± SD ($n = 4$). (*D*) Hematoxylin and eosin-stained sections of kidney cortex, kidney medulla, and liver of non-tumor bearing mice after 7 days of single injection with PBS or 25 mg/kg PLL dendrimer (10X magnification).

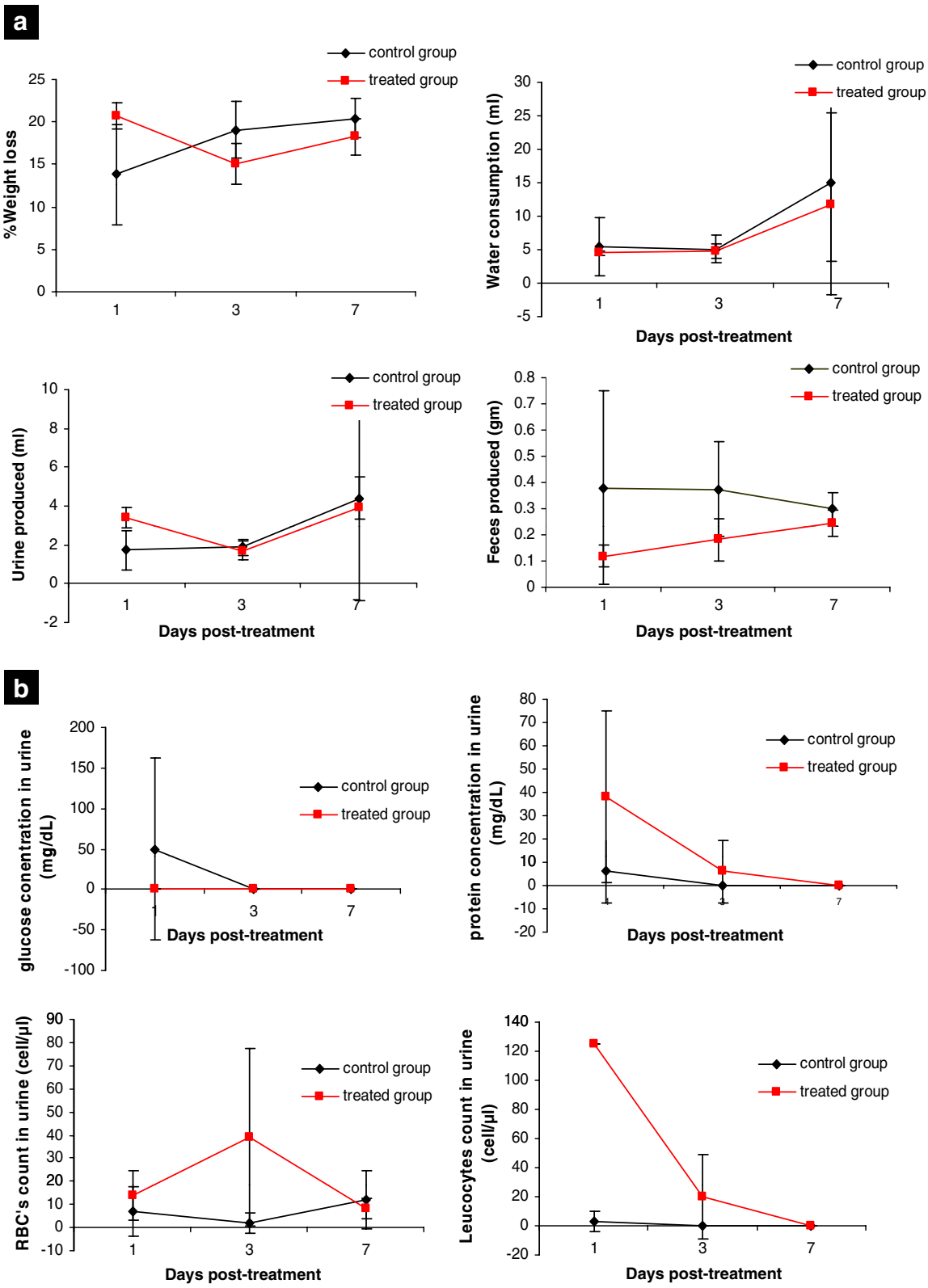

Fig. S5. PLL-dendrimer toxicity after single administration of 25 mg/kg in non-tumor-bearing C57Bl/6 mice. (*A*) Animal weight, water consumption, and urine and feces production. (*B*) Urinalysis.

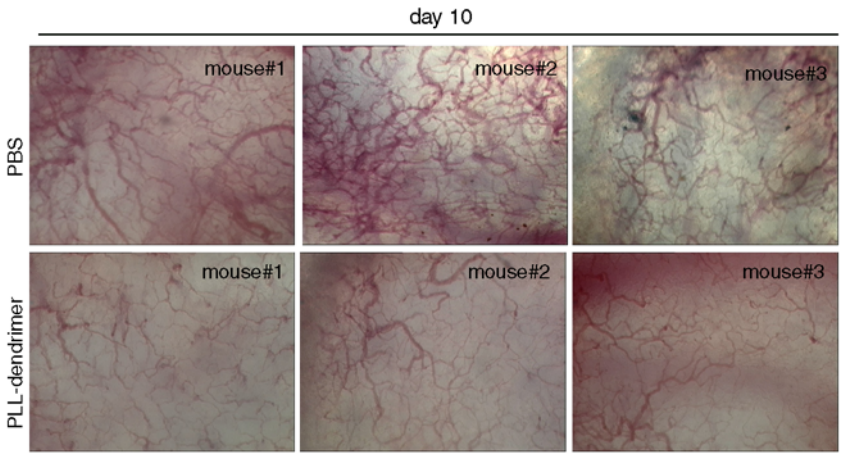

Fig. S6. Tumor microvessels are sparser in a window chamber model after i.v. administration of PLL dendrimer. Transmitted light images from three different mice of naïve (*Upper*) or treated (*Lower*) tumors on day 10 after tumor transplantation. Mice injected via tail vain with 50 mg/kg/day dendrimer (once daily, on days 4 and 7 after tumor transplantation) or with PBS alone. All images using 10x magnification.

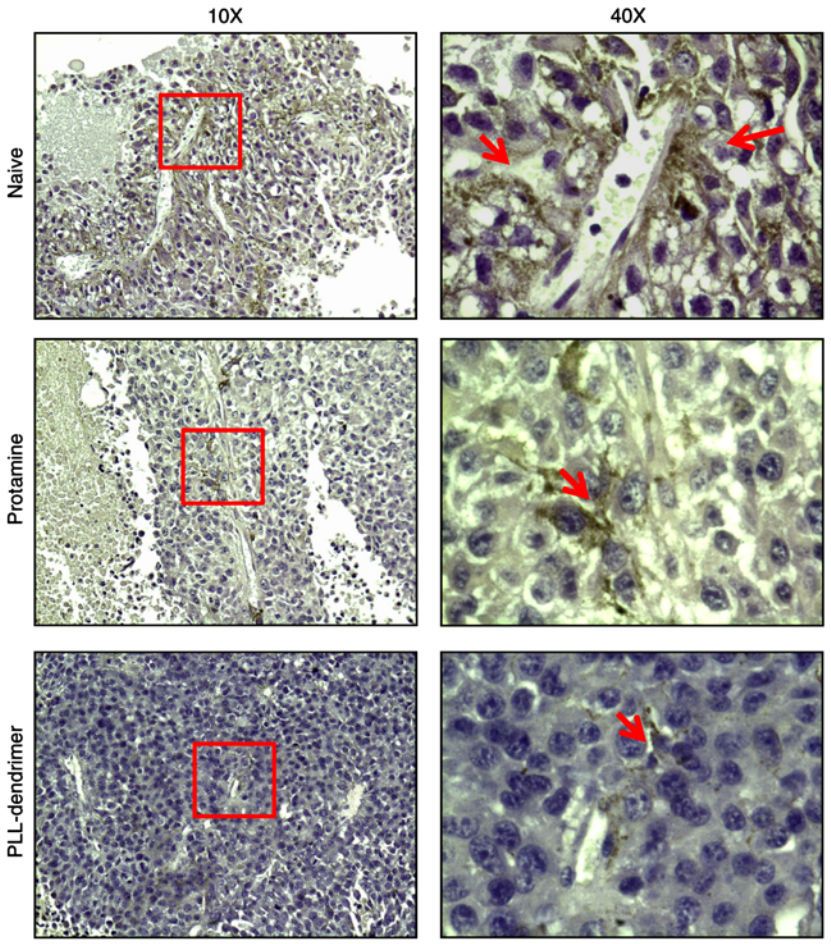

Fig. S7. PLL dendrimer delayed solid tumor growth and reduces CD31+ cells after systemic administration in vivo. Immunohistochemistry of CD31 of tumor sections with arrows pointing at CD31+ cells stained brown and are significantly lower in PLL-dendrimer treated tumors. All images were captured at 10x and 40x magnification.

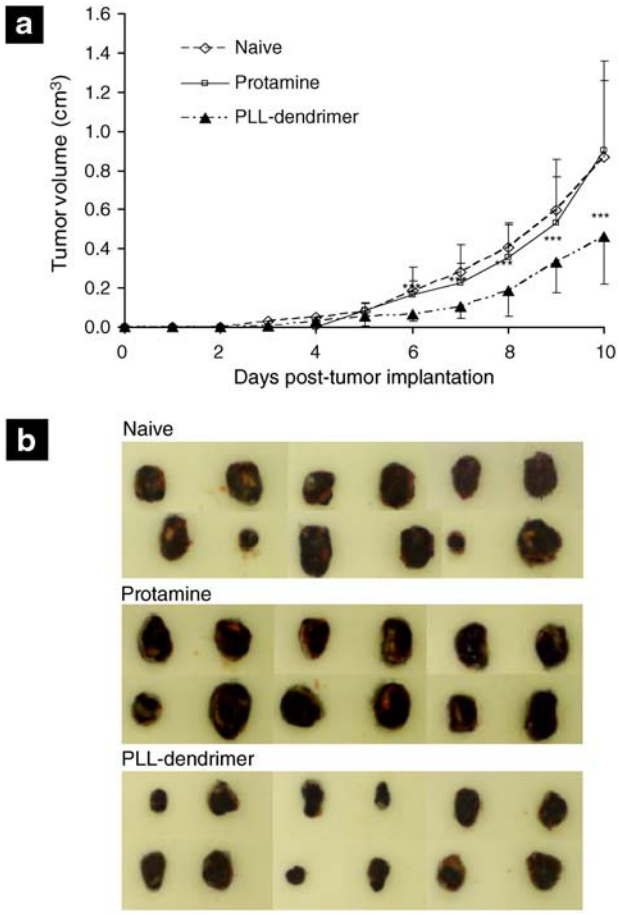

Fig. S8. Systemic administration of PLL-dendrimer delayed solid tumor growth in vivo. (*A*) Growth curves of murine B16F10 melanoma tumors. 50 mg/kg/day PLL dendrimer were injected i.v. twice on days 1 and 2; 60 mg/kg/day protamine were injected s.c. twice daily every day; and PBS alone was injected daily. Injections began on day 1 after tumor inoculation. Data expressed as mean ± SD ($n = 6 - 8$) (***$P < 0.001$). (*B*) Photographs of excised murine B16F10 melanoma tumors when naïve group reached volume 800 – 1, 000 mm^3.

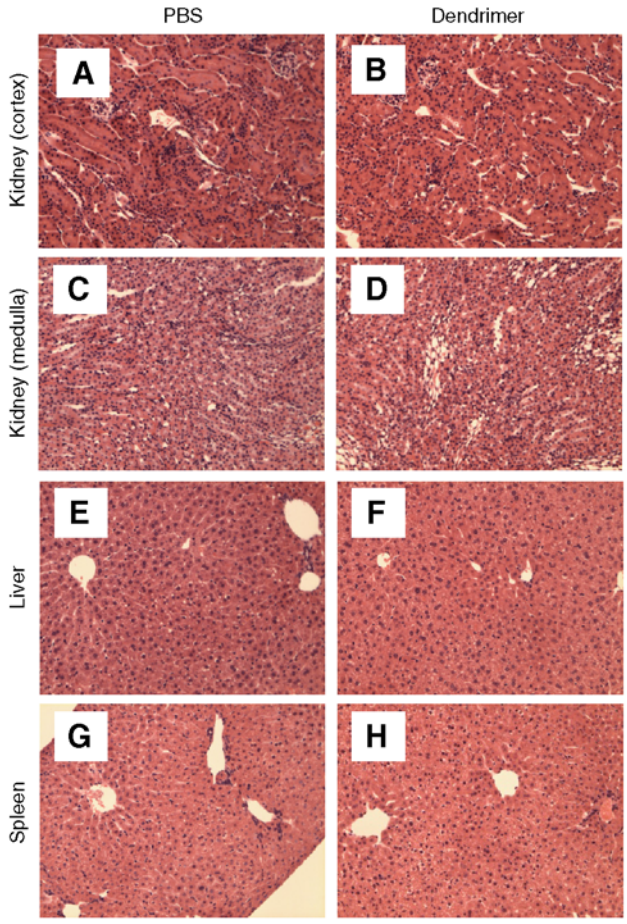

Fig. S9. Hematoxylin and eosin-stained sections of mice organs after i.v. administration of 50 mg/kg PLL dendrimer or PBS on day 1 and 2 post-tumor implanation in tumor-bearing C57Bl/6 mice. Magnifications 10X.

Table S1: Hematological results[a] from female non-tumor bearing C57Bl/6 mice treated with a single dose of the PLL-dendrimer and sampled at 0.5-168 hours after dosing[b]

	Hour of sampling					
	0.5		72		168	
	naive	dendrimer	naive	dendrimer	naive	dendrimer
RBC	7.56	9.25	8.72	8.42	8.58	8.99
HGB	11.57	14.60	13.27	12.83	13.03	13.82
HCT	40.40	46.28	46.40	41.48	45.57	44.48
MCV	52.83	50.00	53.00	49.50	53.00	49.40
MCH	15.27	15.73	15.17	15.20	15.17	15.32
MCHC	29.50	31.53	29.10	30.90	29.20	31.02
PLT	508.33	395.50	564.33	561.00	668.00	810.40
WBC	2.13	4.13	3.06	1.55	1.11	3.02
Neut	0.27	1.64	0.48	0.35	0.16	0.37
Neut%	13.33	36.00	15.67	22.50	13.67	12.20
Lymph	1.75	2.02	2.40	1.11	0.87	2.42
Lymph%	82.00	54.50	78.00	71.25	79.00	81.60
Mono	0.10	0.23	0.17	0.09	0.07	0.16
Mono%	4.33	4.50	5.33	5.50	6.67	4.20
Eo	0.01	0.24	0.02	0.01	0.01	0.07
Eo%	0.33	5.00	1.00	0.50	0.67	2.00
Baso	0.00	0.00	0.00	0.00	0.00	0.00
Baso%	0.00	0.00	0.00	0.00	0.00	0.00

[a] Values are means; n=3 and n=4 for control and treated groups respectively, except n=5 for treated group at 168hrs.

[b]Abbreviations and units: RBC, red blood cells, 10^6/l; Hb, hemoglobin, g/dl; HCT, hematocrit, %; MCV, mean cell volume, fl; MCH, mean cell hemoglobin, pg; MCHC, mean cell hemoglobin concentration, g/dl; platelets, 10^3/l; WBC, white blood cells, 10^3/l; Neut, neutophils, 10^3/l; Lymph, lymphocytes, 10^3/l; Mono, monocytes, 10^3/l; Eo, eosinophils, 10^3/l; Baso, basophils, 10^3/l.

Significantly different to control animals $p=<0.05$