Supplemental Methods and Data

MyD88 Deficiency Attenuates Angiotensin II-induced Abdominal Aortic Aneurysm Formation Independent of Signaling Through Toll-like Receptors 2 and 4

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MATERIALS AND METHODS

Experimental Animals and Diet

MyD88$^{-/-}$ x apoE$^{-/-}$ mice were obtained from Drs. Freeman and Moore.[1] MyD88$^{-/-}$ mice were also bred into LDLR$^{-/-}$ background (N10 to C57BL/6 background) using mice from The Jackson Laboratory. LDLR$^{-/-}$ mice that were either TLR2$^{+/+}$ or $^{-/-}$, or TLR4$^{+/+}$ or $^{-/-}$ were obtained from Drs. Curtiss and Tobias (Scripps Institute)[2] and Dr. Akira (Osaka University).[3]

All mice were bred as littermate controls, and housed in a pathogen-free barrier facility. All MyD88$^{+/+}$ and $^{-/-}$ mice were given autoclaved sterile water ad libitum containing sulfatrim (0.2%). ApoE$^{-/-}$ mice were fed a normal laboratory diet throughout experimentation. LDLR$^{-/-}$ mice were fed a diet enriched with saturated fat (milk fat 21% wt/wt) and cholesterol (0.2% wt/wt; Diet number TD.88137, Harlan Teklad) for 1 week prior to AngII infusion and throughout the duration of this infusion. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Genotyping by Polymerase Chain Reaction

The primers used to genotype mice for apoE, LDLR, MyD88, TLR2, and TLR4 are listed in online Table I. Genotypes were verified by two independent investigators.

Osmotic Minipump Implantation

At 8 to 12 weeks of age, male mice were implanted with Alzet osmotic minipumps (Model 2004, Durect Corporation) subcutaneously into the right flank. Infusion of AngII (1,000 ng/kg/min; Catalog number H-1705, Bachem) continued for 28 days, as described previously.[4]

Irradiation and Bone Marrow-derived Cell Repopulation

This procedure was performed as described previously.[5] Male LDLR$^{-/-}$ mice (6 to 8 weeks old) were lethally irradiated with a total of 900 rads divided into two doses (450 rads/dose, 3 hours apart) from a cesium γ source. Bone marrow-derived cells were harvested from MyD88$^{+/+}$ or $^{-/-}$ and TLR4$^{+/+}$ or $^{-/-}$ male mice (6-8 weeks old) and injected into irradiated, recipient LDLR$^{-/-}$ mice ($1x10^7$ donor cells per animal).

Four weeks after irradiation, recipient mice were fed the saturated fat-enriched diet. AngII infusion was started 1 week later, while mice were maintained on this special diet. At termination, DNA was isolated from bone marrows of the recipient mice and PCR was performed to verify the successful repopulation of donor cells.

Blood Pressure Measurements

Systolic blood pressure (SBP) was measured on conscious mice using either BP-2000 (Visitech Systems) or Coda 8 (Kent Scientific Corp) tail-cuff system as described previously.[6] SBP was measured for 5 consecutive days prior to pump implantation, and during the last week of AngII administration.

Aortic Tissue and Plasma Collection

Twenty-eight days after pump implantation, mice were terminated, and blood was drawn from right ventricles for plasma collection. Aortas were perfused with saline, extracted, and placed into 10% (wt/vol) formalin. These aortas were then carefully cleaned free of adventitia for abdominal aortic aneurysm (AAA) and atherosclerosis analyses.

White Blood Cell Analysis

White blood cells and the subtypes in whole blood were immediately counted at the time of termination on a Hemavet 950 LV veterinary multi-species hematology system (Drew Scientific).

Measurement of Plasma Lipids

Plasma cholesterol concentrations and lipoprotein cholesterol distribution were analyzed as described previously.[7]

Measurements of Abdominal Aortic Aneurysms

Maximal external diameters of suprarenal aortas were measured to define AAAs as described previously.[8]

Quantification of Atherosclerosis

Atherosclerotic lesions were measured on the intimal surface of aortic arches and thoracic aortas using an en face technique, as described previously.[9,10]

Histology and Immunostaining

Abdominal aortas or spleens were embedded in OCT and sectioned in a cryostat. Staining of hematoxylin and eosin was performed in aortic sections. Immunostaining of CD68 was determined in sections of spleens using a rat anti-CD68 antibody (FA-11; Catalog number MCA1957, AbD Serotec), as described previously.[11]

Blood Monocyte and Gr-1/Ly-6C Determination with Flow Cytometry

MyD88$^{-/-}$ x LDLR$^{-/-}$, TLR4$^{-/-}$ x LDLR$^{-/-}$, and LDLR$^{-/-}$ mice (n=8/genotype) were fed the saturated fat-enriched diet for 3 weeks (started 1 week prior to AngII infusion) and infused with AngII for 2 weeks. Peripheral blood was collected into 0.2% EDTA at the termination and mononuclear cells were isolated via lysis of red blood cells with an ammonium chloride solution. In a separate experiment, spleens from MyD88$^{+/+}$ x LDLR$^{-/-}$ and MyD88$^{-/-}$ x LDLR$^{-/-}$ male mice were harvested (n=4/genotype) and split into 4 equal sections. All sections were incubated in RPMI 1640 with fetal bovine serum (10% vol/vol), glucose (1% wt/vol), penicillin/streptomycin (1% vol/vol of each), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 1mM), and sodium pyruvate (1 mM) with either saline or AngII (1 μM) for 24 hours. Subsequently, splenocytes were isolated by crushing the spleen sections into 100 μM cell strainer and all red blood cells were lysed using the amonium chloride solution. Peripheral blood and splenic monocyte populations were determined via fluorescence-activated-cell sorting (FACS^{TM})

on a Becton Dickson LSRII using antibodies against CD115 (phycoerythrin labeled; Catalog number 12-1162-83, eBioscience), F4/80 (biotinylated; Catalog number MCA497B, AbD Serotec), and Gr-1 (PerCp-Cy5.5 labeled; Catalog number 552093, BD Pharmingen). The biotinylated antibody was detected using streptavidin-APC (eBioscience). Monocyte populations were determined as being CD115 and F4/80 double positive. These double positive monocytes were then classified as Ly-6C low (Ly-6C^{low}), intermediate (Ly-6C^{int}), and high (Ly-6C^{hi}), as described previously.[12]

Thioglycollate Elicitation of Peritoneal Macrophages

MyD88$^{-/-}$ x LDLR$^{-/-}$ (n=10), TLR4$^{-/-}$ x LDLR$^{-/-}$ (n=5), and LDLR$^{-/-}$ (n=10) mice were fed the saturated fat-enriched diet for 1 week and subsequently injected intra-peritoneally with thioglycollate broth medium (1 ml; 4% wt/vol). Four days after injection, mice were killed, and peritoneal macrophages were extracted, as described previously.[13] Red blood cells were lysed using the ammonium chloride solution. Cell numbers were calculated using a hemacytometer and normalized to CD68 positive cell population, as stained with a fluorescein isothiocyanate-labeled CD68 antibody (Catalog number MCA1957F, AbD Serotec) 1:10 and analyzed via flow cytometric analysis.

Real-time Polymerase Chain Reaction

Vascular smooth muscle cells (VSMCs) were harvested from aortas of C57BL/6 mice as described previously.[14,15] These cells were incubated with saline (24 hours), AngII (1 μM for 24 hours), or lipopolysaccharide (1 μg/ml for 6 hours; Invitrogen). Cells were washed and RNA was harvested using the Qiagen All Prep RNA/protein kit. All samples were incubated with Turbo DNA-free (Ambion) to remove DNA contamination. Real-time PCR was performed as described previously.[16] Taqman probes were utilized for detection of TLR4 (AB catalog Mm00445273_m1) and MyD88 (AB catalog Mm00440339_g1). Real-time PCR of 18S rRNA was used as the endogenous control (Catalog number 4352930E, Applied Biosystems). Negative controls were performed as described previously.[16]

Statistics

All statistical analyses were performed using either version 3.5 of SigmaStat (SPSS Inc.) or version 8.2 of SAS (SAS Institute). All measurements are represented as the mean±SEM. Two-group comparisons on quantitative variables were performed parametrically (Student's t test) or nonparametrically (Mann-Whitney Rank Sum test) according to the results of preliminary tests for within-group normality and equality of variances. SBP data were analyzed by fitting a linear mixed model with explanatory variables of genotypes and time (prior to and during AngII infusion) along with random effects for individual mice. One Way ANOVA was performed, where indicated, with an appropriate post hoc test. Values with $P<0.05$ were considered statistically significant.

Table I. Primers Used for Genotyping Mice

Gene	Primers
ApoE	5’-GCCGCCCCGACTGCATCT 5’-TGTGACTTGGGAGCTCTGCAGC 5’-GCCTAGCCGAGGGAGAGCCG
LDLR	5'-AGGTGAGATGACAGGAGATC 5'-AGGATGACTTCCGATGCCAG 5'-GCAGTGCTCCTCATCTGACTTG
MyD88	5'-TGGCATGCCTCCATCATAGTTAACC 5'-GTCAGAAACAACCACCACCATGC 5'-ATCGCCTTCTATCGCCTTCTTGACG
TLR2	5'-TTGGATAAGTCTGATAGCCTTGCCTCC 5'-ATCGCCTTCTATCGCCTTCTTGACGAG 5'-GTTTAGTGCCTGTATCCAGTCAGTGCG
TLR4	5'-TGTTGCCCTTCAGTCACAGAGACTCTG 5'-CGTGTAAACCAGCCAGGTTTTGAAGGC 5'-ATCGCCTTCTATCGCCTTCTTGACGAG

Table II. Incidence and Rupture Rate of AngII-induced AAAs in Whole Body Deficient Mice and their Relative Wild Type Controls

Mouse Genotype		Number of Mice	AAA incidence	Death due to Aortic Rupture
$ApoE^{-/-}$	$MyD88^{+/+}$	42	33/42 (79%)	14/42 (33%)
	$MyD88^{-/-}$	27	2/27 (7%) *	1/27 (4%)*
$LDLR^{-/-}$	$MyD88^{+/+}$	22	19/22 (86%)	8/22 (36%)
	$MyD88^{-/-}$	23	2/23 (9%)*	1/23 (4%)*
	$TLR2^{+/+}$	9	8/9 (89%)	2/9 (22%)
	$TLR2^{-/-}$	11	9/11 (82%)	4/11 (36%)
	$TLR4^{+/+}$	10	14/14 (100%)	4/14 (29%)
	$TLR4^{-/-}$	20	5/20 (25%)*	4/20 (20%)

* denotes $P<0.01$ for comparisons of $MyD88^{-/-}$ or $TLR4^{-/-}$ versus their relative wild type controls using Fisher Exact Test.

Table III. Characteristics of Study Mice with Genotypes of MyD88, TLR2, or TLR4

Mouse Genotype		Number of Mice	Plasma Cholesterol Concentrations (mg/dL)	Systolic Blood Pressure (mmHg)		Body Weight (g)
				Baseline	AngII Infusion	
$ApoE^{-/-}$	$MyD88^{+/+}$	42	320 ± 32	112 ± 5	171 ± 4*	29.1 ± 0.4
	$MyD88^{-/-}$	27	306 ± 23	116 ± 3	173 ± 3*	29.0 ± 0.3
$LDLR^{-/-}$	$MyD88^{+/+}$	19	1174 ± 57	127 ± 2	174 ± 4*	27.7 ± 0.9
	$MyD88^{-/-}$	23	1250 ± 72	135 ± 10	164 ± 5*	31.2 ± 0.5†
	$TLR2^{+/+}$	9	1334 ± 57	115 ± 2	157 ± 4*	26.9 ± 1.5
	$TLR2^{-/-}$	11	1275 ± 62	118 ± 4	150 ± 9*	26.5 ± 3.1
	$TLR4^{+/+}$	14	1257 ± 62	142 ± 2‡	168 ± 3*‡	26.5 ± 0.6
	$TLR4^{-/-}$	19	1180 ± 50	131 ± 2	158 ± 3*	27.7 ± 0.5

* denotes $P<0.001$ for comparisons of AngII infusion versus baseline within genotypes using repeated measures analysis with Bonferroni post hoc test.
† denotes $P=0.005$ for comparisons of $MyD88^{-/-}$ versus $^{+/+}$ using Mann-Whitney Rank Sum test.
‡ denotes $P=0.03$ for comparisons of $TLR4^{+/+}$ versus $^{-/-}$ at baseline and AngII-infusion, respectively, using two-way ANOVA with repeated measures.

Table IV. Characteristics of LDLR$^{-/-}$ Mice that were Chimeric for either MyD88 or TLR4

Mouse Genotype	Number of Mice	Plasma Cholesterol Concentrations (mg/dL)	Systolic Blood Pressure (mmHg)		Body Weight (g)
			Baseline	AngII Infusion	
MyD88$^{+/+}$	22	982 ± 63	122 ± 5	165 ± 15*	26.4 ± 0.8
MyD88$^{-/-}$	22	831± 53	126 ± 3	154 ± 5*	28.7 ± 0.8
TLR4$^{+/+}$	22	963 ± 65	123 ± 4	154 ± 6*	26.9 ± 0.5
TLR4$^{-/-}$	23	977 ± 58	126 ± 3	152 ± 6*	28.0 ± 0.7

* denotes P<0.001 for comparisons of AngII infusion versus baseline within genotypes using repeated measures analysis with Bonferroni post hoc test.

Table V. Cell Numbers in Peripheral Blood of LDLR$^{-/-}$ Mice that were Chimeric for either MyD88 or TLR4

Genotype of Donors	Number of Mice	Cell Number (10^3 cells/μl)				
		White Blood Cells	Neutrophils	Lymphocytes	Monocytes	Platelets
MyD88$^{+/+}$	22	3.42 ± 0.3	1.57 ± 0.3	1.54 ± 0.3	0.15 ± 0.02	663 ± 43
MyD88$^{-/-}$	22	4.09 ± 0.5	1.26 ± 0.2	2.48 ± 0.4	0.19 ± 0.03	598 ± 63
TLR4$^{+/+}$	22	4.25 ± 0.5	1.19 ± 0.1	2.63 ± 0.3	0.26 ± 0.03	661 ± 48
TLR4$^{-/-}$	23	4.12 ± 0.5	1.25 ± 0.2	2.40 ± 0.3	0.23 ± 0.03	507 ± 44*

* denotes P=0.04 for comparisons of TLR4$^{-/-}$ versus $^{+/+}$ by Mann-Whitney Rank Sum analysis.

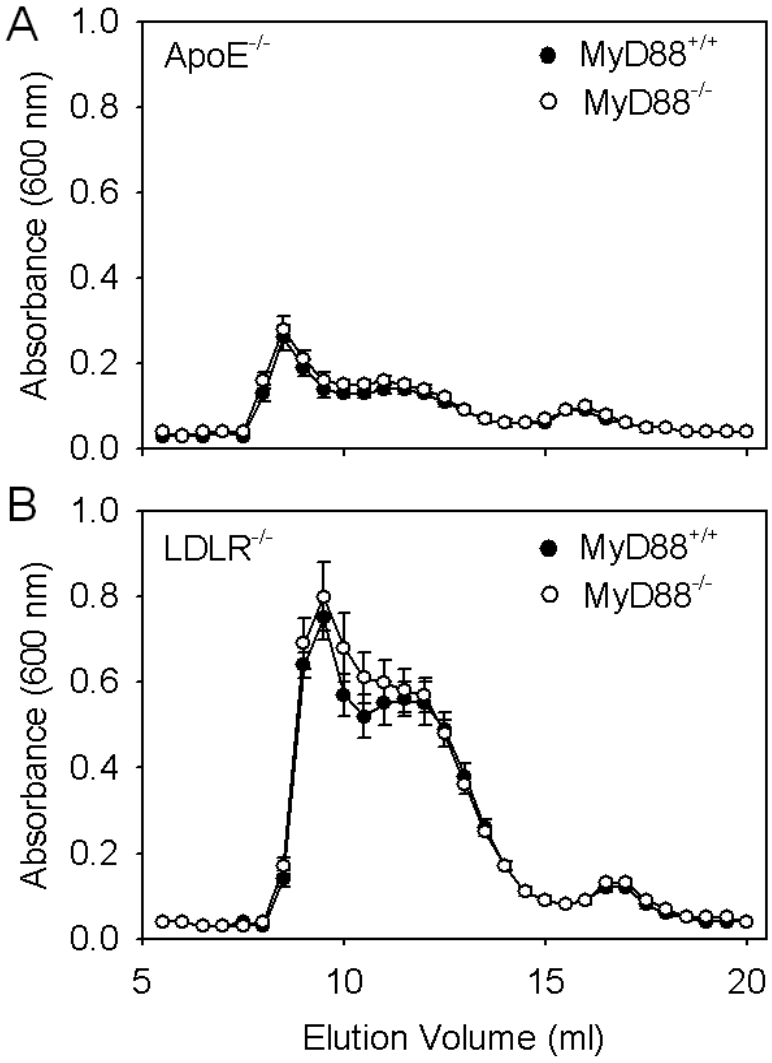

Figure I. **Lipoprotein cholesterol distribution determined by size exclusion chromatography.** (**A**) ApoE$^{-/-}$ and (**B**) LDLR$^{-/-}$ mice. Symbols represent means of 5-10 plasma samples from individual mice, and bars are SEMs.

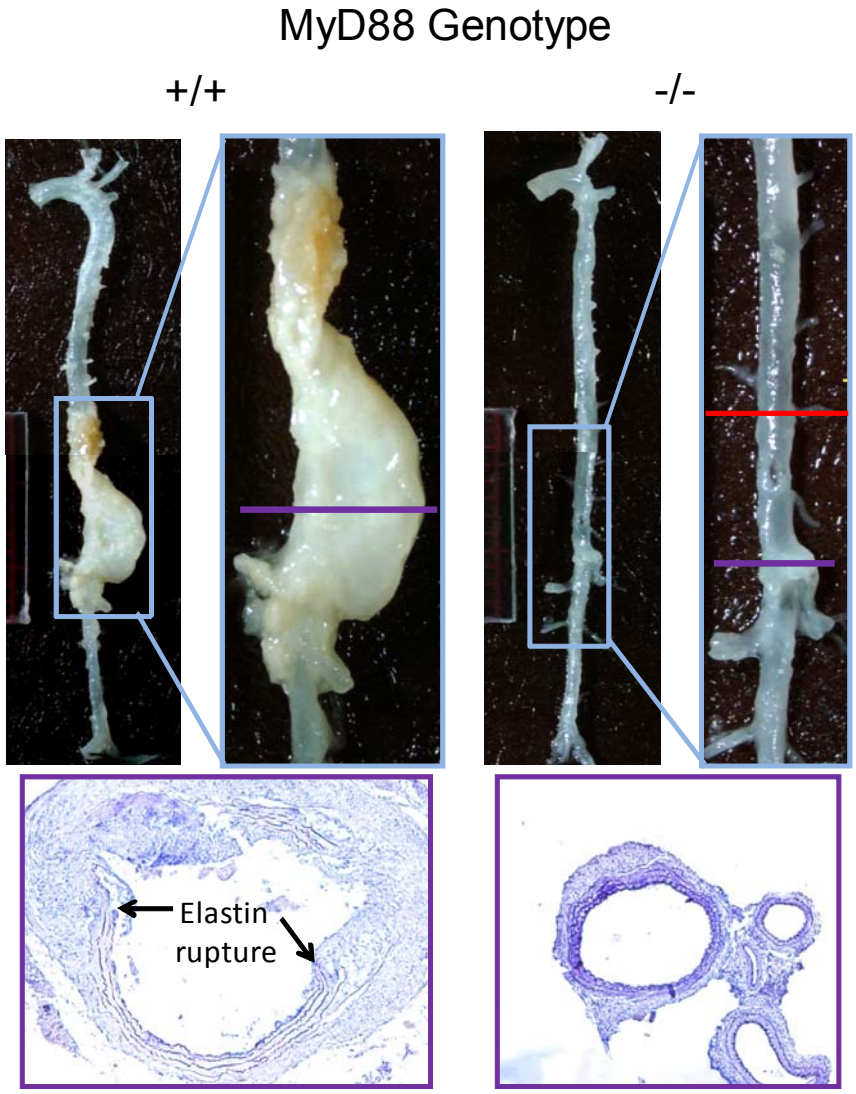

Figure II. **Examples of aortas from AngII-infused MyD88$^{+/+}$ and $^{-/-}$ mice in an LDLR$^{-/-}$ background.** AngII infusion into MyD88$^{+/+}$ mice promoted elastin rupture in the suprarenal aortic region and led to profound luminal expansion. In contrast, aortas maintained normal appearance during AngII infusion in MyD88$^{-/-}$ mice. The upper panels show entire aortas and magnified images of AAA-prone region. The lower panels show hematoxylin and eosin-stained tissue sections from the suprarenal aortas. Tissue sections were derived approximately from the regions noted with purple lines, respectively. Red line indicates the location of the last intercostal arteries.

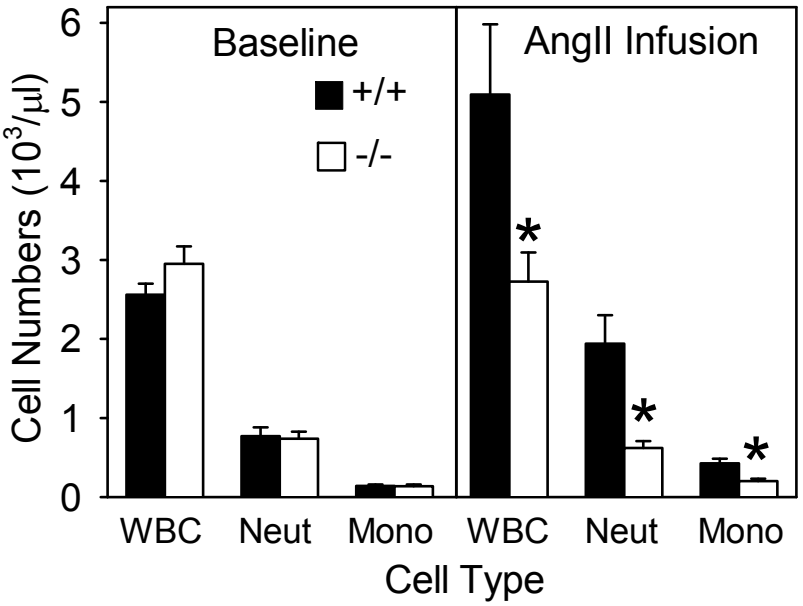

Figure III. **MyD88 deficiency attenuated AngII-induced leukocytosis.** White blood cells (WBCs), neutrophils (Neut), and monocytes (Mono) in peripheral blood were counted in AngII-infused MyD88$^{+/+}$ (n=19) and $^{-/-}$ (n=23) mice (LDLR$^{-/-}$ background) on a Hemavet analyzer. * denotes P<0.01 for comparisons of MyD88$^{-/-}$ versus $^{+/+}$ by Mann-Whitney Rank Sum analysis.

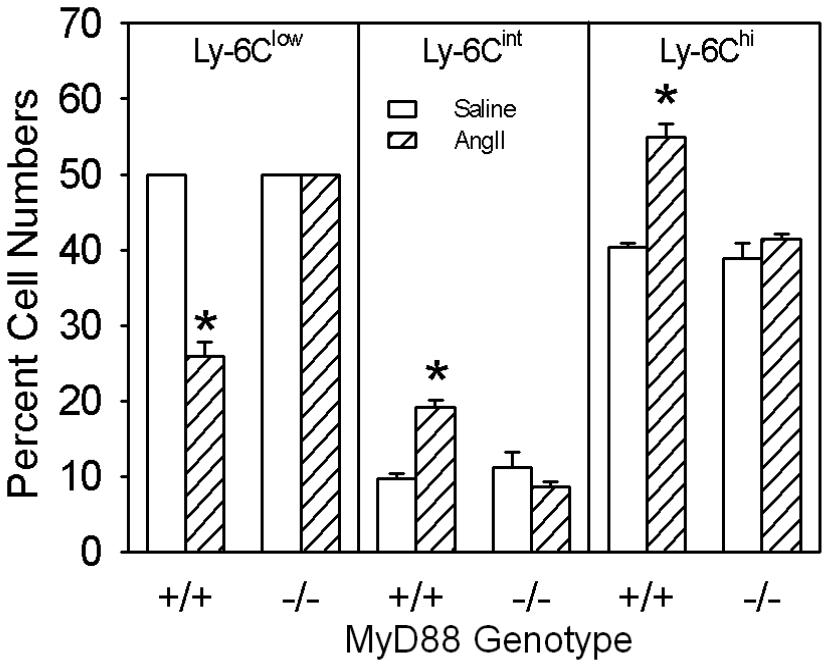

Figure IV. **MyD88 deficiency ablated AngII-induced redistribution of Ly-6C monocyte populations in peripheral blood.** Peripheral blood monocytes ($CD115^{+}$ and $F4/80^{+}$) were divided into Ly-6C low ($Ly\text{-}6C^{low}$), Ly-6C intermediate ($Ly\text{-}6C^{int}$), and Ly-6C high ($Ly\text{-}6C^{hi}$) populations in $MyD88^{+/+}$ (n=8/group for saline and AngII) or $^{-/-}$ (n=8/group for saline and AngII) mice in an $LDLR^{-/-}$ background. * denotes $P<0.001$ for comparisons with $MyD88^{+/+}$ saline infusion, $MyD88^{-/-}$ saline infusion, and $MyD88^{-/-}$ AngII infusion, respectively, within Ly-6C monocyte populations by one way ANOVA on Ranks.

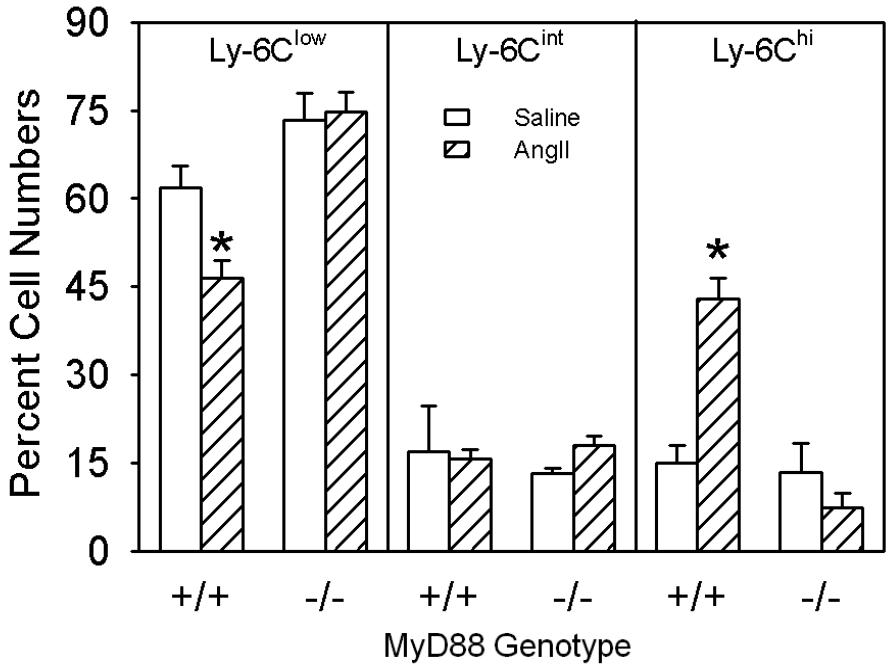

Figure V. **MyD88 deficiency ablated AngII-induced redistribution of Ly-6C monocyte populations in spleen.** Monocytes ($CD115^{+}$ and $F4/80^{+}$) in spleens were divided into Ly-6C low ($Ly\text{-}6C^{low}$), Ly-6C intermediate ($Ly\text{-}6C^{int}$), and Ly-6C high ($Ly\text{-}6C^{hi}$) populations in $MyD88^{+/+}$ or $^{-/-}$ (n=4/genotype) mice in an $LDLR^{-/-}$ background. * denotes $P<0.001$ for comparisons with $MyD88^{+/+}$ saline, $MyD88^{-/-}$ saline, and $MyD88^{-/-}$ AngII, respectively, within Ly-6C monocyte populations by one way ANOVA on ranks.

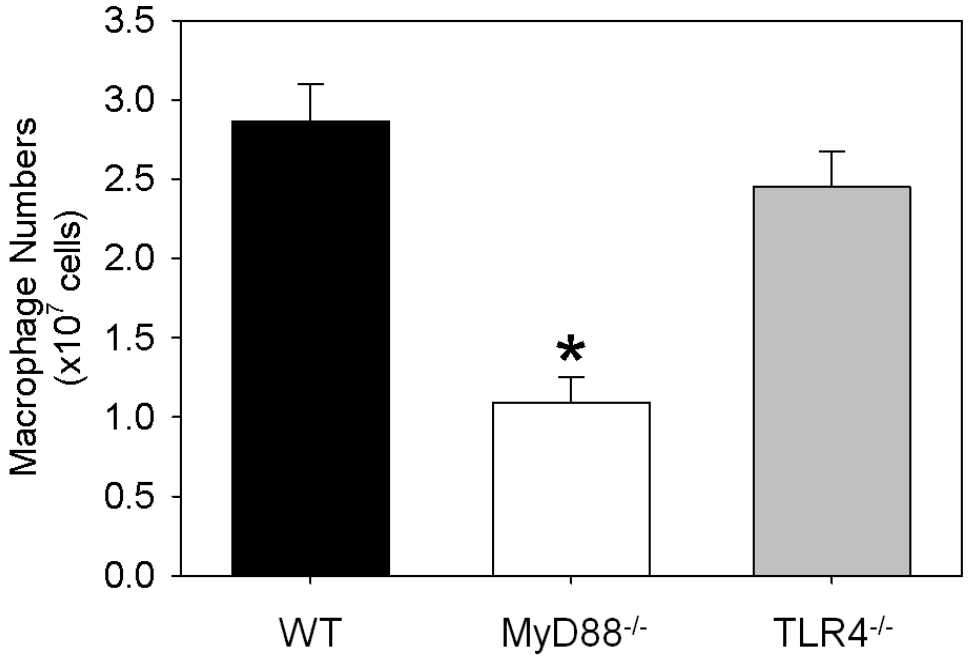

Figure VI. **MyD88 deficiency attenuated macrophage elicitation.** Thioglycollate-elicited $CD68^+$ peritoneal macrophages were quantified in wild type (WT; n=10), $MyD88^{-/-}$ (n=10), and $TLR4^{-/-}$ (n=5) mice in an $LDLR^{-/-}$ background. * denotes P<0.001 for comparisons with WT and $TLR4^{-/-}$ groups.

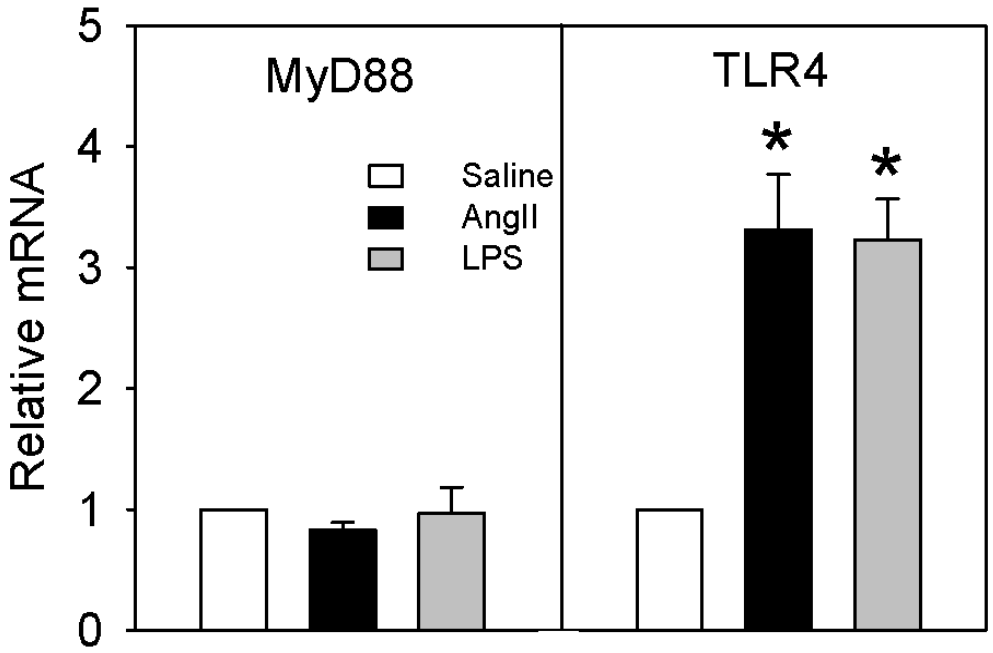

Figure VII. **AngII increased mRNA abundance of TLR4 but not MyD88 in SMCs.** mRNA abundance of MyD88 and TLR4 in SMCs isolated from suprarenal aortic regions of C57BL/6 male mice were determined by real-time PCR. Lipopolysaccharide (LPS; 1 μg/ml) was used as TLR4 agonist. * denotes $P<0.001$ for comparisons with cells incubated with saline.

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