ONLINE SUPPLEMENT

Leukocyte Calpain Deficiency Reduces Angiotensin II-induced Inflammation and Atherosclerosis but not Abdominal Aortic Aneurysms in Mice

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Running Title: Leukocyte Calpain Deficiency Reduces Atherosclerosis

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Table I. Effects of calpastatin overexpression in BM-derived cells in male LDL receptor -/- mice infused with saline or AngII

Groups	CAST WT		CAST Tg	
Infusion	**Saline**	**AngII**	**Saline**	**AngII**
N	5	15	5	15
Body Weight (g)	27.7 ± 1.6	25.0 ± 1.5	27.9 ± 1.0	24.5 ± 1.4
Plasma Cholesterol (mg/dL)	1091 ± 89	1613 ± 33#	1340 ± 96	1402 ± 75
Systolic BP Pre-Infusion (mmHg)	159 ± 3	150 ± 3	160 ± 1	158 ± 3
Systolic BP Post-Infusion (mmHg)	158 ± 3	193 ± 5*	160 ± 2	193 ± 2*
WBC (10^3/µl)	4.9 ± 0.9	5.0 ± 0.3	4.1 ± 0.4	5.8 ± 0.5
RBC(10^6/µl)	8.8 ± 0.5	11.7 ± 0.1	8.6 ± 0.5	10.5 ± 0.5
Platelets(10^3/µl)	617 ± 29	514 ± 19	691 ± 49	587 ± 39

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were determined at termination. Systolic blood pressure was measured prior to (week 0) and during AngII infusion (week 4). Two way repeated measures ANOVA was used to analyze systolic blood pressures. * Denotes $P<0.05$ systolic BP post-infusion vs pre-infusion, by two-way repeated measures ANOVA. # denotes $P<0.05$ for plasma cholesterol CAST WT AngII vs CAST WT Saline, by two-way ANOVA. There were no significant differences between the CAST genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.

Table II. Effects of calpain-1 deficiency in BM-derived cells in male LDL receptor -/- mice infused with AngII

Groups	Calpain-1 +/+	Calpain-1 -/-
N	15	15
Body Weight (g)	25.9 ± 1.6	26.6 ± 1.6
Plasma Cholesterol Concentrations (mg/dL)	1410 ± 68	1344 ± 36
Systolic BP Pre-infusion (mmHg)	142 ± 2	141 ± 3
Systolic BP Post-infusion (mmHg)	171 ± 4*	170 ± 4*
WBC (10^3/µl)	4.0 ± 0.5	3.4 ± 0.6
RBC(10^6/µl)	10.0 ± 0.6	10.1 ± 0.2
Platelets(10^3/µl)	592 ± 54	667 ± 36

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were measured at termination. One way repeated measures ANOVA was used to analyze systolic blood pressures. * Denotes $P<0.001$ for systolic BP post-infusion vs pre-infusion, by one-way repeated measures ANOVA. There were no significant differences between calpain-1 genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.

Table III. Effects of calpain-2 deficiency in leukocytes in male LDL receptor -/- mice infused with AngII

Groups	Cre 0/0	Cre +/0
N	13	13
Body Weight (g)	27.4 ± 0.9	27.3 ± 0.5
Plasma Cholesterol Concentrations (mg/dL)	1820 ± 96	1825 ± 68
Systolic BP Pre-infusion (mmHg)	156 ± 6	157 ± 6
Systolic BP Post-infusion (mmHg)	191 ± 4*	195 ± 6*
WBC (10^3/µl)	5.6 ± 0.6	6.6 ± 0.6
RBC(10^6/µl)	10 ± 0.3	9.9 ± 0.4
Platelets(10^3/µl)	650 ± 36	693 ± 25

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were determined at termination. One way repeated measures ANOVA was used to analyze systolic blood pressures. * Denotes $P<0.001$ systolic for BP post-infusion vs pre-infusion, by one-way repeated measures ANOVA. There were no significant differences between calpain-2 LsyM-Cre genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.

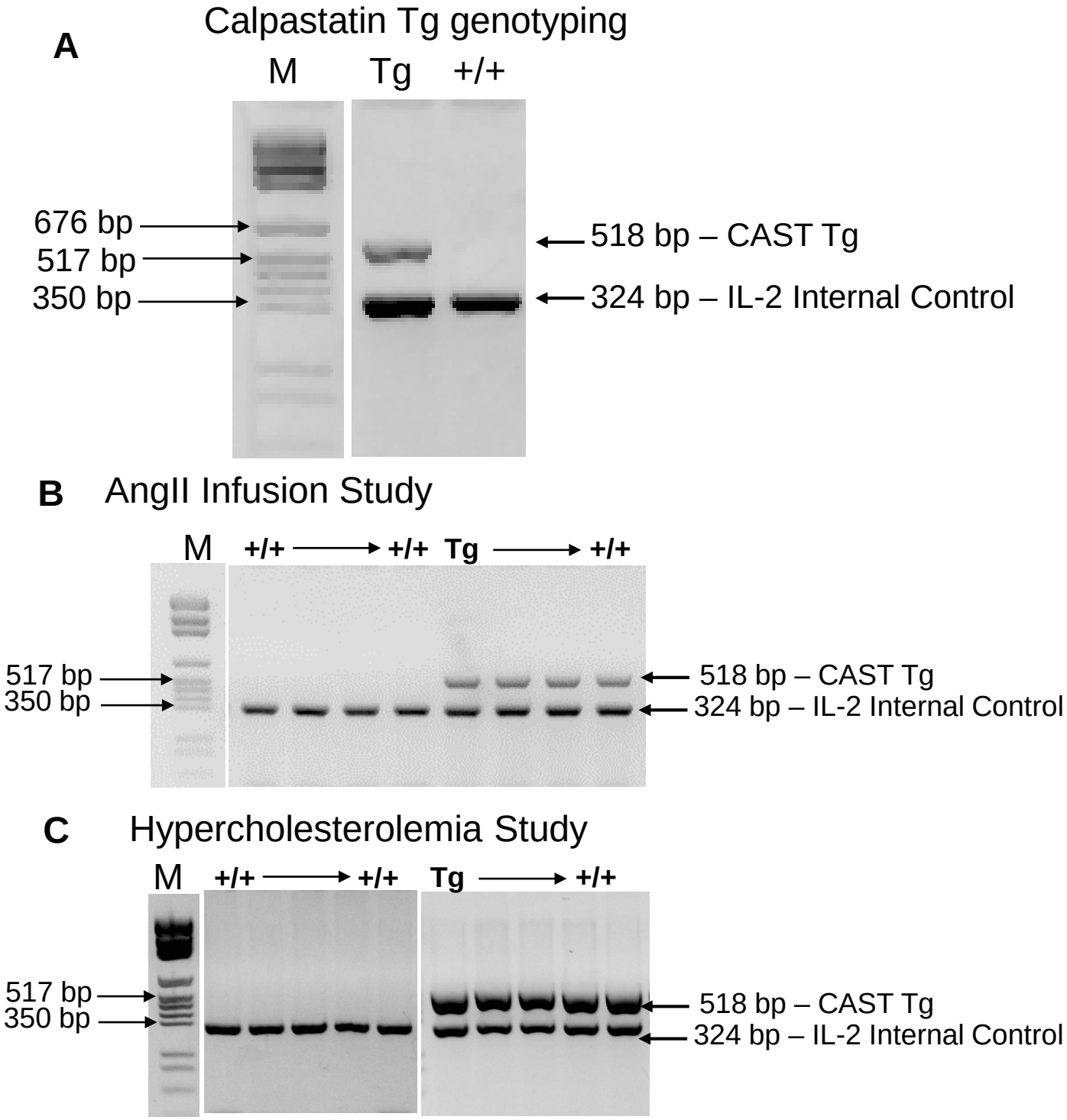
Table IV. Effects of calpastatin overexpression in BM-derived cells in male LDL receptor -/- mice fed with hypercholesterolemic diet

Groups	CAST WT	CAST Tg
N	14	15
Body Weight (g)	33.0 ± 1.0	33.1 ± 1.0
Plasma Cholesterol Concentrations (mg/dL)	1375 ± 93	1511 ± 75
Systolic BP Pre HCD (mmHg)	155 ± 4	148 ± 3
Systolic BP Post HCD (mmHg)	146 ± 4	138 ± 3
WBC (10^3/µl)	6.3 ± 0.8	5.4 ± 0.3
RBC(10^6/µl)	8.1± 0.7	9.0 ± 0.2
Platelets(10^3/µl)	695 ± 49	638 ± 48

Values are represented as means ± SEMs. Body weights, plasma cholesterol concentrations, and blood cell counts were determined at termination. One way repeated measures ANOVA was used to analyze systolic blood pressures. There were no significant differences between the CAST genotypes for body weight, plasma cholesterol, systolic BP and blood cell counts.

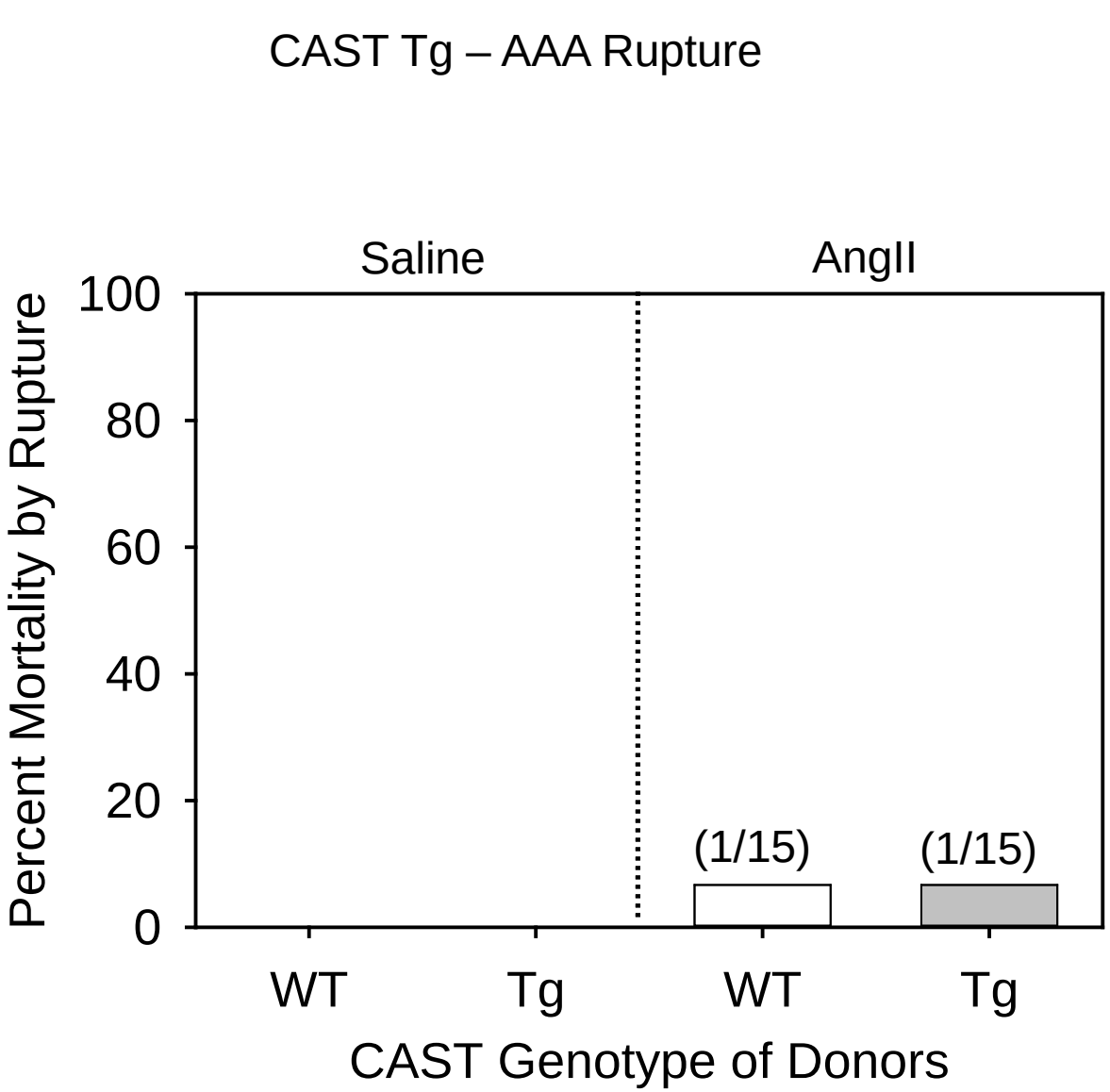
Table V. Primers used for real-time PCR

Gene	Primers	Product size (bp)
MCP-1	5'-CAGCCAGATGCAGTTAACGC 5'-TCTGGACCCATTCCTTCTTG	175
IL-6	5'-GGGAAATCGTGGAAATGAGAAA 5'-AAGTGCATCATCGTTGTTCATACA	167
IL-10	5'-CCAAGCCTTATCGGAAATGA 5'-TCTCACCCAGGGAATTCAAA	190
IKKα	5'-GTCAGGACCGTGTTCTCAAGG 5'-GCTTCTTTGATGTTACTGAGGGC'	118
IKKβ	5'-ACAGCCAGGAGATGGTACG 5'-AGGGTGACTGAGTCGAGAC	296
IKKε	5'-ACCACTAACTACCTGTGGCAT 5'-CCTCCACTGCGAATAGCTTC	214
ABCA1	5'-CTGGTTTGGTGAGGAAATTCA-3' 5'-ACCTTCATGCCATCTCGGTA-3'	150
ABCG1	5'-GCTGGGAAGTCCACACTCAT-3 5'-ATCATGGGTCCTGAAGAGT-3	173
SRA-1	5'-AAGAAGAACAAGCGCACGTGGAAC-3 5'-AGGAGGCCCTTGAATGAAGGTGAT-3	137
CD36	5'-TGCTGGAGGTGTTATTGGTG-3' 5'-TGGGTTTTGCACATCAAAGA-3'	190
ACAT-1	5'-GCATTCAGTGTGGTTGTCCT-3 5'-AGGGCATGAGCCATATGAAC-3	143
LXRα	5'-TGACTTTGCCAAACAGCTC-3 5'-TCACGTCTCCAGAAGCATGAC-3	103
18S	5'-CTCTGTTCCGCCTAGTCCTG 5'-AATGAGCCATTCGCAGTTTC	170

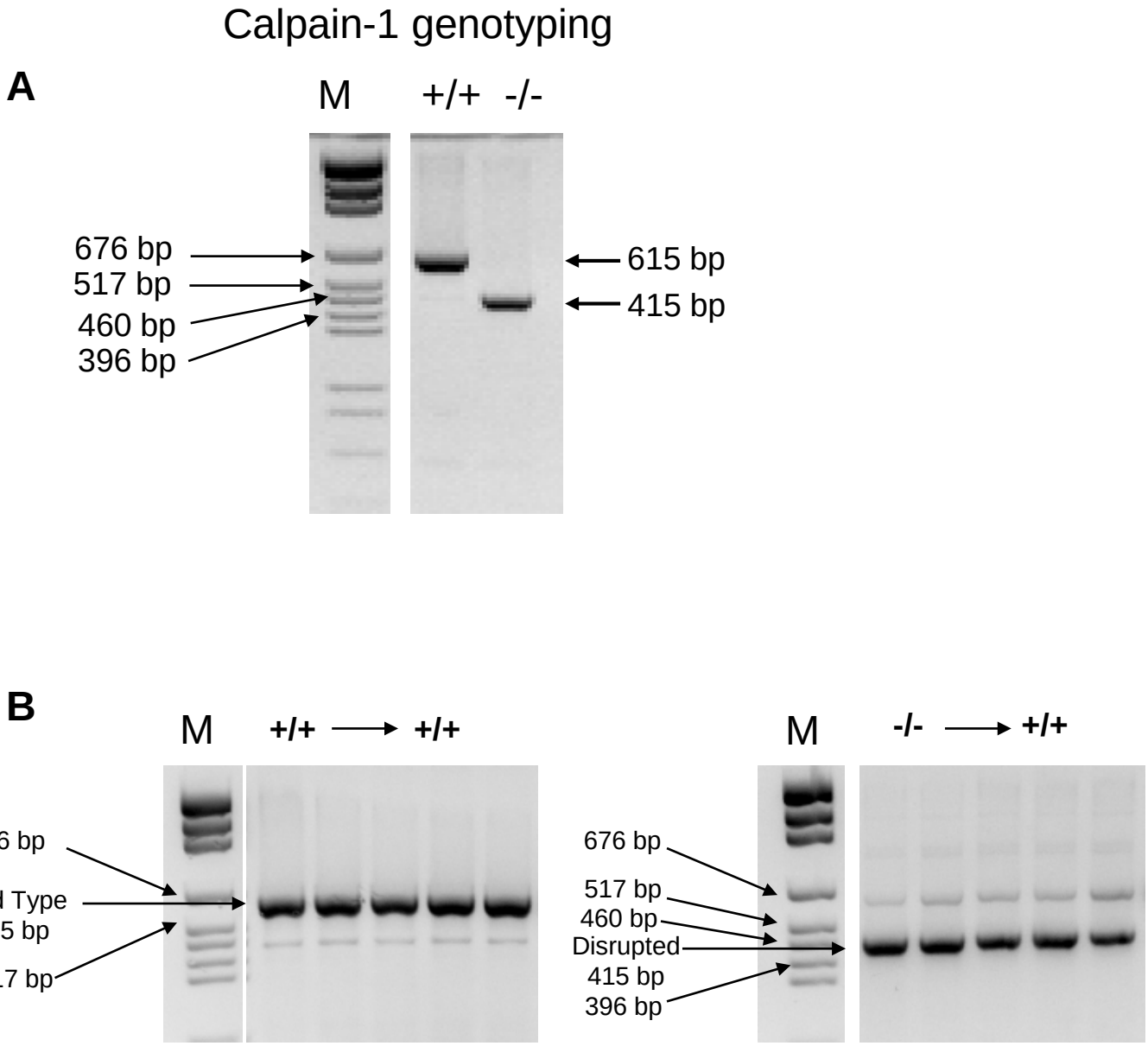


Supplementary Figure I. Genotyping of experimental mice for the CAST transgene (Tg) by PCR.

A. Genomic DNA from BM-derived cells was isolated and screened by PCR for the CAST-Tg allele. IL-2 was used as an internal control. **B and C**. PCR confirmed CAST-Tg genotype in BM-derived cells harvested from chimeric mice. PCR on BM-derived cells yielded amplicons of 518 and 324 bp for CAST-Tg and IL-2 alleles, respectively. (M= Molecular weight ladder)



Supplementary Figure II. CAST overexpression had no effect on AngII-induced aortic rupture.
Mortality due to AAA rupture in AngII infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs. White (WT) and gray bar (Tg).

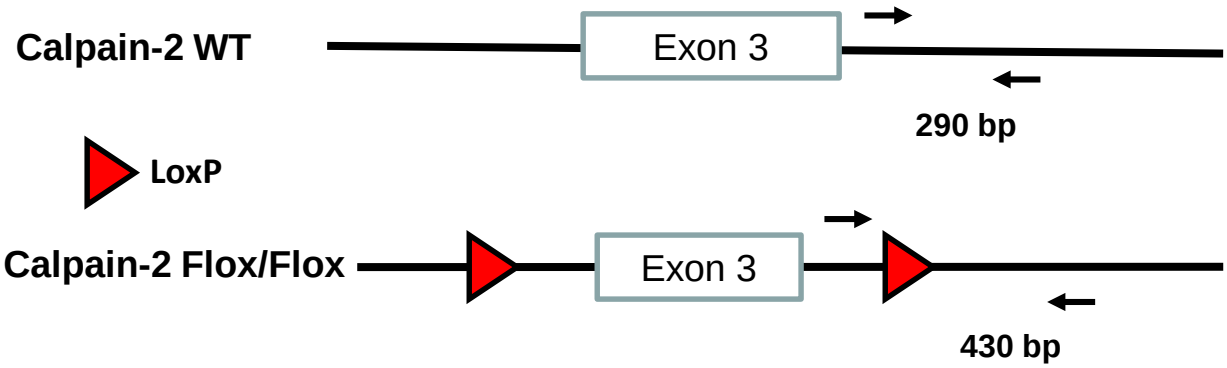


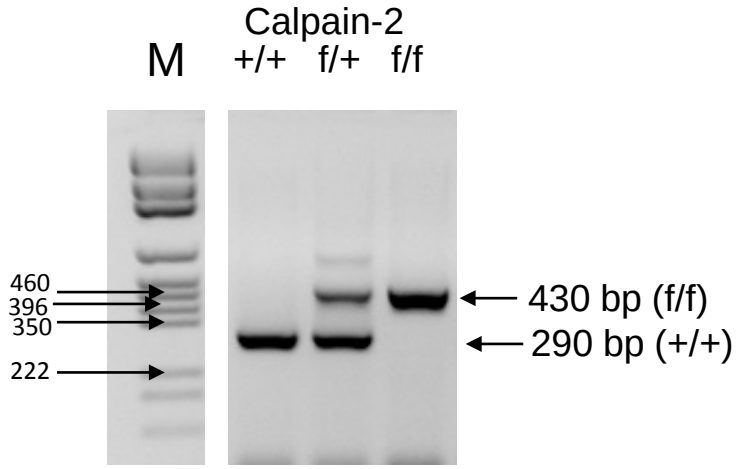
Supplementary Figure III. Genotyping of experimental mice for calpain-1 alleles by PCR.

A. Genomic DNA from BM-derived cells was isolated and screened by PCR for calpain-1 wild type (+/+) and null (-/-) alleles. **B**. PCR confirmed calpain-1 genotypes in BM-derived cells harvested from chimeric mice. PCR on BM-derived cells yielded amplicons of 615 and 415 bp for calpain-1 +/+ and -/- alleles, respectively. (M= Molecular weight ladder)

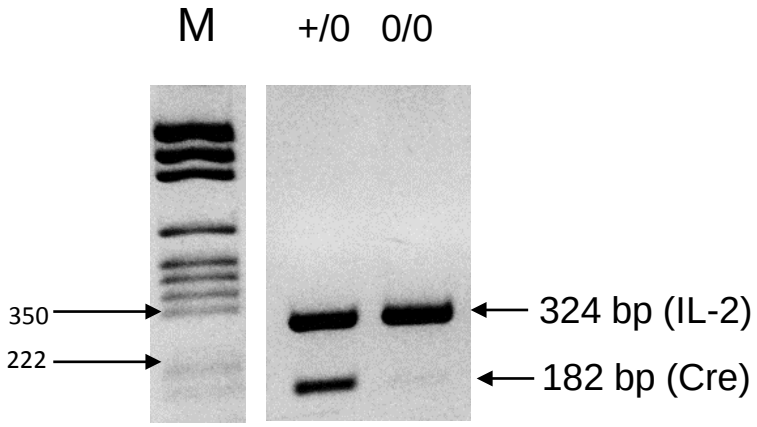
Calpain-2 flox and Cre genotyping

A. Calpain-2 flox

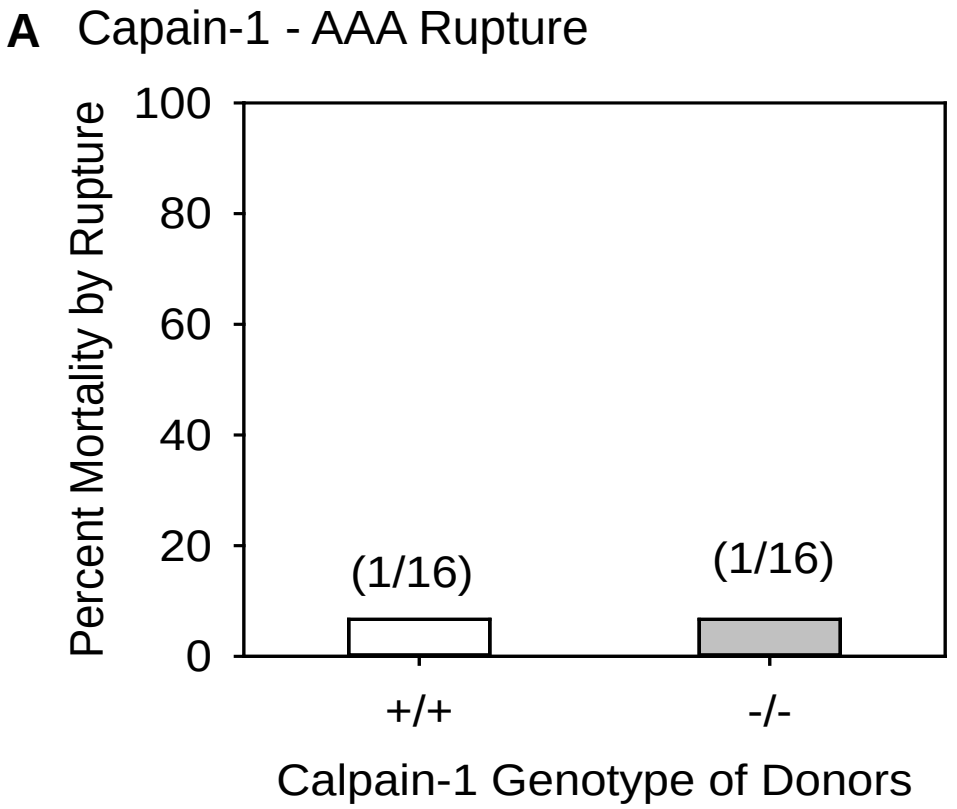


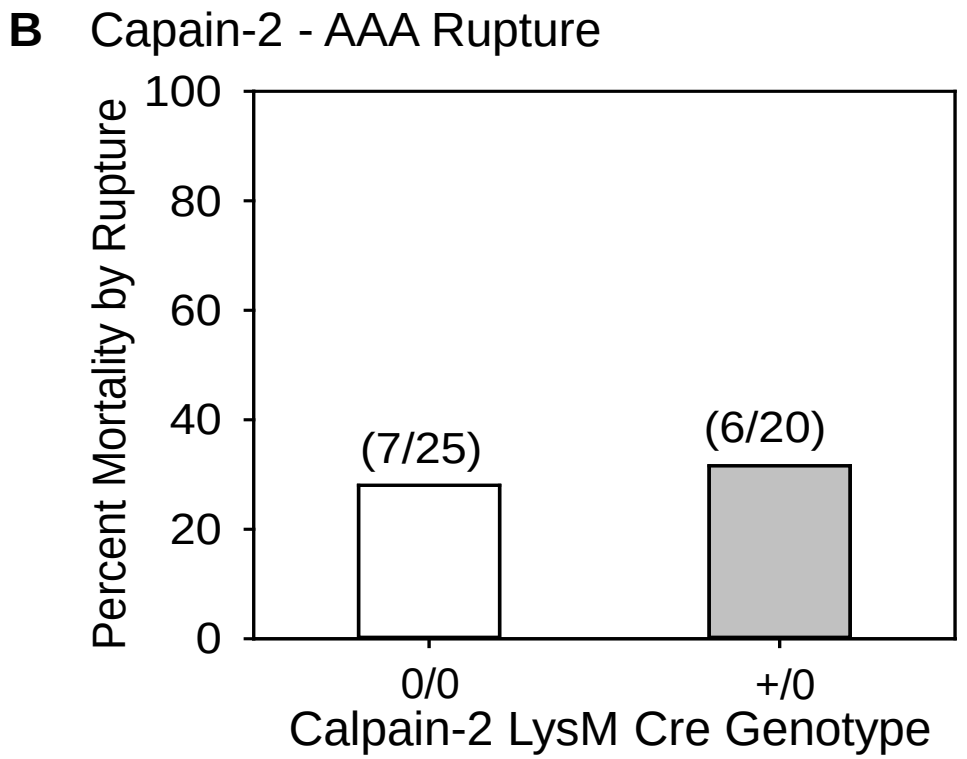


B. Cre

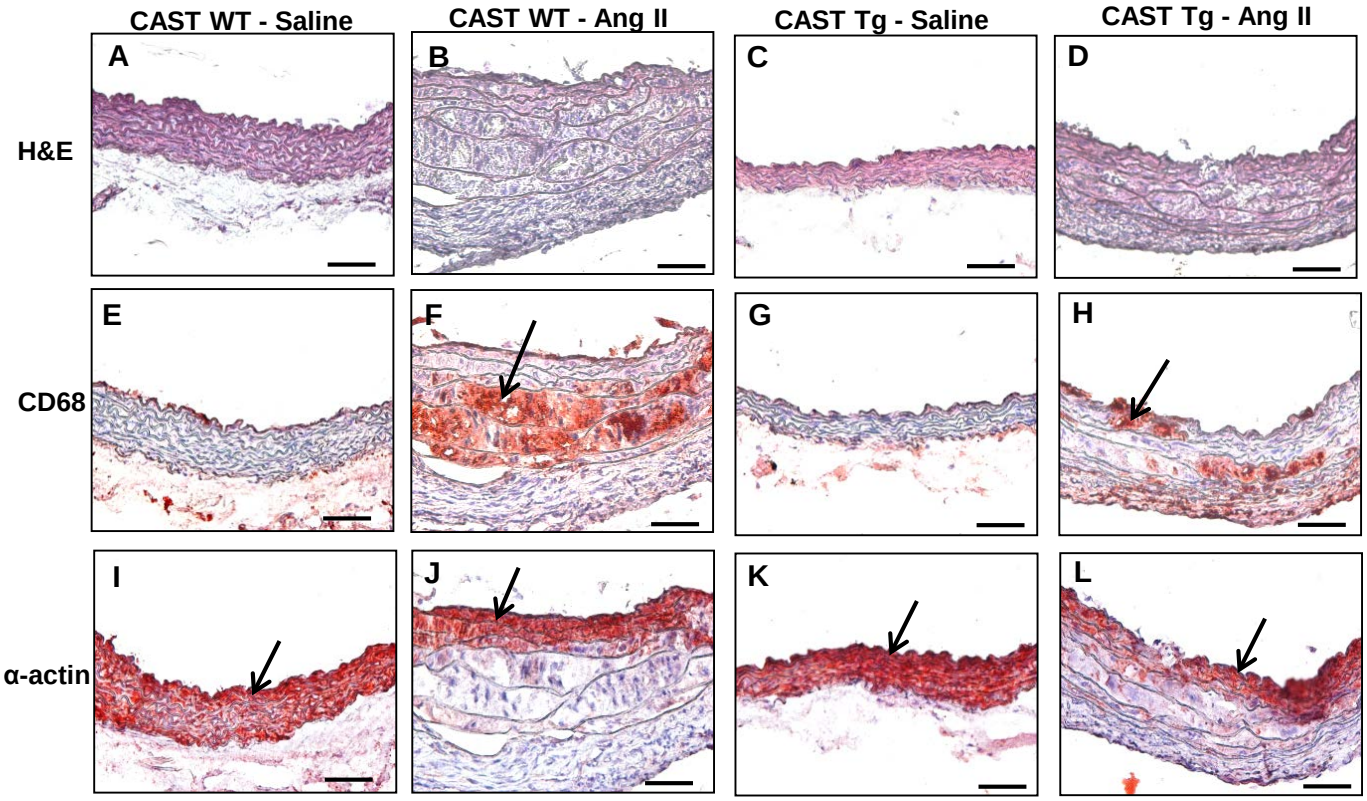


Supplementary Figure IV. Genotyping of experimental mice for the calpain-2 floxed allele and Cre Tg by PCR. A. PCR screening strategy to verify the presence of calpain-2 flox/flox. A 430 bp product was generated from calpain-2 f/f mice, while a 290 bp product was obtained from calpain-2 WT mice. Genomic DNA from tail biopsies was isolated and screened by PCR. **B**.Cre Tg was confirmed by PCR using IL-2 gene as internal control. PCR on genomic DNA yielded amplicons of 182 and 324 bp for Cre Tg and IL-2 alleles, respectively. (M= Molecular weight ladder)





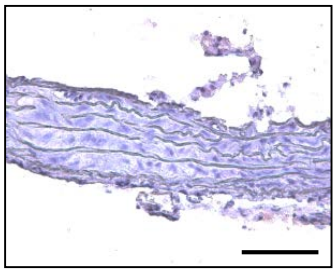
Supplementary Figure V. Calpain-1 or-2 deficiency in myeloid cells had no effect on AngII-induced aortic rupture. **A.** Mortality due to AAA rupture in AngII infused LDL receptor-/- mice transplanted with calpain-1 +/+ or -/- BM cells. **B.** Mortality due to AAA rupture in AngII-infused calpain-2 f/f mice that were either LysM Cre 0/0 or +/0. White (+/+ / Cre0/0) and gray bar (-/- / Cre+/0).



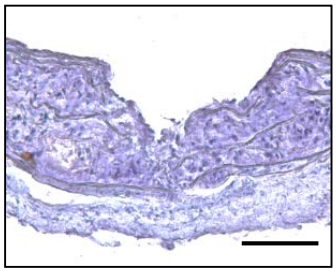
Supplementary Figure VI. CAST overexpression reduced macrophage accumulation in AngII-infused aortic arch.
Representative aortic arch (200x magnification) from AngII-infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs stained with H&E (**A-D**), immunostained for CD68 (**E-H)** and α-actin (**I-L)**. Arrows indicate positive staining (red).

A **For CD 68 Staining**

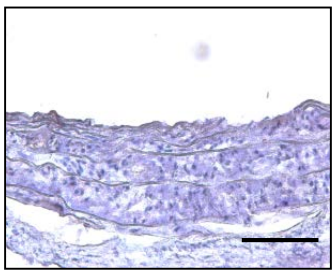
Rat IgG Control



Secondary Ab – Rabbit anti Rat only Control

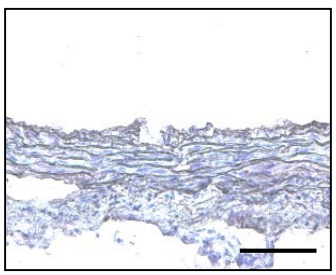


No antibodies Control

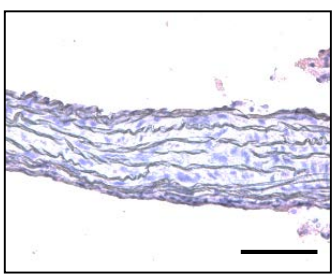


B **For SMC-α actin Staining**

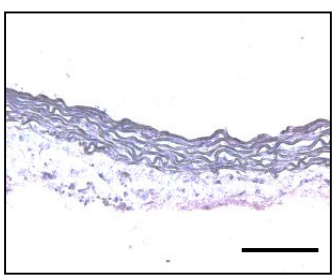
Rabbit IgG Control



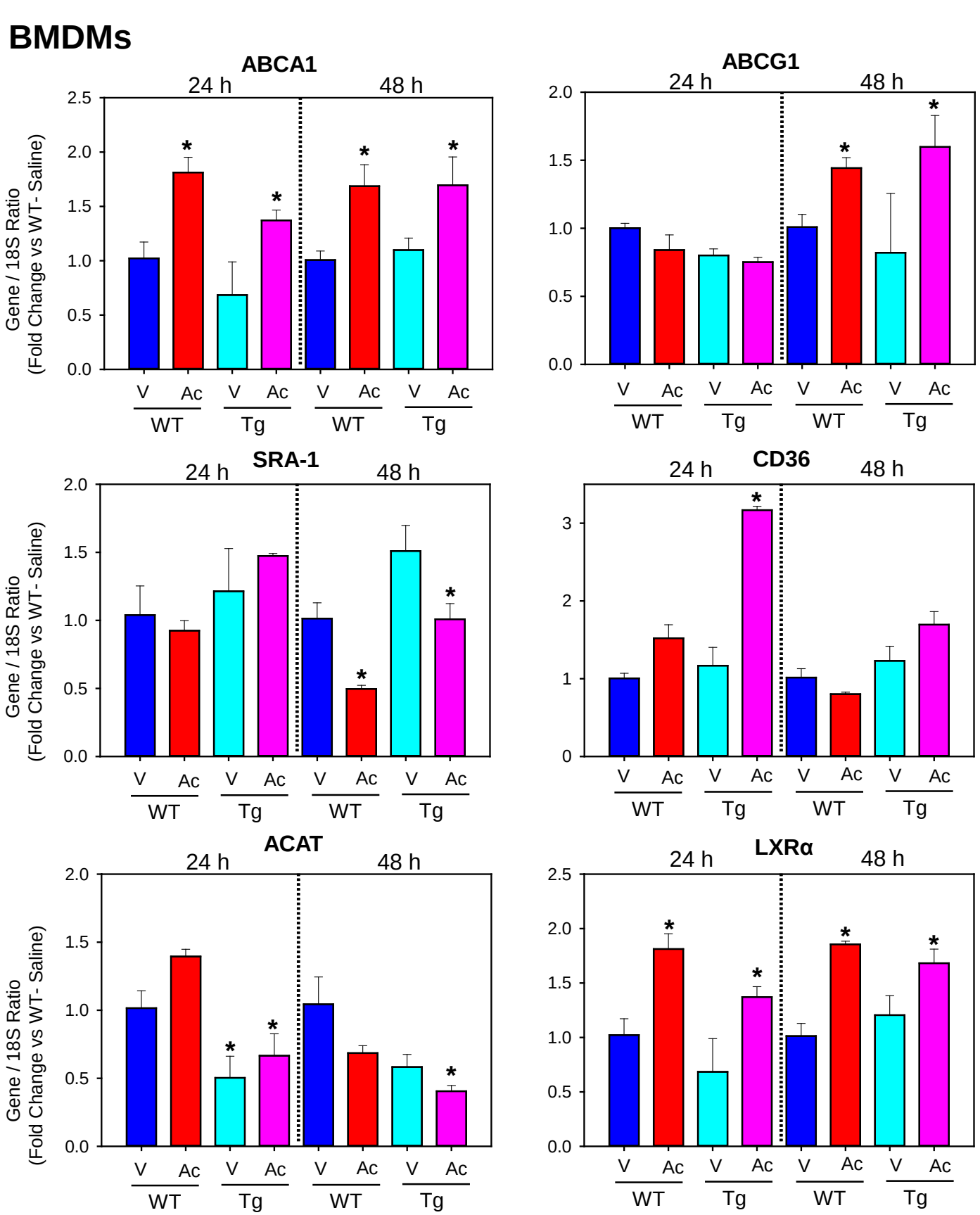
Secondary Ab – Goat anti Rabbit only Control



No antibodies Control



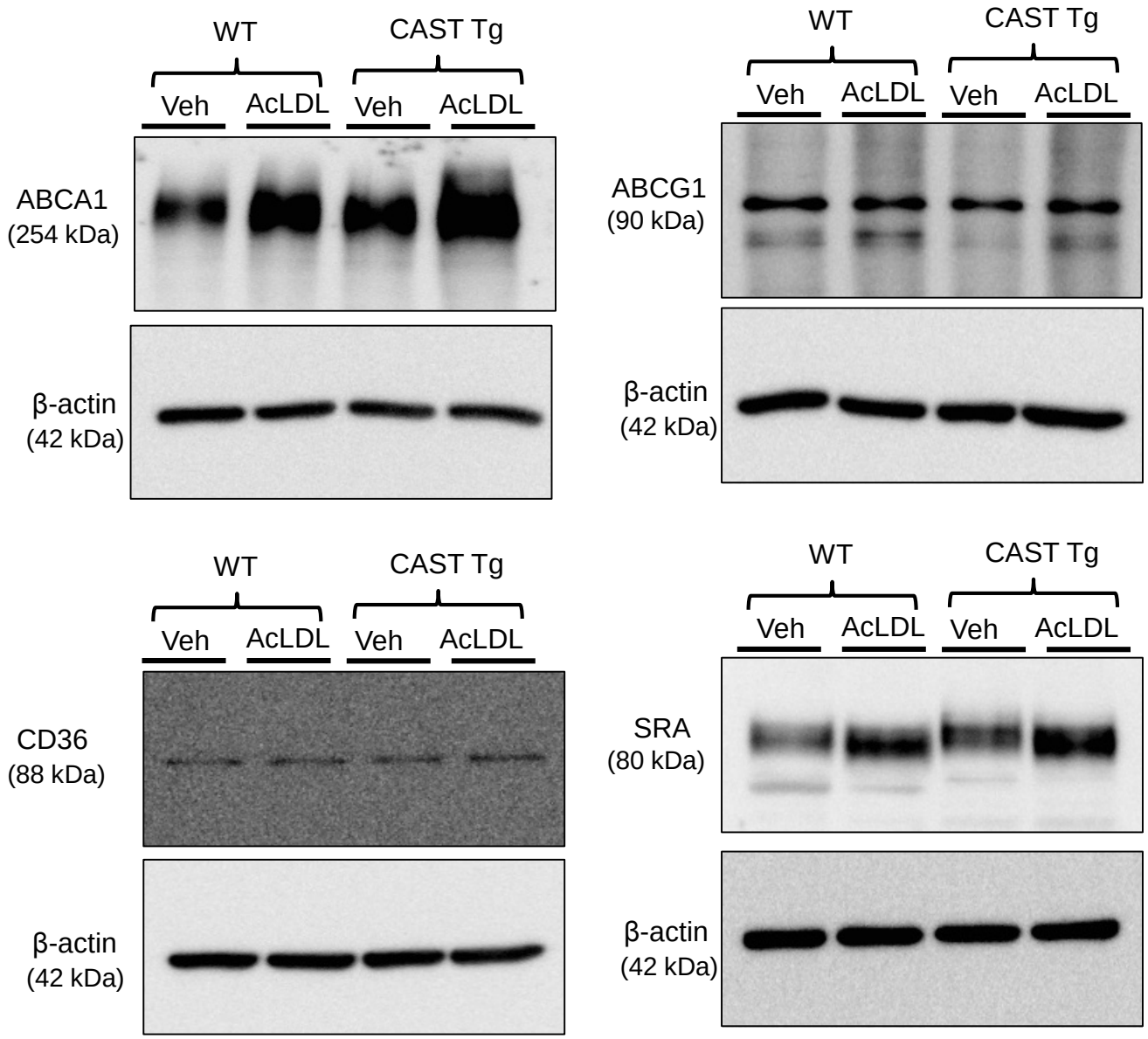
Supplementary Figure VII. Representative images of negative controls for immunostaining on arch atherosclerotic lesions. Representative aortic arch atherosclerotic lesion sections stained with rat IgG (**A**), rabbit IgG (**B**), only secondary antibodies (Rabbit anti-rat or Goat anti-rabbit) and no antibodies. Scale bars correspond to 50 μm (200x magnification).



Supplementary Figure VIII. CAST overexpression had no effect on atherosclerosis-associated genes in lipid-loaded macrophages.

mRNA abundance of ABCA1, ABCG1, SRA-1, CD36, ACAT and LXRα in WT and CAST Tg BMDMs incubated with vehicle (V) or Ac-LDL (Ac;25 μg/ml) (n=4). Values are represented as mean ± SEM. * denotes *P*<0.05 when comparing AcLDL vs Vehicle (Two-way ANOVA with Holm-Sidak post hoc analysis).

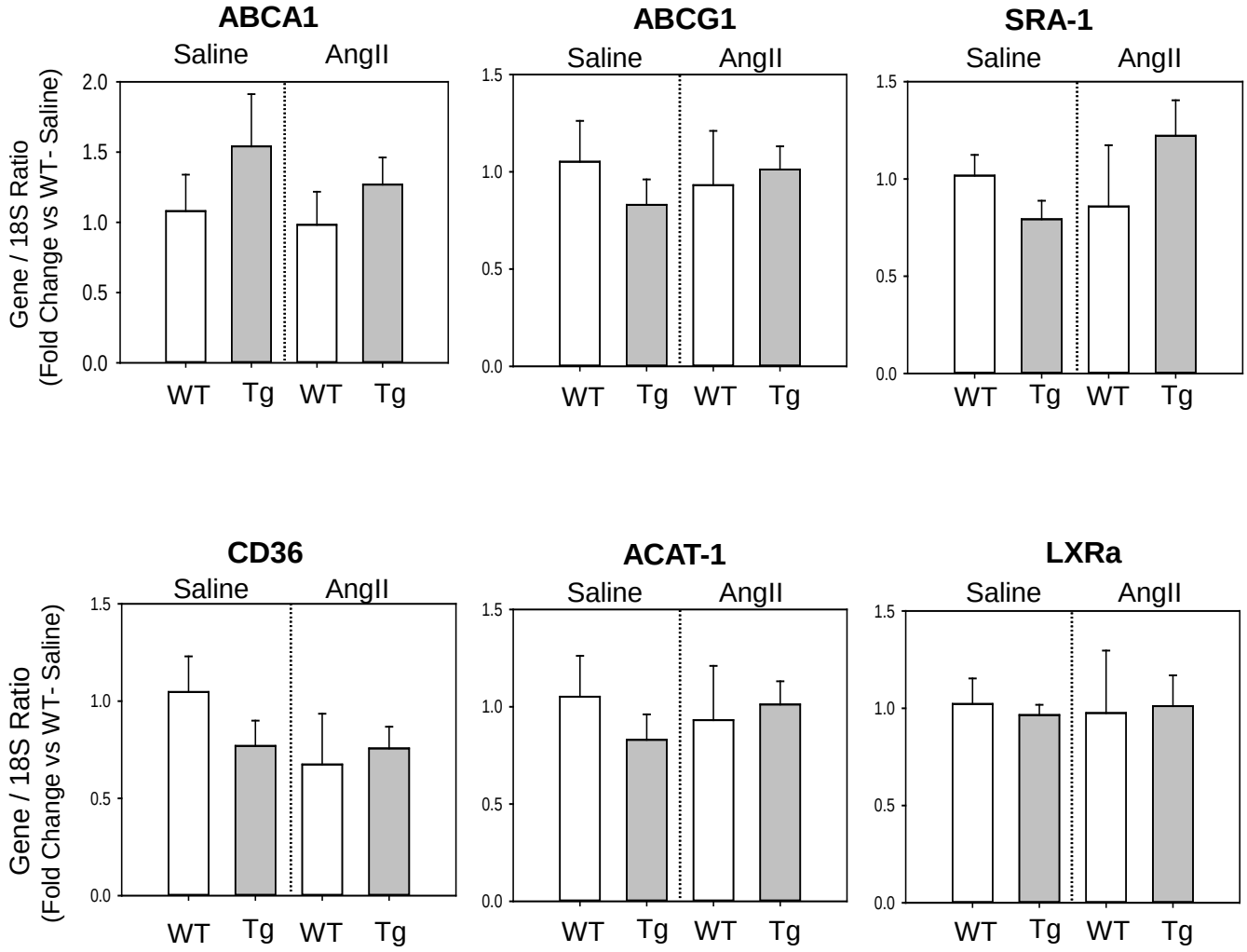
BMDMs



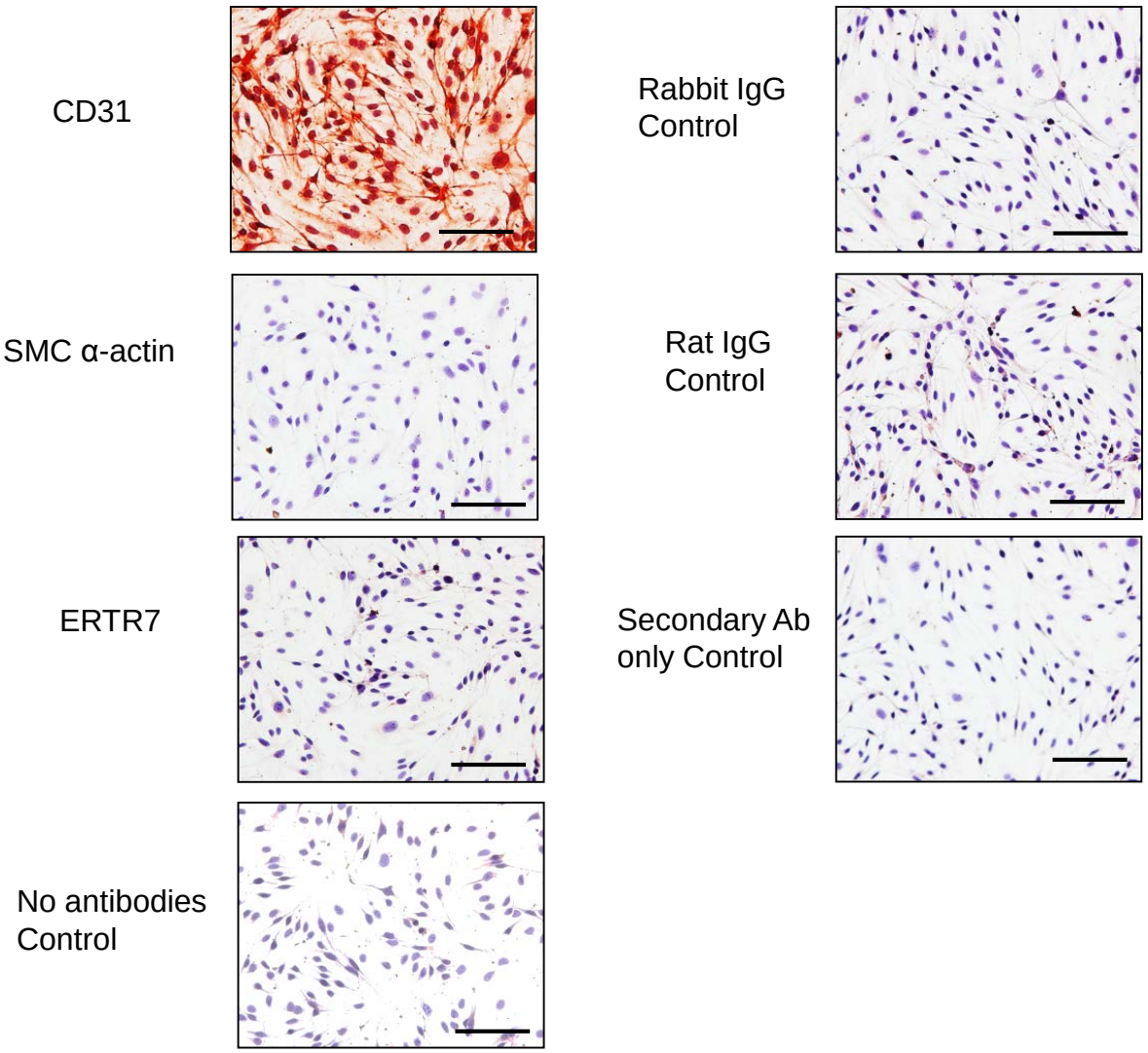
Supplementary Figure IX. CAST overexpression had no effect on protein abundance of ABC transporters, CD36 and SRA in lipid-loaded macrophages.

Western blot analyses of ABCA1, ABCG1, CD36 and SRA in WT and CAST Tg BMDMs incubated with Ac-LDL (25 μg/ml) (n=4).

MPMs



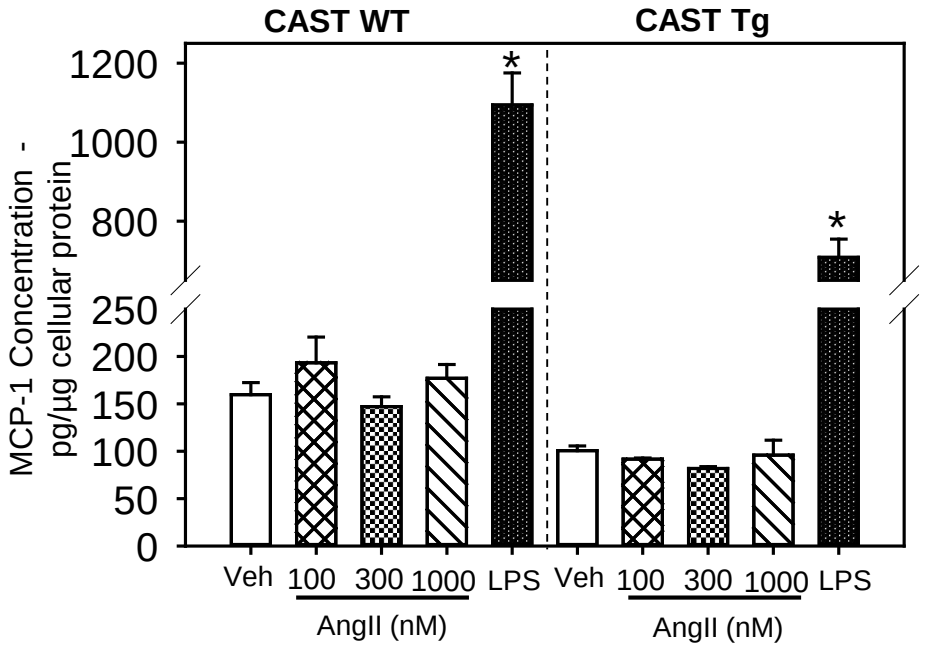
Supplementary Figure X. CAST overexpression had no effect on atherosclerosis-associated genes in mouse peritoneal macrophages. mRNA abundance of ABCA1, ABCG1, SRA-1, CD36, ACAT and LXRα in MPMs from saline and AngII infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs were analyzed by qPCR (n=4-6). Values are represented as mean ± SEM. (Two-way ANOVA with Holm-Sidak post hoc analysis).



Supplementary Figure XI. CD31 staining on mouse aortic endothelial cells. Representative images of aortic endothelial cells immunostained for CD31, SMC α-actin, ERTR7 (fibroblasts) and appropriate IgG, secondary antibodies controls. CD31+ cells stain red. Scale bars correspond to 50 μm (200x magnification).

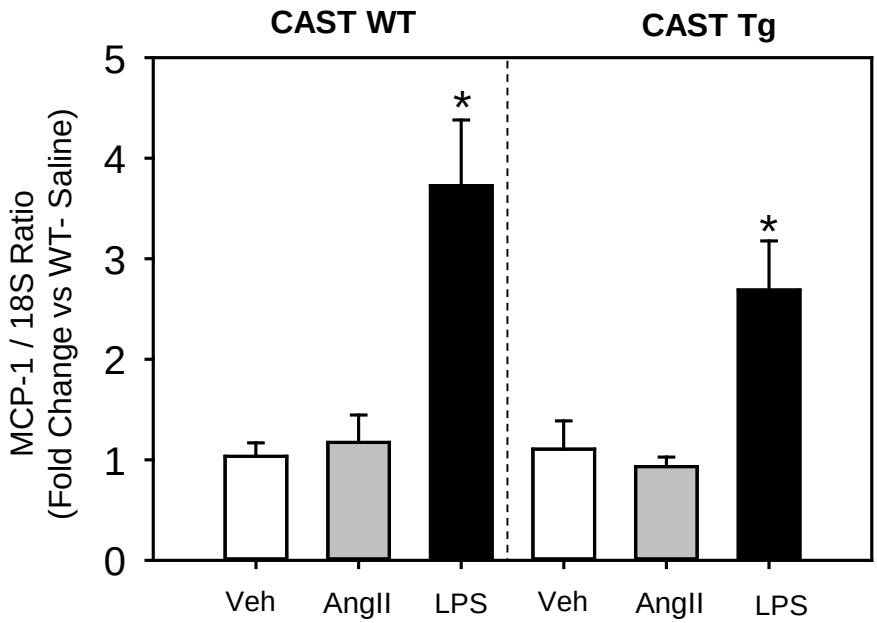
A

BMDMs – MCP-1 ELISA



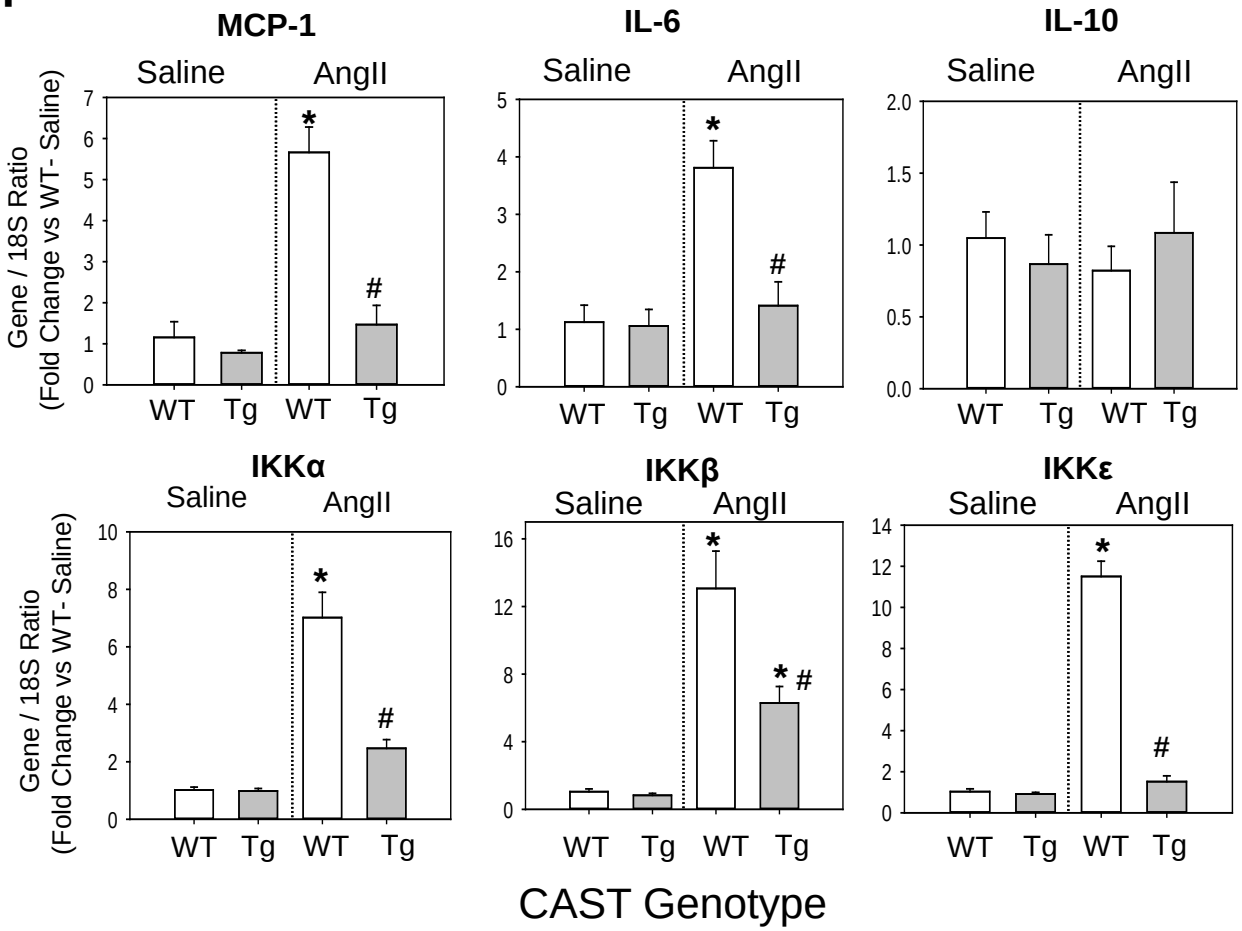
B

BMDMs – MCP-1 qPCR



Supplementary Figure XII. AngII had no effect on MCP-1 production in BMDMs. A. MCP-1 protein accumulated in cell culture media was measured by ELISA (n=4). **B.** MCP-1 mRNA abundance was analyzed by real-time PCR using 18S as an internal control (n=4). Values are represented as means ± SEMs. Statistical analyses were performed using one-way ANOVA followed by Holm-Sidak post hoc tests. * denotes $P<0.05$ when comparing LPS vs vehicle.

Spleen



Supplementary Figure XIII. CAST overexpression reduced AngII-induced inflammation in spleen.
mRNA abundance of MCP-1, IL-6, IL-10, and IKK genes in spleen from saline and AngII infused LDL receptor-/- mice transplanted with WT or CAST-Tg BMs were analyzed by qPCR (n=4-6). Values are represented as mean ± SEM. * and # denotes *P*<0.05 when comparing AngII vs saline and WT vs Tg respectively (Two-way ANOVA with Holm-Sidak post hoc analysis).