Sam68 impedes the recovery of arterial injury by augmenting inflammatory response

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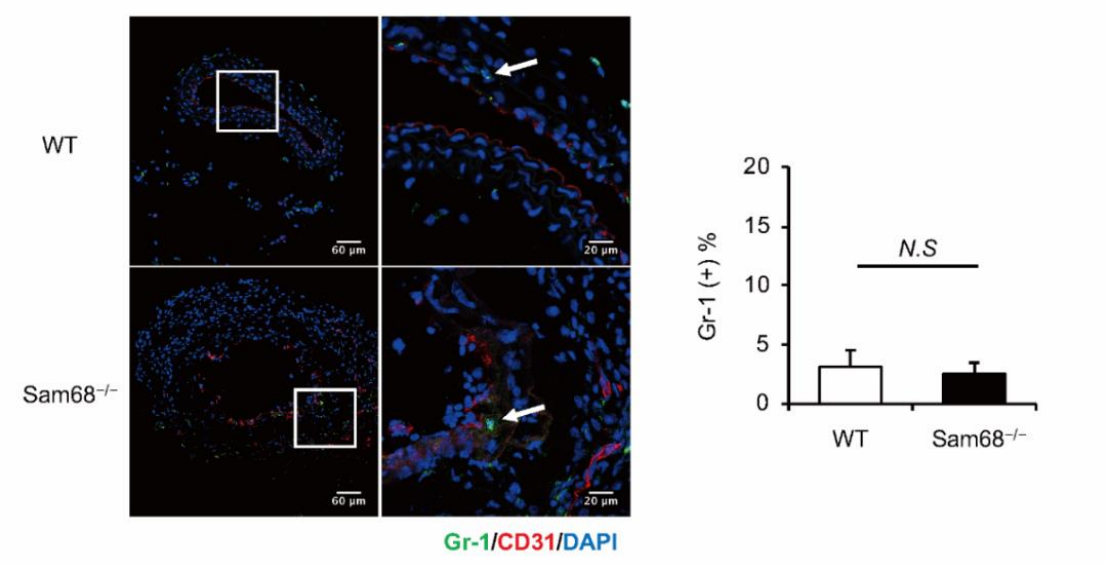
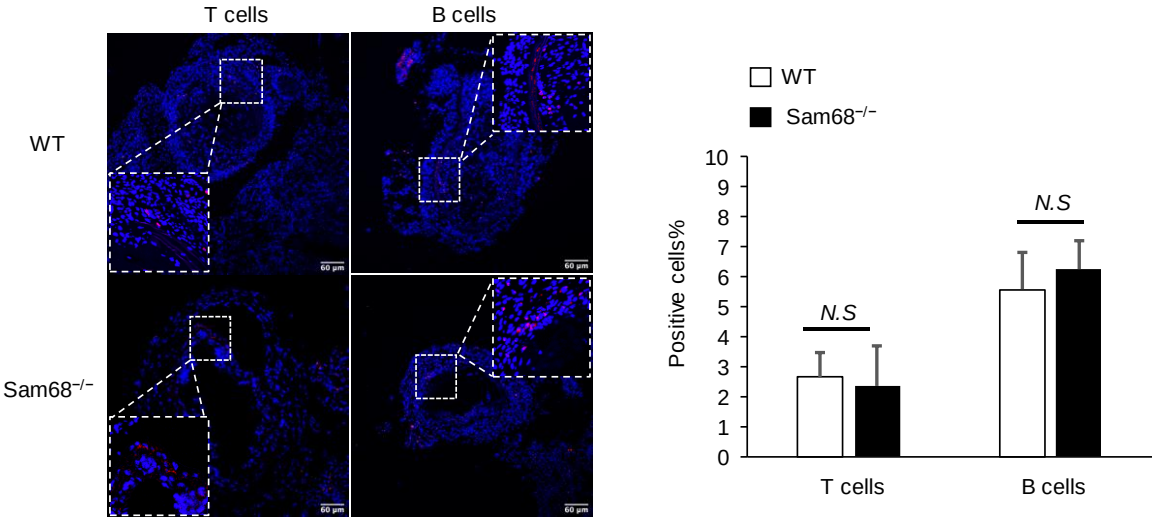
Supplementary Information

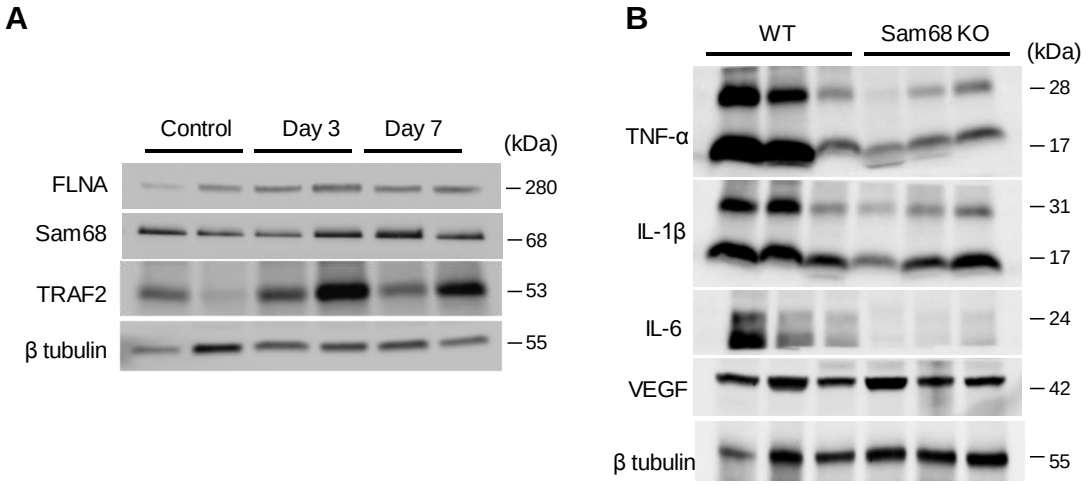
Supplementary Figures



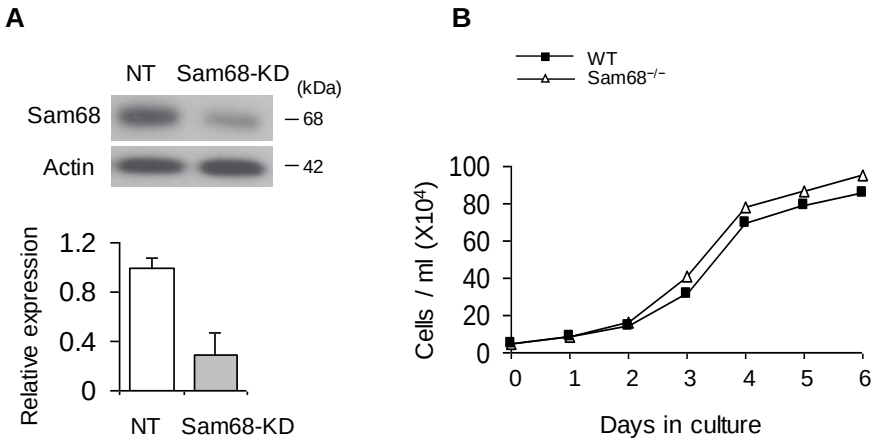
Supplementary Figure I. Neutrophil infiltrations in the injured carotid arteries of WT and Sam68$^{-/-}$ mice are similar. Carotid denudation injury was induced by wire in the left carotid arteries of WT and Sam68$^{-/-}$ mice. Three days later, injured vessels were isolated and analyzed histologically with immunofluorescent staining (*left panel*.), and the infiltrating Gr-1+ neutrophils were quantified and expressed as per 100 nuclei (*right panel*). n=5 per group. N.S., not significant.



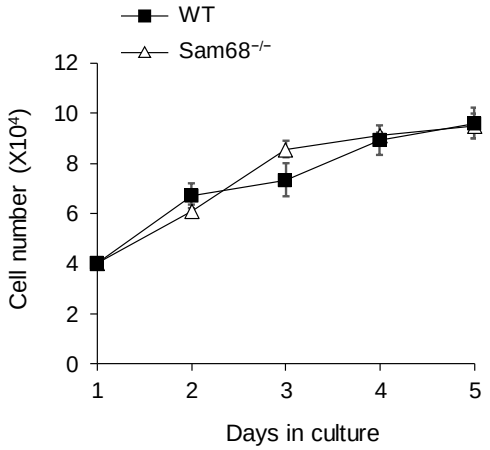
Supplementary Figure II. T and B cell infiltrations in the injured carotid arteries are similar between of WT and Sam68$^{-/-}$ mice. Carotid denudation injury was induced by wire in the left carotid arteries. Three days later, injured vessels were isolated and analyzed histologically with immunofluorescent staining for CD3 and B220 (*left panel*.). The CD3+ T cells and B220+ B cells were quantified and expressed as per 100 nuclei (*right panel*). n=5 per group. N.S., not significant.



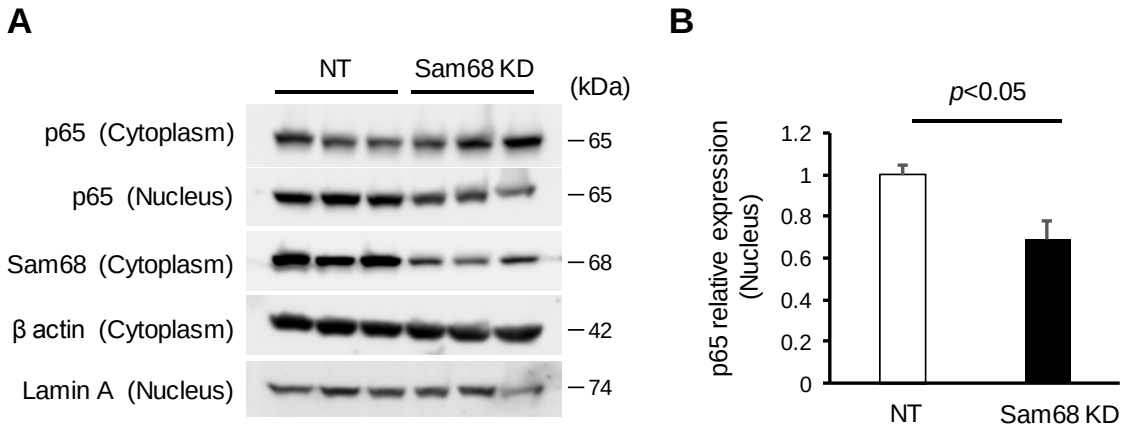
Supplementary Figure III. Sam68 deficiency results in lowered levels of pro-inflammatory cytokine expression in the injured carotid arteries. Carotid denudation injury was induced by wire in the left carotid artery of WT and Sam68$^{-/-}$ mice and at serial time points, the arterial tissues were isolated for Western blotting analyses of protein expression. **(A)** Endogenous Sam68, FLNA, and TRAF2 expression in control uninjured arteries and day 3 and day7 post-injury arteries in WT mice. **(B)** TNF-α, IL-1β, IL-6 and VEGF expression in day 3 post-injury carotid arteries from WT and Sam68$^{-/-}$ mice. n=3 per group.



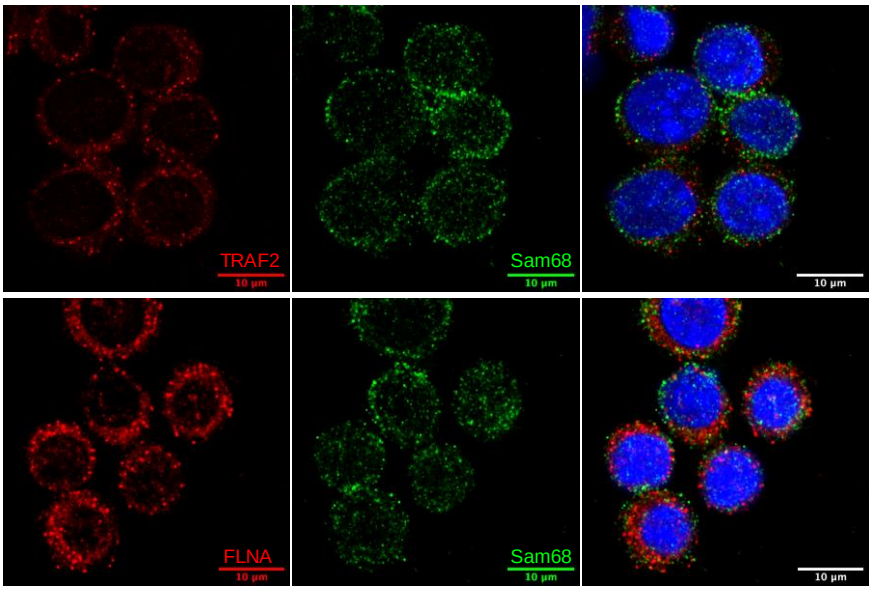
Supplementary Figure IV. Knockdown of Sam68 does not affect endothelial cell growth *in vitro*. MS1 cells were infected with lentiviral vectors coding for Sam68-shRNA or non-targeting (NT)-shRNA, and the transduced cells were selectively enriched in puromycin. **(A)** Representative and quantification of Sam68 protein expression as assessed by Western blotting. **(B)** MS1/Sam68-shRNA cells and MS1/NT-shRNA cells were cultured in DMEM medium supplemented with 10% FBS and 1%P/S. Cell counts were measured at a series of time points. Shown is the mean of Triplicate samples.



Supplementary Figure V. Deletion of Sam68 does not affect vascular smooth muscle cell (VSMC) growth *in vitro*. Primary VSMCs were isolated from WT and Sam68$^{-/-}$ mouse aorta and cultured in DMEM/F-12 medium supplemented with 10% FBS and 1%P/S. Cells at passage 4 were seeded at 4x104 cell/well of 96-well plate, and their proliferation were evaluated using MTT assay by following manufacturer's protocol. n=3 per group per time point.



Supplementary Figure VI. Sam68 knockdown in attenuates TNF-induced NF-κB nuclear translocation in macrophages. (A) Representative Western blotting analysis of p65 in the nucleus and cytoplasm of Raw264.7/NT and Raw264.7/Sam68-KD cells following TNF-α treatment (10 ng/ml) for 30 min. **(B)** Nuclear p65 levels were quantified densitometrically, normalized to Lamin A, and expressed as fold difference in Sam68-KD vs. NT cells. n=3.



Supplementary Figure VII. Immunohistochemical staining of Sam68, TRAF2, and FLNA in macrophage. Raw264.7 cells were treated with TNF-α (10ng/mL) for 30 min, then fixed with 100% methanol for 5 min and blocked with 10% normal donkey serum in 0.1% PBS-Tween for 1 h. Next, the cells were incubated with anti-Sam68 antibody (abcam, ab76471, 1:1000 dilution) together with anti-Filamin A antibody (Millipore, MAB1678, 1:100 dilution) or with anti-TRAF2 antibody (Santa Cruz, sc-7346, 1:100 dilution) overnight at 4°C, and stained with secondary antibody, donkey anti-rabbit Alexa Fluor 488 (Thermofisher, A-21206, 1:1000 dilution) or donkey anti-mouse Alexa Fluor 568 (Thermofisher, A10037, 1:1000 dilution) for 1 h at RT in dark. Lastly, the cells were incubated with DAPI for 10 min to allow for nuclear staining. The slides were examined under confocal microscope. Sam68, green; TRAF2 (red, upper panel); FLNA (red, lower panel).

Supplementary Table

Supplementary Table I: Primer sequences

Experiment	Gene	Forward primer	Reverse primer
Real-Time qRT-PCR for Gene expression	*TNF-α*	AGGGATGAGAAGTTCCCAAATG	GTGAGGGTCTGGGCCATA
	IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
	IL-6	TCGGAGGCTTAATTACACATGTTC	TGCCATTGCACAACTCTTTTCT
	MCP-1	CTGAAGACCTTAGGGCAGAT	AAGGAATGGGTCCAGACATAC
	VCAM1	CCAAATCCACGCTTGTGTTGA	GGAATGAGTAGACCTCCACCT
	ICAM1	TTCACACTGAATGCCAGCTC	GTCTGCTGAGACCCCTCTTG
	18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Genotyping	*Sam68*	GCCACTTCACATTGGCTAAAC	ATGCCTGCTTGCCGAATA

Supplementary Table II: Mass spectrometry identified proteins that interact with Sam68 upon TNF-α treatment in macrophages

Accession	Description	Coverage GQ_3	des	SM GQ	Coverage GQ_4	eptides GQ	PSM GQ_	MW [kDa]
P14131	40S ribosomal protein S16 OS=Mus musculus GN=Rps16 PE=2	15.07	2	4	20.55	3	12	16.4
Q8CBA2	Schlafen family member 5 OS=Mus musculus GN=Slfn5 PE=2 S	3.73	2	4	8.48	5	11	100.8
Q8VI93	2'-5'-oligoadenylate synthase 3 OS=Mus musculus GN=Oas3 P	4.13	3	4	21.79	16	34	126.3
Q3KQM4	Splicing factor U2AF 65 kDa subunit OS=Mus musculus GN=U2	11.07	2	4	29.32	5	15	33.9
B1ARA3	60S ribosomal protein L26 (Fragment) OS=Mus musculus GN=F	15.53	2	4	24.27	3	10	12.2
B7FAV1	Filamin, alpha (Fragment) OS=Mus musculus GN=Flna PE=4 S'	11.90	3	4	13.90	20	38	274.5
D6REF7	Sphingosine-1-phosphate lyase 1 OS=Mus musculus GN=Sgpl1	5.34	2	4	22.18	6	10	54.7
E9Q415	Low affinity immunoglobulin gamma Fc region receptor II OS=M	10.58	2	4	10.58	2	5	35.0
J3QNB1	La-related protein 1 OS=Mus musculus GN=Larp1 PE=2 SV=1	4.38	3	4	8.96	7	18	121.0
Q792Z1	MCG140784 OS=Mus musculus GN=Try10 PE=2 SV=1 - [Q792	8.13	2	4	0.00			26.2
Q8BQ46	Protein Taf15 OS=Mus musculus GN=Taf15 PE=2 SV=1 - [Q8E	14.90	3	4	11.85	3	5	58.6

Note: Raw264.7 macrophages were treated with TNF-α (10 ng/mL) and vehicle for 7 min and lyzed. The Sam68 co-immunoprecipitations were performed. The precipitated proteins were identified and analyzed by mass spectrometry.