Supplemental materials

Platelet IKKβ Deficiency Increases Mouse Arterial Neointima Formation via Delayed Glycoprotein Ibα Shedding

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Extended methods

Mice

PF4-cre [1] and floxed IKKβ mice [2] (C57BL/6 background) were received from Dr. Radek C. Skoda and Dr. Michael Karin. The C57BL/6J and LDLR$^{-/-}$ mice were received from The Jackson Laboratory (Bar Harbor, ME). PF4-cre and floxed IKKβ mice were crossed to generate platelet-specific IKKβ-deficient mice -IKKβ$^{fl/fl}$/PF4^{cre} mice and their IKKβ$^{fl/fl}$ littermates (Supplemental Figure VII). A mouse 384 SNP panel (including markers spread across the genome at approximately 7 Mbp intervals) was used to characterize the genetic background of the breeding IKKβ$^{fl/fl}$/PF4^{cre} mice (Charles River Laboratories International, Troy, NY). IKKβ$^{fl/fl}$/PF4^{cre} and LDLR$^{-/-}$ mice were crossed to generate IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice and

their IKKβ$^{fl/fl}$/LDLR$^{-/-}$ littermates. Supplemental Figure I shows the breeding scheme. All of the animal experiments and care were approved by the Georgia Health Sciences University Animal Care and Use Committee, in accordance with AAALAC guidelines.

Carotid artery wire injury model

Eight-week-old male mice were fed a Western diet (42.0% kcal fat, 42.7% kcal carbohydrate, 15.2% kcal protein) (Harlan Teklad, Madison, WI) for 2 weeks. A guide wire injury was then administered to the carotid artery.[3-5] To quantify the neointimas, each carotid artery was serially sectioned from the bifurcation to the common carotid artery, resulting in 100 slides; each slide had 3 serial sections. Among these slides, 12 slides, including slides 1, 10, 19, 28, 37, 46, 55, 64, 73, 82, 91 and 100, were stained with Movat pentachrome (Sigma, St. Louis, MO). For each carotid artery, 10 sections (one section on each above slide) were analyzed. The areas of the lumen, internal elastic lamina, and external elastic lamina were determined by planimetry using NIH Image Software, and the intimal area, medial area and intima to media (I/M) ratio were calculated.[3-5]

Neutrophil depletion

After being fed a Western diet for 2 weeks, a carotid artery wire injury was performed on the IKKβ$^{fl/fl}$/PF4^{cre}/ LDLR$^{-/-}$ mice and the IKKβ$^{fl/fl}$/ LDLR$^{-/-}$ control mice. A neutrophil-depleting anti-PMN antibody (Accurate Chemical & Scientific, Westbury, NY) was diluted in PBS (1:5), and 0.1 ml of the diluted antibody was injected into the mouse peritoneal cavity daily for 4 weeks after the wire injury.[6] For the control mice, rabbit IgG (Accurate Chemical & Scientific) was injected at the same dose. The efficacy of the anti-PMN antibody on

neutrophil depletion was confirmed by flow cytometry and HESKA CBC-Diff Veterinary Hematology System (data not shown).

Bone marrow transplantation

The bone marrow transplantation was performed as previously described.[5] Bone marrow-derived cells from the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice or IKKβ$^{fl/fl}$/LDLR$^{-/-}$ controls were transplanted into lethally irradiated LDLR$^{-/-}$ mice. Three weeks after the bone marrow transplantation, the mice were fed a Western diet for 2 weeks and then subjected to carotid artery wire injury.

Histological analysis of the injured arteries

Cross sections of the injured arteries were stained with monoclonal antibodies to identify the platelets (MWReg30; Santa Cruz Biotechnology, Santa Cruz, CA), macrophages (F4/80, clone CI:A3-1; Accurate Chemical, Westbury, NY) and neutrophils (anti-mouse neutrophil, clone 7/4; Accurate Chemical). The images were analyzed with Image-Pro Plus.

***In vivo* examination of leukocyte interactions with the injured arterial wall**

The left carotid arteries of the mice were injured with a guide wire to induce vascular damage. After 10 min, the leukocyte interactions with the injured vessel were recorded and analyzed with an intravital epifluorescence microscopy system.[4]

Leukocyte interactions with activated platelets in a micro-flow chamber

The mouse platelets were isolated from the IKKβ$^{fl/fl}$/PF4^{cre} mice and their littermate IKKβ$^{fl/fl}$ mice by gel filtration.[7] The micro-flow chamber was prepared as previously described.[4, 5] Specifically, 2 x 10^9/ml of isolated platelets were loaded into a rectangular glass capillary tube. After adhering to the surface of the capillary tube, the platelets were activated with thrombin (0.1 U/ml). The wild type mice were anesthetized and injected with 1 mg/ml rhodamine 6G (50 µl/30 g body weight) via the tail vein. Then, the blood from the carotid artery was passed through the micro-flow chamber. For some experiments, the mice were first treated with anti-M2 antibody and IgG. Leukocyte rolling and adhesion on the activated platelets was observed and recorded with an intravital epifluorescence microscope system.

Blocking of the GPIbα binding site on Mac-1

An affinity purified peptide-specific polyclonal antibody (termed anti-M2) to the Mac-1 binding site for GPIbα was kindly supplied by Dr. Daniel I. Simon.[8] A total of 100 µg anti-M2 or 100 µg IgG (Accurate Chemical & Scientific) was injected via the tail vein of IKKβ$^{fl/fl}$/PF4^{cre}/ LDLR$^{-/-}$ mice and the IKKβ$^{fl/fl}$/ LDLR$^{-/-}$ control mice that had been fed a Western diet for 2 weeks. Following antibody or IgG treatment, these mice were subjected to wire injury.

Platelet activation, aggregation and secretion

The mouse platelets were isolated by gel filtration.[7] The purity of the platelets was determined by flow cytometry (Supplemental Figure VIII). Platelet activation was achieved by treating the platelets with thrombin (Sigma, St. Louis, MO) at the indicated concentrations, followed by neutralization with an equimolar dose of hirudin (Sigma) if necessary. As previously described,[9] platelet aggregation was measured in a Lumi-Aggregometer model 700 (Chronolog)

at 37°C with stirring (1000 rpm). In parallel with platelet aggregation, platelet secretion was monitored as ATP release with the addition of the luciferin-luciferase reagent to the platelet suspension. Quantification was performed using ATP standards.

Flow cytometric analysis

The anti-Ly6G, anti-CD41 and anti-P-selectin antibodies were purchased from BD Biosciences. The anti-CD115 antibody was from eBioscience (San Diego, CA). The anti-GPIbα, anti-GPV, anti-GPVI, anti-GPIX and anti-αIIbβ3 antibodies were obtained from Emfret Analytics (Wurzburg, Germany). For whole blood flow cytometry, Ly6G and CD115 were used to identify mouse circulating neutrophils and monocytes, respectively. CD41 was used to detect platelets. Flow cytometry was performed using a FACSCalibur (BD Biosciences). The data were analyzed using CellQuest (Tampa, FL) software.

Platelet electron microscopy

Platelets was isolated from whole blood and fixed in glutaraldehyde in White's saline. The platelets were then placed in 1% osmic acid in Zetterquiest's buffer, dehydrated with alcohol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate to enhance the contrast, and the sections were then examined with a Philips 301 electron microscope (F.E.I. Co., Hillboro, OR, USA).

Western blot analysis

The platelets were lysed in a modified RIPA lysis buffer.[4] Primary antibodies against GPIbα (Emfret Analytics, Wurzburg, Germany), ADAM17, active ADAM17 (Abcam,

Cambridge, MA), P38 MAPK, p-P38 (phosphorylated P38 MAPK), and GAPDH (Cell Signaling, Danvers, MA) were added, followed by incubation with alkaline phosphatase–conjugated secondary antibodies. The membranes were developed with a chemiluminescence reagent. The band intensities were quantified using the NIH Image J program.

Blood lipid and leukocyte analysis

Plasma triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and total cholesterol were measured via an automated enzymatic technique (Boehringer Mannheim GmbH). Total and differential leukocyte counts in the blood were quantified using an automated blood cell counter (Hemavet 850FS, CDC Technologies, Oxford, CT).

Statistical analysis

The data are presented as the mean ± SEM. The data were analyzed by either one-way ANOVA followed by a Bonferroni correction post-hoc test or Student's *t*-test to evaluate two-tailed levels of significance. The null hypothesis was rejected at $P < 0.05$.

Supplemental Data

Table I, II

Table I

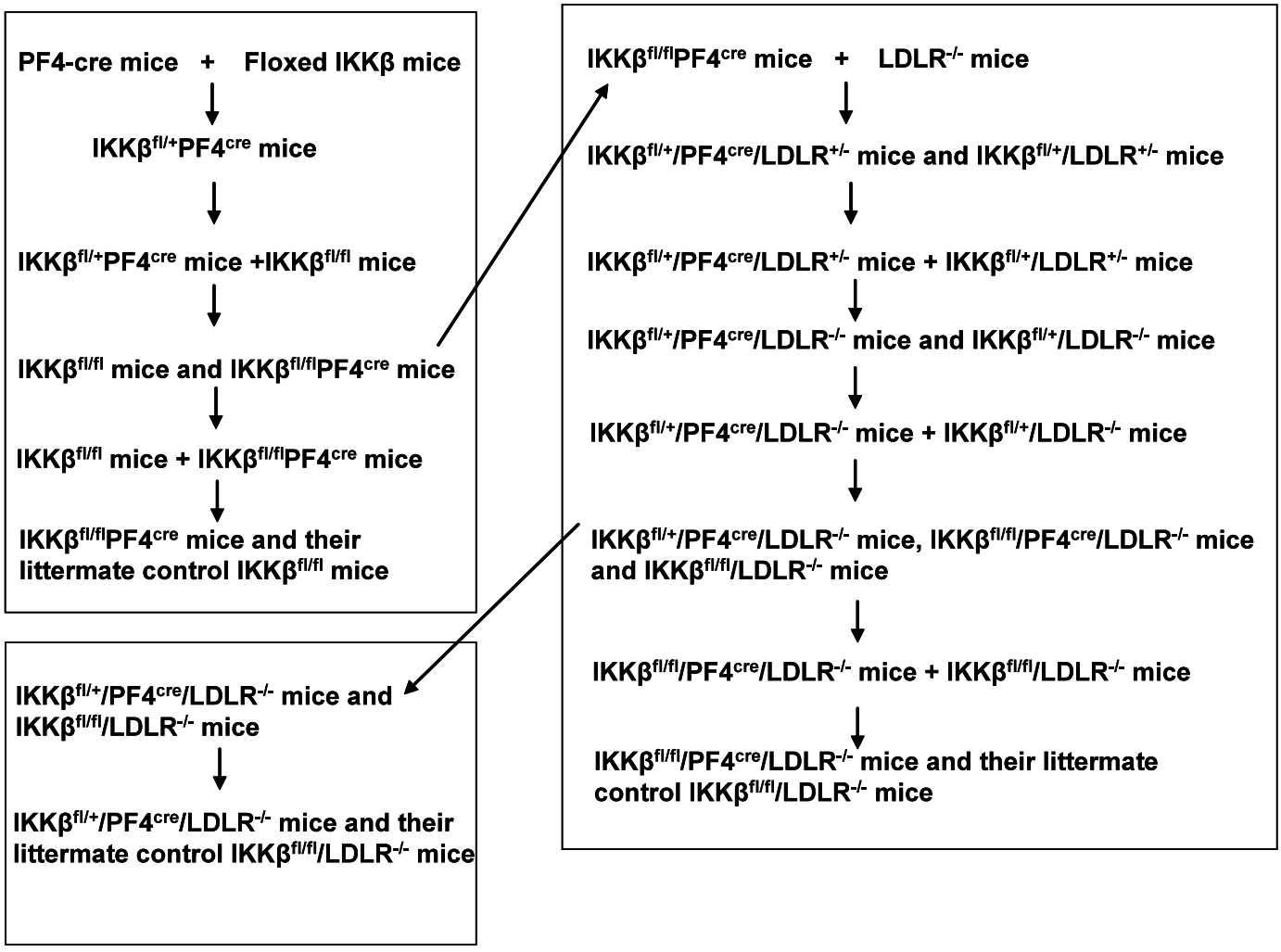
Genotype	n	WBC (k/μL)	NE (k/μL)	LY (k/μL)	MO (k/μL)	NE%	LY%	MO%	PLT (k/uL)
IKKβ$^{fl/fl}$/LDLR$^{-/-}$	10	10.7±0.9	2.8±0.5	5.4±0.8	0.5±0.1	30.0±1.8	58.6±1.5	4.3±0.6	780.8±123.5
IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$	10	9.8±0.6	2.9±0.8	5.1±0.9	0.5±0.1	31.9±1.3	56.8±1.4	5.4±0.5	598.6±95.8

Table II

Genotype	BW (g)	Cholesterol (mg/dl)	Triglyceride (mg/dL)	LDL (mg/dl)	VLDL (mg/dL)	HDL (mg/dL)
IKKβ$^{fl/fl}$/LDLR$^{-/-}$	28.4±7.7	863.4±58.2	338.3±21.2	636.3±47.3	68.4±9.7	159.0±10.4
IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$	27.2±6.4	907.7±57.1	368.7±19.4	667.5±56.4	61.3±8.2	144.5±8.7

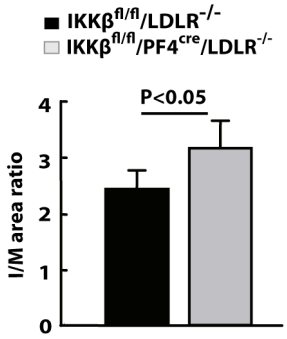
Supplemental Table I and II. Blood cell analysis (Table I) and lipid profile (Table II) of the IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice and the control IKKβ$^{fl/fl}$/LDLR$^{-/-}$mice

Supplemental Figure I



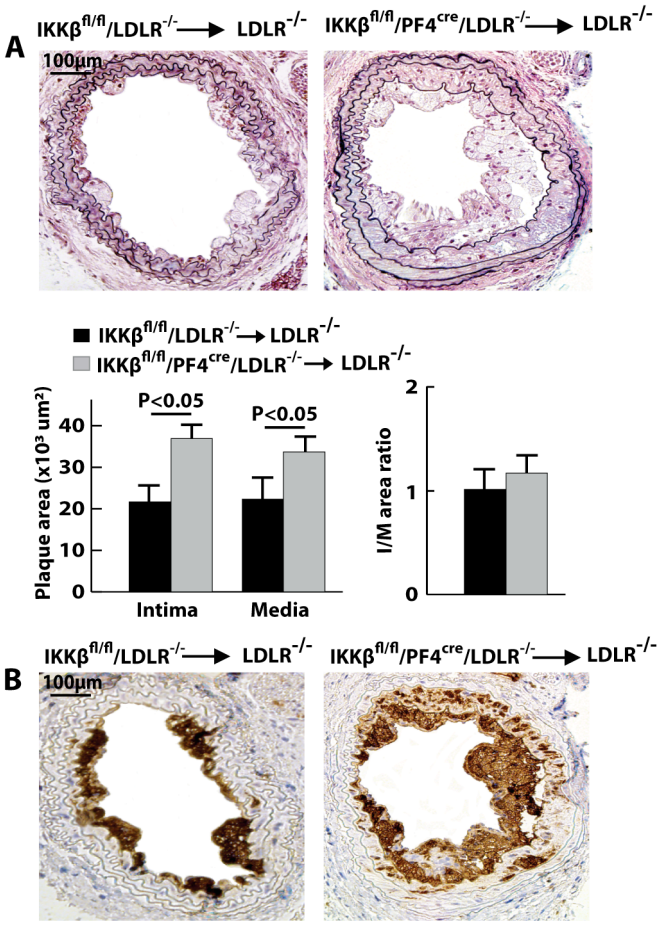
Supplemental Figure I. The breeding scheme of experimental mice.

Supplemental Figure II



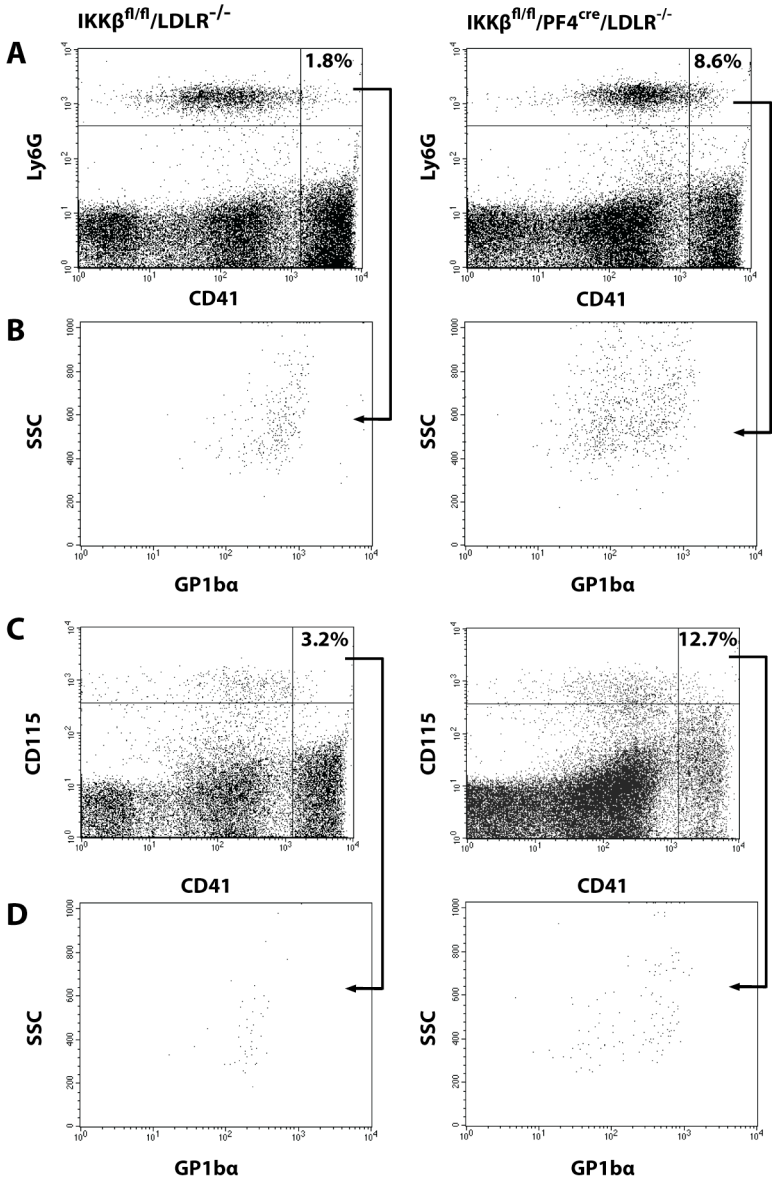
Supplemental Figure II. The size of the neointima and media of the injured carotid arteries. The arterial neointima at 4 weeks after injury was stained with Movat pentachrome, and the ratio of intima (I) to media (M) was calculated (I/M, n = 12 for each group)

Supplemental Figure III



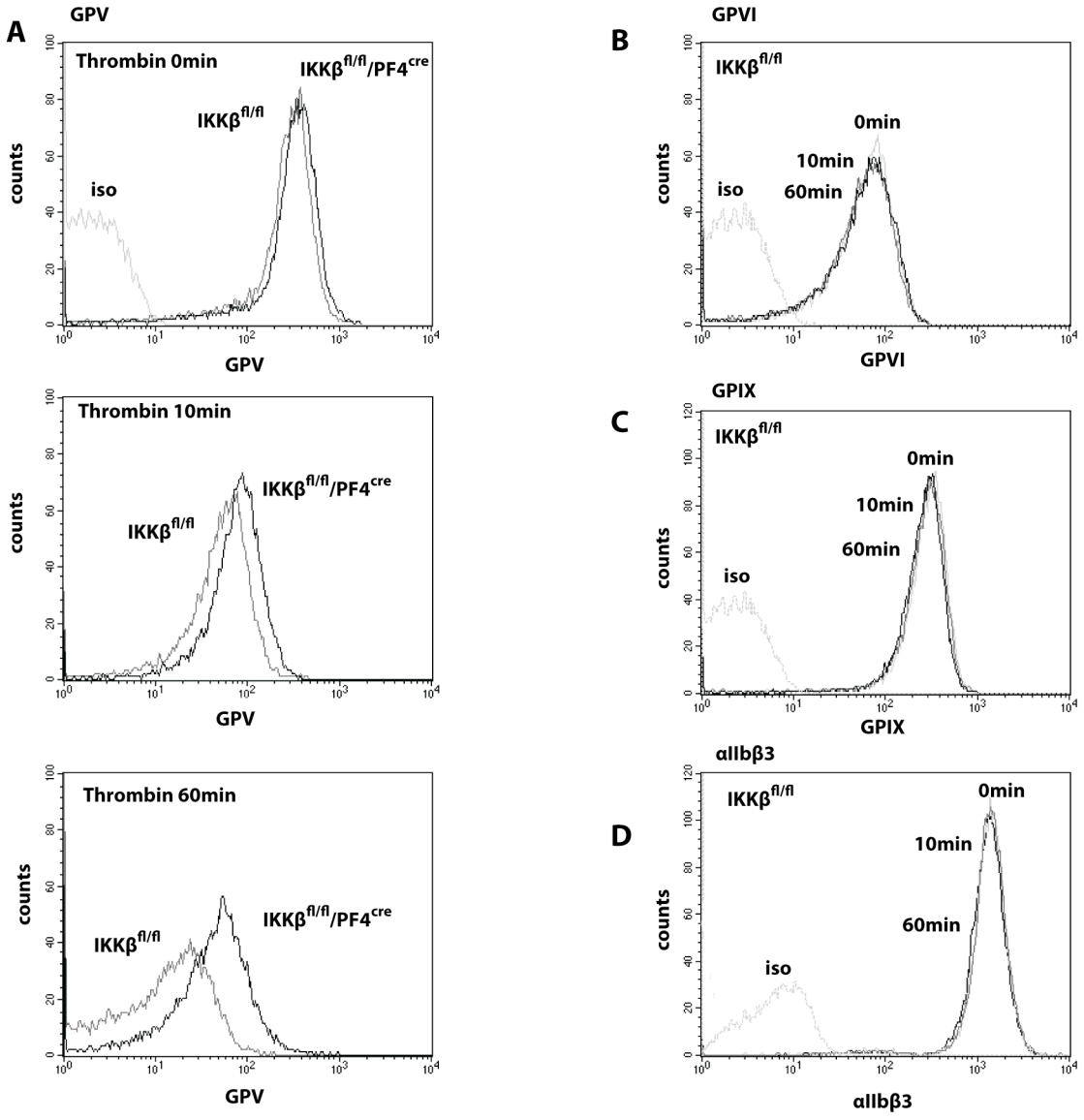
Supplemental Figure III. Platelet IKKβ deficiency increased neointima formation in the wire-injured carotid arteries from the $LDLR^{-/-}$ mice following bone marrow transplantation. The $LDLR^{-/-}$ mice were lethally irradiated and then received bone marrow from the $IKK\beta^{fl/fl}/PF4^{cre}/LDLR^{-/-}$ or $IKK\beta^{fl/fl}/LDLR^{-/-}$ mice. Three weeks after bone marrow transplantation, the mice were fed a Western diet for 2 weeks and then subjected to carotid artery wire injury. A, Movat pentachrome staining of cross sections of the injured artery at 4 weeks after injury. The size of the neointima (I) and media (M) and the ratio of the intima to media (I/M) were quantified (n = 10 for each group). B**,** The infiltrated macrophages were immunostained with anti-F4/80 antibody in the arterial neointima. The percentage of the area that was positive was calculated by dividing the positive area by the measured lesion area (n = 10 for each group).

Supplemental Figure IV



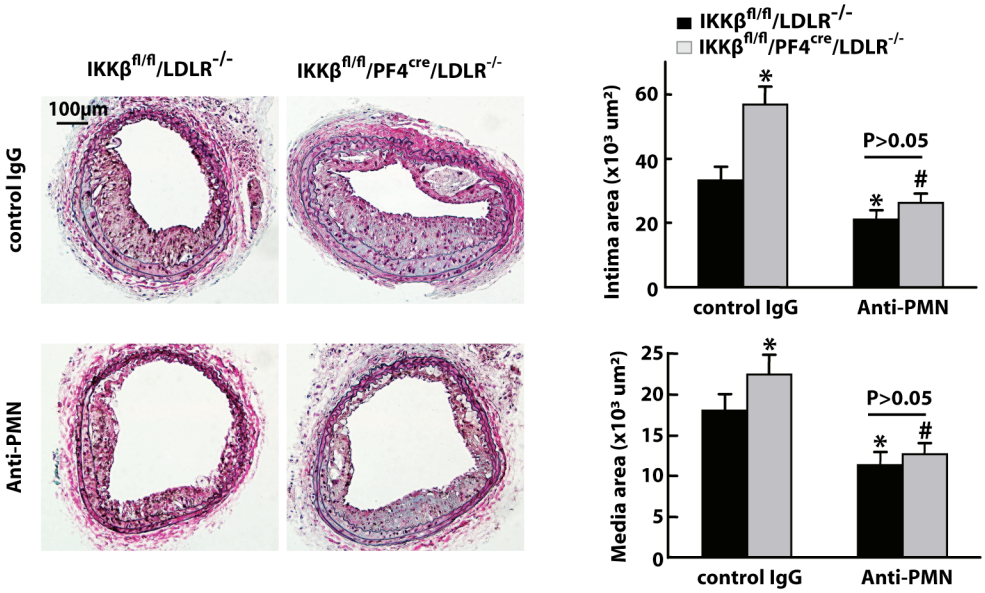
Supplemental Figure IV. Platelet IKKβ deficiency increased platelet-leukocyte aggregates *in vivo*. IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ and IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice were fed a Western diet for 4 weeks. Whole blood was collected from the submandible and used for flow cytometry. Platelet-neutrophil aggregates were defined as cells positive for both CD41 (platelet marker) and Ly6G (neutrophil marker), whereas platelet-monocyte aggregates were defined as cells positive for CD41 and CD115 (monocyte marker). Representative flow cytometry results showing platelet-neutrophil aggregates (A), levels of GPIbα for platelet- leukocyte aggregates (B and D) and platelet-monocyte aggregates (C) (n = 5 for each group).

Supplemental Figure V



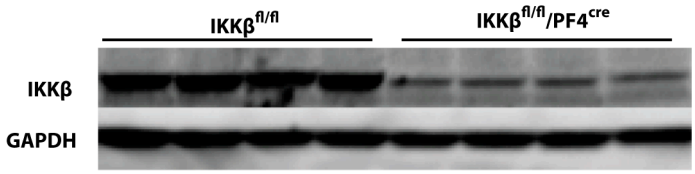
Supplemental Figure V. Effects of IKKβ deficiency on GPV shedding in platelets. The platelets were isolated from the IKKβ$^{fl/fl}$/PF4^{cre} and IKKβ$^{fl/fl}$ mice and stimulated with thrombin at 0.1 U/ml. Representative flow cytometric histograms show the level of GPV (**A)**, GPVI (B), GPIX (C) and αIIbβ3 (D) on the activated platelets. Three separate experiments with different donors were performed.

Supplemental Figure VI



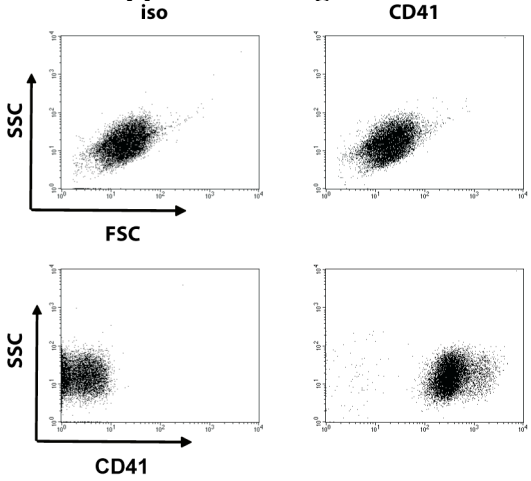
Supplemental Figure VI. Neutrophil depletion decreased neointima formation in wire-injured carotid arteries of both IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice and IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice. The mice were fed a Western diet for 2 weeks and then subjected to carotid artery wire injury. Control rabbit IgG and anti-PMN antibody were intraperitoneally injected into these mice daily. Four weeks later, the carotid arteries were collected, and the size of the neointima and media was analyzed with Movat pentachrome staining. Ten mice were included in each group.*P<0.05 vs. IKKβ$^{fl/fl}$/LDLR$^{-/-}$ mice treated with control IgG; $^{\#}P$<0.05 vs. IKKβ$^{fl/fl}$/PF4^{cre}/LDLR$^{-/-}$ mice treated with control IgG.

Supplemental Figure VII



Supplemental Figure VII. Western blot of isolated platelets showing that PF4-cre effectively knocked out platelet IKKβ expression in IKKβ$^{fl/fl}$PF4^{cre} mice.

Supplemental Figure VIII



Supplemental Figure VIII. The purity of isolated platelets was determined by flow cytometry. Representative data showing that more than 99% platelets are CD41 positive.

References

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