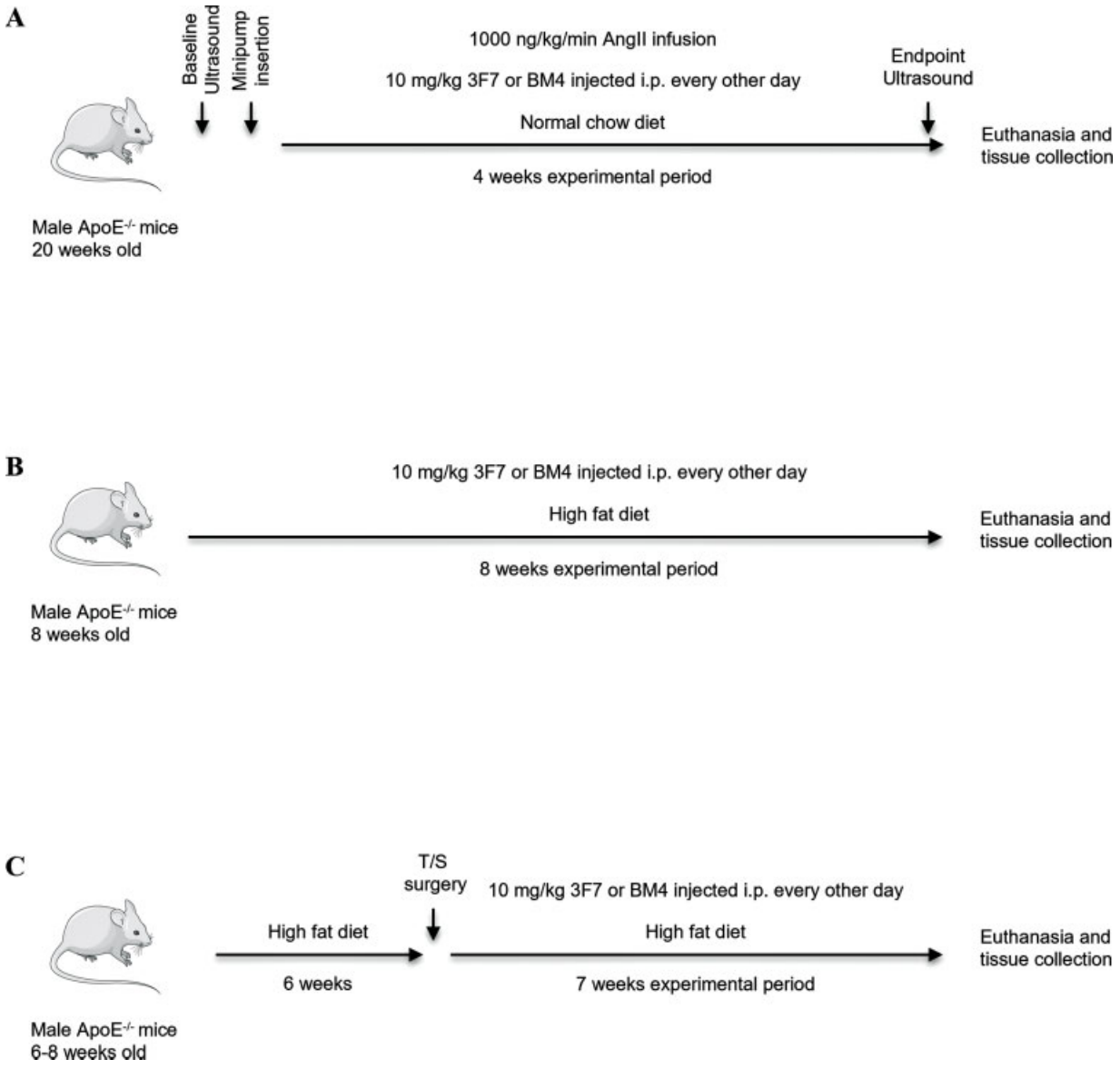
Supplementary Methods

Mouse Experiments and 3F7 Treatment

All animal procedures were approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia, under ethics application numbers E/1658/2016/B and E/1187/2012/B and conform to the current National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. ApoE$^{-/-}$ mice were generated from a C57BL/6 background, bred, and maintained at the AMREP Animal Centre.

Mouse models of abdominal aortic aneurysm (AAA), stable, and unstable atherosclerosis were employed (►**Supplementary Fig. S1**). For the investigation of atherosclerotic plaques, two animal models were employed. In the first group, male 8-week-old ApoE$^{-/-}$mice were randomly assigned to receive either 3F7 or BM4 via intraperitoneal (i.p.) injection. These animals were fed a high-fat diet (HFD) containing 21% fat and 0.15% cholesterol (SF00–219, 21% fat and 0.15% cholesterol, Specialty Feeds, Australia) for 8 weeks. At the age of 16 weeks, the mice were sacrificed and investigated. In the second group, mice underwent tandem stenosis (TS) surgery to generate unstable atherosclerotic plaques (as described below). Animals were randomly assigned to receive either 3F7 or BM4, with the treatment regime beginning directly after surgery.

An angiotensin II (AngII)-infused mouse model was used to investigate AAA, as previously described.[1] Briefly, an



Supplementary Fig. S1 Overview of the animal models used. Three mouse models were investigated: (**A**) the AngII-induced mouse model of AAA, (**B**) the ApoE$^{-/-}$ mouse model of stable atherosclerosis and (**C**) the TS mouse model of vulnerable/unstable atherosclerosis. AngII, angiotensin II; i.p., intraperitoneal; TS, tandem stenosis.

osmotic minipump delivering a constant infusion of AngII (1,000 ng/kg/min) was inserted subcutaneously between the scapulae of 20-week-old ApoE$^{-/-}$ mice for a period of 28 days. The animals were randomly assigned to receive either 3F7 or BM4 via i.p. injection, with the treatment regime starting directly after surgery. Animals received a normal chow diet for the duration of the study. Baseline and endpoint molecular ultrasound imaging was performed to assess the dilatation of the abdominal aorta. All groupings of animals were blinded from the responsible researchers throughout this study.

Therapeutic application of the anti-FXIIa mAb 3F7 in mice has been described previously.[2] The antibody is administered at a dose of 10 mg/kg i.p. every second day. Single-dose pharmacokinetic and pharmacodynamic studies with 3F7 at 10 mg/kg in mice, including the i.p. administration route, support sufficient antibody levels for a robust FXIIa inhibition over the complete treatment period in the performed atherosclerosis and AAA experimental models (data not shown). Both 3F7 and its isotype control, BM4 (MuBM4-MuG1K), were supplied by CSL Limited as murine IgG1 antibodies to minimize potential immunogenicity following repeated administration.

Tandem Stenosis Surgery

This surgery has been previous described.[3,4] Male 6- to 8-week-old ApoE$^{-/-}$mice were fed an HFD (SF00–219, 21% fat and 0.15% cholesterol, Specialty Feeds, Australia) for 6 weeks. At 12 to 14 weeks of age, mice were anaesthetized by a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture through i.p. injection. An incision was made in the neck and the right common carotid artery was dissected from circumferential connective tissues. A TS with 150 µm outer diameter was introduced, with the distal point 1 mm from the carotid artery bifurcation and the proximal point 3 mm from the distal stenosis. The stenosis diameter was obtained by placing a 6–0 blue braided polyester-fiber suture around the carotid artery together with a 150 µm needle that was tied into it and later removed. Animals were euthanized 7 weeks postsurgery.

Various anatomical sections of the aortic tree are referred to by Roman numbers from I to V, as described in depth previously.[3,4] Briefly, segment I refers to the region of carotid vulnerable plaque, segment V refers to the stable plaque within the aortic arch, and segment IV refers to the healthy vasculature of the left common carotid artery.

In Vivo Molecular Ultrasound Imaging

Molecular ultrasound imaging was performed on AngII-induced AAA animals prior to osmotic pump insertion (baseline) and 1 day prior to euthanasia (endpoint) using a Vevo2100 small-animal high-resolution imaging scanner (VisualSonics Inc., Toronto, Canada). Scans were performed using a 22 to 55 MHz high-frequency transducer (lateral resolution 100 µm; transverse resolution 40 µm; focal length 10 mm; low acoustic pressure or mechanical index 0.14). Animals were anesthetized using 1.5 to 2% isoflurane and fur was removed from the abdominal region using shaving cream (Dove, Sydney, Australia). To image the abdominal aorta, the animal was placed onto the imaging station and the transducer was placed over the abdomen with the transducer marker facing 12 o'clock. Fundamental brightness mode (B mode; transmitting power 100%; dynamic range 65 dB) was used for anatomical imaging. Both supra- and infra-renal aortic dilatation measurements were collected, using the position of the renal arteries as an anatomical marker to ensure the measurements were taken at the same area at both baseline and endpoint, ensuring reproducibility.

Endpoint and Tissue Collection

For the investigation of atherosclerosis, animals were sacrificed using pentobarbital sodium/ phenytoin sodium (Euthal, 10 mg/kg, Delvet Limited, Australia) given intraperitoneally. Blood samples were collected by cardiac puncture. A catheter was placed in the left ventricle for perfusion with 10 mL phosphate-buffered saline (PBS), at pH 7.4 under physiological pressure to expel all remaining blood from the vasculature. After perfusion, either aortic roots or the entire aortic arch with the brachiocephalic artery and the right and left carotid arteries was embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical), snap-frozen in liquid nitrogen, and stored at −80°C for subsequent histological analysis. Blood was centrifuged and plasma was collected, snap-frozen, and stored at −80°C for later analysis.

For the assessment of AAA, animals were euthanized via a lethal dose of ketamine/xylazine. A laparoscopic incision was made, and internal organs were shifted aside to expose the abdominal aorta. The connective tissues were dissected from the supra- and infra-renal portions of the abdominal aorta, allowing for macroscopic examination of the AAA using a Canon professional-grade camera. After perfusion with PBS, as mentioned above, a portion of the supra- and infra-renal abdominal aorta was embedded in OCT and snap-frozen using liquid nitrogen for histological analysis.

Tissue Cryoprocessing

The frozen carotid arteries, aorta arch, and aortic sinus, as well as supra- and infra-renal portions of the abdominal aorta were processed for histological analysis in relation to atherosclerosis and AAA, respectively. Sections of 6 µm thick transversal cryosections were prepared using a cryostat (Zeiss MICROM HM 550). For the AAA samples, to ensure an appropriate area of aneurysm was collected for histological analysis, the vessels were trimmed on the cryostat until the AAA size visually correlated to the macroscopic images obtained at the time of euthanasia.

Histology

To assess atherosclerosis, sections were histologically stained with Mayer's hematoxylin and eosin (H&E), Oil-red O, or Picro-Sirius Red (PSR). The total intimal plaque area was quantified on sections stained with Mayer's H&E, which allowed the quantification of other stains to be corrected for plaque size. These included lipid accumulation, necrotic

core areas (acellular areas), and collagen deposition. PSR was also used to measure the relative cap thickness, defined as the ratio of the cap thickness at the shoulder and mid-plaque region divided by maximal intimal thickness. Quantification of histological samples for each segment was performed on sequential 6 µm sections obtained at 120 µm intervals.[5] For each mouse, total intimal plaque size was measured in four sections across 480 µm of the aortic sinus area and the quantification was achieved by averaging those sections.[6] All lesion areas were measured using the internal elastic lamina as the internal border of the lesion.

To assess AAA, sections were histologically stained with H&E for simple morphological analysis, as well as with both PSR and Masson's trichrome to investigate collagen deposition. For each mouse, two sections taken at an interval of ~150 µm were assessed.

Immunohistochemistry

Snap-frozen sections were thawed, fixed, and incubated with one of the following primary antibodies: vascular cell adhesion molecule-1 (VCAM-1, clone sc-1504, 1:100 dilution; Santa Cruz Biotechnology, United States), α-smooth muscle actin (clone 1A4; 1:100 dilution; Sigma Aldrich, United States), CD68 (clone FA-11, 1:100 dilution; AbD Serotec, United Kingdom), or TER-119 (clone TER-119, 1:400 dilution; eBioscience, United States). Detection was achieved using a Vectastain ABC kit and DAB substrate (Vector Laboratories, United States). Isotype control antibodies were used for validation of each immunostaining (rat IgG, ThermoFisher Scientific, United States; rat IgG2b kappa, eBioscience, United States; rabbit IgG, Vector Laboratories, United States).

IVIS In Vivo Imaging System

To study the localized enrichment of FXIIa in the TS mouse model, 3F7 was conjugated with Alexa-Fluor (AF) 546 using a labeling kit (ThermoFisher, Massachusetts, United States) following the commercial SOP. The success of the labeling was tested by fluorescence microscopy and comparison with its control antibody, BM4. Antibodies were injected into TS animals via tail vein and circulated for 30 minutes prior to animal sacrifice. Vessel segments (segment I, segment IV, segment V, and thoracic aorta) were dissected and imaged under an IVIS Lumina III in vivo imaging system (PerkinElmer, Massachusetts, United States).

Immunofluorescence

For in vivo staining of FXIIa, the vessels were cryo-sectioned and acquired using confocal microscopy. For ex vivo staining of FXIIa, snap-frozen sections were thawed, fixed in acetone, and permeabilized using 0.2% Triton-X-100 in PBS for 10 minutes at room temperature. The primary antibody, 3F7-AF647 (conjugated in-house, 1:50 dilution; CSL Limited, Australia), was incubated overnight. Sections were washed in PBS before nuclear counterstaining and slides were mounted with an antifade mounting medium (Vector Laboratories, United States). BM4-IgG AF647 (conjugated in-house; CSL Limited, Australia) was used as the isotype control. Nuclear staining was performed using Hoechst stain.

Immunofluorescence Acquisition

All images were acquired using a Nikon A1r confocal microscope with a 405, 488, 561, and 647 nm laser. Postacquisition analysis was performed using ImageJ software.

Microscopy

Quantification of histological and immunohistological stains was performed using Optimas 6.2 Video Pro-32 software. Representative images of stains were captured using an Olympus BX43 or BX50 microscope (Olympus, Tokyo, Japan) with CellSens software using light microscope settings and, in the case of PSR, polarized light settings.

Lipid Concentration in Mouse Sera

For TS animals, blood samples were taken by cardiac puncture at the time of sacrifice, centrifuged at 2,000 g for 15 minutes, then the serum removed and stored at −80°C for later analysis. Total serum cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol, and triglyceride concentrations were measured using a Roche Cobas B101 system.

Mouse Serum Chemokines and Cytokine Analysis

Quantification of the various cytokines in the atherosclerotic mouse serum was performed with a Millipore Mouse Cytokine/Chemokine Magnetic Bead Panel (Catalog No. MCYTMAG-70K-PX32).[7] Briefly, mouse serum samples were diluted in assay buffer, and incubated overnight with the premixed beads. Standards and controls were combined with the supplied serum matrix and incubated using the same parameters. After washing, detected antibodies were allowed to incubate, followed by an additional incubation with streptavidin-PE. Following a final wash, sheath fluid was used to resuspend the beads before analysis on a Luminex 200 TM with xPONENT software.

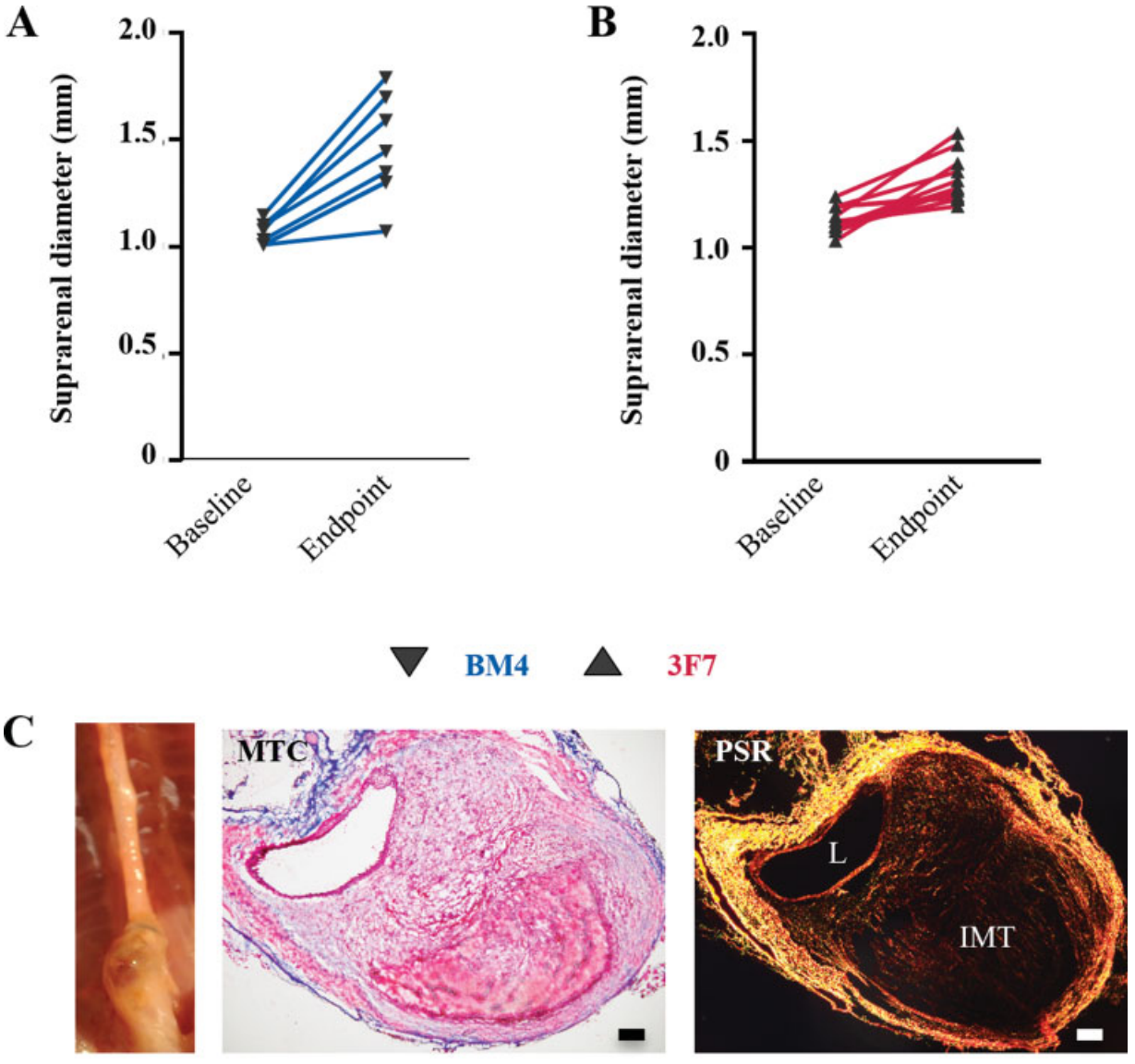
Bradykinin and Breakdown Product Analysis

Four internal standards were used in the assay: (1) [$^2H_5$-D-Phe5]-bradykinin (BK*, Sigma Aldrich) was used as the quantitative internal standard for full-length BK and hydroxyproline bradykinin (HypBK); (2) [$^2H_5$-D-Phe5, $^2H_5$-D-Phe7]-bradykinin (BK**, Sigma Aldrich) was used as the recovery internal standard; (3) [$^2H_5$-D-Phe5]-bradykinin (1–5) (BK_{1-5}*, Sigma Aldrich) was used as the quantitative internal standard for bradykinin fragments and their hydroxylated variants; and (4) Sar-[D-Phe8]-des-Arg9-bradykinin (Sar-BK, Phoenix Pharmaceuticals) was used as the internal standard to check instrument and assay performance. BK* stock solution, BK_{1-5}* stock solution, and Sar-BK stock solution were mixed and diluted to prepare an internal standard 1x stock solution. Blood collected by direct cardiac puncture was immediately transferred to 3x excess of chilled methanol containing internal standard 1x stock solution and thoroughly mixed to effect protein

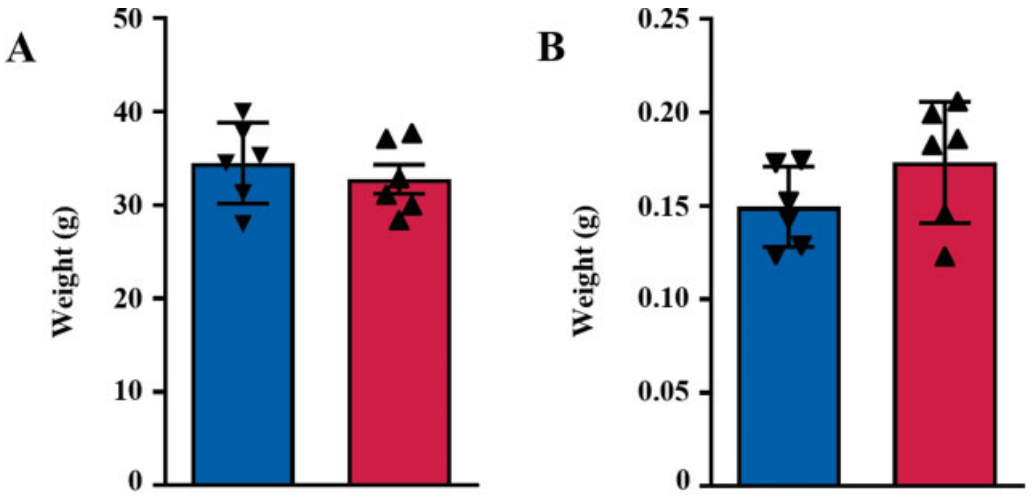
precipitation and BK extraction. The mixture was centrifuged, the blood supernatant was carefully collected, and vacuum dried, reconstituted in internal standard 2x stock solution, centrifuged, and finally transferred to HPLC vials for triplicate liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) measurements. BK** stock solution was diluted to prepare internal standard 2x stock solution. Our LC-MS/MS assay was capable of detecting and quantifying BK and HypBK, as well as BK_{1-5}, $HypBK_{1-5}$, BK_{1-7}, $HypBK_{1-7}$, BK_{1-8}, $HypBK_{1-8}$, BK_{2-9}, and $HypBK_{2-9}$. All LC-MS/MS experiments were performed using a Sciex QTRAP 6500 triple-quadrupole mass spectrometer combined with Agilent 1290 HPLC.

Statistical Analysis

Unless otherwise specified, quantitative data are expressed as mean ± standard deviation. Comparisons of parameters between two groups were made using the unpaired Student's *t*-test after normal data distribution was confirmed. A *p*-value of

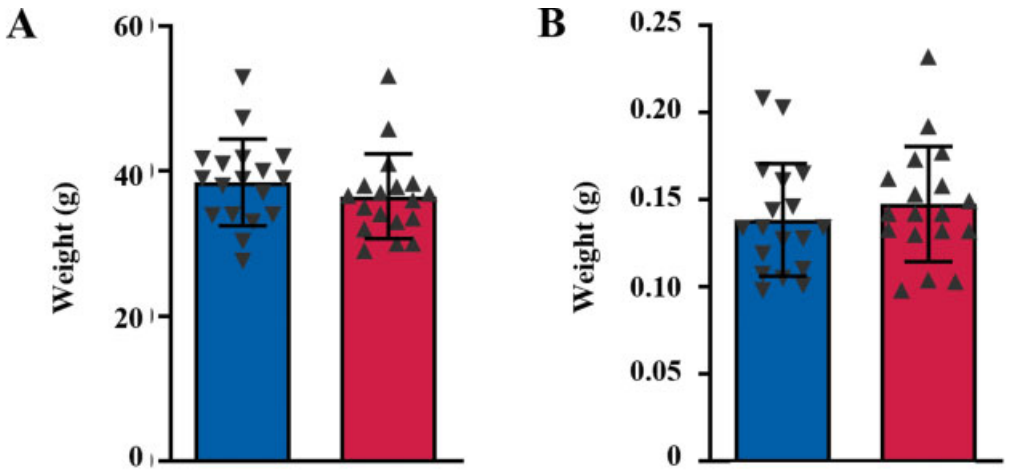


Supplementary Fig. S2 Effect of 3F7 on AAA development and histological examination of a large aneurysm from a BM4-treated mouse. AAA was induced via a constant infusion of AngII over 28 days. The diameter of the abdominal aorta was measured directly above the renal branches at baseline and endpoint using ultrasound. Individual changes in dilatation from baseline to endpoint are plotted for mice receiving (**A**) the control BM4 ($n = 8$) and (**B**) 3F7 ($n = 11$). (**C**) Representative images of MTC and PSR staining of a large aneurysm from a mouse receiving BM4 control. Scale bars = 500 µm. A comparative statistical analysis of BM4- and 3F7-treated mice is provided in ►**Fig. 1D**. AAA, abdominal aortic aneurysm; AngII, angiotensin II; L, lumen; MTC, Masson's trichrome; PSR, Picro-Sirius Red.



	BM4	3F7
Total cholesterol (mmol/L)	21.92 (±4.80)	19.47 (±3.61)
LDL cholesterol (mmol/L)	22.23 (±4.49)	20.80 (±3.92)
Triglycerides (mmol/L)	1.48 (±0.46)	1.0 (±0.28)
HDL cholesterol (mmol/L)	4.85 (±0.58)	5.19 (±1.09)

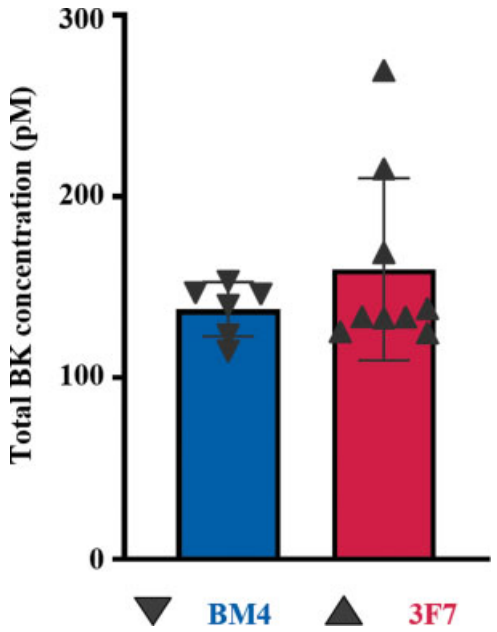
Supplementary Fig. S3 Body and spleen weights, and serum lipid measurements for the ApoE$^{-/-}$ atherosclerosis mouse model. ApoE$^{-/-}$ mice were fed a HFD for 8 weeks while receiving therapeutic 3F7 or BM4 control antibodies. At endpoint, (**A**) the body weights and (**B**) the spleen weights of all animals were recorded (BM4 $n = 6$; 3F7 $n = 6$). (**C**) Serum cholesterol, LDL cholesterol, triglycerides, and HDL were measured (BM4 $n = 6$; 3F7 $n = 6$). Values are mean ± SD. All assays were assessed using unpaired Student's *t*-tests. HDL, high-density lipoprotein; HFD, high-fat diet; LDL, low-density lipoprotein; SD, standard deviation.



C ▼ BM4 ▲ 3F7

	BM4	3F7
Total cholesterol (mmol/L)	28.93 (±6.23)	24.37 (±8.67)
LDL cholesterol (mmol/L)	28.17 (±5.10)	23.91 (±8.13)
Triglycerides (mmol/L)	1.89 (±0.58)	1.76 (±0.85)
HDL cholesterol (mmol/L)	4.48 (±0.53)	4.28 (±0.40)

Supplementary Fig. S4 Endpoint body and spleen weights, and serum lipid measurements for the TS-vulnerable plaque mouse model. ApoE$^{-/-}$ mice were fed an HFD for 6 weeks prior to TS surgery. Following surgery, animals remained on an HFD for 7 weeks and therapeutic 3F7 or BM4 control was administered. At endpoint, (**A**) the body weights and (**B**) the spleen weights of all animals were recorded (BM4 $n = 18$; 3F7 $n = 18$). (**C**) Serum cholesterol, LDL cholesterol, triglycerides, and HDL were measured (BM4 $n = 17$; 3F7 $n = 16$). Values are mean ± SD. All assays were assessed using unpaired Student's *t*-tests. HDL, high-density lipoprotein; HFD, high-fat diet; LDL, low-density lipoprotein; SD, standard deviation; TS, tandem stenosis.



Supplementary Fig. S5 Endpoint bradykinin levels remain unaltered after treatment with 3F7. ApoE$^{-/-}$ mice were fed an HFD for 6 weeks prior to TS surgery and remained on an HFD for 7 weeks following surgery. During the final 7 weeks, animals received either 3F7 or BM4. At the end of this period, circulating BK levels were assessed using LC-MS/MS (BM4 $n = 6$; 3F7 $n = 9$). Values are mean ± SD. The unpaired Student's *t*-test was used for statistical comparison between BM4 and 3F7-treated mice. BK, bradykinin; HFD, high-fat diet; LC-MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; SD, standard deviation; TS, tandem stenosis.

References

1 Wang X, Searle AK, Hohmann JD, et al. Dual-targeted theranostic delivery of miRs arrests abdominal aortic aneurysm development. Mol Ther 2018;26(04):1056–1065
2 Larsson M, Rayzman V, Nolte MW, et al. A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk. Sci Transl Med 2014;6 (222):222ra17
3 Chen Y-C, Bui AV, Diesch J, et al. A novel mouse model of atherosclerotic plaque instability for drug testing and mechanistic/therapeutic discoveries using gene and microRNA expression profiling. Circ Res 2013;113(03):252–265
4 Chen YC, Rivera J, Peter K. Tandem stenosis to induce atherosclerotic plaque instability in the mouse. Methods Mol Biol 2015; 1339:333–338
5 Koay YC, Chen Y-C, Wali JA, et al. Plasma levels of TMAO can be increased with "healthy" and "unhealthy" diets and do not correlate with the extent of atherosclerosis but with plaque instability. Cardiovasc Res 2020;117(02):435–449
6 Kyaw T, Tay C, Khan A, et al. Conventional B2 B cell depletion ameliorates whereas its adoptive transfer aggravates atherosclerosis. J Immunol 2010;185(07):4410–4419
7 Pusic K, Aguilar Z, McLoughlin J, et al. Iron oxide nanoparticles as a clinically acceptable delivery platform for a recombinant blood-stage human malaria vaccine. FASEB J 2013;27(03):1153–1166

