Pharmacological inhibition of Factor XIIa attenuates abdominal aortic aneurysm, reduces atherosclerosis, and stabilizes atherosclerotic plaques

Amy K Searle, Yung Chih Chen, Maria Wallert, James McFadyen, Ana Maluenda, Jonathan Noonan, Peter Kanellakis, Maria T Zaldívar, Angela Huang, Hadi Lioe, Mark Biondo, Marc W Nolte, Paolo Rossato, Alex Bobik, Con Panousis, Xiaowei Wang, Hamid Hosseini, Karlheinz Peter.

Affiliations below.

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Conflict of Interest: LH, MB, and CP are employees of CSL Limited. MWN and PR are employees of CSL Behring Innovation GmbH. Y-CC, CP, HH, MWN, and KP are inventors on patent applications describing antibody-mediated anti-FXIIa therapies.

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Abstract:
Background: 3F7 is a monoclonal antibody targeting the enzymatic pocket of FXIIa, thereby inhibiting its catalytic activity. Given the emerging role of FXIIa in promoting thrombo-inflammation, along with its apparent redundancy for haemostasis, the selective inhibition of FXIIa represents a novel and highly attractive approach targeting pathogenic processes that cause thromboinflammation-driven cardiovascular diseases.

Methods: The effects of FXIIa inhibition were investigated using three distinct mouse models of cardiovascular disease - angiotensin II-induced abdominal aortic aneurysm (AAA), an ApoE-/- model of atherosclerosis, and a tandem stenosis model of atherosclerotic plaque instability. 3F7 or its isotype control, BM4, were administered to mice (10 mg/kg) on alternate days for 4 to 8 weeks, depending on the experimental model. Mice were examined for the development and size of AAAs, or the burden and instability of atherosclerotic plaque and associated markers of inflammation.

Results: Inhibition of FXIIa resulted in a reduced incidence of larger AAAs, with less acute aortic ruptures and an associated fibro-protective phenotype. FXIIa inhibition also decreased stable atherosclerotic plaque burden and achieved plaque stabilization associated with increased deposition of fibrous structures, a >2-fold thicker fibrous cap, increased cap-to-core ratio, and reduction in localized and systemic inflammatory markers.

Conclusions: Inhibition of FXIIa attenuates disease severity across three mouse models of thromboinflammation-driven cardiovascular diseases. Specifically, the FXIIa-inhibiting monoclonal antibody 3F7 reduces AAA severity, inhibits the development of atherosclerosis, and stabilizes vulnerable plaques. Ultimately, clinical trials in patients with cardiovascular diseases such as AAA and atherosclerosis are warranted to demonstrate the therapeutic potential of FXIIa inhibition.

Corresponding Author:
Karlheinz Peter, Baker Heart Research Institute - BHRI, Atherothrombosis and Vascular Biology, PO Box 6492 St Kilda Road Central, 8008 Melbourne, Australia, karlheinz.peter@bakerdi.edu.au

Affiliations:
Amy K Searle, Baker Heart Research Institute - BHRI, Atherothrombosis and Vascular Biology, Melbourne, Australia
Yung Chih Chen, Baker Heart Research Institute - BHRI, Atherothrombosis and Vascular Biology, Melbourne, Australia
Maria Wallert, Baker Heart Research Institute - BHRI, Atherothrombosis and Vascular Biology, Melbourne, Australia
Karlheinz Peter, Baker Heart Research Institute - BHRI, Atherothrombosis and Vascular Biology, Melbourne, Australia

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FXIIa Inhibition in AAA and Atherosclerosis/Searle et al.

Original Article

Pharmacological Inhibition of Factor XIIa Attenuates Abdominal Aortic Aneurysm, Reduces Atherosclerosis, and Stabilizes Atherosclerotic Plaques

Amy K Searle,1,2,* Yung-Chih Chen,1,2,3,* Maria Wallert,1,* James D McFadyen,1,2,3,4 Ana C Maluenda,1 Jonathan Noonan,1,3 Peter Kanellakis,1 Maria TK Zaldivia,1 Angela Huang,1 Hadi
Abstract

Background 3F7 is a monoclonal antibody targeting the enzymatic pocket of FXIIa, thereby inhibiting its catalytic activity. Given the emerging role of FXIIa in promoting thromboinflammation, along with its apparent redundancy for haemostasis, the selective inhibition of FXIIa represents a novel and highly attractive approach targeting pathogenic processes that cause thromboinflammation-driven cardiovascular diseases.

Methods The effects of FXIIa inhibition were investigated using three distinct mouse models of cardiovascular disease - angiotensin II-induced abdominal aortic aneurysm (AAA), an ApoE−/− model of atherosclerosis, and a tandem stenosis model of atherosclerotic plaque instability. 3F7 or its isotype control, BM4, were administered to mice (10 mg/kg) on alternate days for 4 to 8 weeks, depending on the experimental model. Mice were examined for the development and size of AAAs, or the burden and instability of atherosclerosis and associated markers of inflammation.

Results Inhibition of FXIIa resulted in a reduced incidence of larger AAAs, with less acute aortic ruptures and an associated fibro-protective phenotype. FXIIa inhibition also decreased stable atherosclerotic plaque burden and achieved plaque stabilization associated with increased deposition of fibrous structures, a >2-fold thicker fibrous cap, increased cap-to-core ratio, and reduction in localized and systemic inflammatory markers.
Conclusions Inhibition of FXIIa attenuates disease severity across three mouse models of thromboinflammation-driven cardiovascular diseases. Specifically, the FXIIa-inhibiting monoclonal antibody 3F7 reduces AAA severity, inhibits the development of atherosclerosis, and stabilizes vulnerable plaques. Ultimately, clinical trials in patients with cardiovascular diseases such as AAA and atherosclerosis are warranted to demonstrate the therapeutic potential of FXIIa inhibition.

Keywords

3F7 monoclonal antibody
abdominal aortic aneurysm
atherosclerosis
coagulation factor 12
vulnerable plaque
**INTRODUCTION**

The rupture of an atherosclerotic plaque and formation of an occlusive arterial thrombus are the inciting events leading to myocardial infarction and ischaemic stroke, which represent the leading causes of mortality and morbidity worldwide \(^1\). Atherosclerosis, the leading
pathology that causes these ischaemic complications, is fuelled by chronic inflammatory processes. Whilst the role of the coagulation system initiating pathological thrombus formation upon plaque rupture is well appreciated, there is now a growing body of evidence that the coagulation system also directly contributes to atherosclerosis development, including plaque destabilization. Indeed, multiple coagulation factors have been demonstrated to be present within atherosclerotic plaques. Moreover, active coagulation factors such as tissue factor, thrombin, factor X, and factor XII (FXII) have been shown to be present early on during the development of atherosclerotic plaques. Supporting this notion, it has recently been demonstrated that genetic deficiency of factor XII or XI inhibits atherogenesis in a mouse model of atherosclerosis. These findings support the concept of utilizing anticoagulants as a means to prevent atherosclerosis. However, given that all clinically available anticoagulant therapeutics are associated with an inherent risk of bleeding, newer more advanced strategies avoiding bleeding are required.

Factor XII, the zymogen of the serine protease, FXIIa, is activated by several substances including polyphosphate, vascular collagen, misfolded proteins, and NETs. The activation of FXII initiates the intrinsic coagulation pathway and liberates the formation of bradykinin thus promoting inflammation. Significantly, recent data has demonstrated that FXII-/- mice are afforded protection from atherosclerosis. Interestingly, the protective effects of FXII deficiency appear to occur independently of any systemic effects on coagulation, or bradykinin production, thus suggesting an important proinflammatory role of FXII/XIIa within the confines of the atherosclerotic plaque. In this regard, FXIIa induces the production of proinflammatory, proatherogenic cytokines including, IL-6, IL-1b, IL-12 and TNF-a from bone marrow-derived macrophages. However, to date, the effects of pharmacological FXIIa inhibition as an anti-atherosclerotic approach has not been investigated. FXIIa is a highly attractive therapeutic target given the fully recombinant
human 3F7 monoclonal antibody (mAb), which binds to the catalytic site of activated FXII and potently inhibits both FXIIa and βFXIIa has been demonstrated to inhibit thrombosis without impeding haemostasis in preclinical animal models and an affinity-improved version (CSL312) is currently in phase 3 clinical trials for the prevention of hereditary angioedema (ClinicalTrials.gov Identifier: NCT04656418). Therefore, given its key role in mediating pathological thrombosis and a number of inflammatory processes implicated in the development of atherosclerosis and AAA, we hypothesized that the therapeutic inhibition of FXIIa with the mAb 3F7 represents a novel approach to inhibit atherosclerosis and AAA by treating both, coagulation and inflammation, while preserving haemostasis.

In this study, we demonstrate that inhibition of FXIIa with 3F7 has a protective effect in mouse models of AAA and atherosclerosis. Most importantly using a unique mouse model of atherosclerotic plaque instability, we show that FXIIa inhibition via 3F7 leads to the stabilization of vulnerable, unstable atherosclerotic plaques. Taken together, these data indicate that FXIIa inhibition suppresses thrombo-inflammation and thereby exerts beneficial effects in vascular diseases, such as AAA and atherosclerosis.

METHODS

Mouse experiments and 3F7 administration

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 NIH 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.

Briefly, mouse models of AAA, stable, and unstable atherosclerosis were employed (Supplementary Figure 1). For the investigation of atherosclerotic plaques, ApoE−/− and TS mouse models were employed while an angiotensin II (AngII)-infusion mouse model was used for the AAA study. Animals were randomly assigned to receive either 3F7 or its isotype control BM4 every second day via intraperitoneal (i.p) injection, with administration beginning directly after surgery or at 8-weeks old for the ApoE−/− study. Both 3F7 and BM4 (MuBM4-MuG1K), were supplied by CSL Limited as murine IgG1 antibodies.

Detailed information on the methods used in this publication can be found in the Supplemental Material.

**Statistical analysis**

Unless otherwise specified, quantitative data are expressed as mean±SD. 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 <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software.

**RESULTS**

**FXIIa is localized within AAAs**

Several negatively charged surfaces which can activate FXII have been described within the AAA microenvironment. Therefore, we postulated that FXIIa is detectable in AAAs.
Using immunofluorescence (IF), we confirmed the presence of FXIIa within AAAs (Figure 1A, B).

**Anti-FXIIa (3F7) administration results in smaller AAAs and a more stable phenotype**

We next investigated the efficacy of the anti-FXIIa mAb 3F7 to prevent the development of AAA. Mice received 3F7 or BM4 isotype control antibody every 48 hours for 28 days following the implantation of an osmotic minipump containing AngII to induce AAA. Although 3F7 did not significantly alter the incidence of aneurysm formation, it did result in less acute aortic ruptures and a higher proportion of small aneurysms on the lower limit of classification (lower-limit aneurysm 1.2–1.4 mm, aneurysm >1.5 mm; BM4 no aneurysm n=1, lower-limit aneurysm n=4, aneurysm n=3, rupture n=3; 3F7 no aneurysm n=1, lower-limit aneurysm n=9, aneurysm n=1, rupture n=1; Figure 1C). Ultimately, inhibition of FXIIa significantly impeded the progressive dilatation of the abdominal aorta throughout the 28-day experimental period, therefore forming smaller aneurysms as compared to the control animals receiving BM4 (Figure 1D; p=0.0072). Individual plots of the baseline and endpoint dilatation measurements can be found in Supplementary Figure 2A & B.

Studies have suggested that the volatility of an AAA and its risk of acute rupture are correlated to the size and reactivity of the aneurysms’ thrombus. The AngII mouse model of AAA is characterized by the development of an intramural thrombus (IMT) in the absence of an intraluminal thrombus. Although FXIIa inhibition did not impede IMT formation (Figure 1E), morphological analysis of those aneurysms containing an IMT demonstrated a more stable phenotype in animals receiving 3F7 vs the control, BM4. Specifically, the IMT of 3F7-treated animals with lower-limit small aneurysms showed thick deposition of collagen between IMTs and the vessel–lumen interface (Figure 1F), which was not seen in either lower-limit aneurysms (Figure 1G) or aneurysms (Supplementary Figure 2C) from the BM4
control group. The formation of this protective layer of collagen may offer a potential mechanism by which inhibiting FXIIa might protect from AAA rupture.

**Inhibition of FXIIa promotes stable atherosclerosis**

Following the observation of reduced dilatation and rupture, and evidence of increased collagen content in AAA, we next explored the potential for 3F7 to promote stable atherosclerosis. We investigated the effect of FXIIa inhibition on the development of atherosclerosis in ApoE/−/− mice fed an HFD. We found that 3F7 decreased total atherosclerotic plaque size and necrotic core area (Figure 2A–B; p=0.0031; 0.0489) and increased total collagen content (Figure 2C; p=0.0169), as compared to those mice receiving isotype control antibody BM4. No significant changes in lipid profile were observed (Supplementary Figure 3). This demonstrates the protective effect of blocking FXIIa, a finding consistent with a previous study demonstrating that genetic deficiency of FXII in FXII/−/−ApoE/−/− double-knockout mice reduced the development of atherosclerosis 6.

**FXIIa is localized within the microenvironment of mouse unstable atherosclerotic plaque**

To assess the efficacy of 3F7 in a more translationally relevant animal model of atherosclerosis, we utilized a TS mouse model, which is characterized by thin-capped, rupture-prone, unstable carotid artery plaques with a phenotype similar to the culprit lesions often responsible for ischaemic events in patients 17. Anatomical information regarding the location of the TS and the predefined area of plaque instability within this model can be found in the Methods and Chen *et al.* 17.

Prior to investigating the therapeutic capacity of 3F7 in the TS mouse model, we confirmed the accumulation of FXIIa within the unstable atherosclerotic plaques. Fluorescence (AF546)-labelled 3F7 was detected inside the unstable atherosclerotic plaques using both IF
(Figure 3A, B) and IVIS (Figure 3C, D). This is an important finding both, in regard to demonstrating a pathological activation of FXII in unstable plaque and also in regard to the suitability of FXIIa targeting as a selective therapeutic approach.

**3F7 stabilizes vulnerable atherosclerotic plaques**

Inhibition of FXIIa via administration of 3F7 began 24 h after TS surgery. We assessed various markers of plaque stability. At the endpoint of this study, there were no differences in the body weight, spleen weight, or serum lipids between animals receiving 3F7 and BM4 (Supplementary Figure 4). 3F7 administration decreased the total plaque area, necrotic core size, and lipid deposition within the vulnerable plaques, as compared to animals receiving BM4 (Figure 4A–C; p=0.0101; 0.0391; 0.0429). Furthermore, there was a marked increase in total collagen deposition within the vulnerable plaques of those animals receiving 3F7, as well as increases in cap thickness and cap-to-core ratio, two important measurements of plaque instability directly translatable to clinical measurements of plaque instability in humans (Figure 4D–F; p=0.0004; 0.0197; 0.0015). This increase in collagen deposition was mirrored by an increase in intimal smooth-muscle cells, another structural element supporting atherosclerotic plaque stability (Figure 4G; p<0.0001). Another important sign of plaque instability is intraplaque haemorrhage. The antibody TER119 specifically binds to the glycophorin A-associated protein (Ly-76) expressed on erythrocytes and so can be used to detect intra-plaque haemorrhage. Mice treated with 3F7 showed markedly reduced staining of TER119 in segment I of the TS model as compared to those mice receiving the isotype control antibody BM4 (Figure 4H; p=0.0029), again strongly indicating the plaque-stabilizing effect of 3F7.
3F7 administration decreases plaque macrophage content and circulating pro-inflammatory markers, as well as increasing markers associated with fibrosis in vulnerable plaques

Dual inhibition of pro-thrombotic and inflammatory pathways is a unique feature of 3F7. The effect of FXIIa inhibition via 3F7 on inflammation was assessed locally using immunohistochemistry and systemically using a mouse cytokine/chemokine multiplex array.

Assessment of localized inflammation showed a marked decrease in VCAM-1 expression following 3F7 treatment (Figure 5A; p=0.0002), which correlated to a reduction in macrophages infiltrating into the plaque (Figure 5B; p≤0.0001). Multiplexing identified significant downregulation of circulating chemoattractants: monocyte chemoattractant protein-1 (MCP-1, CCL2), eotaxin-1 (CCL11), keratinocyte-derived chemokine (KC, CXCL1), and lipopolysaccharide-induced CXC chemokine (LIX, CXCL5), in the plasma of animals treated with 3F7 (Figure 5C; p=0.0133; 0.0350; 0.0493; 0.0421). In addition, circulating pro-inflammatory markers IL-1α, IL-1β, IL-12p40, and IL-17 (Figure 5D; p=0.0335; 0.0499; 0.0485; 0.0347), and stimulating factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) were downregulated (Figure 5E; p=0.0459; 0.0234), while factors associated with wound healing and fibro-protection, IL-4, IL-13, and IL-5, were all significantly upregulated following treatment with 3F7 (Figure 5F; p=0.0491; 0.0410; 0.0055). Interestingly, the level of circulating BK as determined by (LC-MS/MS, Supplementary Figure 5) was not influenced by 3F7 administration.
DISCUSSION

Cardiovascular disease (CVD) remains a leading cause of mortality and morbidity globally. With the rising diabetes and obesity epidemic in the developed world, in addition to the significant risk of recurrent events for patients with CVD, there remains a pressing need for novel drugs for CVD treatment and prevention. Moreover, therapeutic options for patients with AAA remain limited to surgical options, which are often inappropriate for a significant proportion of patients with AAA and are also associated with high mortality rates. Therefore, given this unmet clinical need, non-surgical preventive and treatment options for AAA are required. The availability of therapies that have the potential to inhibit the development of atherosclerosis and to stabilize unstable atherosclerotic plaques, as well as rupture-prone AAAs, would represent a major clinical need. Atherosclerosis and AAA are both characterized by chronic inflammation, with single cell RNA-sequencing studies beginning to highlight the significant diversity of immune cells in both diseases. Similarly, thrombosis is also a central feature of both pathologies. As a result, therapies targeting both inflammation and thrombosis are of key interest in cardiovascular research. Accordingly, recent evidence has highlighted that FXIIa plays an important role in mediating pathological thrombosis, in addition to possessing a number of previously unappreciated roles in regulating inflammation. Therefore, given the importance of the thrombo-inflammatory process in contributing to atherogenesis and AAA development, we investigated the efficacy of FXIIa inhibition as a potential approach for prevention and treatment of atherosclerosis and AAA.

For AAA, there is increasing evidence that the thrombotic burden is associated with the risk of aneurysm rupture. The highly proteolytic microenvironment of the thrombus is proposed to decrease wall strength and thereby increase the risk of aneurysm rupture. In our study, although similar numbers of animals developed aneurysms and IMTs, the 3F7-treated
cohort experienced less acute ruptures and developed smaller aneurysms in comparison to the control mice. Upon histological examination of collagen deposition within AAAs an indicator of structural stability, we observed a thick, fibrous layer of collagen deposited at the IMT–vessel interface in animals treated with 3F7. Recently, a study by Moran et al. 32 investigated the gene and protein profiles of both an ApoE\(^{-}\)FXII\(^{+}\)double-knockout and an ApoE\(^{-}\)3F7-treated mouse model. The authors reported decreases in ADAM-17, endothelial growth factor receptor, and the matrix metalloproteinases MMP-2 and MMP-9 expression 32, correlating to reduced local inflammation and remodelling, as well as to the preservation of extracellular matrix integrity. As the luminal portion of the IMT is the most inflamed area of an aneurysm, containing the highest proportion of pro-inflammatory infiltrating cells 33, the increased deposition of collagen at this interface is an exciting finding and might contribute to the increased rate of survival associated with 3F7 administration.

3F7 significantly attenuated the development of atherosclerosis in ApoE\(^{-}\) mice. Most strikingly, pharmacological inhibition of FXIIa prevented the development of vulnerable atherosclerotic plaques in the TS model of plaque instability. Mice treated with 3F7 exhibited significantly decreased necrotic core size and lipid deposition. In accordance with our AAA data, we also observed marked increases in collagen deposition, cap thickness, and the cap-to-core ratio. These are particularly important findings, since it is well accepted that the extent of crosslinked collagen deposited within the cap region is proportional to the stability of the plaque and increases in cap thickness confer a more stable atherosclerotic phenotype 34. Moreover, inhibition of FXIIa resulted in increases in intimal smooth-muscle cell content and almost eliminated intraplaque haemorrhage, further highlighting the compelling plaque-stabilizing effects of FXIIa inhibition. The remarkable magnitude of this plaque-stabilizing effect achieved by 3F7 can also be seen in comparison to the effects seen with the use of a
myeloperoxidase inhibitor in the tandem stenosis model, which despite reporting on only small plaque-stabilizing effects attracted broad interest\textsuperscript{35}.

Recent experimental data have highlighted the role of FXII/XIIa in regulating inflammation, including direct regulation of neutrophil function and wound healing\textsuperscript{36} in addition to a central role in the pathogenesis of experimental autoimmune encephalomyelitis\textsuperscript{37}. These findings have served to highlight that pharmacological inhibition of FXIIa can achieve significant anti-inflammatory effects. In this regard, our findings that 3F7 administration significantly decreases VCAM-1 expression and CD68\(^+\) macrophage infiltration point to a potential role of FXIIa inhibition as an anti-inflammatory strategy for the prevention and potential treatment of atherosclerosis.

This concept is further supported by the fact that we observed downregulation of circulating cytokines IL-1\(\beta\), IL-12p40, and MCP-1. These cytokines and chemoattractants play important roles in mediating monocyte/macrophage recruitment and differentiation into pro-inflammatory M1 macrophages, and, specifically for IL-1\(\beta\), foam cell apoptosis\textsuperscript{38}, and likely help explain the reduction in macrophage infiltration and overall disease attenuation. While the present study did not specifically examine the downstream signalling effects of FXIIa, our data are consistent with a recent report demonstrating that genetic deficiency of FXII afforded ApoE\(^{-/-}\) mice protection from atherosclerosis\textsuperscript{6}. Here, FXIIa was shown to stimulate the secretion of pro-inflammatory cytokines, including IL-1\(\beta\) and IL-12, from bone marrow-derived macrophages and antigen-presenting cells, which likely explains the beneficial anti-inflammatory effects of FXIIa inhibition. It is important to note that another important function of FXIIa relates to its ability to activate the KKS to yield the pro-inflammatory oligopeptide BK. However, we found no detectable differences in plasma BK levels between 3F7 and BM4-treated mice, suggesting that the pro-inflammatory effects of FXIIa in the
context of atherosclerosis are either largely localized to the site of the lesion and any BK generated not detectable systemically, or independent of the KKS.

An outstanding issue pertaining to the role of FXII in mediating atherosclerosis relates to what activates FXII within the confines of an atherosclerotic plaque. While this was not a focus of our current study, it is likely that the activation of FXII in atherosclerotic plaques is linked to the multitude of physiological FXII activators that have been previously demonstrated to be abundant within atherosclerotic plaques. Indeed, extracellular traps, misfolded protein aggregates, and activated platelets have all been previously demonstrated to be present within plaques and are well-described physiological activators of FXII.

Further emphasizing the central role of FXII in mediating atherothrombosis, deficiency of FXII or FXIIa inhibition has been demonstrated to diminish thrombus formation on atherosclerotic plaque material ex vivo. Moreover, a recent study investigating pharmacological inhibition of FXI not only implicated FXI/FXIa in the development of atherosclerosis and thereby indirectly supporting the pathological role of FXIIa within the plaque confines, but also provides further evidence that therapeutic targeting of coagulation factors holds immense potential to impact cardiovascular clinical outcomes.

The tandem stenosis mouse model representing unstable plaques as seen in patients is a unique preclinical tool both to develop diagnostic approaches for the detection of unstable plaques as well as for the development and testing of drugs for plaque stabilizing effects. For the latter, the promising effects of FXIIa inhibition hold great promise for clinical translation, particularly as first clinical applications, utilizing the anti-inflammatory and antithrombotic effects of FXIIa inhibition are currently trialled. However, our data also indicate FXIIa targeting can be used for diagnostic approaches. Anti-FXIIa imaging was shown to be a potential means to identify unstable atherosclerotic plaques, which is a long
sought-after diagnostic approach with the strong translational perspective of identifying patients at risk and ultimately preventing myocardial infarctions.

Together, our data demonstrate that inhibition of FXIIa impedes the development of atherosclerosis and stabilizes vulnerable atherosclerotic plaques, in addition to preventing AAA rupture in a process linked to its anti-inflammatory and stabilizing effects. These data, coupled with the anti-thrombotic benefits of FXIIa inhibition without interference with normal haemostasis, identify FXIIa inhibition as a potential novel preventative and therapeutic strategy for unstable atherosclerosis and AAA.

However, our study does have some limitations. Whilst we show for the first time that the specific pharmacological targeting of FXIIa with 3F7 attenuated disease severity, any contributions of zymogen FXII to the observed pathology cannot be ascertained given the specificity of 3F7 for activated forms of FXIIa. As zymogen FXII is reported to directly influence innate immune functions and exerts mitogenic activity in endothelial and smooth muscle cells 14, further studies are required to dissect the contributions of zymogen and activated forms of FXII. In addition, we used only male mice for our investigations which, although these diseases disproportionally affect males, may have led us to miss potential sex-specific effects of FXIIa inhibition. Follow-up studies will need to include female mice to exclude sex differences. Finally, our experimental models are designed to test for prophylactic benefits of 3F7 in developing AAA and atherosclerosis. Further investigations are required to establish the efficacy of 3F7 as a truly therapeutic approach. This includes ascertaining the optimal dose and administration interval.

CONCLUSIONS
We demonstrate that 3F7 administration results in the stabilization of both AAAs and vulnerable atherosclerotic plaques. 3F7 restricts the development of large aneurysms and results in a lower incidence of acute aortic rupture, potentially a result of the demonstrated increased collagen deposition between the IMT and the vessel lumen. In unstable atherosclerosis, we observed less unstable plaque development with 3F7 administration, including increased collagen and smooth-muscle cell density. This finding was accompanied by significant reductions in both systemic and local pro-inflammatory markers. The ability of 3F7 to prevent pathological thrombosis and reduce inflammation without impeding haemostasis is an additional unique and supportive feature of this potential therapeutic approach. Our preclinical data indicates that FXIIa inhibition has the potential to stabilize and prevent the rupture of AAAs and of vulnerable atherosclerotic plaques. Clinical trials are warranted to demonstrate the translatability of our preclinical data to clinically meaningful benefits in patients with AAA and atherosclerosis.

**What is known about the topic?**

- Coronary atherosclerosis and abdominal aortic aneurysm and their resulting complications of myocardial infarction and aortic rupture, respectively, are globally dominant causes of mortality and morbidity.
- Medical therapies that can prevent and stabilize vulnerable atherosclerotic plaques, and rupture prone AAAs are highly sought-after.
- Activated coagulation factor XII (FXIIa) sits at the interface of both coagulation and inflammation.

**What does this paper add?**

- Inhibition of FXIIa via a monoclonal antibody reduces the size and increases the stability of atherosclerotic plaques and abdominal aortic aneurysms.
- FXII inhibition via 3F7 decreases markers of local and systemic inflammation in a mouse model of vulnerable plaque.
- 3F7 mAb and its derivatives warrant further testing as potential drug candidates for the prevention of myocardial infarction and the development and rupture of
AUTHOR CONTRIBUTION STATEMENT

Y-CC, CP, XW, HH, and KP designed the study. AKS, Y-CC, MW, JDM, ACM, JN, PK, MTKZ, AH, HL, MB, MWN, PR, XW, and HH were involved in the acquisition and interpretation or analysis of data. AKS drafted the manuscript. Y-CC, MW, JDM, JN, AB, CP, XW, and KP provided critical review of the intellectual content of the manuscript. All authors approved the final version of the manuscript prior to submission.

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CONFLICT OF INTEREST STATEMENT

LH, MB and CP are employees of CSL Limited. MWN and PR are employees of CSL Behring Innovation GmbH. Y-CC, CP, MWN, HH, and KP are inventors on patent applications describing antibody-mediated anti-FXIIa therapies.

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FIGURE LEGENDS

Figure 1: Presence of FXIIa in AAA and inhibition of FXIIa decreases severity and increases stability of AAA. A) Representative IF images showing FXIIa (3F7-staining) deposition within the AAA. B) BM4 was used as isotype control. The vessel lumen is marked by interrupted lines. ApoE<sup>−/−</sup> mice were infused with Angiotensin-II via an implanted osmotic minipump and then treated with 3F7 or the control BM4. Ultrasound measurements of the dilatation of the abdominal aorta were taken. C) Percentages of ruptured, large, small, and no aneurysm formation (BM4 n=11; 3F7 n=12). D) Ratio change (endpoint/baseline) for mice that developed AAA and survived, i.e. did not experience rupture (p=0.0072; BM4 n=7; 3F7 n=10). E) Table shows the incidence of IMT formation, determined by morphological assessment or necropsy (BM4 n=10; 3F7 n=11). Representative images show the morphological analysis of collagen deposition using MTC and PSR for: F) lower-limit aneurysms from 3F7-treated animals (n=5) and G) a lower-limit aneurysm from BM4 control animals (n=1). Scale bars = 200 µm. AAA = abdominal aortic aneurysm; FXII = factor 12; FXIIa = activated factor 12; IMT = intraluminal thrombosis; L = lumen; MTC = Masson’s trichrome; PSR = Picro-Sirius Red. The unpaired Student’s t-test was used for comparison of BM4 and 3F7-treated mice.

Figure 2: FXIIa inhibition attenuated the development of stable atherosclerosis. ApoE<sup>−/−</sup> mice were placed on an HFD for 8 weeks to develop stable atherosclerosis. A) H&E was used to morphologically assess plaque size (p=0.0031), B) necrotic core area (p=0.0489) and C) the collagen content (p=0.0169) in stable plaques of the aortic sinus, as compared to the BM4 control–treated animals (BM4 n=6; 3F7 n=5). Values are mean±SD. Scale bars = 200 µm. Interrupted lines indicate total lesion area used for collagen content analysis.
Representative images show one valve of the aortic sinus only. All valves were included in the analysis. H&E = haematoxylin and eosin; HFD = high-fat diet. Assays were assessed using unpaired Student’s t-tests.

Figure 3: FXIIa accumulates abundantly in unstable atherosclerotic plaques as compared to stable plaques. TS mice were injected with fluorescently labelled (AF546) 3F7 or BM4 and the presence of FXIIa was detected in unstable and stable atherosclerotic plaques using IF microscopy and IVIS. Representative IF images show: A) the presence of FXIIa detected by *in vivo* labelling within the unstable plaque of a TS mouse; B) a section of unstable plaque from an animal injected with BM4 isotype control. The atherosclerotic vessels, both stable (segment V & TA) and unstable plaques (segment I), as well as healthy vessels (segment IV), were excised and imaged using IVIS (left) and brightfield (right). C) Segment I shows strong accumulation of fluorescently labelled 3F7 but not the isotype control fluorescence antibody. Areas of stable atherosclerosis also reveal some FXIIa binding, but to a lesser extent. FXIIa = activated factor 12; IVIS = *in vivo* imaging system; TA = thoracic aorta; TS = tandem stenosis.

Figure 4: Inhibition of FXIIa via antibody 3F7 stabilizes vulnerable atherosclerotic plaques. ApoE<sup>-/-</sup> mice were placed on an HFD for 6 weeks prior to TS surgery. Following surgery, animals remained on the HFD for 7 weeks and 3F7 or BM4 isotype control were administered. A) H&E was used to morphologically assess plaque size (p=0.0101; BM4 n=16; 3F7 n=17) and B) necrotic core area (p=0.0391; BM4 n=14; 3F7 n=19) in unstable plaques, as compared to the BM4-treated animals. C) Oil-red O staining was used to assess the lipid deposition within the plaques (p=0.0429; BM4 n=15; 3F7 n=18). Picro-Sirius Red was used to assess the degree of protective collagen deposition within the plaques, with
histological examination performed using BF and polarized light. D) The total intimal collagen deposition, E) fibrous cap thickness and F) cap-to-core ratio, as compared to BM4-treated animals (p-values = 0.0004; 0.0197; 0.0015; BM4 n=16; 3F7 n=20). G) Intimal smooth-muscle cell deposition (p=<0.0001; BM4 n=12; 3F7 n=18) and H) intra-plaque haemorrhage, as indicated by erythrocyte/TER119 staining (p=0.0029; BM4 n=12; 3F7 n=11). Isotype refers to the isotype control for the antibody used in the respective immunohistochemical stain. Values are mean±SD. Scale bars = 200 µm. A-SM = alpha smooth muscle; BF = brightfield; FXII = factor 12; FXIIa = activated factor 12; H&E = haematoxylin and eosin; HFD = high-fat diet. Assays were assessed using unpaired Student’s t-tests.

Figure 5: Inhibition of FXIIa via 3F7 reduces plaque-localized and systemic inflammation. Immunohistochemistry of unstable plaques shows: A) reduction in VCAM-1 expression and B) CD68+ macrophage infiltration by 3F7 administration (p=0.002; <0.0001; BM4 n=15–18; 3F7 n=16). Isotype refers to the isotype control for the antibody used in the respective immunohistochemical stain. In addition, further insights into the degree of inflammatory markers circulating in the sera were obtained: C) The chemoattractants MCP-1 (CCL2), eotaxin-1 (CCL11), KC (CXCL1), and LIX (CXCL5) are significantly reduced (p-values = 0.0133; 0.0350; 0.0493; 0.0421), D) the pro-inflammatory markers IL-1α, IL-1β, IL-12p40 and IL-17 are significantly reduced (p-values = 0.0335; 0.0499; 0.0485; 0.0347), E) the stimulating factors GM-CSF and M-CSF (p-values = 0.0459; 0.0234) and F) the factors associated with wound healing and fibrosis, IL-4, IL-13, and IL-5 (p-values = 0.0491; 0.0410; 0.0055), are also significantly reduced (BM4 n=12–24; 3F7 n=17–24). Values are mean±SD. Scale bars = 200 µm. FXIIa = activated factor 12; GM-CSF = granulocyte-macrophage colony-stimulating factor; KC = keratinocyte-derived chemokine; LIX = LPS-
induced CXC chemokine; MCP-1 = monocyte chemoattractant protein-1; M-CSF = macrophage colony-stimulating factor. Assays were assessed using unpaired Student’s t-tests.
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 NIH 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 AAA, stable, and unstable atherosclerosis were employed (Supplementary Figure 1). 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 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 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 osmotic mini-pump delivering a constant infusion of AngII (1000 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 intraperitoneally (i.p.) every second day. Single-dose pharmacokinetic and pharmacodynamic studies with 3F7 at 10mg/kg in mice, including 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 6–8-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 post-surgery.

Various anatomical sections of the aortic tree are referred to by Roman numbers from I–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 22–55 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 1.5–2% 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 were embedded in optimal cutting temperature compound (OCT, Sakura Finetechanical), 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 cryo-processing**

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 haematoxylin 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. For each
mouse, total intimal plaque size was measured in 4 sections across 480 μm of the aortic sinus area and the quantification was achieved by averaging those sections. 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 (MTC) to investigate collagen deposition. For each mouse, two sections taken at an interval of approximately 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, USA), alpha-smooth muscle actin (clone 1A4; 1:100 dilution; Sigma Aldrich, USA), CD68 (clone FA-11, 1:100 dilution; AbD Serotec, UK), or TER-119 (clone TER-119, 1:400 dilution; eBioscience, USA). Detection was achieved using a Vectastain ABC kit and DAB substrate (Vector Laboratories, USA). Isotype control antibodies were used for validation of each immunostaining (rat IgG, ThermoFisher Scientific, USA; rat IgG2b kappa, eBioscience, USA; rabbit IgG, Vector Laboratories, USA).

**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 labelling kit (ThermoFisher, Massachusetts, USA) following the commercial SOP. The success of the labelling 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 min prior to animal sacrifice. Vessel segments (segment I, segment IV, segment V, and thoracic aorta (TA)) were dissected and imaged under an IVIS Lumina III in vivo imaging system (PerkinElmer, Massachusetts, USA).
**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 min at room temperature (RT). 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, USA). 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. Post-acquisition analysis was performed using ImageJ software.

**Microscopy**

Quantification of histological and immunohistological stains were 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 2000 g for 15 min, then the serum removed and stored at −80 °C for later analysis. Total serum cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) 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). 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, detection 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: (i) [\(^{2}\text{H}_{5}\)-D-Phe5]-bradykinin (BK*, Sigma Aldrich) was used as the quantitative internal standard for full-length BK and hydroxyproline bradykinin (HypBK); (ii) [\(^{2}\text{H}_{5}\)-D-Phe5, \(^{2}\text{H}_{5}\)-D-Phe7]-bradykinin (BK**, Sigma Aldrich) was used as the recovery internal standard; (iii) [\(^{2}\text{H}_{5}\)-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 (iv) Sar-[D-Phe\(^{8}\)]-des-Arg\(^{9}\)-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\textsubscript{1-5}, HypBK\textsubscript{1-5}, BK\textsubscript{1-7}, HypBK\textsubscript{1-7}, BK\textsubscript{1-8}, HypBK\textsubscript{1-8}, BK\textsubscript{2-9}, and HypBK\textsubscript{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±SD. 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 <0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software.

**SUPPLEMENTARY FIGURES**

**Supplementary Figure 1: Overview of the animal models used.** Three mouse models were investigated: A) the AngII-induced mouse model of AAA, B) the ApoE\textsuperscript{-/-} mouse model of stable atherosclerosis and C) the TS mouse model of vulnerable/unstable atherosclerosis. AngII = angiotensin II; i.p. = intraperitoneal; TS = tandem stenosis.

**Supplementary Figure 2: 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. AAA = abdominal aortic aneurysm; AngII = angiotensin II; L = lumen; MTC = Masson’s trichrome; PSR = Picro-Sirius Red. A comparative statistical analysis of BM4 and 3F7 treated mice is provided in Figure 1D.
Supplementary Figure 3: Body and spleen weights, and serum lipid measurements for the ApoE<sup>−/−</sup> atherosclerosis mouse model. ApoE<sup>−/−</sup> mice were fed a HFD for 8 weeks whilst 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. HDL = high-density lipoprotein; HFD = high-fat diet; LDL = low-density lipoprotein. All assays were assessed using unpaired Student’s t-tests.

Supplementary Figure 4: Endpoint body and spleen weights, and serum lipid measurements for the TS vulnerable plaque mouse model. ApoE<sup>−/−</sup> 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 were 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. HDL = high-density lipoprotein; HFD = high-fat diet; LDL = low-density lipoprotein; TS = tandem stenosis. All assays were assessed using unpaired Student’s t-tests.

Supplementary Figure 5: Endpoint bradykinin levels remain unaltered after treatment with 3F7. ApoE<sup>−/−</sup> 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. BK = bradykinin; HFD = high-fat diet; LC-MS/MS = liquid chromatography–mass spectrometry/mass spectrometry; TS = tandem stenosis. The unpaired Student’s t-test was used for statistical comparison between BM4 and 3F7-treated mice.

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Figure 5

A

**YCAI-1 area** (% of total lesion area)

B

***CD68 area** (% of total lesion area)

C

MCP-1 (CCL2, pg/mL)  
Eotaxin-1 (CCL11, pg/mL)  
KC (CXCL1, pg/mL)  
LIN (CXCL5, pg/mL)

D

IL-1α (pg/mL)  
IL-1β (pg/mL)  
IL-12p40 (pg/mL)  
IL-17 (pg/mL)

E

GM-CSF (pg/mL)  
M-CSF (pg/mL)

F

IL-4 (pg/mL)  
IL-13 (pg/mL)  
IL-5 (pg/mL)

BM4  
3F7

* p < 0.05  
** p < 0.01  
*** p < 0.001
Supplementary Figure 1

A

Baseline Ultrasound
Minipump insertion

Male ApoE<sup>−/−</sup> mice
20 weeks old

1000 ng/kg/min AngII infusion
10 mg/kg 3F7 or BM4 injected i.p. every other day

Normal chow diet

4 weeks experimental period

Euthanasia and tissue collection

B

Male ApoE<sup>−/−</sup> mice
8 weeks old

10 mg/kg 3F7 or BM4 injected i.p. every other day

High fat diet

8 weeks experimental period

Euthanasia and tissue collection

C

T/S surgery

High fat diet

6 weeks

10 mg/kg 3F7 or BM4 injected i.p. every other day

High fat diet

7 weeks experimental period

Euthanasia and tissue collection
Supplementary Figure 2

A

B

C

Suprarenal diameter (mm)

Baseline

Endpoint

BM4

3F7

MTC

PSR

L

IMT
Supplementary Figure 3

A

B

C

BM4  ▼

3F7  ▲

|                | 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 Figure 4

|                      | 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 Figure 5

Total BK concentration (pM)

- BM4
- 3F7