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Pharmacological inhibition of BLT1 diminishes early abdominal aneurysm formation

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Abstract

Leukotriene B_4 (LTB_4) is a pro-inflammatory lipid mediator generated by the enzymes 5-lipoxygenase (5-LO) and LTA_4-hydrolase. LTB_4 signals primarily through its G protein-coupled receptor BLT1, which is highly expressed on specific leukocyte subsets. Recent genetic studies in humans as well as knockout studies in mice have implicated the leukotriene synthesis pathway in several vascular pathologies. Here we tested the hypothesis that pharmacological inhibition of BLT1 diminishes abdominal aortic aneurysm (AAA) formation, a major complication associated with atherosclerotic vascular disease. Chow-fed *Apoe*$^{-/-}$ mice were treated with a 4 week infusion of Angiotensin II (AngII, 1000 ng/kg/min) beginning at 10 weeks of age, in a well-established murine AAA model. Administration of the selective BLT1 antagonist CP-105,696 beginning simultaneously with AngII infusion reduced the incidence of AAA formation from 82% to 40% ($p<0.05$). There was a concordant reduction in maximal aortic diameter from 2.35 mm to 1.56 mm ($p<0.05$). While administration of the antagonist on day 14 after the onset of AngII infusion diminished lesional macrophage accumulation, it did not significantly alter the size of AAA by day 42. Thus, pharmacological inhibition of BLT1 may ultimately hold clinical promise, but early intervention may be critical.

Introduction

A mounting body of correlative evidence has linked the leukotriene synthesis pathway to several chronic inflammatory conditions, including atherosclerosis. 5-LO and its derivatives are highly expressed within human carotid, aortic, and coronary artery plaques[1-3]. Furthermore, genetic studies have associated particular variants of 5-LO, its accessory protein FLAP, as well as the enzyme LTA_4-hydrolase with stroke and myocardial infarction in humans[4-6].

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LTB_4 is an eicosanoid lipid mediator generated downstream of 5-LO and LTA_4-hydrolase. It is known to activate multiple leukocyte subsets leading to cell recruitment, production of reactive oxygen species, and induction of gene expression. LTB_4 signals primarily through its high-affinity G protein-coupled receptor (GPCR) BLT1, which is highly expressed in specific subsets of circulating peripheral blood leukocytes, as well as on non-leukocytes including endothelial cells and smooth muscle cells[7-9]. A low-affinity receptor, BLT2, is also ubiquitously expressed at low levels in many human tissues[10, 11].

Functional studies in murine models have begun to address the role of bioactive lipid products in atherogenesis and related vascular complications. Two groups have demonstrated as much as a two-fold reduction in atherosclerotic plaques in *Apoe*$^{-/-}$/*Blt1*$^{-/-}$ compound-deficient mice as compared to *Apoe*$^{-/-}$ controls[7, 12]. Animal studies have also addressed the role of the leukotriene pathway in abdominal aortic aneurysm (AAA) formation[13]. In contrast to luminal plaques, AAAs are characterized by pathological changes in the aortic wall that lead to segmental weakening, progressive dilation, and spontaneous rupture[14]. Chronic inflammation of the aortic wall and proteinase-mediated degradation of structural matrix proteins are believed to contribute to the development of AAA[15], though the precise molecular mechanisms that underlie vessel wall changes remain poorly understood. Zhao et al. demonstrated that 5-LO plays a striking role in aortic aneurysm formation in a cholate-induced model[16], though a recent study by the same group using a pharmacological inhibitor of 5-LO had a non-significant effect on AngII-induced AAA[17]. Our group has demonstrated that *Apoe*$^{-/-}$/*Blt1*$^{-/-}$ mice have decreased aneurysm formation in an Angiotensin II-triggered model of murine AAA formation[13]. Thus, interventions downstream of 5-LO, specifically isolating the LTB_4-BLT1 axis, may have more consistent effects on this pathology.

However, studies performed in mice in which there is complete BLT1 deletion may be limited by developmental confounders. Pharmacological blockade of the receptor represents an important tool to test the role of BLT1-mediated signaling in the adult animal. Such studies are of potential clinical importance, as there are currently no pharmacological interventions available for AAA. Here we used a specific BLT1 antagonist to test whether blockade of the BLT1 receptor in adult animals can modulate the development of AAA, and whether the disease can be reversed once it is established.

Materials and methods

Mice

Male *Apoe*$^{-/-}$ mice were purchased from Jackson Laboratory, Bar Harbor, ME and fed a chow diet (Prolab). All procedures were approved by Massachusetts General Hospital Center for Comparative Medicine.

Ang II Infusion

Male *Apoe*$^{-/-}$ mice fed a standard chow diet were treated with AngII (1000 ng/kg/min, Sigma Chemical Co., St. Louis, MO) via 4 week Alzet osmotic pumps (Model 2004; Durect Corporation, Cupertino, CA) beginning at 10 weeks of age as previously described[14]. Implanted AngII pumps remained in place through subsequent treatment with the inhibitor or vehicle, as indicated.

CP-105,696 compound treatment

The BLT1 inhibitor CP-105,696 was provided by Pfizer[18, 19]. 100 mg/kg/day of the BLT1 inhibitor was dissolved in 10% ethanol, 0.5% methylcellulose, and 0.5% Tween 80. The CP-105,696 compound was administered daily via gavage concomitant with AngII infusion

for 4 weeks in the inhibition study. In the reversal study, the BLT1 inhibitor was also co-administered with the AngII infusion, but began at the end of week 2/start of week 3.

Blood and Plasma Analysis

A total of 1 mL of blood was obtained from experimental mice by right ventricular puncture upon euthanasia. A small aliquot of blood was analyzed for complete blood count (Drew Scientific Group, Oxford, CT), and the remainder was used for enzymatic colorimetric analysis of total cholesterol (Roche Diagnostics, Indianapolis, ID).

Morphometric Analysis

Aortas were dissected from mice, pinned on a black wax board and images were obtained with a Nikon Coolpix camera (Nikon Inc, Melville, NY) attached to an inverted microscope as previously described[13]. Aortic diameter and area were determined by a Java-based image processing program (ImageJ software, US National Institutes of Health). Measurements were conducted by a single trained observer blinded to the genotype and treatment of the mice.

Chemotaxis assays

For splenocyte isolation, spleens from vehicle- and inhibitor-treated mice were dissected out and passed through a 70 μm filter. 25,000 cells were placed on the top of a 96-well modified Boyden chemotaxis chamber (Neuro-Probe). 32 μL of 10-fold serial dilutions of LTB_4 (Cayman Chemical) and SDF-1 (Peprotech) were placed in the bottom wells, separated from the cells by an 8-μm-pore, polyvinylpyrrolidone-free, polycarbonate filter. Following incubation at 37°C in 5% CO_2 for 90 minutes, cells that had migrated into the lower chamber were manually counted using a Nikon microscope with a 10× objective.

Ultrasound analysis of mouse aortas

Mice were anesthetized with ketamine/xylazine and laid supine. Aortic imaging was performed using a RMV 704 scanhead with a center frequency of 40MHz (Vevo 770, VisualSonics, Toronto, Canada) as previously described[20, 21]. The transducer was first oriented parallel to the aorta and the vessel was scanned from the femoral bifurcation to the aortic arch. The renal arteries were identified and long axis images of the infra- and suprarenal aorta were acquired. The transducer was then placed perpendicularly to the aorta and multiple short axis images were acquired at all levels of the aorta. Maximal aortic suprarenal diameter was measured on the short axis view two weeks after AngII infusion, and at 2 and 6 weeks after the start of BLT1 drug inhibitor.

Immunohistochemistry

Suprarenal aortas were embedded in OCT and serial 10 μm sections were cut through the aneurysmal aorta. Immunohistochemistry was performed with an antibody to identify macrophages (Mac-3, BD Biosciences) as previously described[13, 22]. Gelatinase-A (MMP-2) antibody was kindly provided by Alan Daugherty, University of Kentucky. Negative controls were prepared by substitution with an isotype-matched control antibody or non-immune sera. Quantitative analysis was performed with NIS-Elements AR 2.30 software (Melville, NY). Area of positive staining for Mac-3 and MMP-2 antibody was divided by total lesion area to account for variability in AAA size.

Statistical analysis

Data were analyzed with SigmaStat (Systat software) using Student's *t*-test and one- or two-way ANOVA as indicated. AAA incidence was analyzed by Fisher's exact test. A probability

value of <0.05 was considered statistically significant. All data are reported as mean ± SEM as indicated.

Results

Functional inhibition of BLT1 by CP-105,696

We first established the lowest dose of the drug necessary to functionally inhibit the BLT1 receptor. Of note, prior studies have suggested that doses of CP-105,696 compound greater than 10 mg/kg/day in mammals would ensure plasma levels of at least 5-6 ug/ml, which are necessary to inhibit leukocyte activation[19, 23]. Following the establishment of dose-response curves of splenocyte chemotaxis to SDF-1 and LTB_4 (Fig. 1A, B), we performed chemotaxis experiments on splenocytes isolated from mice that had been treated with the well-described BLT1 inhibitor, CP-105,696[18, 19] across a spectrum of doses (0, 10, 100, and 200 mg/kg/day) for 7 days (Fig. 1 C, D, E, F). As expected, LTB_4 induced robust chemotaxis of splenocytes from untreated mice in a dose-dependent manner, with the maximal effect reached at 100 nM (Fig. 1B). Splenocyte chemotaxis was relatively unaffected by treatment of mice with CP-105,696 at 10 mg/kg/day (Fig. 1D). By contrast, splenocytes from mice treated with 100 or 200 mg/kg/day CP-105,696 had significantly inhibited chemotaxis to LTB_4 (Fig. 1 E, F). We verified the specificity of the antagonist by documenting that chemotaxis to SDF-1, which activates a distinct chemoattractant receptor (CXCR4), was unchanged by administration of CP-105,696. We thus determined that the lowest therapeutic concentration of CP-105,696 to inhibit the LTB_4-BLT1 axis was 100 mg/kg/day (Fig. 1E).

Aneurysm inhibition by CP-105,696

To assess the effect of early inhibition of BLT1 in aneurysm formation, we infused AngII into 10 week-old *Apoe*$^{-/-}$ mice receiving CP-105,696 daily at 100 mg/kg/day (n=10 mice) or vehicle (n=11 mice) concomitant with the onset of AngII infusion. Mice were sacrificed after 4 weeks. After Ang II infusion, drug and vehicle treated mice had similar body weights, cholesterol concentrations, and white blood cell counts, thus excluding several potential confounding variables (Table I, Online only Supplementary Material).

Several metrics were used to quantify aneurysm formation in *Apoe*$^{-/-}$ mice. We first assessed the incidence of aneurysms in both groups, defining an aneurysm as a dilation of the suprarenal aorta greater than 150% of the diameter of the infrarenal aorta, which is entirely spared from enlargement in this model[14]. Consistent with previously reported findings in the *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Blt1*$^{-/-}$ mice[13], the AAA incidence in the CP-105,696-treated group was 40% as compared to 82% in vehicle-treated mice ($p<0.05$). We also assessed the extent of aortic dilation in *Apoe*$^{-/-}$ mice by measuring the maximal suprarenal diameter (Fig. 2B), and found a significant reduction in drug-treated mice compared to the vehicle controls. Representative images from vehicle- and inhibitor-treated mice are shown in Figure 2C. Taken together, these data suggest that pharmacological inhibition of BLT1 abrogates AAA formation in this murine model.

The effect of CP-105,696 in the progression of aortic dilation

To assess the extent to which BLT1 inhibition might ameliorate the course of aortic dilation once established, we infused mice with AngII and followed them with serial examinations using ultrasound (Fig. 3), which can be used to accurately monitor AAA formation in this model[20, 21]. The transducer was oriented in the short axis and sequential images were taken from the infrarenal segment to the thoracic aorta, and maximal aortic suprarenal diameter was measured from the acquired images. After 2 weeks of AngII infusion, mice were then randomly split in two groups: n=10 mice received the BLT1 inhibitor, while n=12 mice received the vehicle daily for 6 weeks. All of the mice developed AAA as assessed by ultrasound.

Ultrasound was performed again after 2 and 6 weeks of daily CP-105,696 treatment, at which time the mice were sacrificed. As in the prevention trial, both groups of mice had similar body weights, cholesterol concentrations, and white blood cell counts (Table II Online only Supplementary Material). Both groups of mice had similar aortic diameters at the start of the BLT1 inhibitor treatment. We observed a modest increase in aortic expansion on top of the previously established AAA in both groups of mice after 2 weeks of drug or vehicle treatment. After 6 weeks, we documented a highly significant reduction in aortic macrophage infiltration and MMP-2 expression in the animals receiving the BLT1 inhibitor (Fig. 4 A, B, C, D). We did not detect any positive stain in the isotype-matched IgG control-stained AAA tissues (Fig. 4 A, B, right panels). However, the reduction in inflammation did not translate into modulation of aortic size, as aortic diameters were the same in both the vehicle- and drug-treated groups at the 6 week time point (Fig. 5A). Of note, *in vivo* (ultrasound) and *ex vivo* (pathology) measurements taken at 6 weeks were highly correlated (R=0.918, Fig. 5B). Representative ultrasound images of aortas acquired at the beginning of treatment, and at 2 and 6 weeks of treatment are shown in Fig. I Online only Supplementary Material.

Discussion

Animal and human studies have increasingly implicated the leukotriene synthesis pathway in chronic inflammatory diseases, including atherosclerosis and its related complications. Here we have shown that BLT1 blockade in the adult animal confers a decrease in aneurysm incidence as well as a concordant reduction in aortic dilation. While BLT1 inhibition diminished aortic macrophage content in established AAA, it did not reverse AAAs at 6 weeks after treatment.

Our findings are consistent with recent studies linking leukotrienes specifically with AAA formation. In a cholate diet-triggered model of AAA, 5-LO deficiency strikingly diminished aneurysmal dilation in a hyperlipidemic mouse background[16]. However, emerging evidence suggests that atherogenesis and aneurysm formation may be inherently different processes, and thus modulation of the same signal in two disease models may not necessarily demonstrate concordant results[22, 24]. While 5-LO deficiency markedly attenuated aneurysm formation, there was no significant effect on the formation of lipid-rich atherosclerotic plaques in a comprehensive analysis[16]. Recent studies have demonstrated that 5-LO generates both pro-inflammatory as well as anti-inflammatory products [e.g., LXA(4) metabolites].[25] Such work underscores the need for precise interventions in the leukotriene pathway to best target inflammatory processes. Further downstream of 5-LO, genetic deficiency of the BLT1 receptor decreased both hyperlipidemia-induced atherosclerosis[7] and AngII-induced AAA[13]. The abrogation of BLT1 signaling had similar effects on both overall plaque development and AAA formation—in contrast to results seen with “upstream” 5-LO inhibition. Thus, pharmaceutical intervention aimed at this receptor may have multiple salutary effects on the vasculature.

The use of knockout mice in the prior studies limited the scope of our prior investigation to alterations that precede the onset of the disease model. Furthermore, developmental confounders and aberrant compensatory pathways can also affect studies in knockout mice. The present study thus extends prior work, by demonstrating that BLT1 blockade in the adult animal also modified AAA formation. We were interested to find that the effects of the drug on AAA incidence and size were extremely similar to the genetic modulation of the LTB_4-BLT1 axis. Thus, the effects seen in the *Blt1*$^{-/-}$ mice in previous work were likely due to modulation of the response to injury in the adult animal, and not secondary to effects on developmental pathways that preceded the onset of the stressor.

While institution of the inhibitor with the onset of the AngII infusion blunted the AAA response, treatment with the inhibitor beginning two weeks after the start of AngII treatment

failed to engender AAA reversal. By contrast, administration of a JNK inhibitor caused regression of aneurysmal diameter in two models of murine AAA[26, 27]. After 6 weeks of therapy in our study, there was a reduction in macrophage recruitment and MMP-2 expression in aortas of mice treated with the BLT1 inhibitor, though aortic size did not change. We hypothesize that the anti-inflammatory effects of the BLT1 inhibition occurred after the initiation of AngII-induced elastin degradation and as such were not sufficient to engender aortic aneurysm regression. Our findings are consistent with the notion that the pathogenesis of aneurysmal disease is complex and multifactorial. For example, it has been shown that macrophages are present in the early stages of AngII-induced aneurysms and precede the medial degradation suggesting a contributing role[24]. However other studies have demonstrated that macrophage depletion actually predisposes mice to aortic intra-mural hematomas[28]. Thus, the timing of interventions in complex inflammatory diseases is critical, and further work is clearly necessary.

Future studies must also determine whether longer treatment can ultimately cause regression of aortic dilation. Whether a potent anti-inflammatory response might ultimately translate into diminished aortic rupture must ultimately be examined as well. In conclusion, our data have extended the role of the LTB_4-BLT1 axis as critical to aneurysm formation in mice. As with classic peptide chemokines, the pleiotropic effects of lipid chemoattractants in disease contexts are increasingly appreciated. These findings are clinically relevant because small-molecule antagonists to BLT1 are under development and would thus target multiple vessel wall cell types and multiple vascular pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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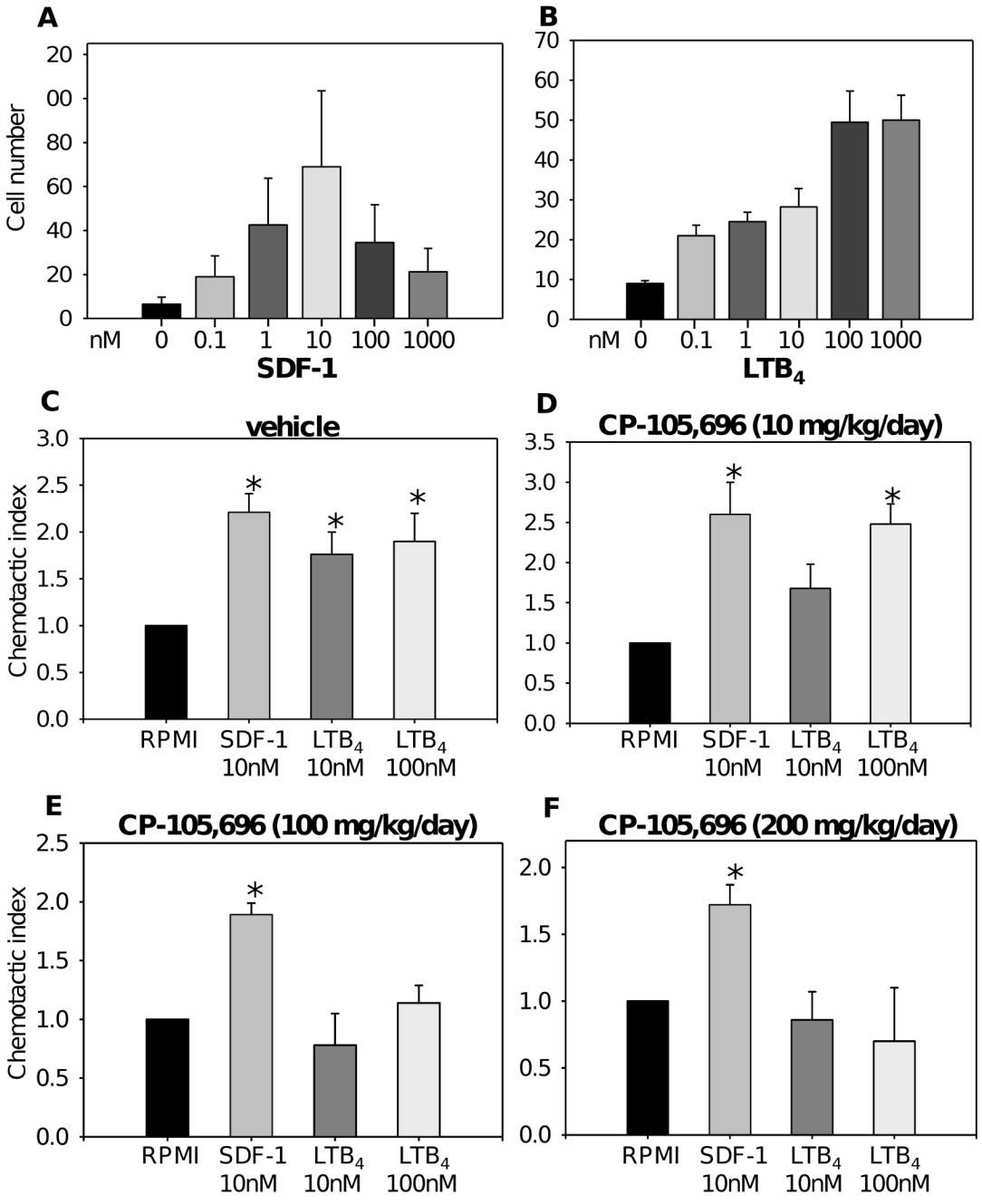

Figure 1. Inhibition of BLT1-mediated chemotaxis

(A, B) Chemotactic response curves of mouse splenocytes to SDF-1 and LTB_4.

(C, D, E, F) Chemotaxis assays of isolated splenocytes from mice treated with CP-105,696 for 7 days at the indicated dose. Isolated splenocytes were placed in modified Boyden chambers and chemotaxis was assessed in response to SDF-1 (10 nM) and LTB_4 (100 nM). Data shown are from two experiments and are presented as cell number or mean chemotactic index (cells per four-high power fields in duplicate wells moving in response to SDF-1 or LTB_4 relative to media alone). *$p<0.05$ versus media alone.

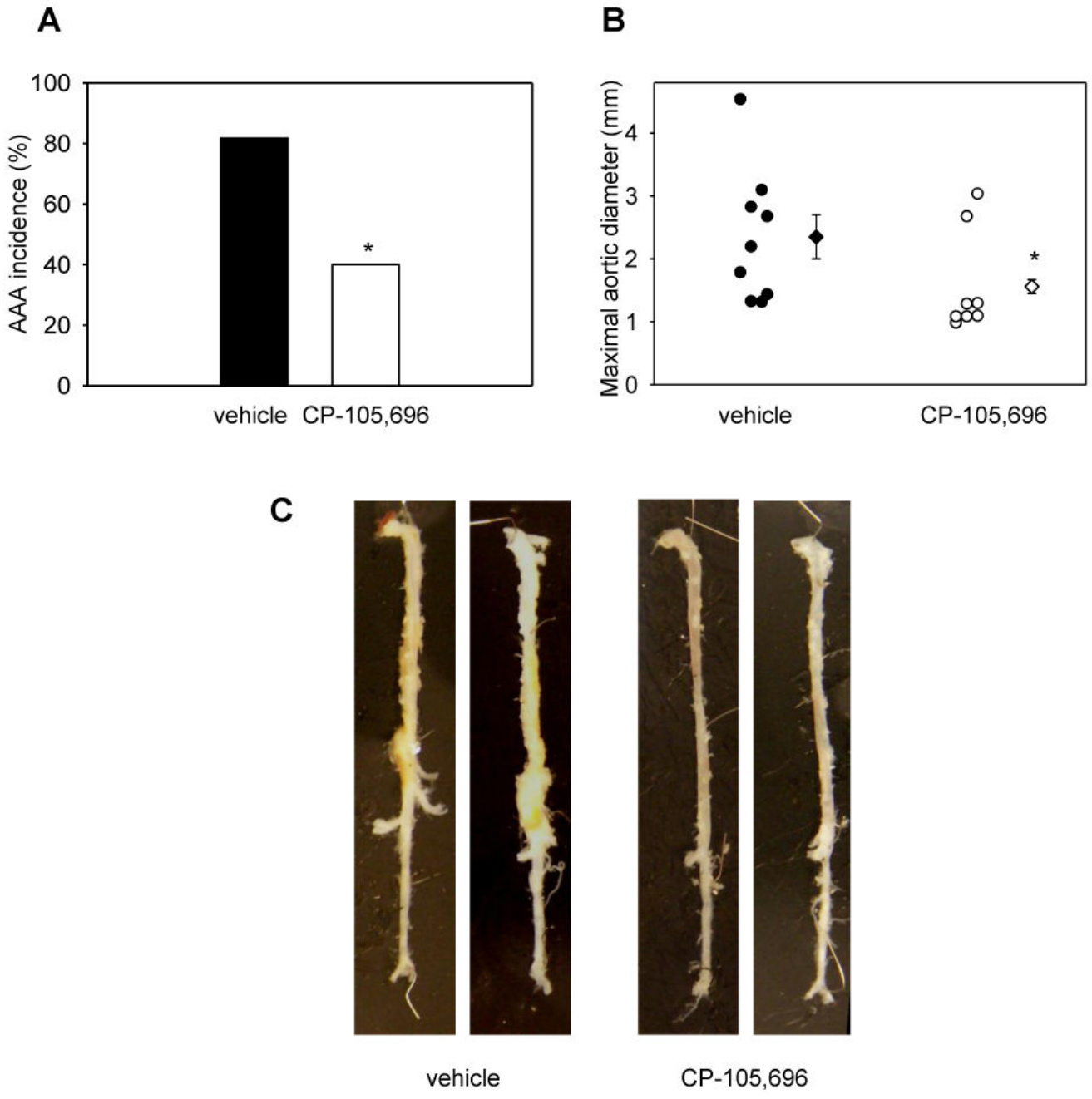

Figure 2. AAA formation in AngII-infused mice treated concomitantly with vehicle or CP-105,696

Apoe$^{-/-}$ mice were treated with AngII concomitant with vehicle or CP-105,696 for 4 weeks. **(A)** AAA incidence (*p<0.05), **(B)** maximal aortic diameter (*p<0.05), (**C)** Representative images of aortas from mice treated with vehicle or CP-105,696 from the prevention study. Circles represent individual mice, diamonds represent means and bars represent SEMs. The experiment included n=11 mice in the vehicle group, and n=10 mice in the CP-105,696 group. Two mice from the vehicle-control and two mice from the inhibitor-treated group died of AAA rupture.

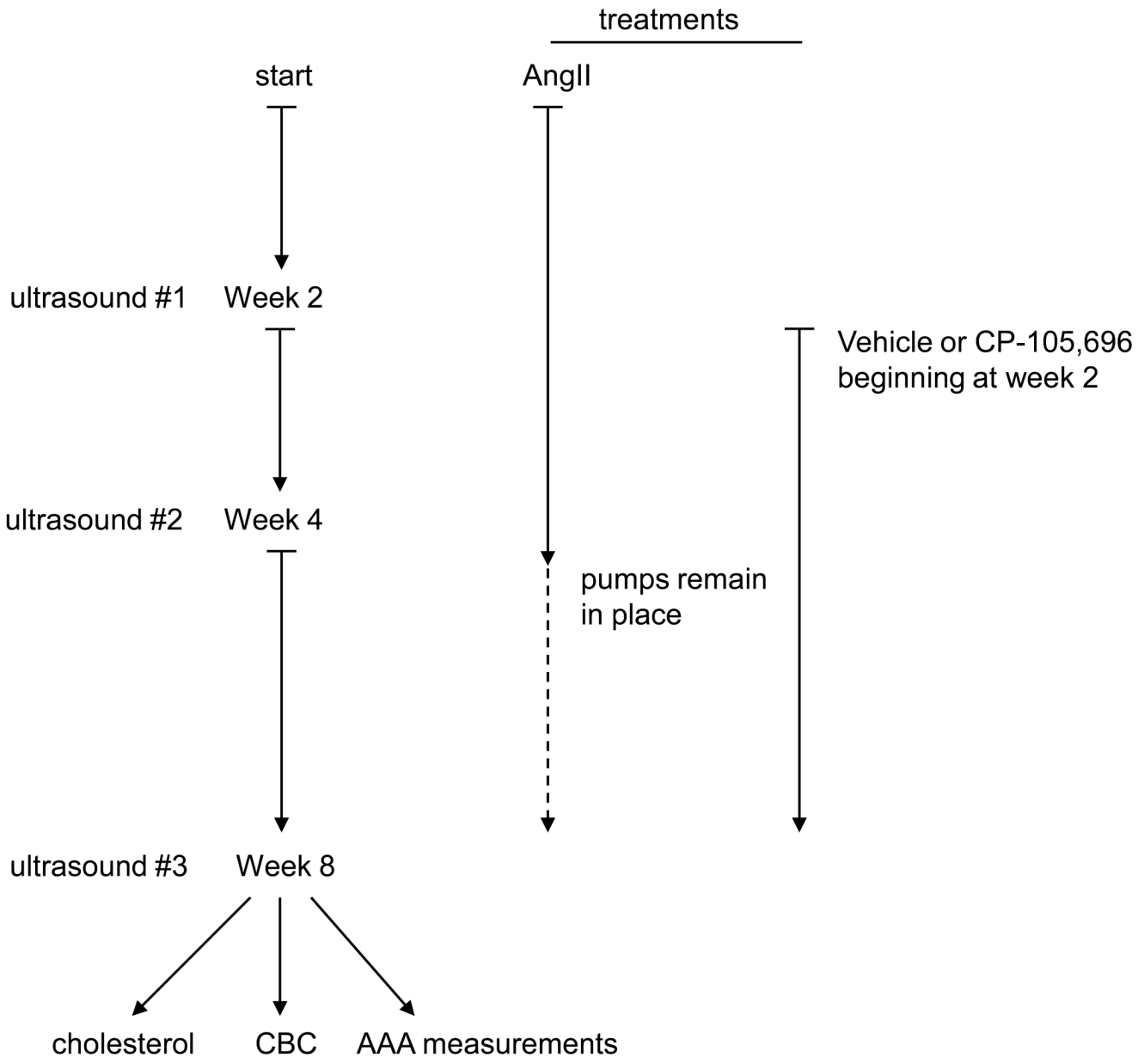

Figure 3. Experimental design of AAA reversal study

Mice were infused with AngII for 2 weeks and then evaluated by ultrasound. Mice that had developed AAA at 2 weeks were randomly assigned to CP-105,696 (n=10) or vehicle control (n=12). All mice were evaluated again by ultrasound at weeks 4 and 8 after the onset of AngII infusion, and sacrificed at week 8 and evaluated for CBC, total cholesterol and aortic quantitation. The dashed line represents the AngII pumps that remained in place throughout the treatment with CP-105,696 for a total of 8 weeks.

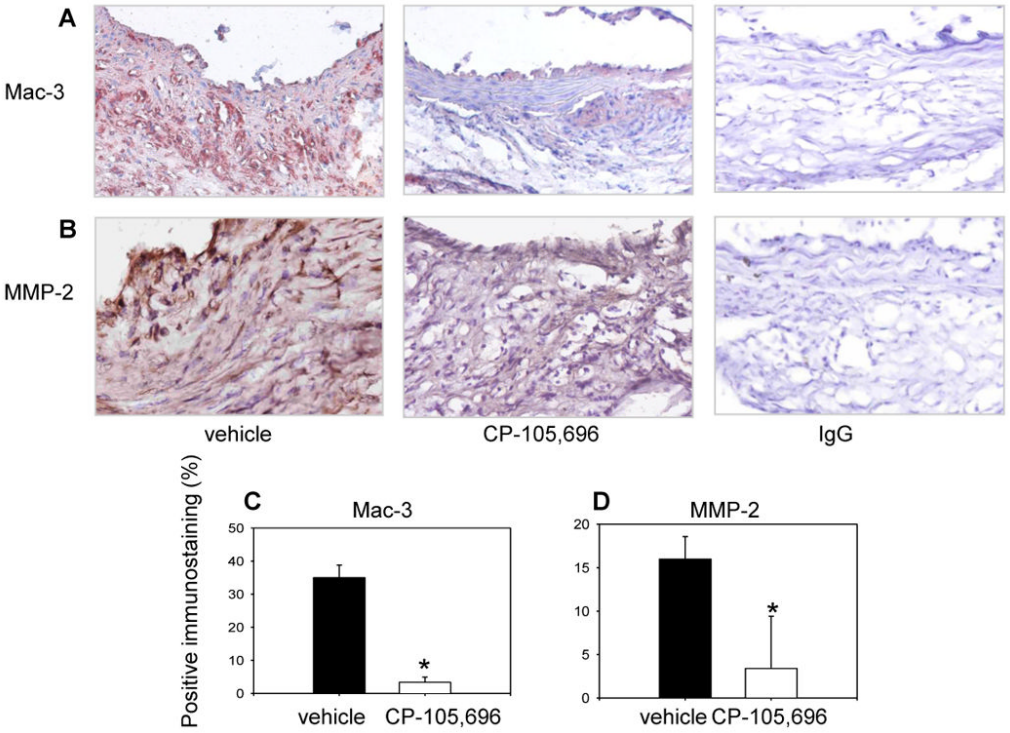

Figure 4. Diminished macrophage accumulation and MMP-2 expression in aortas of mice treated with CP-105,696

(A) Representative images of macrophage (Mac-3) and **(B)** MMP-2 stain show intense macrophage infiltration and MMP-2 expression in the vehicle control and less macrophage accumulation and MMP-2 expression in the inhibitor-treated aorta (middle panels). No positive stain is detected in the isotype-matched control IgG for Mac-3 and MMP-2 stains (right panels). Magnification 20×. **(C, D)** Quantification of Mac-3 and MMP-2 immunostaining from vehicle- and inhibitor-treated AAA (*$p<0.001$).

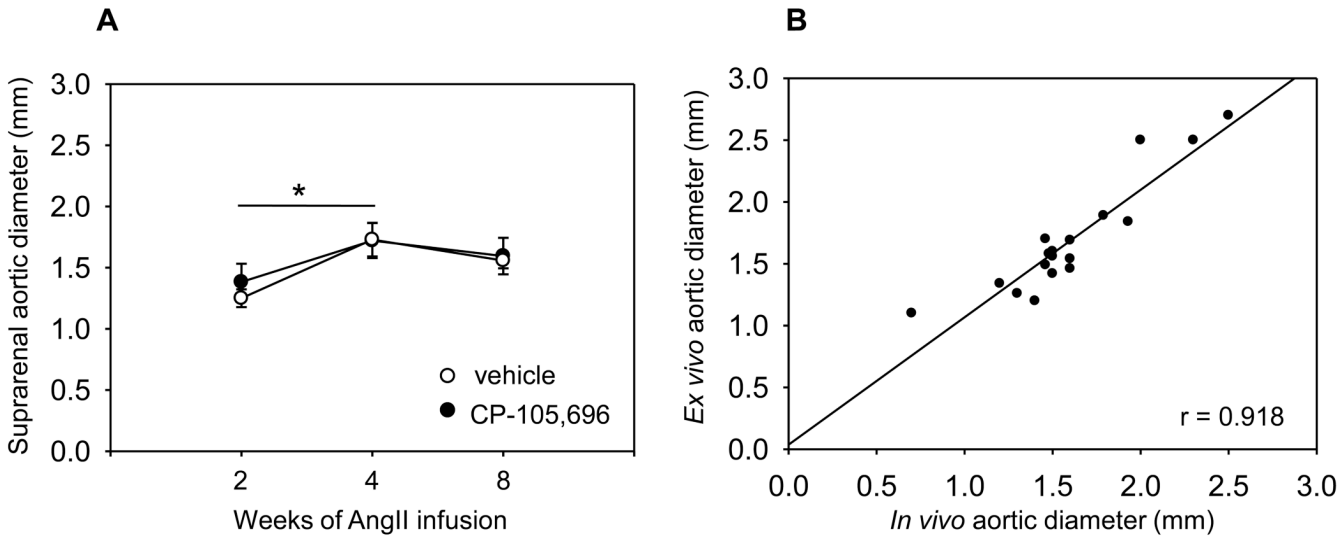

Figure 5. Ultrasound measurements of aortas from mice treated with vehicle or CP-105,696 beginning 2 weeks after AngII infusion

N=12 mice were treated with vehicle and n=10 mice were treated with CP-105,696 at 2 weeks after AngII pumps were placed. **(A)** Maximal aortic diameters were measured periodically in the suprarenal aorta throughout treatment with vehicle or CP-105,696. Circles represent means and bars represent SEMs. **(B)** Correlation of maximal aortic diameter measurements by ultrasound versus histological measurements after dissection at 8 weeks (R=0.918).