Peptide Inhibitor of CXCL4–CCL5 Heterodimer Formation, MKEY, Inhibits Experimental Aortic Aneurysm Initiation and Progression

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Objective—Macrophages are critical contributors to abdominal aortic aneurysm (AAA) disease. We examined the ability of MKEY, a peptide inhibitor of CXCL4–CCL5 interaction, to influence AAA progression in murine models.

Approach and Results—AAAs were created in 10-week-old male C57BL/6J mice by transient infrarenal aortic porcine pancreatic elastase infusion. Mice were treated with MKEY via intravenous injection either (1) before porcine pancreatic elastase infusion or (2) after aneurysm initiation. Immunostaining demonstrated CCL5 and CCR5 expression on aneurysmal aortae and mural monocytes/macrophages, respectively. MKEY treatment partially inhibited migration of adaptively transferred leukocytes into aneurysmal aortae in recipient mice. Although all vehicle-pretreated mice developed AAAs, aneurysms formed in only 60% (3/5) and 14% (1/7) of mice pretreated with MKEY at 10 and 20 mg/kg, respectively. MKEY pretreatment reduced aortic diameter enlargement, preserved medial elastin fibers and smooth muscle cells, and attenuated mural macrophage infiltration, angiogenesis, and aortic metalloproteinase 2 and 9 expression after porcine pancreatic elastase infusion. MKEY initiated after porcine pancreatic elastase infusion also stabilized or reduced enlargement of existing AAAs. Finally, MKEY treatment was effective in limiting AAA formation after angiotensin II infusion in apolipoprotein E–deficient mice.

Conclusion—MKEY suppresses AAA formation and progression in 2 complementary experimental models. Peptide inhibition of CXCL4–CCL5 interactions may represent a viable translational strategy to limit progression of human AAA disease. (Arterioscler Thromb Vasc Biol. 2013;33:718-726.)

Key Words: abdominal aortic aneurysm ■ CXCL4 ■ CCL5 ■ chemokines ■ mice

Abdominal aortic aneurysm (AAA) is a potentially life-threatening degenerative vascular condition. Histologically, AAA disease is characterized by transmural aortic macrophage infiltration, medial neovascularization, elastin degradation, and smooth muscle cell attenuation.1 Accumulating evidence suggests that medial and adventitial macrophage activation promotes aneurysmal degeneration via production of matrix-degrading metalloproteinases (MMPs), proinflammatory cytokines, reactive oxygen species, and lipid-derived mediators.2–10 To date, no pharmacological treatment strategy has proven effective in limiting aneurysm enlargement or rupture. Surgical repair of late-stage disease, with its attendant morbidity, remains the only effective method of reducing aneurysm-related mortality.11 Given the crucial role of macrophages in AAA pathogenesis, pharmacological strategies to reduce transmural monocyte migration and macrophage localization may provide an effective alternative for medical disease management.

Transmural leukocyte migration is tightly controlled by receptor interactions with adhesion molecules and chemokines in vascular structures. These interactions mediate leukocyte tethering and rolling, activation-dependent arrest or firm adhesion, and eventual transendothelial diapedesis.12 In human AAA disease, serum and tissue levels of monocyte-attracting chemokines, such as CCL2/monocyte chemotactic protein-1 and CCL5/RANTES, are significantly elevated,13–16 and CXCL4, a platelet-derived chemokine, is overexpressed in intraluminal aneurysm thrombus.13,14 Chemokine receptor polymorphisms, such as CCR2 heterozygote V64l and CCR5 delta 32 deletion, are also associated with increased AAA disease risk.17,18 In experimental models, increased CCL2 and CCL5 mRNA and protein expression is present in aneurysmal aortic tissue,19–23 and either leukocyte CCR2 gene deletion or siRNA-mediated expression knockdown attenuates AAA development.24–28 Although the significance of CCL2–CCR2 interaction in AAA pathogenesis is well described, the role of CCL5/CCR5 remains controversial and uncertain.29

See accompanying editorial on page 670
Formation of a C-type CXCL4–CCL5 heterodimer substantially augments CCL5-mediated monocyte adhesion, arrest, and transmigration in vitro. MKEY, a mouse CCL5-based synthetic cyclic peptide, prevents CXCL4–CCL5 heterodimer formation by competition with CXCL4 for CCL5-binding sites and releases CXCL4 from existing heterodimers. In in vitro assays, MKEY inhibits CCL5-mediated monocyte chemotaxis and arrest on activated endothelial cells. In apolipoprotein E-deficient (ApoE−/−) mice, MKEY inhibits monocyte recruitment to atherosclerotic lesions. The critical importance of macrophage localization and activation in AaA pathogenesis led us to hypothesize that MKEY, on the basis of its known inhibitory effects in atherosclerosis-prone mice, might alter the course of aneurysm pathogenesis as well. We thus designed a series of experiments to evaluate the efficacy of MKEY in limiting initiation and progression of experimental AAA disease.

Materials and Methods

Mice
Male C57BL/6J mice and ApoE−/− mice on C57BL/6J genetic background at 10 weeks of age were purchased from The Jackson Laboratory, Bar Harbor, Maine. Experimental procedures and care for laboratory animals were conducted in compliance with Stanford Laboratory Animal Care Guidelines and approved by the Administrative Panel on Laboratory Animal Care (labanimals.stanford.edu).

Experimental Aneurysm Creation
In most experiments, AAs were created via intra-aortic porcine pancreatic elastase (PPE) infusion as previously described. Briefly, under inhaled anesthesia with operative magnification, the infrarenal abdominal aorta was exposed and controlled proximally and distally with 6-0 silk suture. Heat-tapered PE-10 tubing was inserted into the controlled segment just proximal to the aortic bifurcation. PPE was infused for 5 minutes into the controlled segment (30 μL of 1.5 U/mL type I PPE in saline, Catalog No. 098K7008, Sigma-Aldrich, St. Louis, MO). After PPE infusion, the residual infusion was aspirated, the tubing was withdrawn, and the aortotomy was closed using 10-0 nylon suture. In additional experiments, AAAs were created in ApoE−/− mice via a 28-day subcutaneous infusion of angiotensin II (Ang II; 1000 ng/kg per minute) via implanted osmotic minipumps (Azet model 2004, Durect Corporation, Cupertino, CA). After recovery from surgery and anesthesia, mice were housed in separated cages with free access to chow and water.

Serial Aortic Diameter Determination via Ultrasound Imaging
Aneurysm formation and progression were monitored by serial aortic diameter measurements using transabdominal ultrasound at 40 MHz (Vevo 770; Visualsonics, Toronto, Canada), as previously described by ourselves and others, beginning immediately before PPE infusion (day 0) and at 3, 7, and 14 days postoperation. For the Ang II/ApoE−/− experiments, measurements were obtained at day 0 (before initiating Ang II infusion) and at 3, 7, 14, 21, and 28 days after PPE infusion. The presence of an aneurysm was defined as >50% increase in infused aortic segment diameter in the PPE model and either a >50% diameter increase or the onset of suprarenal aortic dissection in the Ang II/ApoE−/− mice. All diameter measurements were performed by a single investigator blinded to study group assignment, with <2% variation of repeated measurements.

Analysis of CCR5 Expression on Single Leukocyte Suspensions
In selected PPE-infused mice, aortae were harvested at 14 days, digested using elastase and collagenase, and passed through 40-μm filters to obtain single-cell populations. In additional experiments, leukocytes were also isolated from whole blood for analysis. Single leukocyte suspensions were stained with monoclonal antibodies against CD45, CD11b, and CCR5 and analyzed using flow cytometry (BD FACSCalibur, BD Biosciences, San Diego, CA). Data are presented as the percentage of CCR5+ cells in CD45–CD11b– cells (monocytes/macrophages) or CD45–CD11b+ leukocytes.

In Vivo Leukocyte Migration Assays
Donor leukocytes were isolated from spleens and bone marrow of mice 2 weeks after PPE infusion and labeled with fluorescent dye 5-(and 6)-carboxylfluorescein succinimidyl ester (CFSE) as previously described.3 Additional aneurysmal recipient mice were injected intravenously with either vehicle alone or MKEY (Formula CT-2009ca, Carolas Therapeutics, Inc, San Diego, CA) at 20 mg/kg in vehicle. Thirty minutes thereafter, 5×10^5 CFSE-labeled donor leukocytes were intravenously injected into both the vehicle and MKEY groups. To ensure sufficient MKEY levels for blocking CXCL4–CCL5 interaction in vivo, an additional 20 mg/kg was given intravenously at the time of donor cell transfer. Recipient mice were euthanized 2 hours after cell transfer. CFSE+ donor leukocytes in the spleen, peripheral lymph nodes, and blood of each recipient mouse were determined by flow cytometric analysis. At least 5×10^5 total leukocytes were analyzed for each sample. Donor cells in lymphoid tissues and blood of recipient mice were calculated as the percentage of total leukocytes. Donor cells in aneurysmal lesions were identified via fluorescence microscopy and calculated as the number of donor cells/aortic cross section. At least 10 aortic sections, 50 μm apart, were evaluated per mouse aorta. Donor cell migration in MKEY-treated group is expressed as the percentage of that in vehicle-treated mice, in which migration was set at 100.

MKEY Influence on AAA Progression
Both the PPE and AngII/ApoE−/− models were used to examine the effect of MKEY on AAA formation and progression. In the PPE/C57BL/6J model, mice were injected intravenously with 10 or 20 mg/kg MKEY daily starting 3 days before PPE infusion for 17 days or starting 5 days after PPE infusion. In the Ang II/ApoE−/− model, mice were treated daily with 10 mg/kg MKEY for 3 days before Ang II pump implantation and for 27 days thereafter. Infrarenal and suprarenal aortic diameters were recorded for 14 days after PPE infusion and for 28 days after Ang II pump implantation using transabdominal ultrasound.

Tissue Analysis and Immunostaining
At euthanization, aortae were harvested, fixed with 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned (4 μm in thickness). Selected aortic tissues were embedded in optimal cutting temperature medium for frozen sectioning. Elastic-Masson staining and immunohistochemical staining were performed as previously described. The primary antibodies for immunohistochemistry were a rabbit anti-mouse SMC α-actin polyclonal antibody (Laboratory Vision, Fremont, CA), a rat anti-mouse MAC-2 monoclonal antibody (Clone M3/38, Cedarae Laboratories, Burlington, Ontario, Canada), a goat anti-mouse CCL5 polyclonal antibody or normal goat IgG (R&D systems, Minneapolis, MN), a rabbit anti-mouse CD31 polyclonal antibody (Laboratory Vision), a rabbit anti-mouse MMP2 polyclonal antibody, and a rabbit anti-mouse MMP9 polyclonal antibody (Chemicon International Inc, Temecula, CA). Other reagents, including biotinylated anti-goat, rat, or rabbit secondary antibodies, streptavidin–peroxidase conjugates, and peroxidase substrate kits (3-amino-9-ethylcarbazole and 3,3-diaminobenzidine), were purchased from the Vector Laboratories, Burlingame, CA. Destruction of medial elastin and SMCs was graded as I (mild) to IV (severe). Data on mural macrophage infiltration and angiogenesis were provided as the number of MAC2+ cells and CD31+ blood vessels per aortic cross section, respectively.

Statistical Analysis
Data are represented as mean±SD. Depending on the type of data analyzed, nonparametric Mann–Whitney test or 2-way ANOVA
CCL5 and Its Receptor CCR5 Are Strongly Expressed in Aneurysmal Aortae

An anti-CCL5 antibody was used to stain frozen aortic sections from PPE- or saline (control)-infused C57BL/6j mice (Figure 1A). There was no CCL5 staining in the control aorta. In contrast, strong medial and adventitial anti-CCL5 antibody staining was present in aneurysmal aortae. Normal goat IgG (negative control for CCL5 antibody) did not stain aneurysmal or control aortae. Immunostaining for CD45, CD11b, and CCR5 was performed on isolated tissue leukocytes from aneurysm specimens. As seen in Figure 1B, 32% of CD45⁺CD11b⁺ cells expressed CCR5, versus 4% of CD45⁻CD11b⁻ cells, suggesting that most of the CCR5-expressing cells were monocytes/macrophages. In contrast, only 5% of circulating CD11b⁺ leukocytes (Figure 1C and 1D) and CD11b⁻ leukocytes (not shown) expressed CCR5. Thus, CD11b⁺CCR5⁺ monocytes/macrophages seem to be preferentially localized within aneurysm tissue in PPE-infused mice.

CCL5 Expression Influences Transmural Aortic Leukocyte Migration in Aneurysmal Aortae

In vivo short-term leukocyte migration assays were performed to determine the significance of CCL5 expression in aortic leukocyte (including monocyte) recruitment in PPE-induced AAAs. Pretreatment with MKEY reduced aortic accumulation of injected, CFSE-labeled, mixed spleen and bone marrow cells by 25%, without apparent affect on peripheral lymph node and bone marrow migration (Figure 2). These results indicate that CCL5 expression influences, at least to some degree, transaortic leukocyte migration in this model.

MKEY Treatment Suppresses the Development and Progression of AAAs

Based on the apparent functional consequences of CCL5 expression in aneurysmal aortae, we hypothesized that CCL5 inhibition would influence the formation and progression of AAAs after PPE infusion. To test this, mice were intravenously injected with vehicle or MKEY starting 3 days before aortic

Results

Expression of CCL5 and its leukocyte receptor CCR5 in aneurysmal aortae. A, Acetone-fixed frozen sections from mice 2 weeks after porcine pancreatic elastase (PPE; left) or saline (right) infusion were stained with a goat anti-mouse CCL5 polyclonal antibody (upper) or an equal amount of normal goat IgG (negative control, lower). This staining pattern was reproduced in at least 3 mice. Original magnification, ×400. B, Single leukocyte suspensions from enzyme-digested aneurysmal aortae were stained with the monoclonal antibodies against CD45, CD11b, and CCR5 (or its negative control antibody) and analyzed using flow cytometry. The percentages of CCR5⁺ cells in CD45⁺CD11b⁺ (monocytes/macrophages) and CD45⁻CD11b⁻ cells (other leukocytes) are shown in the left and right histograms, respectively. Each flow cytometric histogram is the overlay image for the staining of anti-CCR5 mAb (unshaded) and its negative control antibody (shaded). This experiment was repeated 3×, and the cells pooled from 3 mouse aortae were used for each staining. C and D, Whole-blood leukocytes from mice 2 weeks after PPE infusion were stained with CD11b and CCR5 (or its negative control antibody) and analyzed using flow cytometry. Both small and large leukocytes expressed CD11b (C). A representative flow cytometric plot shows that 5% of CD11b⁺ leukocytes expressed CCR5 and most of them were small leukocytes as indicated by side scatter size (D). This experiment was repeated in 5 mice.
PPE infusion and continuing daily for the next 14 days. After PPE infusion, aortic diameters enlarged in a progressive fashion from day 3 onward (Figure 3A). In MKEY-treated mice, at either dose of 10 mg/kg or 20 mg/kg, PPE infusion produced significantly smaller aneurysms (Figure 3A). At 20 mg/kg, aneurysm formation was nearly completely obliterated. At both 7 and 14 days, mean aortic diameters in both MKEY-treated groups were significantly smaller than their respective controls (Figure 3B). Mean aortic diameter in the 20 mg/kg MKEY-treated group was also significantly smaller than that in the 10 mg/kg MKEY-treated group (Figure 3B). In terms of aneurysm incidence after PPE infusion, AAAs (defined by a >50% or further increase in aortic diameter) developed in all vehicle-treated mice (8/8) within 7 days (Figure 3C). In contrast, AAA developed in 3 mice (60%, 3/5) and 1 mouse (14%, 1/7) treated with 10 and 20 mg/kg MKEY, respectively, within 14 days (Figure 3C). Although AAA incidence in both MKEY-treated groups was lower than that noted in the vehicle-treated group, a significant difference was only seen between vehicle- and 20 mg/kg MKEY-treated groups. These results indicate that MKEY therapy suppresses experimental AAA formation and progression in a dose-dependent fashion.

MKEY Treatment Preserves Aortic Mural Integrity
To identify the mechanisms responsible for MKEY-mediated AAA suppression, elastic-Masson staining and SMC immunostaining were performed on aortic sections. As illustrated in Figure 4A, PPE infusion severely reduced medial elastin density and SMC cellularity in vehicle-treated mice. Both elastin fragmentation and SMC depletion were significantly attenuated in MKEY-treated mice (Figure 4B and 4C). Although the mean scores were lower in the 20 mg/kg MKEY group than those in the 10 mg/kg MKEY group, the difference between the 2 groups did not reach statistical significance.

In addition to mural SMC and elastin preservation, only a small number of aortic MAC2+ cells and CD31+ vessels were observed in MKEY-treated mice. By semiquantitative histological analysis, monocyte/macrophage infiltration and mural neovascularization were significantly reduced in MKEY-treated mice compared with control (Figure 4D and 4E). Consistent with reduced aortic accumulation of monocytes/macrophages, immunostaining for MMP2 and MMP9 was attenuated in the aortae from MKEY-treated mice compared with vehicle-treated mice (online-only Data Supplement). Thus, reduction of monocyte/macrophage infiltration, resultant MMP2 and MMP9 expression, and mural neoangiogenesis may contribute to MKEY-mediated AAA suppression.

MKEY Treatment Stabilizes Existing AAAs
To gain insight into the translational value of MKEY-mediated suppression of existing aneurysms, AAA mice were treated with 20 mg/kg MKEY beginning 5 days after PPE infusion, for 9 additional days. As shown in Figure 5A and 5B, aortic diameters in PPE-infused, vehicle-treated mice continued to enlarge during this time frame. In contrast, further enlargement was nearly completely suppressed in PPE-infused mice by the third day of MKEY treatment. Consistent with the observed effect on aneurysm diameter, qualitatively there seemed to be reduced elastin degradation and SMC depletion.
in delayed-treatment mice, although the impact on SMC density did not reach statistical significance (Figure 5C and 5D). Mural macrophage and neovessel density were significantly reduced in delayed-treatment compared with those in vehicle-treated mice (Figure 5E and 5F). These results indicate that MKEY treatment stabilizes existing AAAs by limiting mural monocyte/macrophage infiltration and further elastin degradation and angiogenesis.

MKEY Treatment Suppresses Ang II Infusion–Induced AAAs in ApoE−/− Mice

Finally, the translational value of MKEY-mediated aneurysm suppression was further examined in the AngII/ApoE−/− AAA model. This represents a mechanistically distinct model, complementary to the PPE model in C57BL/6J mice. MKEY treatment starting before Ang II infusion significantly lowered AAA incidence (20%) compared with vehicle treatment (60%; Figure 6A). AAA-associated mortality in MKEY-treated ApoE−/− mice (20%) was also lower than that in vehicle-treated mice (40%), although there was no statistical difference between the 2 treatment groups (Figure 6B). Consistent with its influence on AAA incidence and mortality, MKEY treatment inhibited aortic enlargement during the first 14 days after Ang II infusion compared with vehicle treatment. There was a significant difference in aortic diameters between the 2 treatment groups on day 3 after Ang II infusion. These results extend our findings in the PPE model, suggesting that CXCL4–CCL5 heterodimer–mediated aortic monocyte migration may be a common mechanism for aneurysm formation in multiple AAA models.

Discussion

These experiments demonstrate that CCL5 and its receptor CCR5 are expressed in experimental aneurysm tissue and mural CD45+CD11b+ monocytes/macrophages, respectively. MKEY, a peptide inhibitor to CXCL4–CCL5 heterodimer formation, significantly inhibits migration of adoptively transferred donor leukocytes into recipient aneurysmal aorta. Furthermore, MKEY treatment initiated after aneurysm formation stabilizes aortic mural architecture and limits further aneurysm progression. The relevance of these findings to aneurysm disease was generally validated by the observation that MKEY suppressed aneurysms initiated by Ang II infusion in male ApoE−/− mice. Together, these experiments highlight the significance of CCL5 in experimental aneurysm pathogenesis and underscore its potential role in human AAA disease.

Chemokines CCL2 and CCL5 recruit inflammatory monocytes into target tissues, promoting macrophage-driven inflammatory conditions, such as vascular disease. Previous work suggests significant potential roles for CCL2 and CCR2 in experimental aneurysm pathogenesis. In the current experiments, using similar models, we demonstrated significant CCL5 production in infiltrating leukocytes, mostly monocytes/macrophages. Its production may occur from other
constitutive or infiltrative aortic cell types as well.\textsuperscript{14,22} Although both are clearly overexpressed in experimental aneurysm tissue, whether CCL2 and CCL5 exert redundant or coordinated roles in AAA pathogenesis remains to be determined.

A previous study reported no apparent impact of targeted CCR5 deletion on aneurysm initiation or progression in mice.\textsuperscript{25} In that study, aneurysms were created via abluminal aortic application of calcium chloride. This apparent discrepancy is likely attributable to pathological features that distinguish the PPE and Ang II/ApoE$^{-/-}$ models from the calcium chloride application model.\textsuperscript{43,44} In the former 2 models, aneurysm formation is accompanied by abundant transmural monocyte/macrophage infiltration and marked medial elastin and SMC depletion. Aneurysms in the calcium chloride model are distinguished primarily by elastin fragmentation, however, with relative preservation of total elastin content and medial SMC density. In their pathological features, aneurysms created by either luminal PPE infusion or systemic Ang II administration have substantially more fidelity to the human condition than those generated by calcium chloride. In addition, reciprocal upregulation of CCR2 activity and expression may occur after CCL5 deletion, sustaining the ability to form aneurysms after calcium chloride–induced injury. Regardless of the alternative potential explanations considered, however, in these experiments, in either C57BL/6J or ApoE$^{-/-}$ mice, MKEY was highly effective in suppressing aneurysm formation.

Monocytes/macrophages are significant contributors to AAA pathogenesis in both human and experimental diseases.\textsuperscript{3–8,10,33,45} In this study, MKEY therapy significantly reduced aortic monocyte/macrophage infiltration while suppressing AAA formation and progression. Previously, intravenous MKEY has been shown to inhibit arrest/firm adhesion of adaptively transferred donor monocytes onto inflamed arteries, binding to both leukocytes and endothelial cells.\textsuperscript{30} In these experiments, in either C57BL/6J or ApoE$^{-/-}$ mice, MKEY was highly effective in suppressing aneurysm formation. A previous study reported no apparent impact of targeted CCR5 deletion on aneurysm initiation or progression in mice.\textsuperscript{25} In that study, aneurysms were created via abluminal aortic application of calcium chloride. This apparent discrepancy is likely attributable to pathological features that distinguish the PPE and Ang II/ApoE$^{-/-}$ models from the calcium chloride application model.\textsuperscript{43,44} In the former 2 models, aneurysm formation is accompanied by abundant transmural monocyte/macrophage infiltration and marked medial elastin and SMC depletion. Aneurysms in the calcium chloride model are distinguished primarily by elastin fragmentation, however, with relative preservation of total elastin content and medial SMC density. In their pathological features, aneurysms created by either luminal PPE infusion or systemic Ang II administration have substantially more fidelity to the human condition than those generated by calcium chloride. In addition, reciprocal upregulation of CCR2 activity and expression may occur after CCL5 deletion, sustaining the ability to form aneurysms after calcium chloride–induced injury. Regardless of the alternative potential explanations considered, however, in these experiments, in either C57BL/6J or ApoE$^{-/-}$ mice, MKEY was highly effective in suppressing aneurysm formation. Monocytes/macrophages are significant contributors to AAA pathogenesis in both human and experimental diseases.\textsuperscript{3–8,10,33,45} In this study, MKEY therapy significantly reduced aortic monocyte/macrophage infiltration while suppressing AAA formation and progression. Previously, intravenous MKEY has been shown to inhibit arrest/firm adhesion of adaptively transferred donor monocytes onto inflamed arteries, binding to both leukocytes and endothelial cells.\textsuperscript{30} In these experiments, MKEY partially but significantly inhibited donor leukocyte migration into aneurysmal aortae of recipient mice. Because of the heterogeneity of the donor cells used in the in vivo short-term leukocyte migration assays, the efficacy
of monocyte inhibition specifically could not be determined. Flow cytometric analysis, however, confirmed that the majority of circulating CCR5+ cells in these mice were small CD11b+ myeloid cells, likely monocytes rather than neutrophils or lymphocytes. Thus, it seems that attenuation of inflammation after PPE infusion in C57BL/6J mice resulted at least, in part, from MKEY-mediated inhibition of aortic monocyte migration. Furthermore, consistent with previous studies, these results demonstrate that chemokines, such as CCL5 or CCL2, are produced by infiltrating leukocytes, including monocytes/macrophages.14,22 Inhibition of monocyte migration by MKEY may reduce the density of chemokine-producing cells and, thus, the regional chemokine gradient available for attracting additional leukocytes.

MKEY therapy also substantially attenuated aortic adventitial neovessel formation in these experiments. Mural neangiogenesis is a salient histological feature of both experimental and human AAA diseases.32,46,47 Subsets of monocytes/macrophages produce the proangiogenic cytokine vascular endothelial growth factor-A,46,49 and CCR5 deletion results in sustained inhibition of experimental corneal neovascularization.50 In addition, CCR5 deficiency in a skin wound–healing model is associated with both reduced local vascular endothelial growth factor-A levels and endothelial progenitor accumulation,51 both prevalent in experimental AAA.52 Thus, the anti-inflammatory influences of MKEY therapy may also be related to influences on aortic mural endothelial progenitor cell accumulation. MKEY may also exert antiangiogenic effects in a vascular endothelial growth factor-A– and endothelial progenitor–independent manner via influences on monocyte migration or phenotypic differentiation of infiltrative macrophages.53

MKEY treatment downregulated PPE-induced aortic MMP2 and MMP9 expression, proteases essential for extracellular matrix degradation in experimental and clinical aneurysm disease.2,7,28 Macrophages are recognized sources of MMP production during aneurysmal degeneration. The specific mechanism by which MKEY minimizes MMP expression, however, remains uncertain. No existing evidence indicates that MKEY directly inhibits MMP production in macrophages or other cells.30 Thus, it is more likely that reduced MMP expression in MKEY-treated aneurysmal aortae results from attenuated monocyte/macrophage migration. Regardless of the mechanism, however, reduced MMP expression in response to MKEY treatment will likely limit elastin degradation, monocyte/macrophage migration, and consequent aneurysmal degradation.32,44,54

The ability of delayed MKEY treatment to stabilize or attenuate established experimental AAAs has substantial clinical implications. Most, if not all, inhibitor studies reported to date, regardless of the agent being tested, initiated therapy before aneurysm creation, a situation at odds with the clinical reality of AAA diagnosis and presurgical disease management. The ability to impair or arrest existing aortic mural inflammation, rather than preventing initiation, is a critical requirement for successful medical AAA inhibition strategies. Compared with CCL2/CCR2 axis antagonists, MKEY and similar CCL5/CCR5 inhibitors offer distinct advantages. First, CCL5/CCR5 inhibition will have less impact on host innate immunity compared with CCL2/CCR2 inhibition.12,55–57

In a previous study, MKEY did not suppress T cell proliferation, viral clearance, or macrophage survival.30 In the present study, MKEY suppressed transmural aortic leukocyte migration without affecting migration of leukocytes/lymphocytes into lymph nodes and the spleen, secondary lymphoid organs critical for adaptive immunity. Second, because of prevalent previous studies of CCL5 in atherosclerosis and macrophage-tropic HIV-1, more clinical trials on CCL5/CCR5 antagonists have been completed or are ongoing compared with those on CCL2/CCR2 inhibitors (www.clinicaltrial.gov).37 Maraviroc, a CCR5 antagonist, is approved for clinical use in HIV-1 patients. Thus, CCL5/CCR5 inhibition therapies are primed for translational application in AAA disease.

MKEY, as a mouse CCL5-based antagonist for CXCL4–CCL5 heterodimer formation, cross-reacts with its human homolog (our unpublished data). CKEY2, a human peptide ortholog of MKEY, disrupts human CXCL4–CCL5 heterodimer and has previously been shown to suppress human monocyte chemotaxis to CCL5, as well as CCL5–triggered monocyte arrest in endothelial cells in vitro.30 The experimental method used in these experiments did not allow for assessment of the influence of CKEY therapy on experimental or clinical aneurysm progression. In a previous human study, however, CXCL4 and CCL5 were noted to be colocalized in aneurysmal aortae, released from aortic tissue and intraluminal thrombus, and elevated in plasma obtained from AAA patients.34 Further studies are required to examine whether MKEY or CKEY2 alters the formation and progression of AAAs in the murine model in which entire mouse hematopoietic lineages are replaced with human hematopoetic cell lineages. Alternatively, once all safety data for CKEY2 are acquired, a clinical trial may be indicated to evaluate its therapeutic efficacy in human AAA disease.

In conclusion, these experiments demonstrate for the first time the ability of the peptide CXCL4–CCL5 heterodimer inhibitor MKEY to both suppress experimental AAA initiation and stabilize existing aneurysms through mechanisms related to impaired mural monocyte/macrophage infiltration and angiogenesis. These findings add to previous findings suggesting that CXCL4–CCL5 inhibition may hold substantial translational value for both atherosclerotic and aneurysmal arterial diseases.

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Disclosures

At the time these experiments were planned and performed, Dr Joshua R. Schultz and Dr Court R. Turner were employees of Carolus Therapeutics, Inc. At the time the manuscript was completed, Dr Turner continued to be employed by Carolus Therapeutics, Inc. The page charges for this article were also supported in part by Carolus Therapeutics, Inc. All other authors have no disclosures.

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Effect of MKEY treatment on the expression of MMP2 and MMP9 in the aortae of PPE-infused mice

Infrarenal aortae were harvested from aneurysmal mice treated with vehicle or MKEY 14 days after PPE infusion, sectioned, and fixed with cold acetone. The sections were sequentially incubated with a rabbit anti-mouse MMP2 or MMP9 antibody, a biotinylated anti-rabbit IgG, streptavidin-peroxidase, and peroxidase substrate AEC. Representative images showed marked attenuation of MMP2 and MMP9 expression in a PPE-infused, MKEY-treated mouse aorta as compared to a PPE-infused, MKEY-treated mouse aorta. These staining patterns were reproduced in 3 mice for each treatment group. Red color in each panel indicated positive staining for MMP2 or MMP9. Original magnification for all images is 200X.