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Angiotensin II infusion promotes ascending aortic aneurysms: attenuation by CCR2 deficiency in apoE$^{-/-}$ mice

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ABSTRACT

AngII (angiotensin II) induces atherosclerosis and AAAs (abdominal aortic aneurysms) through multiple proposed mechanisms, including chemotaxis. Therefore, we determined the effects of whole-body deficiency of the chemokine receptor CCR2 (CC chemokine receptor 2) on these diseases. To meet this objective, apoE (apolipoprotein E)$^{-/-}$ mice that were either CCR2$^{+/+}$ or CCR2$^{-/-}$, were infused with either saline or AngII (1000 ng · kg^{-1} of body weight · min^{-1}) for 28 days via mini-osmotic pumps. Deficiency of CCR2 markedly attenuated both atherosclerosis and AAAs, unrelated to systolic blood pressure or plasma cholesterol concentrations. During the course of the present study, we also observed that AngII infusion led to large dilatations that were restricted to the ascending aortic region of apoE$^{-/-}$ mice. The aortic media in most of the dilated area was thickened. In regions of medial thickening, distinct elastin layers were discernable. There was an expansion of the distance between elastin layers in a gradient from the intimal to the adventitial aspect of the media. This pathology differed in a circumscribed area of the anterior region of ascending aortas in which elastin breaks were focal and almost transmural. All regions of the ascending aorta of AngII-infused mice had diffuse medial macrophage accumulation. Deficiency of CCR2 greatly attenuated the AngII-induced lumen dilatation in the ascending aorta. This new model of ascending aortic aneurysms has pathology that differs markedly from AngII-induced atherosclerosis or AAAs, but all vascular pathologies were attenuated by CCR2 deficiency.

INTRODUCTION

Many studies have demonstrated that chronic AngII (angiotensin II) infusion into hypercholesterolaemic mice augments development of atherosclerosis and promotes formation of AAAs (abdominal aortic aneurysms) [1–8]. Both vascular pathologies are characterized by macrophage accumulation. In AngII-induced atherosclerosis, the macrophage accumulation is restricted to the intima, while this cell type is initially present as a focal accumulation in the media within the AAA-prone region. As AAAs progress, abundant numbers of macrophages accumulate in the adventitia [9]. Despite the differences in arterial regions that accumulate macrophages in AngII-induced atherosclerosis and AAAs, both pathologies are reduced by deficiency in the chemokine receptor CCR2 (CC chemokine receptor 2) on bone-marrow-derived cells [5]. The original intent of the present study was to define

Key words: angiotensin, aneurysm, aorta, chemokine, elastin, macrophage.

Abbreviations: AngII, angiotensin II; AAA, abdominal aortic aneurysm; apoE, apolipoprotein E; AT$_1$ receptor, AngII type 1 receptor; CCR2, CC chemokine receptor 2; MCP, monocyte chemoattractant protein; TGF-β, transforming growth factor-β.

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the effects of whole-body deficiency of CCR2 in the development of AngII-induced atherosclerosis and AAAs. During the present study, we also observed region-specific changes localized to the ascending aortic region.

Ascending aortic dilatation is a salient feature of several disease states including Marfan's syndrome and related disorders that have been associated with mutations in fibrillin-1 [10]. Evidence for this causal role has been provided by development of mice with manipulations of the fibrillin-1 gene. Mice that are hypomorphic for fibrillin-1 develop aortic dilatation associated with elastin fragmentation and macrophage infiltration [11]. Fibrillin-1 has also been manipulated in another mouse model by creation of heterozygous mice expressing a mutation generated by substitution of Cys→Gly at position 1039 (C1039G), and these mice also exhibit aortic dilatation [12]. Although mutations in fibrillin-1 were initially thought to exert their effects through structural deficiencies of connective tissue, it is now known that these variants lead to enhanced activity of TGF-β (transforming growth factor-β) [13]. The causal role of enhanced TGF-β signalling in the development of ascending aortic aneurysms was demonstrated by the ablation of the pathology by administration of a TGF-β-neutralizing antibody [12]. Ascending aortic aneurysms were also ablated by administration of losartan into fibrillin-1 mutant transgenic mice. Losartan was the first developed antagonist against AT_1 (AngII type 1) receptors [14], and it has several other well-defined effects, including influencing the production of prostaglandins and NO and inhibiting the effects of thromboxane A2, tachykinin and imidazoline receptors [15]. Currently, no other AT_1 receptor antagonists prevent ascending aortic arch aneurysms in transgenic mice expressing the C1039G fibrillin-1 mutation. Since losartan has well defined ancillary effects, additional studies are needed to determine whether AngII directly promotes ascending aortic aneurysms.

Given the interest in a potential role for AngII in the development of experimental and human ascending aortic arch aneurysms [12,16], we focused on characteristics and mechanisms of aneurysm formation in the ascending aorta. This occurred in the same mice that had the previously described vascular effects of AngII of augmented atherosclerosis and development of AAAs. The vascular tissue characteristics of ascending aortic aneurysms were distinctly different from aneurysms formed in the abdominal aorta. This included an abundance of macrophages throughout the aortic media of the aneurysmal regions. Since CCR2 has been invoked as a chemoattractant mechanism of other AngII-induced vascular pathologies [5,17,18], we examined the role of this chemokine receptor on the development of ascending aortic aneurysms. Moreover, we contrasted effects of CCR2 deficiency on ascending aortic aneurysms to atherosclerosis and AAAs.

MATERIALS AND METHODS

Animals

$ApoE^{-/-} \times CCR2^{+/-}$ mice (backcrossed ten times in a C57BL/6 background) were developed in Dr Charo's laboratory and have been described previously [19,20]. Male littermates with either $CCR2^{+/+}$ or $CCR2^{-/-}$ genotypes were derived from the breeding of $apoE^{-/-} \times CCR2^{+/-}$ parental pairs. Mice were housed under barrier conditions with food and water provided *ad libitum*. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Diet and AngII infusions

Mice used in studies were initially fed a standard laboratory diet. To promote a hypercholesterolaemic state, the diet was changed to one containing 0.15 % cholesterol and 21 % (w/w) milk fat diet (TD 88137; Harlan Teklad) 1 week prior to pump implantation and throughout infusion. AngII ($1000\ ng \cdot kg^{-1}$ of body $weight \cdot min^{-1}$) or saline were infused subcutaneously via Alzet mini-osmotic pumps (Model 2004; Durect) for 28 days as described previously [1].

Blood pressure measurement

Systolic blood pressure was measured in conscious mice using a computerized tail cuff method (BP-2000; Visitech Systems) [21]. All mice were acclimated to the system for 1 week prior to the start of the study.

Serum lipids and lipoprotein determination

Blood was collected by cardiac puncture in anaesthetized (ketamine/xylazine, 100/10 mg/kg of body weight, intraperitoneally) mice and was centrifuged at 376 *g* for 20 min. Serum was subsequently collected and frozen at −80 °C until assayed. Serum total cholesterol concentrations were determined using enzymatic assay kits (cat. no. 439-17501; Wako). Lipoprotein cholesterol distributions were evaluated in individual serum samples (50 μl) from at least five mice in each group after fractionation by size-exclusion chromatography on a single Superose 6 column [22]. Fractions were collected, and cholesterol concentrations were determined with an enzyme-based kit.

Vascular pathology

After blood collection, saline was perfused through the left ventricle of the heart. Aortas from the ascending region to the bifurcation of the femoral arteries were dissected free and fixed in formalin overnight. Following fixation, adventitial tissue was removed, and aortas were cut longitudinally and pinned. Photographs of aortic intimas were acquired using a Nikon SMZ800 dissecting

Table 1 Effects of CCR2 deficiency on apoE$^{-/-}$ mice infused with either saline or AngII

Values are presented as means ± S.E.M. $^{*}P < 0.05$ and $^{\dagger}P < 0.001$ for comparisons of AngII and saline infusion within genotypes; $^{\ddagger}P < 0.001$ for comparisons of AngII infusion between genotypes.

Infusion	Genotype	*n*	Body weight (g)	Serum cholesterol (mg/dl)	Blood pressure (mmHg)
Saline	CCR2$^{+/+}$	9	28.5 ± 0.5	703 ± 18	120 ± 3
	CCR2$^{-/-}$	9	28.5 ± 0.6	688 ± 35	128 ± 4
AngII	CCR2$^{+/+}$	16	27.4 ± 0.5	656 ± 24	142 ± 4*
	CCR2$^{-/-}$	19	28.1 ± 0.8	820 ± 20†‡	142 ± 5*

scope and Nikon digital DXM 1200 camera. The extent of dilatation and area was quantified in aortic arches using Image-Pro software (MediaCybernetics) [23,24]. Diameters were calculated from measurements of aortic arch circumferences. Intimal areas were quantified from regions of ascending arches protruding from the ventricle to 3 mm proximal from the subclavian artery. After histological staining, cross-sections of ascending aortas were magnified × 400 and photographed using a Nikon Optiphot-2 microscope and Spot camera (RT Color Diagnostics). Medial thicknesses of ascending aortas were measured from the inner to outer elastic laminae ($n = 3$–6 mice/group). Aneurysms in abdominal aortas were measured at the maximal width of the external surface [25]. Detailed descriptions of measurement of *en face* and aortic root atherosclerosis have been described previously [23,24]. Briefly, atherosclerosis in the intima surface was determined by measurements of areas covered by grossly discernable lesions. In sections from aortic roots, measurements were performed on Oil-Red-O-stained serial tissue sections that are 80 μm apart that encompassed the entire lesion (generally, nine sections spanning 720 μm).

Histology and immunostaining were performed as described previously [25]. The following histological stains were used: haematoxylin and eosin for gross morphology and Gomori trichrome for collagen and smooth muscle cells. Macrophages were immunostained using an antiserum (dilution 1:1000, cat. no. AIAD31240; Accurate Chemical). We also attempted to immunostain for MCP-1 (monocyte chemoattractant protein-1) (cat. no. SC-1784 and SC-1785; Santa Cruz Biotechnology; cat. no. AB72012.50; Abcam; cat. no. AAM43; Serotec) and CCR2 (cat. no. 2068-1; Epitomics; cat. no. AVARP6003; Avia; cat. no. GTX45788; GeneTex; cat. no. sc-6227 and sc-6228, Santa Cruz Biotechnology).

Statistics

Means and S.E.M. were calculated for each parameter. Statistical analyses were performed using SigmaStat. In experiments with more than one experimental group, differences were evaluated using a two-way ANOVA, and significant interactions analysed using a Tukey post-hoc test for all pairwise comparisons. Data were analysed to ensure appropriate use of parametric or non-parametric statistics. Fisher's exact test was used to determine differences among groups in the incidence of aneurysm formation. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Whole-body CCR2 deficiency attenuated AngII-induced atherosclerosis and AAAs

Infusion of AngII (1000 ng · kg^{-1} of body weight · min^{-1}) into apoE$^{-/-}$ mice fed a saturated-fat-enriched diet had no significant effect on body weight, serum cholesterol concentrations or lipoprotein cholesterol distribution, but increased systolic blood pressure (Table 1), as described previously [7,8,21,26]. CCR2 deficiency had no effect on AngII-induced increases in blood pressure, while causing a small increase in serum cholesterol concentrations (Table 1). Size-exclusion chromatography demonstrated that the increase was due to increased LDL (low-density lipoprotein)-cholesterol (results not shown).

Infusion of AngII into hypercholesterolaemic mice augmented atherosclerosis and led to development of AAAs, as described previously [7,8]. Whole-body deficiency of CCR2 reduced the size of atherosclerotic lesions in both the en face measurement of aortic intima (Figure 1A) and in tissue sections from the aortic sinus (Figure 1B), and attenuated development of AAAs (Figure 1C). The extent of these changes were similar to that described previously in irradiated apoE$^{-/-}$ mice that were repopulated with bone-marrow-derived cells harvested from CCR2$^{-/-}$ mice [5].

AngII infusion promoted ascending aortic aneurysms

Compared with saline-infused apoE$^{-/-}$ mice, infusion of AngII led to gross changes that were present from the ascending aorta to just proximal of the subclavian artery (Figure 2). These changes included dilatation of the ascending aorta and thinning of the anterior aspect of this region.

The composition of aortic tissues was defined on tissue sections of ascending aorta as shown in Figure 3. Medial

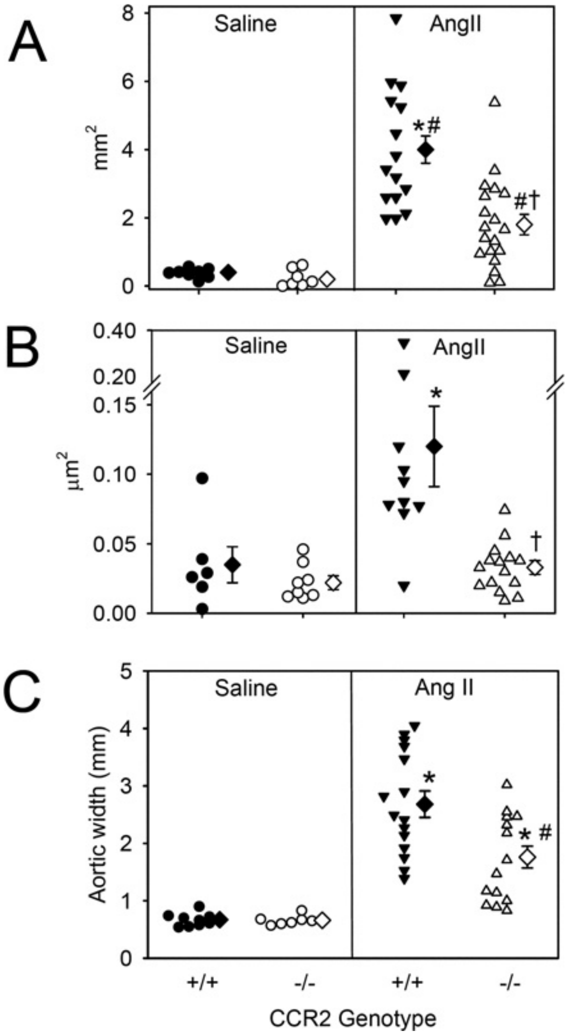

Figure 1 Deletion of CCR2 attenuates the development of AngII-induced atherosclerosis and AAAs

Each symbol represents individual animals, diamonds represent means, and bars are S.E.M. (A) Area of aortic intima covered with atherosclerotic lesions as measured by en face analysis. $*P < 0.001$ and $\#P < 0.005$ for comparisons of saline and AngII infusions within genotypes; $\dagger P < 0.001$ for comparisons of CCR2$^{+/+}$ and CCR2$^{-/-}$ within AngII-infused groups. (B) Mean lesion area per section in the aortic root. $*P = 0.003$ for comparison of saline and AngII infusion within CCR2$^{+/+}$ groups; $\dagger P < 0.001$ for comparisons of CCR2$^{+/+}$ and CCR2$^{-/-}$ within AngII-infused groups. $P < 0.005$ for comparisons of saline and AngII infusions within genotypes. (C) Aneurysm size was measured as width of abdominal aorta. $*P < 0.001$ for comparisons of saline and AngII infusions; $\#P < 0.001$ for comparisons of CCR2$^{+/+}$ and CCR2$^{-/-}$ within AngII-infused groups.

hypertrophy, collagen deposition and macrophage accumulation were not seen in ascending aortic tissue sections from saline-infused mice (Figures 3A, 3D and 3G respectively). Tissue sections from ascending aortas of AngII-infused mice exhibited hypertrophy of the intra-lamellar spaces. The intra-lamellar spaces on the adventitial aspect of vessels exhibited the most extensive hypertrophy, collagen deposition and macrophage accumulation (Figures 3B, 3C, 3E, 3F, 3H and 3I). Areas with medial changes were not associated with the presence of atherosclerosis in adjacent intima. Macrophage accumulation was predominantly in areas that had pronounced hypertrophy and extracellular matrix disruption. Unlike the effects we observed previously during AngII infusion in osteopetrotic mice [27], we did not observe the presence of erythrocytes between elastin layers with ascending aortic pathology. At the anterior aspect of aortic arches, there was frequent destruction of elastin fibres, and the integrity of the artery was largely maintained by collagen fibres in the adventitia (Figures 3C, 3F and 3I). Unlike AAAs [9], we did not detect the presence of T-lymphocytes in ascending aortic aneurysms (results not shown). We were also unable to detect immunostaining on sections of ascending aortic aneurysms that was specific for either CCR2 or MCP-1 using multiple antibodies.

Whole-body CCR2 deficiency attenuated AngII-induced pathological changes in the ascending aorta

With the exception of the anterior region of ascending aortas, AngII infusion increased aortic wall thickness, which presumably accounted for the increased vessel opacity. Aortic wall thickness was measured from the inner to outer elastic lamina. AngII infusion increased aortic wall thickness (Figure 4) that was ablated in CCR2-deficient mice ($P = 0.007$ when compared with AngII-infused CCR2$^{+/+}$ mice). Increased vessel opacity did not result from augmented atherosclerosis, since atherosclerosis was not uniformly distributed throughout the ascending aorta. There was minimal change in the aortic width in the descending aortic region of AngII-infused mice beyond the branch of the subclavian artery.

The severity of dilatation in the ascending aorta was determined by measuring luminal diameters and intimal areas. Saline-infused apoE$^{-/-}$ mice had ascending aortic diameters of 0.81 ± 0.03 mm, while AngII infusion increased diameters to 1.22 ± 0.07 mm (Figure 5A; $P < 0.05$). CCR2 deficiency decreased luminal diameters of ascending aortas during AngII infusion to 0.9 ± 0.02 mm ($P < 0.05$). The en face intimal areas of aortic arches were measured from the ascending aorta to 3 mm proximal from the left subclavian branch. Ascending aortic areas of saline-infused genotypes were between 14 and 15 mm^2, while AngII infusion in CCR2$^{+/+}$ mice increased the area to 24.6 ± 0.8 mm^2 (Figure 5B; $P < 0.001$). CCR2 deficiency reduced ascending aortic intima areas to 18.5 ± 0.5 mm^2 ($P < 0.001$).

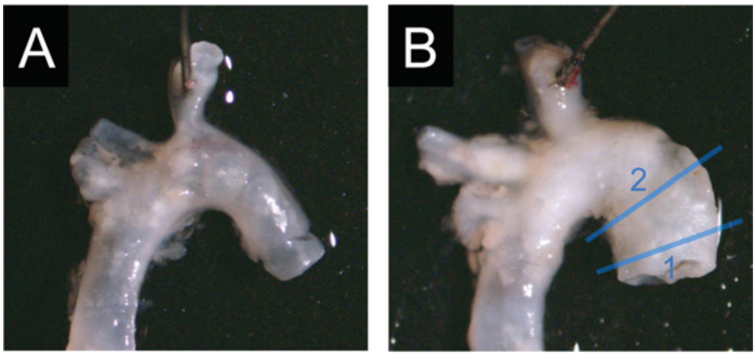

Figure 2 **AngII infusion induces ascending aortic aneurysms**

Examples of aortic arches from apoE$^{-/-}$ mice infused with either saline (A) or AngII (B). The blue lines designated as 1 and 2 represent the areas sectioned and represented in Figure 3.

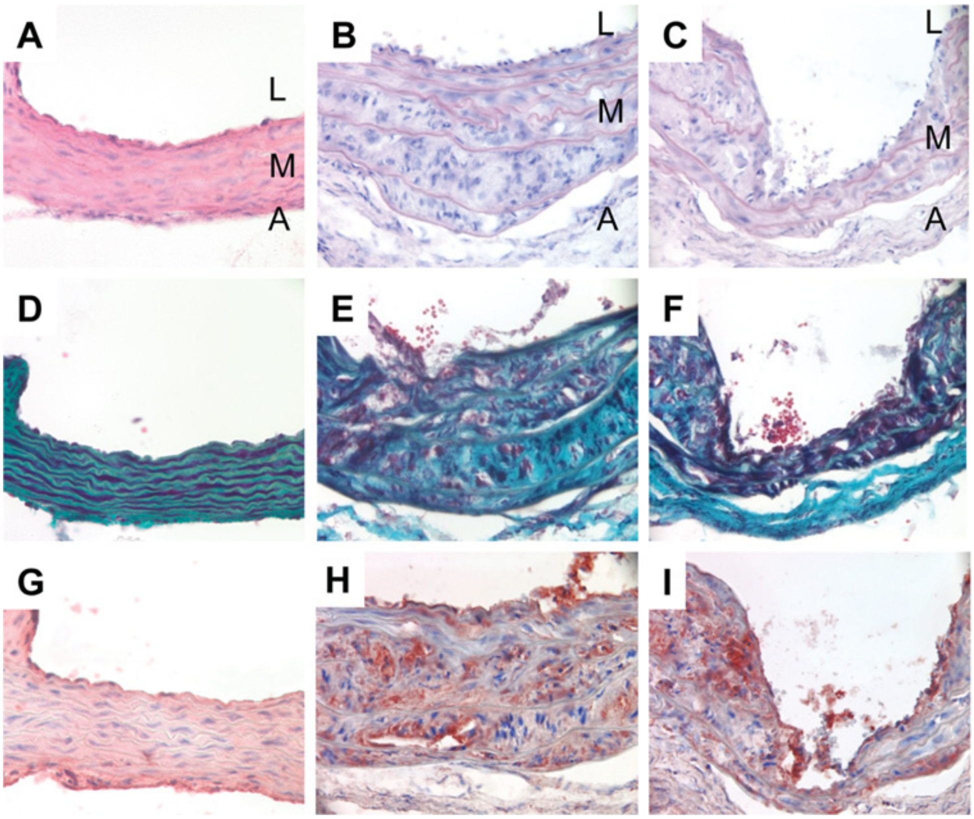

Figure 3 **Histological and cellular characteristics of AngII-induced ascending aortic aneurysms**

(A), (D) and (G) are harvested from a saline-infused CCR2$^{+/+}$ mouse. Sections derived from the area defined by line 1 in Figure 2 are shown in (B), (E) and (H), and the anterior portion of the region in line 2 is represented in (C), (F) and (I). Sections were stained with haematoxylin and eosin (A–C), Gomori trichrome (D–F) and immunostained for macrophages (positive cells are red; G–I). The orientation of aortas is described in (A), (B) and (C) by L, lumen; M, media; A, adventitia. Magnification, × 400.

DISCUSSION

CCR2 is the only known functional receptor for MCP-1 in macrophage migration [18,28]. Deficiency of this receptor on bone-marrow-derived cells reduces atherosclerosis in hypercholesterolaemic mice [19,29,30]. One study has also demonstrated that CCR2 deficiency in chimaeric mice developed by bone marrow transplantation attenuated AngII-induced atherosclerosis and AAAs [5]. However, bone marrow

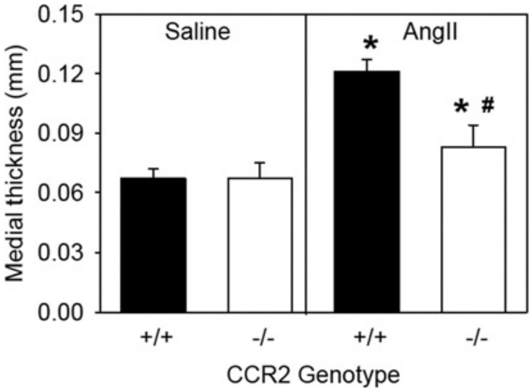

Figure 4 **Deletion of CCR2 attenuates AngII-induced ascending aorta medial expansion**

Ascending aortic thickness was measured from images ($n = 3$–6 mice/group) using image analysis software. Histobars represent groups, and error bars represent S.E.M.. $^*P = 0.002$ for comparison of saline and AngII. $\#P = 0.007$ for comparison of $CCR2^{+/+}$ and $CCR2^{-/-}$ within AngII infusion.

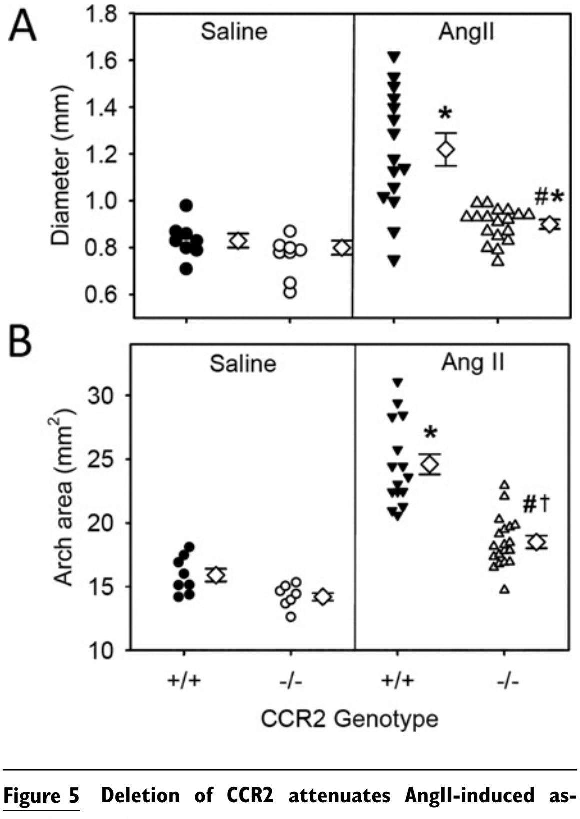

Figure 5 **Deletion of CCR2 attenuates AngII-induced ascending aortic aneurysms**

Ascending aortic diameters (A) and intimal areas (B) of arch regions were measured from images of pinned aortas using image analysis software. Circles and triangles represent individual mice, diamonds are means and bars represent S.E.M. (A) $^*P < 0.001$ for comparison of AngII and saline infusion in both genotypes. $\#P < 0.001$ for comparison of genotype within AngII infusion groups. (B) $^*P < 0.001$ for comparison of AngII and saline infusion in $CCR2^{+/+}$ mice. $\#P < 0.001$ for comparison of $CCR2^{+/+}$ and $CCR2^{-/-}$ mice infused with AngII. $\dagger P < 0.001$ for comparison of saline and AngII infusion in $CCR2^{-/-}$ mice.

transplantation can lead to differences in the development of atherosclerosis and AAAs [25,31]. No previous studies have demonstrated the effects of whole body CCR2 deficiency on the development of AngII-induced vascular diseases. In the present study, it was demonstrated that whole-body CCR2 deficiency attenuated both AngII-induced atherosclerosis and AAAs.

Ascending aortic dilatation, with subsequent rupture, is the most life-threatening manifestation of Marfan's disease [32]. Previous studies in mice expressing a fibrillin-1 mutant have demonstrated a similar pathology to that generated during AngII infusion in the present study [12]. These similarities include dilatation that is localized to the ascending aorta, medial thickening and elastin fragmentation. Thus, the results of the present study are complementary to the previously described effect of losartan in fibrillin-1 mutant mice being due to antagonism of the effects of AngII.

Many publications have demonstrated that AngII infusion leads to aneurysmal formation in the abdominal aorta [1,4,33–36]. In the present study, we demonstrated that AngII infusions led to large lumen dilatations of the ascending aorta, as has been described previously for the abdominal aorta [37]. However, there are marked differences in the pathological characteristics of aneurysmal formation in these two regions. AAAs form rapidly as a consequence of a highly localized transmural elastin disruption that colocalizes with focal medial macrophage accumulation [9]. Adventitial thrombi form adjacent to medial ruptures that promote an intense inflammatory response with the recruitment of macrophages. During subsequent remodelling, aneurysmal tissue contains abundant macrophages, with the accumulation of both T- and B-lymphocytes [9]. In contrast, ascending aortas exhibit extensive elastin fragmentation following infusion of AngII, but not transmural as seen in AAAs. In addition, the distance between the elastin layers progressively increases towards the adventitial side of ascending aortas. Regions of greatest elastin disruption and intralaminar expansion were associated with macrophage accumulation. Furthermore, unlike AAAs in which macrophages have been detected at focal sites in the aortic media at an early phase of the disease, AngII promoted macrophage accumulation throughout the medial layers of ascending aortas. At the anterior aspect of ascending aortas, a different pathology was observed in which there was a focal thinning compared with the surrounding hypertrophic areas. Furthermore, we did not detect thrombotic material at this anterior region. Thus, the highly contrasting pathologies between the aneurysms in abdominal aortas compared with ascending aortas are indicative of different mechanisms by which AngII generates these diseases.

AngII-induced pathology in ascending aortas extended proximal to the subclavian artery, while the descending portion of the thoracic aorta was spared. This localization strongly resembles the distribution of dilatation observed in mice expressing a mutation of fibrillin-1 [12]. The basis for specificity of the dilatation is presumably due to regional differences that exist throughout the aorta. The aorta has considerable functional diversity of smooth muscle cells that may be based on developmental origin [31]. Using mice expressing Cre under the control of Wnt1 in combination with a floxed Rosa26 reporter, it has been demonstrated that the region from the ascending aorta to just distal of the subclavian artery are populated by smooth muscle cells of neural crest origin [38]. This distribution is strikingly similar to the region of ascending aortic aneurysms promoted by AngII that are shown in Figure 2. It remains to be determined what property of these cells enable AngII to promote this localized pathology.

Previous studies using fibrillin-1 mutant transgenic mice demonstrated that while losartan and propranolol administration resulted in comparable haemodynamic effects, only losartan administration reduced ascending aortic aneurysms [12.] Thus, blood pressure did not appear to contribute to aortic aneurysms in this mouse model of Marfan's syndrome. In the present study, the infusion of $1000\,\mathrm{ng} \cdot \mathrm{kg}^{-1}$ of body weight $\cdot\,\mathrm{min}^{-1}$ of AngII was associated with an increase in systolic blood pressure. However, the AngII-induced increases in systolic blood pressure were not influenced by whole-body CCR2 deficiency, despite the attenuated size of the ascending aortic aneurysms. Thus, in a similar manner to previously described for AngII-induced atherosclerosis [2] and AAAs [39], the increase in blood pressure from AngII infusion is not responsible for the development of ascending aortic aneurysms.

Ascending aortas from AngII-infused mice had considerable accumulation of macrophages throughout the media that have a preponderance on the adventitial side of the vessel. Infusion of AngII into C57BL/6 mice has previously been shown to promote macrophage accumulation predominantly in the adventitia [17]. However, that study was performed in the descending thoracic aorta, which did not exhibit dilatation. Thus, CCR2 may be the major stimulus for the recruitment of macrophages to the aortic adventitia. Therefore, the combined results of Bush et al. [17] and the present study demonstrate that another mediator contributes to migration of macrophages from the adventitia into the media. The identification of this putative mediator is unknown.

It is of interest that AngII infusion generates three distinct vascular pathologies within the aorta that have specific locations and pathological characteristics. A unified finding in the AngII-induced vascular diseases was macrophage accumulation. However, even within this unified finding, there are many interesting differences. For AngII-induced atherosclerosis, macrophages are only recruited to the intima and are rarely present in the media. In AngII-induced AAAs, there are initial small focal regions of macrophage accumulation in the media. In contrast, we describe macrophage accumulation in AngII-induced ascending aortic aneurysms occurring throughout the circumference of the artery and predominantly on the adventitial side of the aorta. It will be a fascinating challenge to determine why CCR2 deficiency attenuates macrophage accumulation in these aortic regions.

In summary, the present study is the first description of AngII infusion leading to a highly localized pathology of dilatation and medial remodelling and destruction that is localized to the ascending aortic region. A role of AngII in ascending aortic aneurysms of Marfan's patients is consistent with the benefits of ACE (angiotensin-converting enzyme) inhibition on aortic dilatation in these patients [40,41]. Further evidence will be derived from a currently ongoing clinical trial that is assessing the effects of losartan in Marfan's patients [42]. The pathology of AngII-induced ascending aortic aneurysms is distinct from that described previously for AAAs and will require further study to fully elucidate the basis for the disparate pathological characteristics of aneurysms that are localized to ascending compared with abdominal aortic regions.

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