

A CCR2/CCR5 Antagonist Attenuates an Increase in Angiotensin II-Induced CD11b⁺ Monocytes from Atherogenic ApoE^{-/-} Mice

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Abstract

Objective Monocyte infiltration into the vessel wall, a process primarily mediated by the interaction between monocyte chemoattractant protein-1 (MCP-1) and its receptor, CCR2, is a key step in atherogenesis. Angiotensin II (Ang II) enhances this monocyte infiltration by increasing the endothelial binding integrin, CD11b. However, the modulation of the Ang II-induced CD11b expression in monocytes is not clear. The aim of this study was to determine if MCP-1/MCP-2 receptor (CCR2) interaction regulates monocyte CD11b expression after 7 days of Ang II infusion.

Methods and results In ApoE^{-/-} mice continuous subcutaneous infusion of Ang II (0.75 mg/kg/day) for 7 days significantly increased CD11b expression in circulating monocytes as measured by flow cytometry. CD11b expression in ApoE^{-/-} was increased from 135±9 to 176±12 mean fluorescent intensity (MFI), control and Ang II-treated, respectively while in C57B/J wildtype mice CD11b increased from 128±13 to 174±8 MFI, control and Ang II-treated, respectively. Interestingly, co-infusion of either

MCP-1 neutralizing antibody (25 µg/kg/day) or a CCR2 antagonist (500 µg/kg/day) with Ang II for 7 days effectively inhibited monocyte CD11b expression and this inhibition was accompanied by a down-regulated vascular infiltration of Mac-2 positive monocyte-derived macrophages.

Conclusion Our data in the atherogenic ApoE^{-/-} mouse demonstrates that the Ang II induced increase in both monocytic CD11b integrin expression and monocyte vascular infiltration occurs early in atherogenesis. These Ang II-induced monocytic changes are in part regulated through the MCP-1/CCR2 interaction.

Key words Angiotensin II · Integrin · CD11b · CCR2 · Monocyte chemoattractant protein-1 (MCP-1) · ApoE^{-/-} mouse · Macrophage · Atherosclerosis

Monocyte infiltration into the vessel wall and its further differentiation into foam cell are in the center of atherogenesis [1–10]. The key role of the monocyte chemoattractant protein-1 (MCP-1)/CCR2 system in mediating monocyte migration in atherosclerosis and other inflammatory processes is well-established [11–14]. Up-regulation of MCP-1 in the vessel wall can be induced by physical factors like fluid shear stress and chemical factors including angiotensin II (Ang II) [5, 15–28]. Previous studies have confirmed that MCP-1 induces phosphorylation of CCR2, JAK2, and Stat3, as well as CD11b and CCR2 surface expression in cultured monocytic cell lines [29]. Furthermore, a new CCR2 antagonist, inhibited both MCP-1 induced CCR2 phosphorylation and CD11b up-regulation in vitro [30]. We reasoned that the MCP-1-induced in vitro alterations in monocyte CD11b upregulation might also occur in vivo. Further, this upregulated CD11b expression in circulating monocytes might be a marker reflecting anti-monocyte infiltration mediated through the MCP-1/CCR2

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pathway. The possible role of the MCP-1 receptor, CCR2, for Ang II enhancement of monocyte integrin expression such as CD11b *in vivo* remains unexplored.

To test the hypothesis that the level of vascular macrophage infiltration and CD11b expression on circulating monocytes is regulated by the MCP-1/CCR2 pathway, we have adopted a model of Ang II-enhanced monocyte infiltration in the atherogenic-prone apo E^{-/-} mouse vascular wall. These data supported our hypothesis that both monocyte CD11b expression and vascular infiltration enhanced by Ang II is dependent upon CCR2 and MCP-1. Our results also indicate the correlation between increased levels of CD11b expression on circulating monocytes and the increased numbers of monocyte vascular infiltration in early atherogenesis.

Methods

Reagents and antibodies Human angiotensin II was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest purity available. The rat antibodies for mouse CD11b FITC and mouse scavenger receptor PE(CD204) were obtained from Serotec (Raleigh, NC). The rat antibody for mouse CD16/CD32 (Fc Block) was purchased from Becton Dickinson Pharmingen (San Jose, CA). Mouse isotype controls for IgG FITC and IgG PE were obtained from Serotec. For CCR2 determination, the mouse Fluorokine kit which included mouse MCP-1 (JE) conjugated to biotin and avidin FITC from R&D Systems was used. Alzet mini-osmotic pumps were obtained from Alza Corp. (model 1002, Palo Alto, CA). The CCR2/CCR5 antagonist, a published compound from Takeda Chemical Industries, Ltd. (Osaka, Japan) [30], which was prepared by the Pfizer Inc. Chemistry Department and an antibody toward the mouse MCP-1 protein (Becton Dickinson Pharmingen San Jose, CA) were utilized for the attenuation of monocyte CD11b expression and infiltration into the aortic vessel wall. Purified anti-mouse MAC-2 monoclonal antibody (Cedarlane Laboratories LTD Hornby, Ontario, Canada) was used for immunohistochemical analysis of aortic wall monocyte/macrophage infiltration.

The animal model The animal handling and imaging procedures were approved by the Institutional Animal Care and Use Committee. A total of 41 ApoE^{-/-} and 27 C57B/J mice were used in this study. Sterile micro-osmotic pumps containing Ang II, a CCR2 antagonist, or anti-MCP-1 antibody were implanted subcutaneously into male apoE^{-/-} mice (Charles River laboratories, MA) that had been anesthetized with Telazol (30 mg/kg, Fort Dodge Laboratories, Inc. Fort Dodge, Iowa). The delivery rates for each chemical were: Ang II, 0.75 mg/kg/day; CCR2 antagonist compound (Takeda), 500 µg/kg/day; anti-MCP-1, 25 µg/kg/day.

Animals were allowed to recover and maintained for periods ranging from 1 to 7 days. blood sample was drawn through a cardiac puncture.

Determination of blood pressure Systolic blood pressure and derived pulse rate were obtained prior to and after the 7 day Ang II infusion in conditioned, conscious ApoE^{-/-} and C57B/J wild type mice using a tail-cuff apparatus and PC-based data acquisition system (Visitech BP-2000, Apex, NC). A minimum of five measurements were obtained from each mouse.

Blood samples Mouse whole bloods were collected in a heparin-rinsed syringes by cardiac puncture at the end of each experiment. Blood samples were used within 2 h of collection to avoid any artifactual upregulation of monocyte CD11b *ex vivo* [31].

Flow cytometry To determine CD11b expression, 100 µL blood aliquots were directly prepared for cell surface staining of CD11b and other appropriate cell surface markers. For mouse whole blood cell staining, 1 µL of mouse Fc Block (anti-CD16/CD32) was first added and incubated for 5 min at 4°C. At this point, saturating concentrations (10 µL) of anti-mouse CD11b FITC and anti-mouse scavenger receptor (CD204) PE antibodies were added and incubated for 30 min at 4°C. Lysing of red blood cells was accomplished by adding 2 mL of FACSlysing Buffer (Becton Dickinson San Jose, CA), gentle vortexing and then incubating for 10 min at room temperature in the dark. After red blood cell lysing, centrifugation at 1,100 rpm at 4°C for 5 min pelleted the stained leukocytes. Two more wash steps were done with dPBS-wash and the cells were then resuspended in 250 µL of freshly prepared 1% formaldehyde buffer until ready for flow cytometric analysis. For determining mouse CCR2 expression (MCP-1 receptor), an indirect staining of mouse CCR2 receptors was done. Biotinylated mouse MCP-1 (28.6 µL) was first incubated with 100 µL of whole mouse blood for 60 min at 4°C. A secondary conjugate of avidin-FITC was added (28.6 µL) and incubated for 30 min at 4°C. The rest of the staining procedure is similar to that for CD11b staining described above. A FACSCalibur flow cytometer (Becton Dickinson San Jose, CA) was used for the acquisition of flow data and the CellQuest software used for data analysis. Cell populations were identified for data collection by their forward scatter (FSC) and side scatter (SSC) light profiles. For each sample, 30,000 total events were collected. Fluorescence intensity of immunostaining was quantitated by dot plot analysis. Fluorescent intensity was expressed as the geometric mean channel fluorescence in the appropriate quadrant of the dot plot. The data was then expressed as either percent of the isotype control or as percent change over the unstimulated controls.

Immunohistochemistry In order to evaluate macrophage infiltration into the wall of the thoracic aorta, an immunohistological staining procedure was performed. Briefly, thoracic aortic samples were placed in methanol-Carnoy fixative also referred to methacarn solution (methanol/chloroform/acetic acid 6:3:1) for 12–24 h, embedded in paraffin and cut into 5–6- μ m thick sections. Microscopic section staining consisted of arterial elastic fibers being labeled with Verhoeff's stain and the macrophages being stained by purified anti-mouse MAC-2 monoclonal antibody (Cedarlane Laboratories Ontario, Canada) followed by secondary staining via the Vector ABC kit (Vector, CA). The antibody dilutions that were used were as per the manufacturer's recommendation. The macrophages infiltration into the arterial intima and media (inside EEL) was determined using a $\times 20$ microscopic objective.

Statistical analysis

Data are expressed as mean \pm SEM. Comparison between vehicle-treated and various treated groups were analyzed by the student *T*-test method or one-way ANOVA with the multiple comparison of means. All statistical analyses were performed using the statistical program SAS JMP (SAS Institute Cary, NC). Values of $P < 0.05$ were considered statistically significant.

Results

Systolic blood pressure (SBP) effects of subcutaneously administered Ang II (0.75 mg/kg/day) over 6 days as measured via tail-cuff in control C57B/J and the atherogenic-prone mouse model, ApoE^{-/-}, are shown in Table 1. Ang II significantly increased SBP in both C57B/J wild type mice

Table 1 Effects of Ang II infusion on systolic blood pressure in Apo E^{-/-} or C57B/J wild type mice

Animal	Systolic Blood Pressure (mmHg)	
	- Ang II	+ Ang II
Apo E ^{-/-}	115 \pm 2 (n=12)	127 \pm 5* (n=9)
C57B1/6	121 \pm 4 (n=8)	131 \pm 3* (n=8)

Values are means \pm SEM.

* $p < 0.05$ Student's *t*-test; ApoE^{-/-} vs. C57B/J mice.

The subcutaneous infusion of Ang II was at a rate of 0.75 mg/kg/day. One group of mice for each strain was used for the control and Ang II treatment. The data are means \pm SEM. Statistical significance is denoted above each bar, no Ang II control vs 6 days after Ang II infusion. N size=8 control and 8 Ang II-treated C57B/J wild type mice with 12 control and 9 Ang II-treated Apo E^{-/-} mice.

(121 \pm 4 to 131 \pm 3 mmHg; $P < 0.05$) and ApoE^{-/-} mice (115 \pm 2 to 127 \pm 5 mmHg; $P < 0.05$). Heart rate for the wild type and the ApoE^{-/-} mice were unchanged by Ang II compared to their respective controls.

To ascertain the differential expression of the leukocyte adhesion integrin, CD11b, and the MCP-1 receptor, CCR2, fluorescence-activated cell sorting (FACS) analysis was performed on wild type and ApoE^{-/-} circulating monocytes. As shown in Fig. 1a, a representative FSC versus SSC dot plot is shown for mouse whole blood. The gated region is the representative of the circulating monocyte subpopulation and this gate was used in all subsequent FACS analysis for CD11b expression (Fig. 1b,d). The CD11b expression was without any CD204 expression (Fig. 2d) or modified low density lipoprotein receptor (i.e., scavenger receptor) which rules out any activation of circulating monocytes into macrophages. Additionally, the monocytes were evaluated for the MCP-1 receptor or CCR2 chemokine receptor expression via FACS analysis. As shown in Fig. 1c, the analysis involved the separation of unstained monocytes (M1 region) from the CCR2 positive monocytes (M2 region). The comparisons for both CD11b and CCR2 monocyte expressions involved the effects of Ang II treatment in both wild type and ApoE^{-/-} mice.

The concentration–response of the small molecule, non-peptide CCR2 antagonist (Fig. 2a) to inhibit the 10 nM MCP-1-induced CD11b expression in both the ApoE^{-/-} and C57 wildtype mice is shown in Fig. 2b. The CCR2 antagonist at 10 μ M significantly reduced the MCP-1-induced upregulation of CD11b back to untreated levels for both the ApoE^{-/-} and C57 wildtype mice.

Ang II significantly increased the expression of CD11b in both C57B/J and ApoE^{-/-} mice circulating monocytes after 7 days of treatment (Figs. 3a, vehicle). No significant differences with the level of CD11b expression were observed between the wild type and ApoE^{-/-} monocytes before or after the Ang II treatments. The expression of the MCP-1 receptor, CCR2, was significantly increased by the 7-day Ang II infusion but no change in CCR2 expression was observed in ApoE^{-/-} mice monocytes (Fig. 3b, vehicle).

In order to understand if the Ang II-induced increases in CD11b and CCR2 expressions on circulating monocytes were mediated by MCP-1 and the activation of the CCR2 receptor, a specific CCR2 antagonist or a neutralizing antibody to MCP-1 were co-infused with the Ang II treatment. As shown in Fig. 3a the Ang II-enhanced CD11b expression was significantly reduced by both the CCR2 receptor antagonist (500 μ g/kg/day) as well as the MCP-1 antibody (25 μ g/kg/day) in the ApoE^{-/-} mouse. The MCP-1 antibody, however, significantly increased the Ang II-enhanced CD11b expression in the wild type mouse. The neutralizing antibody for MCP-1 prevented the Ang II-enhancement on CCR2 expression in ApoE^{-/-} monocytes

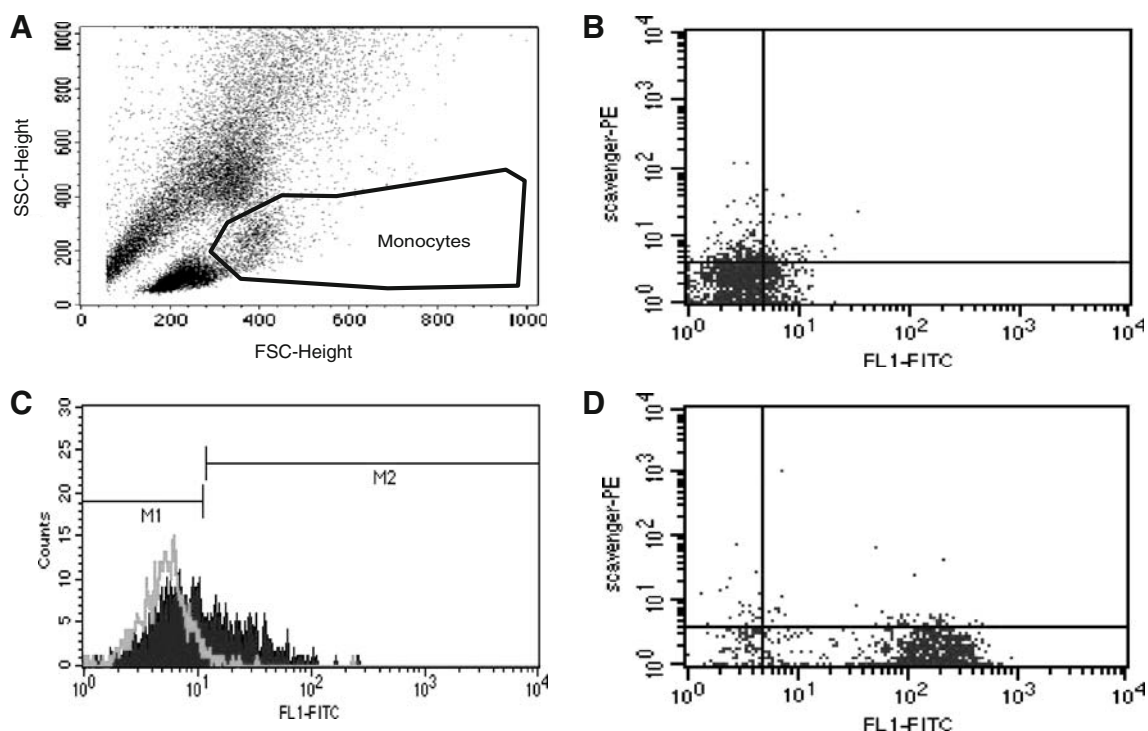


Fig. 1 Representative FACS analysis plots for circulating blood cell subpopulations with gated monocytes (FSC vs SSC; **a**), isotype control for unstained circulating monocytes (**b**), CD11b monocyte expression using rat anti-mouse CD11b FITC antibody conjugate (**d**)

and CCR2 monocyte expression using indirect method of measuring the level of mouse MCP-1 binding (**c**). All FACS analyses used the gated FSC/SSC plot for monocytes and 100 μ L of whole mouse blood was used for each determination

Fig. 2 Concentration–response of specific CCR2 receptor antagonist (**a**) on MCP-1-induced monocyte CD11b expression in C57B/J wild type and ApoE^{-/-} mice. **b** The data are means \pm SEM. * P <0.05, MCP-1 alone vs MCP-1+CCR2 antagonist. The specific MFI data is after the isotype control value was subtracted from each CD11b MFI value

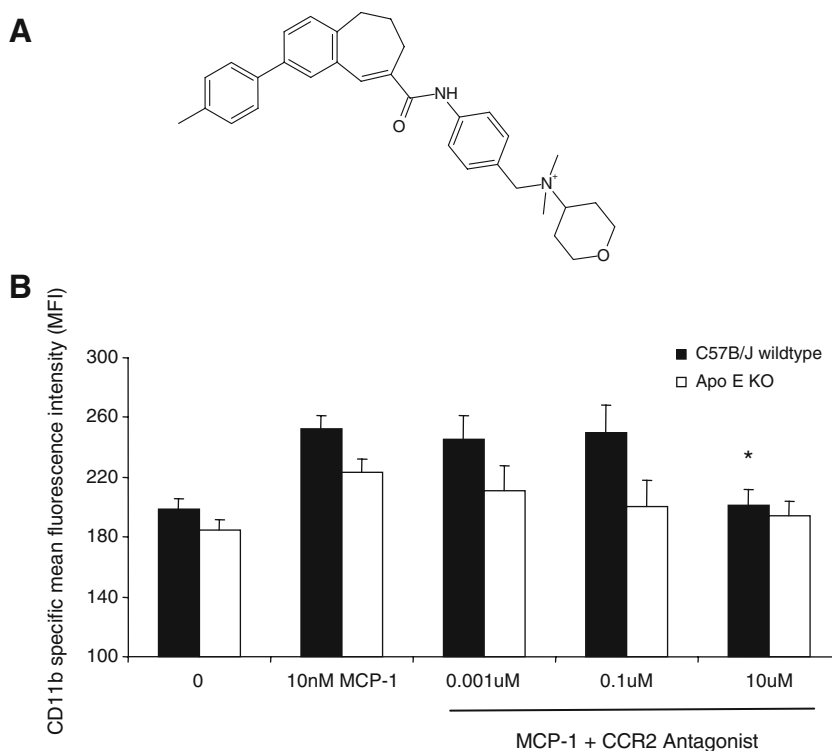
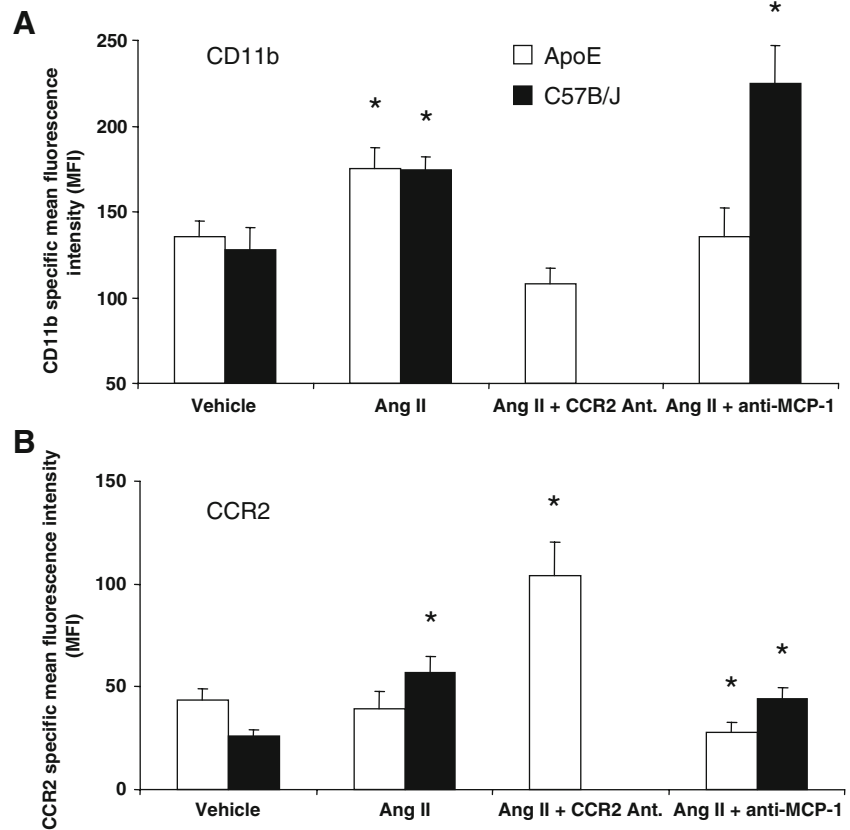


Fig. 3 Effects of the specific CCR2 receptor antagonist or neutralizing antibody to MCP-1 on Ang II-induced monocyte CD11b and CCR2 expression in C57B/J wild type and ApoE^{-/-} mice. **a** Specific monocyte CD11b mean fluorescence intensity (MFI) after 7 days Ang II infusion (0.75 mg/kg/day) with or without CCR2 antagonist (500 µg/kg/day) or MCP-1 antibody (25 µg/kg/day) in C57B/J and ApoE^{-/-} mice. **b** Monocyte CCR2 mean fluorescence intensity (MFI) after 7 days Ang II infusion (0.75 mg/kg/day) with or without CCR2 antagonist or MCP-1 antibody in C57B/J and ApoE^{-/-} mice. The data are means±SEM. **P*<0.05, vehicle vs Ang II alone; #*P*<0.05, Ang II alone vs Ang II + CCR2 antagonist or MCP-1 antibody. The specific MFI data is after the isotype control value was subtracted from each CD11b or CCR2 MFI value



but did not prevent the enhancement on monocytes in the Ang II-treated wild type mice (Fig. 3b).

To determine if the increase in CD11b expression on circulating monocytes translates into increased monocyte-macrophage infiltration into the aortic vascular wall, immunohistological analysis of thoracic aorta sections was accomplished after 7 days of Ang II infusion in the ApoE^{-/-} mice. Representative photomicrographs from vehicle and Ang II-treated mice show a significant increase in monocyte/macrophage infiltration, i.e., an increase in Mac-2 staining, (Fig. 4b) compared to the vehicle treated ApoE^{-/-} mice (Fig. 4a). As predicted from the observed decrease in the Ang II-induced monocyte CD11b expression, both the CCR2 antagonist (Fig. 4c) and the neutralizing MCP-1 antibody (Fig. 4d) also attenuated the monocyte/macrophage aortic wall infiltration compared to Ang II alone. Interestingly, there appeared to be no effect of either the CCR2 antagonist or the neutralizing MCP-1 antibody on the level of adventitial monocyte/macrophage infiltration induced by Ang II.

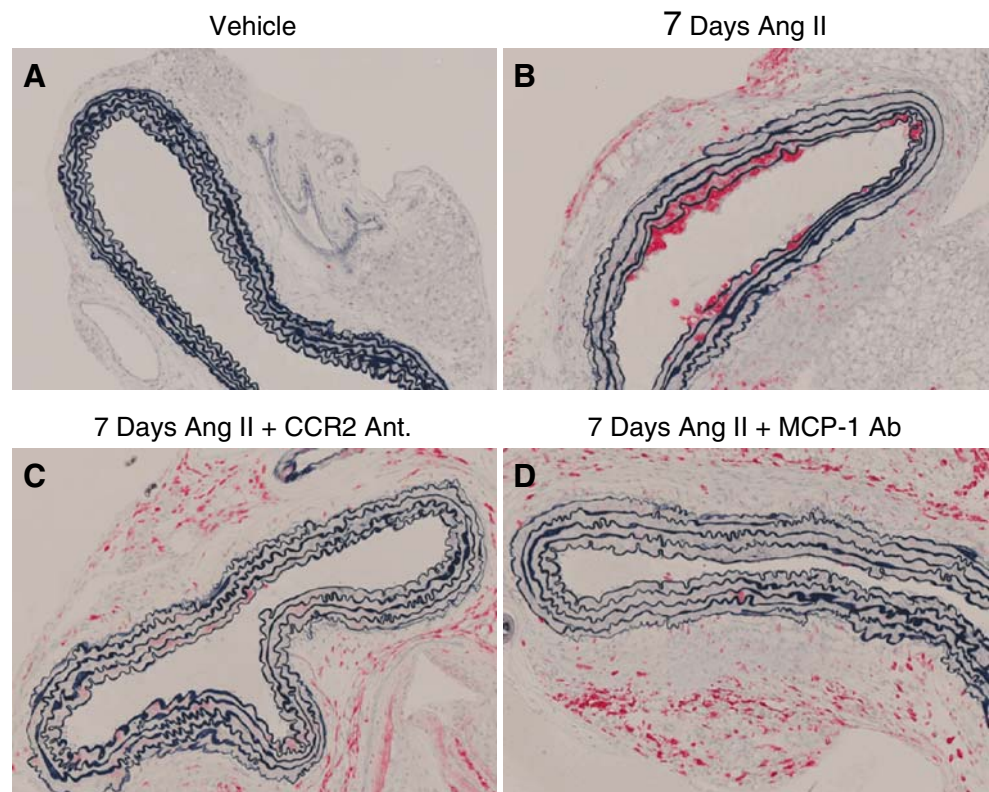
Discussion

We demonstrated that Ang II-induced monocyte infiltration into the vascular wall strongly correlates with increases in

both the adhesion surface integrin, CD11b, and the surface chemotactic receptor, CCR2, on the circulating monocyte in an atherogenic mouse model, namely the ApoE^{-/-} mouse. In addition, the monocytic expression of CD11b was upregulated early (i.e., 7 days) after Ang II induction and based on previously published data may represent one of the initiating events in the long process of Ang II-enhanced atherogenesis.

Monocyte infiltration into the vessel wall is of importance in different pathophysiological states such as atherosclerosis and hypertension where Ang II seems to play a crucial role and has been extensively investigated [1, 5, 11, 14, 15, 21, 28, 32–38]. However, even though the mechanism of Ang II induction of monocyte infiltration into the vessel wall is relatively understood, namely through the activation of the AT₁ receptor, the early inflammatory effects of this stimulation have not been clearly elucidated. The present paper shows, for the first time, that the upregulation of CD11b by Ang II actually is dependent on the activation of the chemoattractant receptor, CCR2. By using a CCR2 antagonist, the Ang II-induced expression of CD11b and the relating monocyte infiltration into the vascular wall was attenuated. Ang II has previously been shown to increase the vascular wall levels of the chemoattractant, MCP-1, which is the endogenous ligand of the CCR2 receptor [28, 39]. These data correlate with our

Fig. 4 Representative microphotographs of the effects of the specific CCR2 receptor antagonist and MCP-1 antibody on Ang II-induced monocyte infiltration into thoracic aorta sub-endothelial space. Cross sectional sections were prepared after 7 days of the SQ infusion of vehicle (a), 0.75 mg/kg/day Ang II plus vehicle (b), 0.75 mg/kg/day Ang II plus 500 μ g/kg/day CCR2 antagonist (c) and 0.75 mg/kg/day Ang II plus 25 μ g/kg/day MCP-1 antibody (d). Monocytes are depicted by the red-colored stain. Microscopic objective $\times 20$ magnification. Both the CCR2 antagonist and the MCP-1 antibody did not show any monocyte infiltrates in the aortic vascular wall but monocytes are present in adventitial tissue compared to the Ang II-infused mice



results in that Ang II upregulates CD11b expression which is, in part, dependent upon the activation of the MCP-1 receptor, possibly through increased MCP-1 levels. Interestingly, Capers et al. showed that the increased levels of MCP-1 due to Ang II infusion could occur within 7 days and confirms our timeline for Ang II-mediated CD11b upregulation. This increase in MCP-1 expression due to Ang II infusion was found to be hemodynamic force-related in that elevated blood pressure and mechanical strain on cultured vascular smooth muscle cells upregulated MCP-1 [39]. Since our paper also shows that an Ang II infusion modestly but significantly increased blood pressure in both the ApoE^{-/-} and wild type mice, an upregulation of Ang II-mediated CD11b expression on circulating monocytes is most likely supported by an increased level of vascular MCP-1. Even though our work did not measure MCP-1 expression, the increased Ang II-induced CD11b expression observed most likely is through increased MCP-1 levels because the observed levels of the MCP-1 receptor, CCR2, were not increased after 7 days of Ang II infusion (Fig. 3b). Further investigation into the signaling pathway between the angiotensin type I and the CCR2 receptors and their combined signaling paths on the monocyte CD11b expression still needs elucidation.

CD11b expression on circulating monocytes is shown to be upregulated, in part, by a relatively short infusion of Ang II (i.e., 7 days) in both wild type C57B/J and ApoE^{-/-} mice. To date, the mediation of monocyte adhesion molecule

regulation and function has been incomplete but the present data for the first time helps elucidate that Ang II can increase monocyte adhesion molecule expression, namely CD11b, not only through a direct angiotensin type I receptor activation as shown by other investigators, but also indirectly through the activation of the chemokine receptor, CCR2. Previous studies have shown that the regulation of CD11b on monocytes by Ang II is increased or unchanged. Treatment of hypercholesterolemic monkeys with the angiotensin type I receptor antagonist losartan for 15 weeks suppressed peripheral blood and bone marrow monocyte CD11b expression [40]. Circulating monocytes from hypertensive patients showed an increased monocyte adhesion to endothelial cells and an increased expression of CD11b. However, when these patients were treated with the angiotensin type 1 receptor antagonist, telmisartan, monocyte adhesion to endothelium decreased but the CD11b and other integrin expressions were unchanged or slightly increased [41]. The angiotensin type 1 receptor antagonist, losartan, for 8 weeks of treatment, caused no change in leukocyte CD11b and other adhesion molecule expressions in patients with CAD [23]. Ang II infusion has also been shown to promote a marked increase in the extent of atherosclerotic lesions and the number of macrophages present in the adventitial tissue underlying these lesions in young female ApoE^{-/-} mice and co-infusion of the angiotensin type I receptor antagonist, losartan, reduced both lesion extent and adventitial macrophages [42]. All

these data indicate that other mechanisms other than just angiotensin type I receptor activation appear to regulate the monocyte CD11b adhesion molecule expression and the level of monocytic adhesion in inflamed tissue. The CCR2 integrin receptor may be, in part, mediating the CD11b expression on circulating monocytes induced by Ang II. Co-infusion of an angiotensin type I receptor antagonist with the novel CCR2 antagonist may prove to ultimate controlling mediators of monocyte CD11b expression.

Monocytes have been shown to express more CCR2 chemokine receptors than any other chemokine receptor and upregulate CD11b preferentially to MCP-1 [43]. Since Ang II has been shown to upregulate the ligand for the CCR2 receptor, i.e., MCP-1 and that attenuating the CCR2 receptor with a CCR2 receptor antagonist, as shown in this paper, it is hypothesized that the upregulation of CD11b on circulating monocytes induced by Ang II is through the CCR2 receptor. Our study confirms the results of Bush et al. that CCR2-deficient mice had no increase in macrophage infiltration or vascular hypertrophy after 7 days of subcutaneous infusion of Ang II [44]. CCR2 antagonism, however, was shown in this paper not to totally normalize the circulating monocyte CD11b expression nor completely remove adventitial macrophages in the ApoE^{-/-} mice. Other monocyte adhesion molecule receptor activation induced via Ang II may be involved. However, based on additional results of this study, the involvement of CCR2 receptor activation in mediating the monocyte CD11b expression is further supported due to the attenuation of the CD11b expression by a neutralizing antibody to MCP-1 to a similar level as the CCR2 receptor antagonist.

In summary, this study has shown a link between the Ang II-induced CD11b expression on circulating monocytes and the activation of the integrin receptor, CCR2, in ApoE^{-/-} mice. This upregulation of this monocyte adhesion molecule appears to occur in early atherogenesis (i.e., 7 days of Ang II infusion) and these results appear to be the first account of this link. CCR2 antagonism may provide a novel therapeutic approach to reducing the effects of Ang II or any CCR2 receptor activator on atherosclerosis.

Conflict of interest The affiliation of all authors to the sponsor, Pfizer, Inc., is that all authors were former employees of Pfizer and now have either retired or are in new research positions. No conflict of interest has existed or exists now for this submitted work by any of the authors.

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