Macrophage Polarization by Angiotensin II-Type 1 Receptor Aggravates Renal Injury-Acceleration of Atherosclerosis

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Objective—Angiotensin II is a major determinant of atherosclerosis. Although macrophages are the most abundant cells in atherosclerotic plaques and express angiotensin II type 1 receptor (AT1), the pathophysiologic role of macrophage AT1 in atherogenesis remains uncertain. We examined the contribution of macrophage AT1 to accelerated atherosclerosis in an angiotensin II-responsive setting induced by uninephrectomy (UNx).

Methods and Results—AT1−/− or AT1+/+ marrow from apolipoprotein E deficient (apoE−/−) mice was transplanted into recipient apoE−/− mice with subsequent UNx or sham operation: apoE−/−/AT1+/+→apoE−/−+sham; apoE−/−/AT1++→apoE−/−+UNx; apoE−/−/AT1−/−→apoE−/−+sham; apoE−/−/AT1−/−→apoE−/−+UNx. No differences in body weight, blood pressure, lipid profile, and serum creatinine were observed between the 2 UNx groups. ApoE−/−/AT1+/+→apoE−/−+UNx had significantly more atherosclerosis (16907±21473 versus 116071±8180 μm², P<0.05). By contrast, loss of macrophage AT1 which reduced local AT1 expression, prevented any effect of UNx on atherosclerosis (77174±9947 versus 75714±1333 μm², P=NS). Although UNx did not affect total macrophage content in the atheroma, lesions in apoE−/−/AT1−/−→apoE−/−+UNx had fewer classically activated macrophage phenotype (M1) and more alternatively activated phenotype (M2). Further, UNx did not affect plaque necrosis or apoptosis in apoE−/−/AT1−/−→apoE−/−+UNx whereas it significantly increased both (by 2- and 6-fold, respectively) in apoE−/−/AT1++→apoE−/− mice. Instead, apoE−/−/AT1−/−→apoE−/− had 5-fold-increase in macrophage-associated apoptotic bodies, indicating enhanced efferocytosis. In vitro studies confirmed blunted susceptibility to apoptosis, especially in M2 macrophages, and a more efficient phagocytic function of AT1−/− macrophages versus AT1+/+.

Conclusion—AT1 receptor of bone marrow-derived macrophages worsens the extent and complexity of renal injury-induced atherosclerosis by shifting the macrophage phenotype to more M1 and less M2 through mechanisms that include increased apoptosis and impaired efferocytosis. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiotensin ii ■ atherosclerosis ■ kidney ■ macrophages

Experimental studies have established the proatherogenic effects of angiotensin II (AII).1-3 Conversely, ample experimental and clinical evidence suggests that antagonism of all actions by angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ARB) provides antiatherogenic benefit.3-7 The proatherogenic effects of AII and the antiatherogenic effects of angiotensin-converting enzyme inhibitors and ARB have been linked to modulation of macrophage functions, including chemotaxis, intimal recruitment, inflammation, neovascularization, and proteolysis.3,4,8,9 All can modulate macrophage-specific functions, as supported by observations that monocyte/macrophages express components of the angiotensin system, including the all type 1 receptor (AT1), and that cellular exposure to AII promotes lipid accumulation, migration, and cytokine production.4,8,9 Nonetheless, the specific contribution in vivo of the macrophage AT1 receptor to atherogenesis has been controversial, ranging from little influence observed in some studies10 to significant proatherogenic effects reported by others, especially in the setting of infusion of exogenous AII.11,12

The variable impact of macrophage AT1 on atherogenesis may reflect heterogeneity in macrophages, with multiple functions differently expressed during progression of atherosclerosis.13 The classically activated M1 macrophage phenotype, stimulated by lipopolysaccharide and interferon-γ, enhances proinflammatory cytokines, including tumor necrosis factor-α, C-C chemokine receptor type 7 (CCR7), inducible nitric oxide synthases (iNOS), interleukin (IL)-1β, and IL-6. The alternatively activated M2 macrophage phenotype, stimulated by IL-4 and IL-13, is linked to inflammation resolution, tissue repair, and endocytic clearance with activation of antiinflammatory cytokines, including arginase-1 and Ym-1. Such antiinflammatory and endocytic functions contribute to efferocytosis, the clearance of apoptotic cells by phagocytes.
which is an essential mechanism for vascular repair and remodeling. Modulation of macrophage phenotype transformation by AT1 was recently documented in a model of antiluminal basement membrane glomerulonephritis. In this study, macrophages infiltrating glomeruli were predominantly of M1 phenotype in untreated rats and of M2 phenotype in rats receiving ARB-treatment, which also caused attenuation of renal injury. We also found that ARB treatment skewed the intrarenal macrophage population from M1 to M2 phenotype in an obesity-related kidney injury model. Previously, we showed that reduction in renal mass dramatically exacerbates atherosclerosis via a mechanism that is clearly responsive to ARB. Because absolute and relative abundance of M1 and M2 macrophages determines extent and composition of the atherosclerotic plaque, we examined the impact of modulating the macrophage AT1 on renal injury-enhanced atherogenesis, focusing on the macrophage phenotype and the mechanisms that influence plaque architecture.

**Methods**

**Experimental Groups**
Female apoE⁻/⁻ mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) maintained on normal mouse chow (R5001; PIM Feeds, St. Louis, MO) served as recipients in bone marrow transplantation studies. All type 1a receptor (AT1a) deficient mice on a C57BL/6 background previously generated in our laboratory have been backcrossed more than 10 times with mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME) maintained on normal mouse chow (R5001; PIM Feeds, St. Louis, MO) served as recipients reconstituted with apoE⁻/⁻ mice.12 Others.13,14 NC). Mice were euthanized under phenobarbital anesthesia and perfused with PBS through the left ventricle. The heart with the proximal aorta was embedded in OCT and snap-frozen in liquid nitrogen. Ten-micrometer thick cryosections were cut from the proximal aorta beginning at the end of the aortic sinus with modifications specific for computer analysis.1,2,3 Cryosections were stained with Oil-Red-O to assess lipid deposition. Quantitative analysis of lesions was performed using Imaging System KS300 (Release 2.0; Kontron Elektronik GmbH, Poway, CA). To assess necrotic areas, cryosections were stained with Harris H&E (Sigma, St. Louis, MO). Both stained and acellular/anuclear areas in intimal lesions were included in quantitation of the total atherosclerotic lesions. Necrotic area was calculated as the ratio between a nuclear area and total lesion area as previously described.23,27,28 Quantitative analysis of lesions was performed using Imaging System KS300. The assessment was done without awareness of group assignment.

**Assessment of Macrophage Content and Phenotype, AT1 Receptor Expression in Atherosclerotic Lesions**
Serial, 5-μm thick cryosections of proximal aorta were fixed in acetone and incubated with monoclonal rat antibody to mouse macrophages (MOMA-2; Serotec, Raleigh, NC) to measure macrophage-positive area within atherosclerotic lesions as previously described.2,3,12,13 Rat anti-mouse CD68 (AbD Serotec, UK) and nuclear DAPI were used to stain macrophages. Rat anti-mouse CCR7 and iNOS (BD bioscience) were used to stain for M1 macrophage phenotype.29–31 Whereas rabbit anti-mouse Ym-1 (stemcell Technologies) or arginase 1 (BD bioscience) were used to stain for M2 macrophage phenotype.23,24 The percentage of M1 or M2 subtypes was determined as the ratio of positive cells for each phenotype marker to total CD68 positive cells. To assess expression of AT1 receptor, immunofluorescence was performed on OCT-embedded aorta sections (Supplement, available online at http://atvb.ahajournals.org).34

**Assessment of Macrophage Apoptosis and Efferocytosis in Atherosclerotic Lesions**
Apoptotic cells in lesions were detected by TUNEL (Tdt-mediated dUTP nick end labeling) using the in situ cell death detection kit (Roche, Indianapolis, IN). Nuclei were counterstained with rabbit antimacrophage cytoplasm antibody (Accurate Chemical and Scientific), goat anti-rabbit biotinylated conjugated secondary antibody, and Alexa Fluor 488 (Molecular Probes).35 Macrophage efferocytosis was evaluated by colocalization of apoptotic cells with intact macrophages.25,35 In situ quantitation of free versus macrophage-associated apoptotic cells in individual lesion sections was performed as described by us and others. Increased ratio of free to macrophage-associated apoptotic cells within the lesion represents inefficient efferocytosis.

**Assessment of Apoptosis, Efferocytosis, and Biomediator Activity In Vitro**
Thioglycollate-elicited peritoneal macrophages from apoE⁻/⁻/AT1⁻/⁻ and apoE⁻/⁻/AT1⁻/⁺ mice were seeded at 1.0×10⁶ cells/well in 2-well chamber slides. After overnight incubation in DMEM with 1% fetal bovine serum, macrophages were reacted with 50 ng/mL lipopolysaccharide for 24 hours. Apoptotic cells were detected by TUNEL using the in situ cell death detection kit (Roche) according to the manufacturer’s instruction. TUNEL positive cells versus total cells were quantitated in triplicate chamber slide wells.
assessing at least 10 fields per well. Proand antiapoptotic markers were assessed in apoE<sup>-/-</sup>/AT1<sup>--</sup> and apoE<sup>-/-</sup>/AT1<sup>+/+</sup> macrophages seeded at 0.5×10<sup>6</sup> cells/well and exposed to lipopolysaccharide (50 ng/mL) or IL-13 (10 ng/mL) for 6 hours. Macrophage total mRNA was extracted and gene expression was assessed by real time PCR with probes for bcl-2 (Mm00477631_m1), caspase-3 (Mm01195085_m1) and 18S rRNA obtained from Applied Biosystems.

To assess efferocytosis, peritoneal macrophages were seeded in 100-mm plates at 2×10<sup>6</sup> in DMEM, and were added to the lesion area of apoE<sup>-/-</sup> wells assessing at least 10 fields per well. Visualization of engulfed apoptotic cells was done with fluorescence microscopy. CFDA-SE cell tracer positive phagocytes were seeded macrophages. After 2 hours incubation, the seeded macrophages were vigorously washed with PBS to remove noningested apoptotic cells, fixed in 4% paraformaldehyde, and counterstained with DAPI for visualization of cell nuclei (Vector Labs, Burlingame, CA). Visualization of engulfed apoptotic cells was done with fluorescence microscopy. CFDA-SE cell tracer positive phagocytes versus total phagocytes were quantitated in triplicate chamber slide wells assessing at least 10 fields per well.

To assess biomediator activity, we assessed peritoneal macrophages with intact or deficient AT1. Macrophage total RNA was extracted and gene expression was assessed by real time PCR with probes for bcl-2 (Mm00477631_m1), caspase-3 (Mm01309900_m1), tumor necrosis factor-α and 18S rRNA (Hs99999901_m1) were obtained from Applied Biosystems.

**Statistical Analysis**

Results are expressed as means±SEM. Statistical difference was assessed by a single-factor ANOVA followed by Tukey-Kramer’s HSD. P<0.05 was considered to be significant.

**Results**

**Systemic Parameters**

Table 1 shows the systemic parameters at time of sacrifice. There were no differences in body weight or systolic BP among the groups. Uninephrectomy increased serum creatinine and total cholesterol levels in both apoE<sup>-/-</sup>/AT1<sup>++</sup> and apoE<sup>-/-</sup>/AT1<sup>-/-</sup> mice. In agreement with previous reports,3,23 the intervention did not affect serum triglyceride levels.

**Atherosclerotic Lesions**

Uninephrectomy significantly increased atherosclerosis in mice reconstituted with AT1<sup>++</sup> marrow (Figure 1). The cross-sectional lesion area in the aortic sinus was 16907±21473 μm<sup>2</sup> in apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + UNx mice and 116071±8180 μm<sup>2</sup> in apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + sham mice (P<0.05). By contrast, UNx caused no increase in mice reconstituted with AT1<sup>-/-</sup> marrow (77174±9547 μm<sup>2</sup> in apoE<sup>-/-</sup>/AT1<sup>-/-</sup>→apoE<sup>-/-</sup> + sham versus 75714±1133 μm<sup>2</sup> in apoE<sup>-/-</sup>/AT1<sup>-/-</sup>→apoE<sup>-/-</sup> + UNx). Interestingly, reconstitution with AT1<sup>-/-</sup> cells reduced progression of atherosclerosis even in mice with intact kidneys. As shown in Figure 1, cross-sectional lesion area of apoE<sup>-/-</sup>/AT1<sup>-/-</sup>→apoE<sup>-/-</sup> + sham was 35% less than in apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + sham. AT1 immunostaining within the atherosclerotic plaque was reduced in atherosclerotic lesions of uninephrectomized mice repleted with AT1-deficient marrow (Supplemental Figure, available online at http://atvb.ahajournals.org). Notably, AT1 expression in the vascular media was not reduced in mice repleted with AT1-deficient marrow.

**Macrophage Phenotype in Atherosclerotic Lesions**

Macrophage-positive area was not significantly different among groups (apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + sham, 73999±7988 μm<sup>2</sup>; apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + UNx: 88644±6660 μm<sup>2</sup>; apoE<sup>-/-</sup>/AT1<sup>-/-</sup>→apoE<sup>-/-</sup> + sham: 66953±8079 μm<sup>2</sup>; apoE<sup>-/-</sup>/AT1<sup>-/-</sup>→apoE<sup>-/-</sup> + UNx: 78390±6032 μm<sup>2</sup>). Nevertheless, macrophage phenotypes were altered within the atherosclerotic lesions. In particular, in mice reconstituted with AT1<sup>++</sup> bone marrow, UNx significantly increased macrophages with markers of the M1 phenotype, including CCR7 (63.6±2.8% versus 53.0±2.3%, P<0.05, Figure 2A) and iNOS (80.3±1.7% versus 31.4±3.7%, P<0.05, Figure 2B). Lesions of apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + UNx also had fewer cells with markers of the M2 phenotype, including Ym-1 (38.7±3.5 versus 64.0±4.2%, P<0.05, Figure 2C) and Arg1 (48.9±3.3 versus 65.6±4.2%, P<0.05, Figure 2D) compared with apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + sham. By contrast, in mice reconstituted with AT1<sup>-/-</sup> cells, UNx had no effect on CCR7 expression, and only modestly increased the number of iNOS positive cells (Figure 2B).

**Characteristics of Atherosclerotic Lesions**

UNx dramatically increased apoptotic cells in lesions of apoE<sup>-/-</sup>/AT1<sup>++</sup>→apoE<sup>-/-</sup> + UNx (274.9±127.3 versus...
1750.0±236.0, P<0.05, Figure 3A). By contrast, UNx in mice reconstituted with AT1−/− bone marrow showed no change in the number of apoptotic cells (195.6±114.2 versus 423.6±231.9, p=NS, Figure 3A). Free versus macrophage-associated apoptotic cells (an index of efferocytosis) were significantly increased in lesions of apoE−/−/AT1+/+→apoE−/−+UNx versus apoE−/−/AT1+/+→apoE−/−+sham (1.71±0.4 versus 0.31±0.08, P<0.05, Figure 3B). By con-
trast, macrophage-associated apoptotic cell ratio was not different in lesions of apoE−/AT1−/−→apoE−/+UNx versus apoE−/AT1−/−→apoE−/+sham (0.56±0.16 versus 0.49±0.17, p=NS, Figure 3B).

Expression of biomarkers was significantly increased in peritoneal macrophages from apoE−/AT1−/−→apoE−/+UNx versus apoE−/AT1−/−→apoE−/+sham (IL-1β; 0.99±0.03 versus 0.43±0.10, P<0.05, TNF-α; 1.57±0.17 versus 0.68±0.05, P<0.05, iNOS; 0.27±0.04 versus 0.16±0.02, P<0.05). By contrast, expression of inflammatory biomarkers was not different in peritoneal macrophages from apoE−/AT1−/−→apoE−/+UNx versus apoE−/AT1−/−→apoE−/+sham (IL-1β; 0.41±0.08 versus 0.12±0.04, P=NS, TNF-α; 1.02±0.12 versus 0.64±0.15, P=NS, iNOS; 0.12±0.04 versus 0.08±0.01, P=NS).

In vitro experiments paralleled the in vivo impact of macrophage AT1 on apoptosis and efferocytosis following UNx. Peritoneal macrophages lacking AT1 were significantly less susceptible to apoptotic stimuli and showed more efficient efferocytosis compared with AT1+ macrophages (Figure 4A and B). In vitro studies confirmed both blunted susceptibility to apoptosis and a more efficient phagocytic function of AT1−/− macrophages. Thus, AT1−/− macrophages had 6.2±0.5% TUNEL-positive cells versus 12.4±1.0% in AT1+ macrophages (P<0.05, Figure 4A). AT1 also modulated gene expression of apoptotic markers. Macrophages lacking AT1 showed significantly less apoptosis and shifted the ratio of proapoptotic caspase 3 versus antiapoptotic bcl-2 (5.78±1.21 versus 9.24±2.43, P<0.05). Further, in AT1−/− macrophages showed 13.2±1.2% positive uptake for apoptotic cells versus 7.9±0.9% in AT1+ macrophages (P<0.05, Figure 4B).

Figure 5A illustrates clustering of apoptotic cells (both free and macrophage-associated) around anuclear (presumably necrotic) areas within an atherosclerotic lesion of apoE−/AT1−/−→apoE−/+UNx. These necrotic areas (Figure 5B) were significantly larger in apoE−/AT1−/−→apoE−/+UNx versus apoE−/AT1−/−→apoE−/+sham (20.18±2.29 versus 9.14±2.19, P<0.05, Figure 5B). In contrast, no such increase occurred when UNx was done in apoE−/AT1−/−→apoE−/− mice (7.42±1.54 versus 8.31±1.31, P=NS, Figure 5B).

**Discussion**

Here we report the novel observation that macrophage AT1 plays a pivotal role in the accelerated progression of atherosclerosis induced by renal damage in renal mass. The underlying mechanisms are independent of BP, serum lipids, or renal function. Instead, the data indicate that macrophage AT1 skews the distribution of the macrophage population within the atherosclerotic lesion toward the proinflammatory M1 phenotype.

Although macrophages are central in initiation and progression of atherosclerosis and possess components of the renin-angiotensin system, the specific role of macrophage AT1 receptors in the pathogenesis of atherosclerosis has not been
defined. The current study shows a key role for macrophage AT1 receptor in both development and progression of atherosclerosis in the AII-responsive setting of renal damage. The worsening of atherosclerosis caused by uninephrectomy in AT1-replete mice was completely abrogated in mice lacking the AT1 receptor in macrophages apoE−/−/AT1−/−→apoE−/−+UNx (Figure 1). This benefit was independent of BP, lipid levels, or renal function, although it is possible that systemic, but not specifically BP, effects are perturbed by UNx which in turn influence the local angiotensin activity within the plaque.35 Although macrophages are central to development of atherosclerosis, bone marrow transplantation reconstitutes more than monocyte/macrophages. Therefore, it is possible that AT1 on other leukocytes also modulates plaque development and progression. AT1 receptor is also expressed on resident vascular cells, including endothelial and smooth muscle cells, which have important roles in atherogenesis and demonstrate antitherapeutic response to systemic receptor antagonism in vivo and in cell culture.38 Thus, although both the resident vascular and infiltrating cells participate to atherogenesis, the relative contribution of AT1 on each of these cell types remains to be determined.

The current data complement previous findings of accelerated atherosclerosis in response to a sustained infusion of AII in normal mice reconstituted with AT1+/+ bone marrow, but not AT1−/−.12,39 Interestingly, previous reports show that AII infusion into mice globally deficient in AT1 reconstituted with AT1+/+ marrow amplified atherosclerosis and caused decreased collagen content, consistent with a more unstable lesion.22 Our studies also fit with the finding that hypertensive humans express high levels of dimeric AT1 in monocytes, a parameter that correlates with greater AII-dependent adhesion to endothelial cells.40,41 Increased expression of angiotensin converting enzyme in monocytes of hemodialysis patients correlates with cardiovascular events and mortality.42,43 and has been specifically linked to atherosclerotic vasculopathy.44 Taken together, these observations suggest that macrophage AT1 has an important role in advancing atherosclerosis caused by dyslipidemia and renal damage.

In addition, this study also reveals a direct role of macrophage AT1 in atherogenesis, even in the absence of administration of exogenous AII or reduced renal mass. In fact, even with intact kidneys, AT1 deficient mice had less atherosclerosis compared with AT1 replete mice (Figure 1). Previous studies concerning the impact of macrophage AT1 on atherogenesis reported variable effects.10,12,39 For example, reconstitution with AT1−/− bone marrow reduced atherosclerosis in apoE−/− mice,12 but it had no significant effects in LDLR−/− mice.10,39 It is possible that these differences reflect the more subtle lesions prevailing in LDLR−/− mice (versus apoE−/− mice) and the substantially shorter duration of followup (18–20 weeks in LDLR−/− mice versus 32 weeks in apoE−/− mice). The current observation reveals a significant impact of macrophage AT1 on atherogenesis over the long term (24 weeks). The findings complement our previous studies that pharmacological antagonism of angiotensin receptor at a time of established atherosclerosis (24 weeks) lessens progression and stabilizes the plaque.3 The
current data also suggest the importance of the AT1 receptor at the earliest stages of atherogenesis. Bone marrow transplantation was performed at 8 weeks, a time before substantial lipid deposition is seen. Thus, it appears that AT1 receptor modulates atherogenesis throughout the atherosclerotic process. These observations underscore the potential clinical benefit of angiotensin actions antagonism at different stages of atherosclerotic development.

The current study shows that renal injury modulates the M1/M2 phenotype and that the macrophage AT1 influences the phenotypic balance in the artery wall. The proinflammatory M1 is increased by UNx in AT1-replete mice, whereas the antiinflammatory M2 phenotype is decreased (Figure 2). On the other hand, in AT1 deficient mice, UNx had a markedly minor to no effect on M1 as determined by the iNOS and the CCR7 markers, respectively (Figure 2A and B). Moreover, UNx in AT1-deficient mice showed no decrease in the M2 phenotype (Figure 2C and D). These results suggest that both UNx and macrophage AT1 determine macrophage phenotype. Renal damage modulates M1, and in the face of macrophage AT1-deficiency, UNx did not completely preclude increasing the M1 phenotype. In contrast, macrophage AT1-deficiency completely prevented UNx-induced fall in the M2 macrophages, findings that appear pivotal to abrogating advancement of atherosclerosis. Indeed, gene expression of apoptotic factors in AT1-intact and -deficient peritoneal macrophages polarized to M1 or M2 phenotype revealed that cells deficient in AT1 had significantly greater expression of antiapoptotic bcl-2. Taken together, the results support the idea that reduced atherosclerosis in mice repleted with AT1-deficient marrow reflects not only decreased population of M1 and persistence of M2 macrophages in the atherosclerotic plaque, but also resistance of AT1-deficient M2 macrophages to apoptosis. In view of the central role of NF-κB in apoptosis, and the potent effect of lipopolysaccharide to activate this signaling pathway, it is possible that resistance of AT1R-deficient macrophages to apoptotic stimuli reflects variable activation in NF-κB in these cells. Interestingly, systemic antagonism of AT1 with ARB was recently shown in a rat model of antiglomerular basement membrane glomerulonephritis model to suppress renal M1 macrophages and increase M2 macrophages together with suppression in M1-promoting proinflammatory mediators and significant inhibition in M1 cell generation.15 Interestingly some macrophages bore both M1 and M2 markers, suggesting the possibility of local switching of macrophage phenotypes from M1 to M2 within the ARB-treated glomeruli. Our data suggests this redistribution macrophage phenotype is modulated, at least in part, by susceptibility to apoptosis; although local switching of cellular phenotype is also feasible. Previous, we showed that systemic inhibition of AT1 reduces macrophage infiltration and promotes compositional changes that stabilize the plaque.3,4 Current results support a key role for macrophage AT1 in such stabilization. Whereas apoptosis was significantly increased in AT1 replete mice with UNx, lesions of AT1−/− macrophage mice showed no such increase after UNx (Figure 3A). Our studies also find that renal damage impairs macrophage efferocytosis, evidenced by the observation of increased number of apoptotic cells not associated with macrophages (Figure 3B) that can further accelerate apoptosis and necrosis in the lesions in apoE−/−/AT1−/−→apoE−/−+UNx mice, but not apoE−/−/AT1−/−→apoE−/−+UNx. Furthermore, AT1−/− macrophages were less susceptible to in vitro apoptosis and showed more efficient capacity to engulf apoptotic cells (Figure 4). Together, these results suggest that macrophage AT1 is linked to impairment of the macrophage clean-up function induced by renal damage because efferocytosis in AT1−/− macrophages is maintained with in vivo UNx and in vitro.

Increased apoptosis and impairment in macrophage efferocytosis have been shown to promote necrosis in atherosclerotic lesions in LRP-1 deficient mice27 and in mice with selective deficiency in macrophage p38α MAPK, a cellular pathway activated by AII.28,35,45 Although renal damage accelerates atherosclerotic lesions rich in necrotic areas,23 whether this involves AII regulation of macrophage p38α MAPK or other cell signaling pathways known to affect AII actions, such as Akt or small G-proteins, remains to be determined. Previously, we showed that uninephrectomy-induced activation of NF-κB activity in peritoneal macrophages was regulated by AT1.8 NF-κB is a major regulator of inflammatory and immune responses and has a pivotal role in inflammatory macrophages, including downregulation in ABCA1 expression. This suggests that the NF-κB’s effect on inflammation and lipid handling can be regulated by AT1. In addition, the LXR agonist, GW3965, increases ABCA1 expression and enhances efferocytosis,46 whereas ABCA1/ABCG1 knockout macrophages have enhanced apoptosis in vitro.47 LXR activation lessens expression of AT1.48 These observations indicate that AT1 can regulate ABC transporters through both NF-κB and LXR pathways, which predict that upregulation of ABC transporters expected in AT1-deficient cells would enhance macrophage efferocytosis. In early atherosclerosis, effectorcysis is believed to be efficient and contribute to resolution of inflammatory reactions and inhibition in plaque progression whereas impaired effectorcysis is the hallmark of advanced atherosclerosis. Such processes are consistent with previous data that the predominant macrophage phenotype in early atherosclerotic lesion is the antiinflammatory M2, which changes to the predominance of the proinflammatory M1 phenotype in advanced lesions.13 The observations are also consistent with studies showing that M2 macrophages are much more efficient at effectorcysis compared to M1 macrophages and suggest the macrophage phenotypic change is crucial both in development and acceleration of atherosclerosis.49–51 In summary, our study shows that reduction in renal mass by UNx accelerates atherosclerosis in mice reconstituted with AT1−/− bone marrow increases M1 and decreases M2 macrophage phenotypes. On the other hand, loss of macrophage AT1 prevents UNx-induced acceleration in atherosclerosis with blunted increase of M1 and abrogated decrease of M2 macrophages. Loss of macrophage AT1 lessens renal ablation-induced apoptosis and necrosis, and restores efficient effectorcysis. Thus, by shifting the M1/M2 phenotype, AT1 on macrophages causes impaired effectorcysis and enhanced necrosis, which are pivotal in renal injury-induced acceleration of atherosclerosis.
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Disclosures

None.

References

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Supplement Material

Figure 1. AT1 immunofluorescence. AT1 expression in atherosclerotic lesions of uninephrectomized mice repleted with bone marrow from AT1-intact (A) or AT1-deficient mice (B).

Methods and Results of AT1 immunofluorescence. To assess expression of AT1 receptor, immunofluorescence was performed on OCT-embedded aorta sections. After blocking with 5% BSA/PBS for 1hr at room temperature, sections were incubated with primary rabbit anti-AT1 antibody (Abcam, ab18801, 1:200) overnight at 4°C. Secondary Alexa Fluor 488-conjugated chicken anti-rabbit IgG (1:500 dilution) was applied for 1hr at room temperature then washed prior to coverslipping. Images were captured with a Zeiss Axioplan 2 camera.¹

AT1 immunostaining within the atherosclerotic plaque was reduced in atherosclerotic lesions of uninephrectomized mice repleted with AT1-deficient marrow. Notably, AT1 expression in the vascular media was not reduced in mice repleted with AT1-deficient marrow.

Supplemental Discussion. Uninephrectomy-induced amplification of atherosclerosis in AT1-replete mice was completely abrogated in uninephrectomized mice lacking the AT1 receptor in macrophages (Figure 1). Although this benefit was independent of BP, lipid levels or renal function it is possible that depletion of macrophage AT1 affected systemic parameters that were not detected by our measurements. Thus, the tail cuff method of blood
pressure measurements used in the current study parallels the intermittent pressure measurements that have largely been used in humans, nonetheless, we recognize these measurements may not detect circadian or subtle changes in pressure over time. Reduced AT1 expression within the atherosclerotic plaque of uninephrectomized mice repleted with AT1-deficient marrow also attest to specific reduction in the local angiotensin activity. While our studies show that macrophage AT1 modulation of atherogenesis is not linked to systemic blood pressure, it is possible that systemic, but not specifically blood pressure, effects are perturbed by UNx which in turn influence the local angiotensin activity within the plaque. For example, reconstitution with AT1-deficient marrow may impact atherogenesis, plaque composition and cellular phenotype within the plaque by modulation a more proximal event such as stem cell differentiation, monocyte kinetics and maturation, and/or infiltration of a particular macrophage phenotype. In this regard, transplantation with AT1-deficient marrow has been shown to generate cells with decreased TNF-α expression compare to AT1-intact cells, findings which could impair potential for transitioning to the M1 phenotype in the circulation and/or the atherosclerotic plaque.2