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Deletion of the myeloid endothelin-B receptor confers long-term protection from angiotensin II-mediated kidney, eye and vessel injury



OPEN

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The endothelin system may be an important player in hypertensive end-organ injury as endothelin-1 increases blood pressure and is pro-inflammatory. The immune system is emerging as an important regulator of blood pressure and we have shown that the early hypertensive response to angiotensin-II infusion was amplified in mice deficient of myeloid endothelin-B (ET_B) receptors (LysM-Cre*Ednrbl*ox/lox). Hypothesizing that these mice would display enhanced organ injury, we gave angiotensin-II to LysM-Cre*Ednrbl*ox/lox and littermate controls (*Ednrbl*ox/lox) for six weeks. Unexpectedly, LysM-Cre*Ednrbl*ox/lox mice were significantly protected from organ injury, with less proteinuria, glomerulosclerosis and inflammation of the kidney compared to controls. In the eye, LysM-Cre*Ednrbl*ox/lox mice had fewer retinal hemorrhages, less microglial activation and less vessel rarefaction. Cardiac remodeling and dysfunction were similar in both groups at week six but LysM-Cre*Ednrbl*ox/lox mice had better endothelial function. Although blood pressure was initially higher in LysM-Cre*Ednrbl*ox/lox mice, this was not sustained. A natriuretic switch at about two weeks, due to enhanced ET_B signaling in the kidney, induced a hypertensive reversal. By week six, blood pressure was lower in LysM-Cre*Ednrbl*ox/lox mice than in controls. At six weeks, macrophages from LysM-Cre*Ednrbl*ox/lox mice were more anti-inflammatory and had greater phagocytic ability compared to the macrophages of *Ednrbl*ox/lox mice. Thus, myeloid cell ET_B receptor signaling drives this injury both through amplifying hypertension and by inflammatory polarization of macrophages.

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KEYWORDS: endothelin; hypertension; macrophages; organ injury

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Translational Statement

Hypertension is the single largest contributor to the global burden of disease and to global mortality. It is a major risk factor for chronic kidney disease, atherosclerosis and retinopathy, and end-organ effects with devastating consequences. Preventing this injury is the principal therapeutic goal in patients with hypertension. Our data provide novel insights into the roles of the immune and endothelin systems in the hypertensive end-organ injury seen in the kidney, eye, and vasculature, providing a rational basis for further investigation into the modulation of these pathways for therapeutic gain. These and other successful studies may encourage the industry to take a lead in this relatively orphan area, potentially resulting in a more rational prescribing of endothelin receptor antagonists for hypertension.

Hypertension is the biggest single contributor to the global burden of disease and to global mortality.¹ Currently, it is estimated that a quarter of the world's adult population is hypertensive, projected to reach approximately 30% by 2025.² Arterial hypertension is a major risk factor for atherosclerosis, coronary artery disease, stroke, and chronic kidney disease, which underpin its devastating effects.³ Thus, preventing end-organ damage is the principal therapeutic goal in patients with hypertension. The last few decades have seen few advances in the treatment of hypertension and its complications. An improved understanding of the mechanisms underlying hypertensive end-organ damage might provide new therapeutic opportunities, particularly for those resistant to conventional blood pressure (BP)-lowering strategies.

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In this context, animal models provide an important discovery platform.⁴ Chronic angiotensin II (ANG II) infusion is widely used to capture the cardinal features of clinical disease,^{4,5} and studies extending the model beyond 4 weeks demonstrate stable hypertension and significant cardiac, renal, and vascular injury.⁴ Earlier work shows that these are blunted by endothelin-1 (ET-1) receptor antagonism.^{6,7} Indeed, ET receptor antagonism is an emerging therapeutic option in a number of clinical conditions including chronic kidney disease⁸ and resistant hypertension.⁹ This partly reflects a hemodynamic benefit because ET-1 exerts powerful, sustained vasoconstriction¹⁰ via activation of endothelin-A (ET_A) receptors present on vascular smooth muscle. In addition, ET_A receptor activation is directly proinflammatory and profibrotic, so BP-independent benefits of ET-1 antagonism may also be therapeutically important.

There has been less focus given to the role of the endothelin-B (ET_B) receptor, which exerts a countervailing influence on BP. Endothelial ET_B activation causes vasodilation and activation of ET_B in the distal renal tubule promotes natriuresis.¹¹ In addition, the ET_B receptor plays a key role in clearing ET-1 from the circulation and curtailing its bioactivity.¹² Indeed, we recently showed that ET_B receptor-mediated clearance of ET-1 by myeloid cells protected against the early development of experimental hypertension.¹³ Other studies also point to the innate immune system, particularly macrophages, as an important modulator of the development and progression of hypertension.^{14–18} Whether this also modulates target organ injury is less clearly defined. We therefore hypothesized that targeted deletion of the mouse myeloid ET_B receptor would exaggerate hypertensive end-organ injury. In the current study, we infused ANG II for 6 weeks and examined injury in the kidney, eye, heart, and vasculature. Surprisingly, myeloid ET_B receptor deficiency protected from end-organ injury. These effects partly reflected a reduction in BP but also involved a tuning of macrophages to an anti-inflammatory phenotype. Thus, targeting the myeloid ET system may provide an attractive and novel therapeutic strategy in the context of hypertensive end-organ damage.

RESULTS

Myeloid ET_B receptor deficiency protects from ANG II-mediated renal injury

After 6 weeks of ANG II infusion, renal excretory function was no different between mice with a myeloid-specific disruption of the *Ednrb* gene (LysM-Cre *Ednrb*^{lox/lox}) and littermate controls (mice with no expression of Cre recombinase *Ednrb*^{lox/lox}) (Figure 1a). *Ednrb*^{lox/lox} mice developed progressive, heavy albuminuria over this period. By contrast, LysM-Cre *Ednrb*^{lox/lox} mice displayed only a modest rise in urinary albumin excretion that did not progress between week 3 and week 6 (at 6 weeks: 564 ± 86 vs. 139 ± 44 g/M, *P* < 0.001, Figure 1b). Consistently, there was less glomerulosclerosis at 6 weeks in LysM-Cre *Ednrb*^{lox/lox} mice compared with controls (61 ± 4 vs. 34 ± 2%, *P* < 0.0001, Figure 1c and d). In addition, there was a greater preservation of glomerular ultrastructure—less

glomerular basement membrane thickening and podocyte foot process broadening and effacement—in LysM-Cre *Ednrb*^{lox/lox} mice compared with control mice (Figure 1e).

The protective effects of the myeloid ET_B receptor extended to ANG II-mediated tubular injury with LysM-Cre *Ednrb*^{lox/lox} mice demonstrating fewer tubular casts after 6 weeks of ANG II compared with *Ednrb*^{lox/lox} (Figure 1f and g). This was supported by lower cortical and medullary *Kim-1* expression in knockout mice compared with controls at 6 weeks (Figure 1h).

Myeloid ET_B receptor deficiency reduces ANG II-mediated renal inflammation

At baseline, there was no difference in the number of renal resident macrophages between LysM-Cre *Ednrb*^{lox/lox} and *Ednrb*^{lox/lox} mice. Six weeks of ANG II increased the number of renal macrophages to a greater extent in *Ednrb*^{lox/lox} mice than in those deficient in myeloid ET_B receptors (at 6 weeks, F4/80+ area/total area: 1.5 ± 0.3 vs. 3.8 ± 0.4%, *P* < 0.001, Figure 2a). Similarly, the number of renal T lymphocytes was no different between the 2 groups at baseline and increased to a greater extent in *Ednrb*^{lox/lox} mice after 6 weeks of ANG II than in Lys M-Cre *Ednrb*^{lox/lox} mice (at 6 weeks, CD3+ area/total area: 3.2 ± 0.6 vs. 6.9 ± 1.1%, *P* < 0.01, Figure 2b).

Myeloid ET_B receptor deficiency promotes an anti-inflammatory renal environment in response to ANG II

We went on to further examine the effects of ANG II on the balance between a proinflammatory (*Tnfα* and *Il-6*) and anti-inflammatory (*Mrc1* and *Arg1*) environment within the kidney. At baseline, and in both cortex and medulla, *Tnfα*, *Il-6*, *Mrc1*, and *Arg1* expression did not differ between LysM-Cre *Ednrb*^{lox/lox} and *Ednrb*^{lox/lox} mice (Figure 3). After 6 weeks of ANG II, cortical and medullary *Il-6* expression increased approximately 4- to 6-fold and medullary *Tnfα* approximately 3-fold in control mice, whereas LysM-Cre *Ednrb*^{lox/lox} mice displayed a modest increase in medullary *Il-6* but no change in *Tnfα* in either compartment (Figure 3a and b). Conversely, whereas *Ednrb*^{lox/lox} mice demonstrated no changes in cortical or medullary *Mrc1* and *Arg1* expression after ANG II, knockout mice upregulated cortical *Arg1* and medullary *Mrc1* (Figure 3c and d).

Myeloid ET_B receptor deficiency protects from ANG II-mediated retinal injury

Prolonged uncontrolled hypertension leads to the development of retinal hemorrhages and immune cell infiltration. Deletion of the ET_B receptor on myeloid cells alone significantly reduced the number of retinal hemorrhages seen after ANG II infusion by approximately 90% (Figure 4a). Six weeks of ANG II led to vessel rarefaction in the inner retina plexus in *Ednrb*^{lox/lox} mice, whereas the vessel density of LysM-Cre *Ednrb*^{lox/lox} mice was preserved (Figure 4b). In addition, control mice demonstrated a greater number of activated microglia in the inner retina in response to 6 weeks of ANG II compared with knockout mice (Figure 4c). Activated microglia exhibited a typical round shape with larger cell body and short neurites.

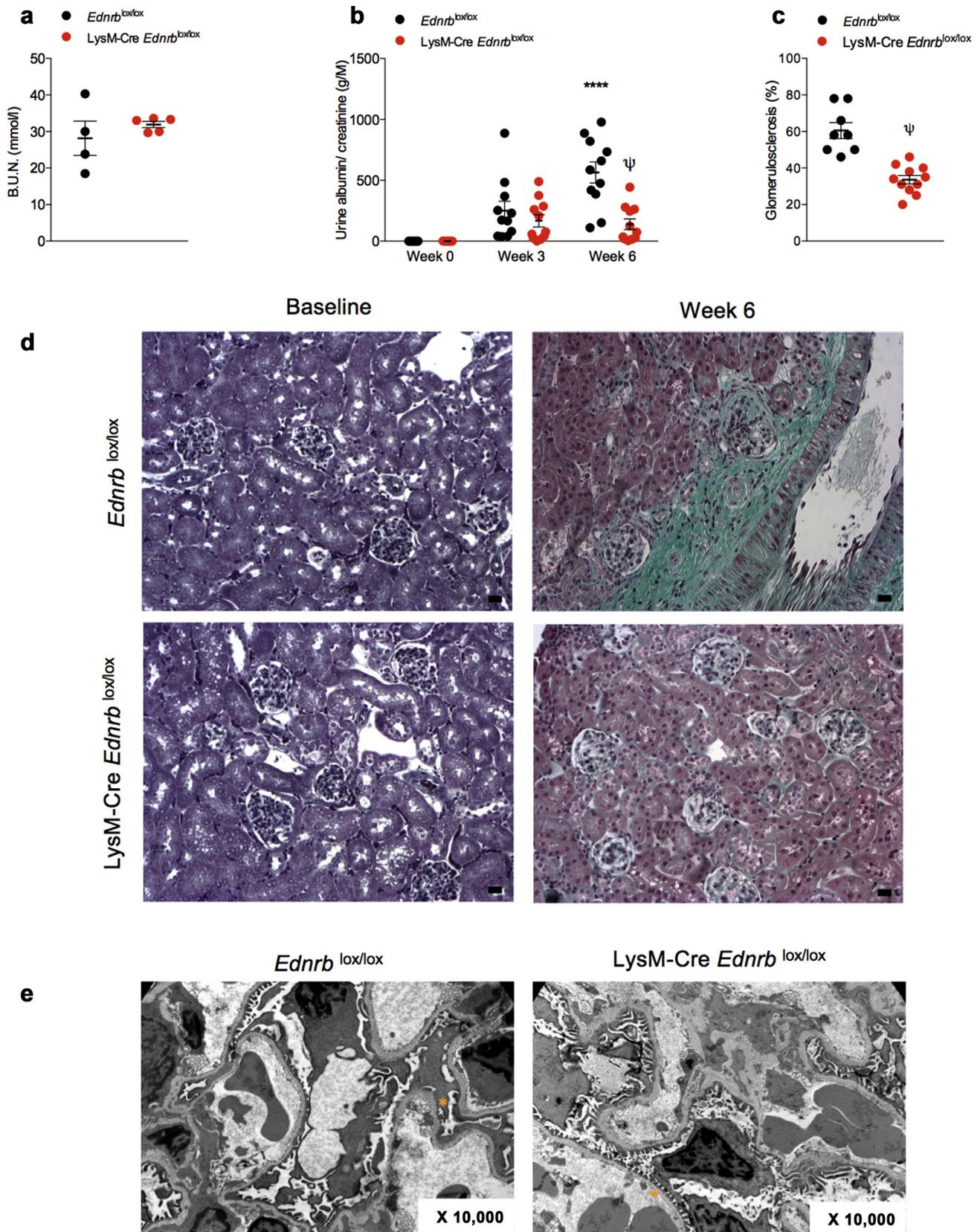


Figure 1 | Specific deletion of *Ednr^b* from myeloid cells protects from angiotensin II (ANG II)-mediated renal injury. (a) Blood urea nitrogen concentration at 6 weeks in *Ednr^b lox/lox* and *LysM-Cre Ednr^b lox/lox* mice after ANG II. (b) Representative images of Masson's trichrome-stained kidney cortex from *Ednr^b lox/lox* and *LysM-Cre Ednr^b lox/lox* mice after 6 weeks of ANG II. (c) Representative transmission electron micrographs of glomeruli from *Ednr^b lox/lox* and *LysM-Cre Ednr^b lox/lox* at 6 weeks. *An area of podocyte foot process effacement and fusion in *Ednr^b lox/lox* mice and an area of normal podocyte ultrastructure in *LysM-Cre Ednr^b lox/lox* mice. (Continued)

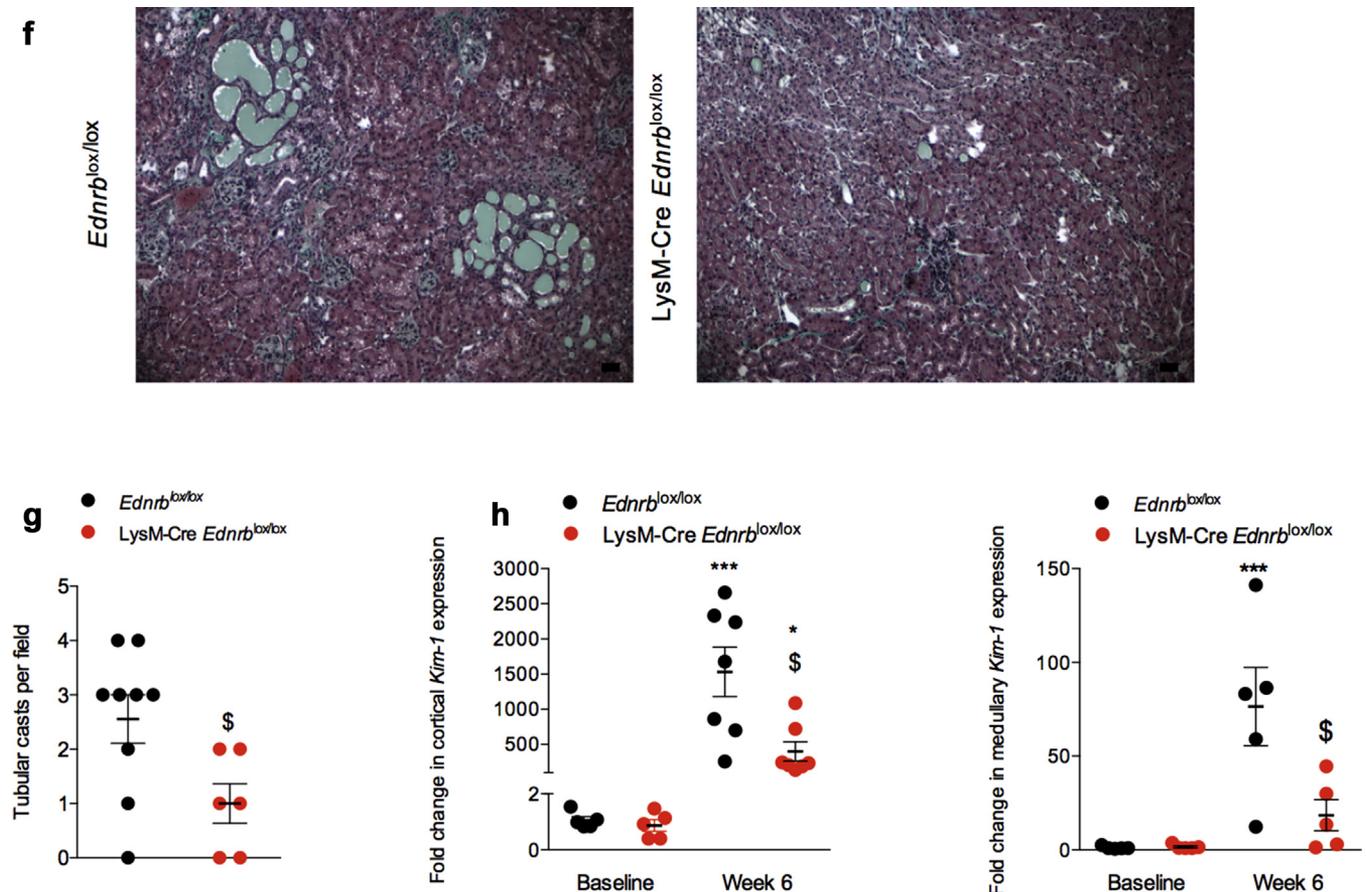


Figure 1 | (Continued) **(f)** Representative images of Masson’s trichrome–stained kidney medulla from *Ednrbllox/lox* and *LysM-Cre Ednrbllox/lox* mice after 6 weeks of ANG II with **(g)** associated quantification of tubular casts. **(h)** Quantitative reverse transcription polymerase chain reaction analysis of cortical and medullary *Kim-1* message at baseline and after 6 weeks. Data represent mean ± SEM of n = 10 mice/group. **P* < 0.05, ****P* < 0.001, and *****P* < 0.0001 for baseline compared with week 6 within the same genotype; §*P* < 0.05 and †*P* < 0.001 for one genotype compared with the other at the same time point. Between-group comparisons for normally distributed data were by a *t* test (**c,g**) or 2-way analysis of variance with Sidak post-test of plan comparisons (**b,h**). For between-group comparisons of non-normally distributed variables, a Mann-Whitney test (**c**) or Kruskal-Wallis test was employed. Bars = 20 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Myeloid ET_B receptor deficiency does not affect ANG II-mediated cardiac hypertrophy and dysfunction

Six weeks of ANG II led to changes in cardiac structure that were in keeping with the development of left ventricular hypertrophy; these did not differ between *LysM-Cre Ednrbllox/lox* and *Ednrbllox/lox* mice (data not shown). ANG II infusion led to a similar increase in heart rate (Figure 5a) and reduction in cardiac output (Figure 5b) in both groups. *LysM-Cre Ednrbllox/lox* and littermates also showed a similar reduction in left ventricular shortening fraction (Figure 5c).

Myeloid ET_B receptor deficiency protects from ANG II-mediated endothelial dysfunction

As expected, 6 weeks of ANG II infusion reduced renal artery blood flow by approximately 50%, and this fall was no different between *LysM-Cre Ednrbllox/lox* and control mice (Figure 5d). ANG II did not affect basilar artery blood flow (Figure 5e) in either group. Interestingly, *LysM-Cre Ednrbllox/lox* displayed greater basilar artery vascular reactivity at baseline compared with controls (20 ± 1 vs. 11 ± 2%, *P* < 0.01).

After 6 weeks of ANG II, and in keeping with the development of significant vascular dysfunction, this response was abolished in *Ednrbllox/lox* mice, whereas *LysM-Cre Ednrbllox/lox* mice retained a significant, albeit reduced, basilar artery vasodilatory capacity that remained within the physiological range (10 ± 2 vs. 0 ± 3%, *P* < 0.05, Figure 5f). In keeping with these data, whereas there were no differences in the responses of isolated mesenteric vessels from knockout and control mice to acetylcholine (endothelium-dependent) and sodium nitroprusside (endothelium-independent) vasodilation at baseline, 6 weeks of ANG II led to impairment of both but with less marked effects in myeloid ET_B receptor-deficient mice compared with controls (Figure 6).

Myeloid ET_B receptor deficiency has a biphasic effect on long-term ANG II-mediated hypertension and promotes natriuresis

LysM-Cre Ednrbllox/lox mice demonstrated an exaggerated BP response to ANG II during the first 2 weeks of infusion, confirming our previous work.¹³ Extending the protocol, we

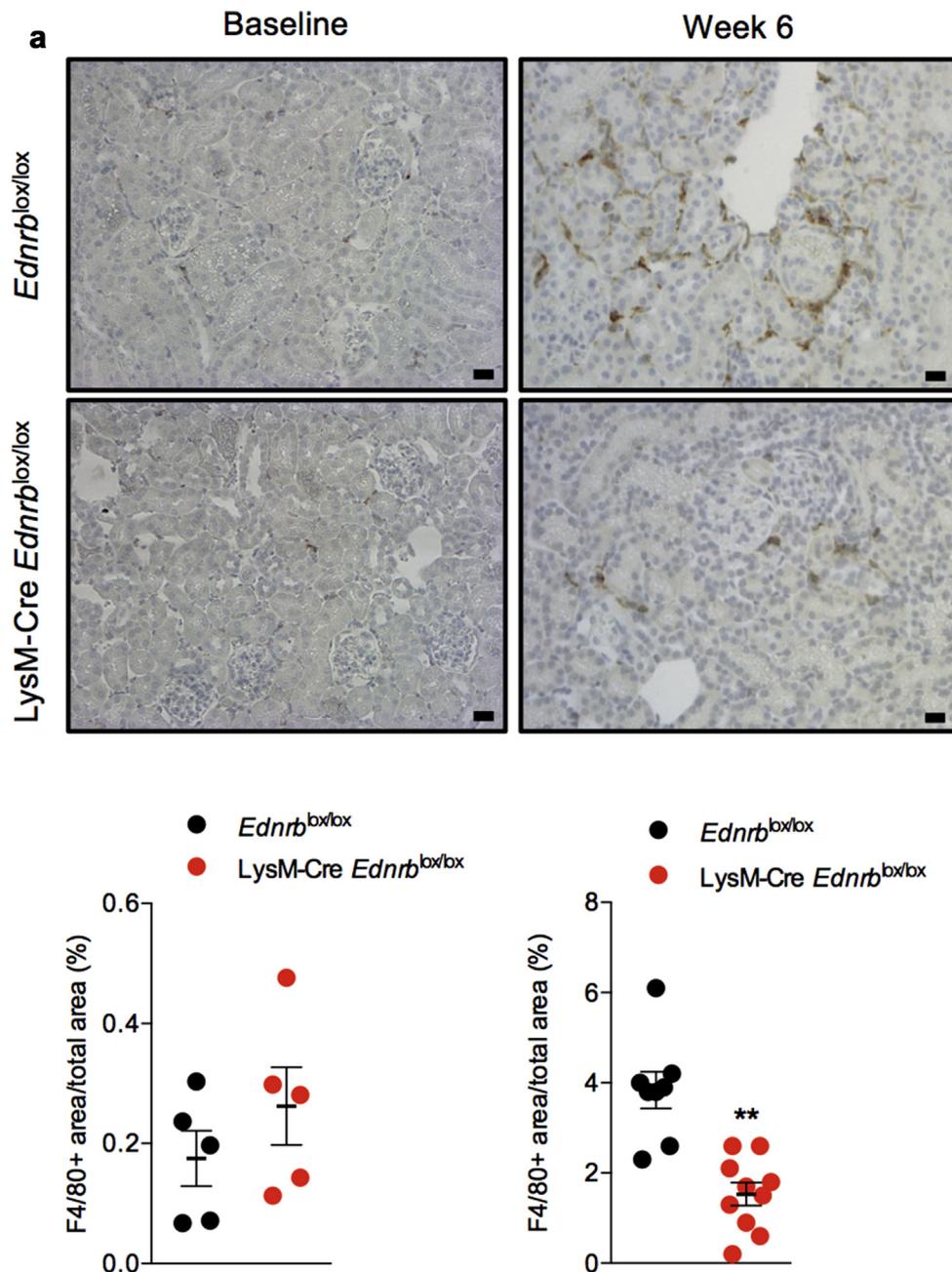


Figure 2 | Specific deletion of *Ednrb* from myeloid cells reduces angiotensin II (ANG II)-mediated renal inflammation. Representative images of renal (a) F4/80 and (continued)

demonstrated that this exaggerated BP response was not sustained. Indeed, there was a reversal during weeks 3–4 such that in weeks 5 and 6, knockout mice now had a significantly lower BP compared with controls (Figure 7a).

Next, we examined renal salt excretion at key time points over the course of the 6-week study. Before ANG II infusion, sodium excretion was no different between genotypes. Infusion of ANG II induced an increase in natriuresis in control mice, and this was sustained through the 6-week course of the study. The response was different in myeloid ET_B receptor-deficient mice: the early natriuresis was blunted (Figure 7b), concomitant with the exaggerated hypertensive response to ANG II; by

week 6, the natriuresis was significantly greater than in controls, coincident with the reversal of BP. ET_B receptors are expressed in the principal cell of the renal tubule. Activation of ET_B here inhibits the epithelial sodium channel (ENaC) to promote natriuresis. We therefore examined renal expression of the ET_B receptor and α ENaC subunit (the rate-limiting step for functional ENaC formation). Before ANG II, there was no difference between genotypes in the expression of either ET_B or α ENaC mRNA. At 6 weeks, control mice showed a significantly increased abundance of α ENaC mRNA in both the cortex and medulla, which was not evident in *LysM-Cre Ednrb^{lox/lox}* mice. Notably, only *LysM-Cre Ednrb^{lox/lox}* mice showed a significant

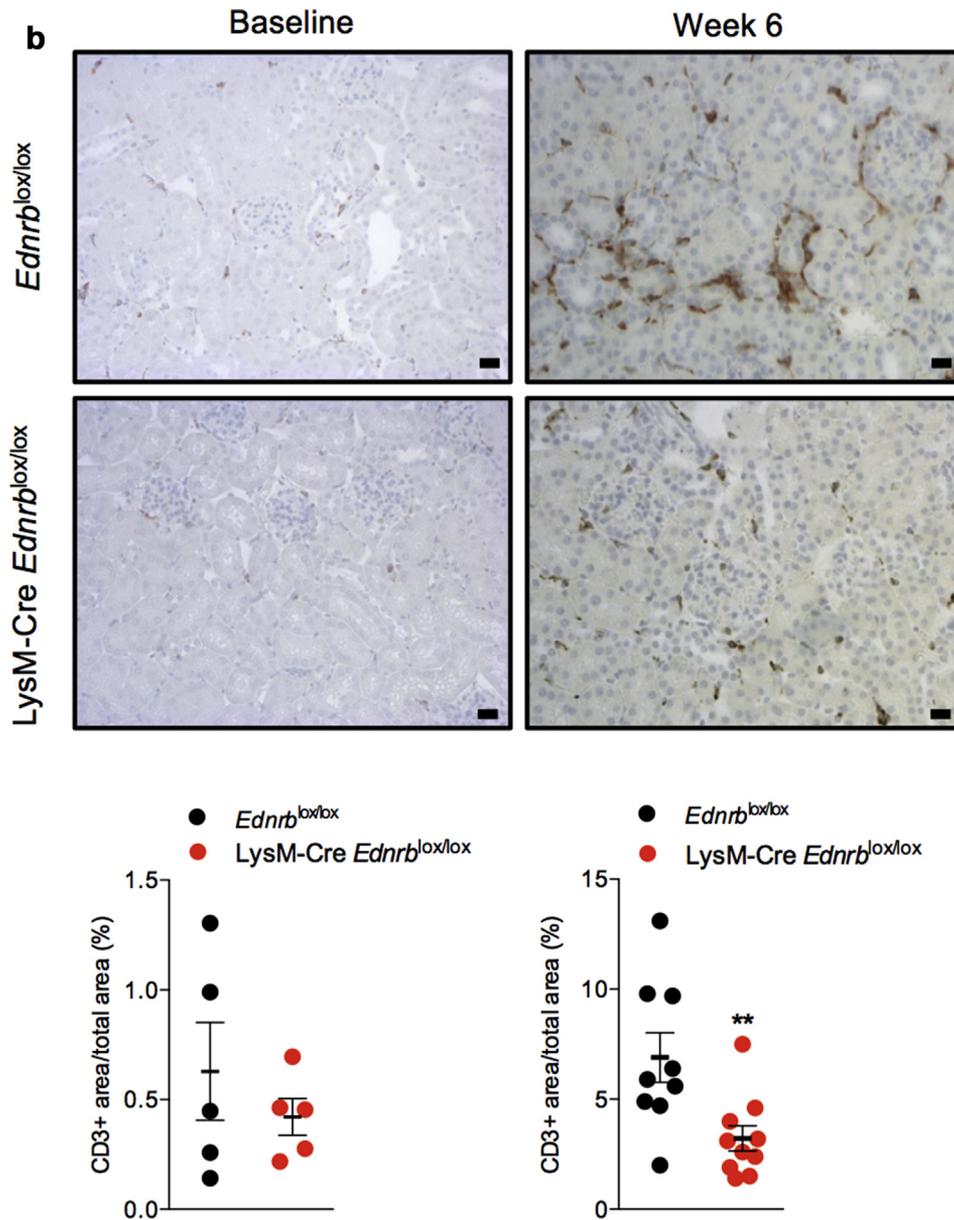


Figure 2 | (continued) (b) CD3 staining on kidney sections from *Ednrb*^{lox/lox} and *LysM-Cre Ednrb*^{lox/lox} mice at baseline and after 6 weeks of ANG II infusion. Data represent mean ± SEM of n = 8 mice/group. ***P* < 0.01 for one genotype compared with the other. Between-group comparisons were made by an unpaired *t* test. Bars = 20 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

increase in ET_B expression after 6 weeks of ANG II infusion (Figure 7c).

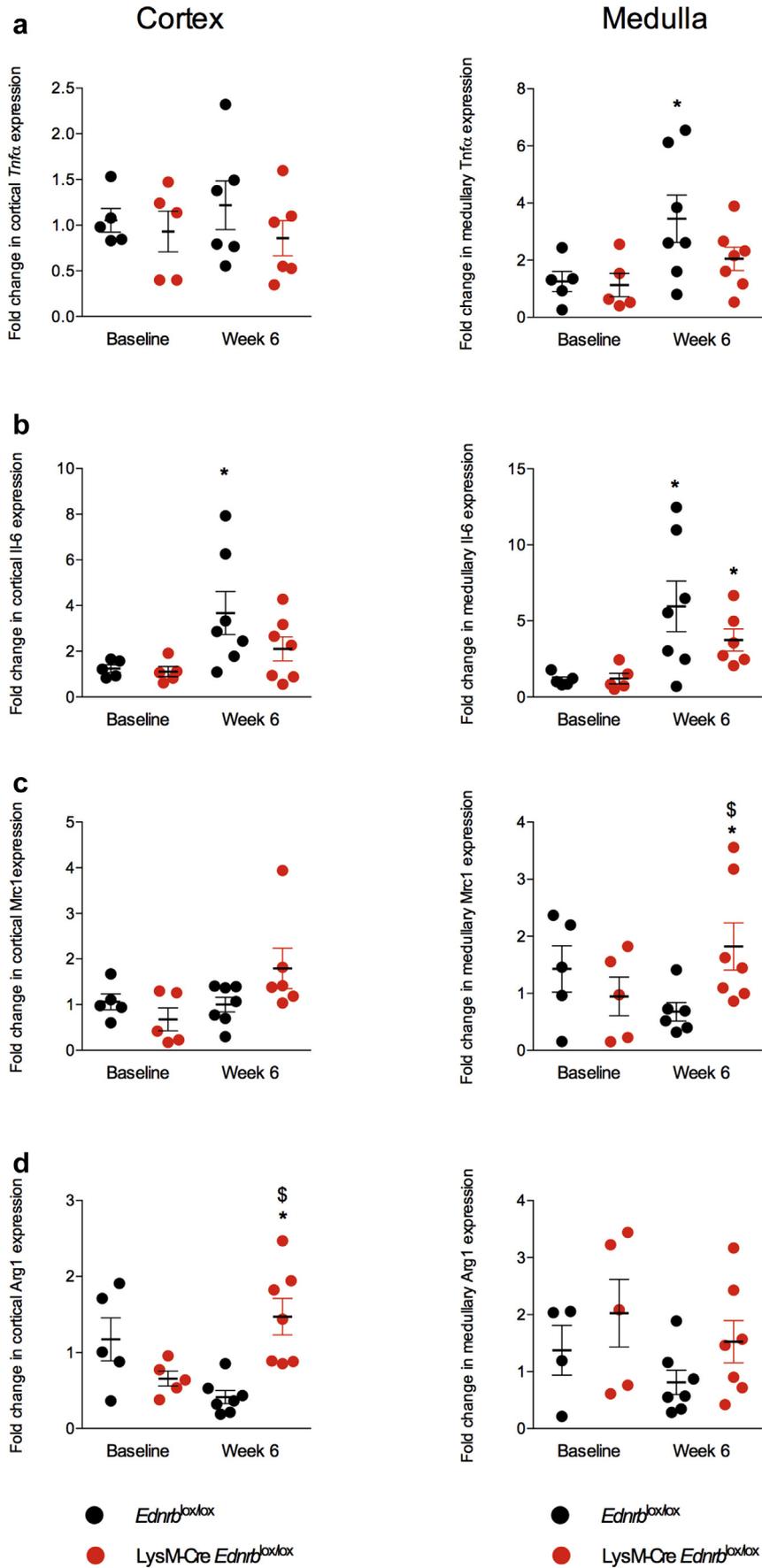
Myeloid ET_B receptor deficiency promotes an anti-inflammatory macrophage and neutrophil phenotype in response to ANG II

Next, we explored the effects of ANG II and myeloid ET_B receptor deficiency on macrophage and neutrophil phenotype. At baseline, there were no differences seen between macrophages from *Ednrb*^{lox/lox} or *LysM-Cre Ednrb*^{lox/lox} mice in terms of their ability to polarize to an inflammatory (M1) or anti-inflammatory (M2) phenotype, or their phagocytic ability (Supplementary Table S1). Interestingly, after 6 weeks of exposure to ANG II, macrophages

harvested from *LysM-Cre Ednrb*^{lox/lox} mice demonstrated less of an M1 phenotype and more of an M2 phenotype than macrophages from littermate controls (Figure 8a–c). Knockout macrophages also displayed a greater phagocytic capacity than controls at this timepoint (Figure 8d). Neutrophils from *Ednrb*^{lox/lox} or *LysM-Cre Ednrb*^{lox/lox} mice did not differ at baseline in terms of cytokine production. However, after exposure to 6 weeks of ANG II, neutrophils from knockout mice were less inflammatory compared with control cells (Figure 9).

DISCUSSION

Damage to the kidney, heart, eye, and arteries underpins the devastating consequences of sustained hypertension. In this



study, we purposefully used an extended-duration ANG II infusion to model such end-organ injury with stable hypertension. In the kidney, injury to both the glomerular and interstitial compartments was apparent and the gradual development of proteinuria and glomerulosclerosis is analogous to that observed in human progressive chronic kidney disease. We examined how ET-1/ET_B signaling in myeloid cells contributed to the development of organ injury. Our study had 3 main findings. First, we have demonstrated, for the first time, that activation of the myeloid ET_B receptor makes a significant contribution to renal, retinal, and vascular injury induced by ANG II. Second, the injurious role of myeloid cell ET-1/ET_B signaling may reflect polarization of macrophages to a proinflammatory phenotype. Third, myeloid cell ET_B receptors modulate the BP response to ANG II infusion in a biphasic way, a Janus-faced role of macrophages that is relevant to progressive human disease.

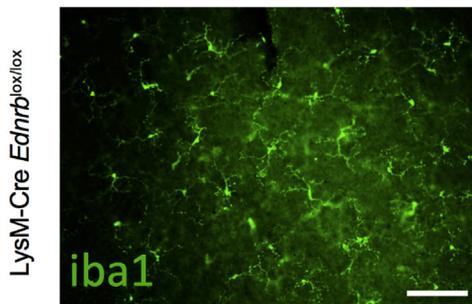
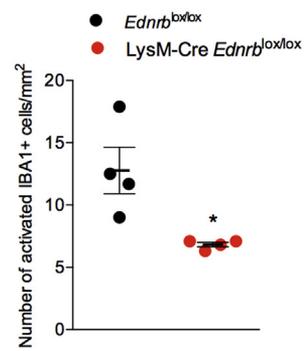
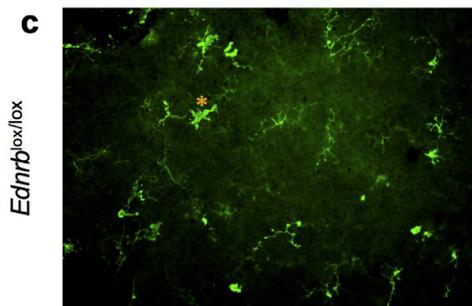
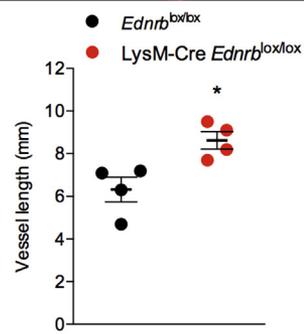
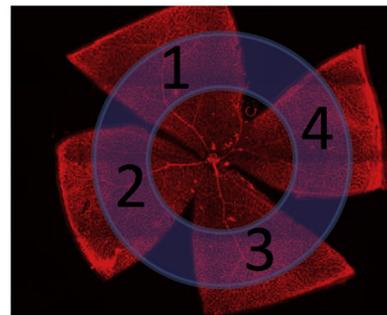
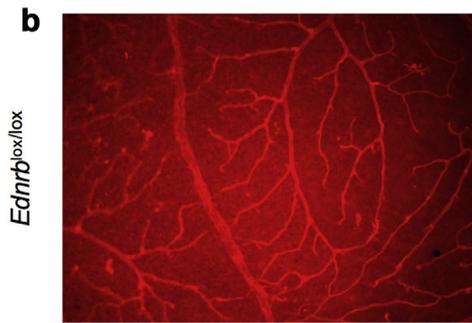
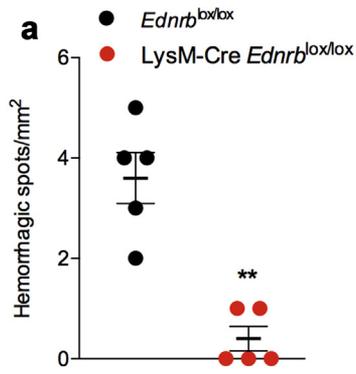
Current research shows that macrophages play diverse roles in hypertension. Crowley *et al.*¹⁶ showed that mice lacking ANG type 1 receptors on bone marrow-derived cells develop exaggerated hypertension and proteinuria during ANG II infusion, whereas Rickard *et al.*¹⁷ studied mice with a macrophage-specific knockout of the mineralocorticoid receptor and found that they were protected from deoxycorticosterone acetate/salt-induced hypertension. As opposed to altering macrophage phenotype, Wenzel *et al.*¹⁵ elegantly depleted macrophages and found that this markedly blunted virtually all consequences of chronic ANG II infusion, including BP elevation, superoxide production, vascular dysfunction, and fibrosis (some of these effects may have been due to modulation of the ET system). We also found that macrophages provide a buffering mechanism in the development of hypertension. The presence of a functional ET-1/ET_B signaling cascade in macrophages slowed the initial rise in BP induced by a 2-week infusion of either ET-1 or ANG II.¹³ The hypertension induced by ANG II is partly mediated by ET-1, and we found in both models that the initial BP-lowering effect of macrophages reflected clearance of ET-1, dependent on an unblocked ET_B receptor and intact endocytosis.¹³ We now show that this “protective” role of ET-1/ET_B in macrophages is not sustained. The BP differential between mice with and without macrophage ET_B receptors is lost beyond 2 weeks and indeed reverses after 4 weeks of ANG II treatment. An “injurious” role for macrophage ET_B ultimately emerges such that specific deletion of this receptor substantially reduces organ injury in the kidney, eye, and vasculature, at least in the setting of ANG II hypertension. This remains to be confirmed in an ET-1 infusion model. Similarly, we have not yet explored whether the myeloid ET_A

receptor, expressed at much lower levels than the ET_B receptor,¹³ contributes to injury progression.

By extending our examination of ANG II hypertension beyond 2 weeks, we highlight diverse and changing roles for macrophages in BP regulation. The hypertensive reversal after 3–4 weeks was associated with a marked improvement in the natriuretic response. Our data point to a mechanism involving increased tubular ET-1/ET_B signaling and downregulation of ENaC-mediated sodium reabsorption in the distal nephron. Moreover, the magnitude of the natriuresis is large, and it is likely that transporters in other segments of the nephron are downregulated. Defining how the nephron functionally remodels sodium transport could be assessed by examining the natriuretic response to a panel of diuretics¹⁹ and is a future direction of our research. Similarly, how deletion of myeloid cell ET_B receptor leads to upregulation of the same system in the principal cell is currently unclear. Recent studies show that renal epithelial cells produce chemokines to orchestrate intrarenal distribution of monocyte-derived cells.^{20,21} Whether our data uncover direct “myelotubular” crosstalk within the normal operation of the ET-1 pathway requires further investigation.

Chronic ANG II infusion also promotes renal T-cell and macrophage infiltration, shown here and by others.^{22,23} This infiltration was substantially reduced by myeloid ET_B deficiency, which also shifted the renal corticomedullary environment from an inflammatory to a more anti-inflammatory one. Together these suggest that the macrophage ET-1/ET_B signaling system operates to promote inflammation and injury in the long term. Certainly, sustained ANG II treatment upregulates the vascular and renal ET system.^{6,7} We have previously shown that macrophages display ET_B-dependent chemokinesis toward ET-1. It is plausible that the increase in renal macrophages seen after 6 weeks of ANG II infusion reflects recruitment of monocytes to the kidney in chemokinetic response to a high systemic-intrarenal ET gradient. Moreover, effective ET_B blockade disrupts the myeloid ET_B sensing mechanism, leading to fewer circulating monocytes transitioning to the kidneys. Others have explored renal inflammation and the contribution that the ET system makes to this. Boesen *et al.*²⁴ showed that during a 2-week infusion of ANG II in mice, ET_A receptor activation mediated a BP-independent increase in renal cortical T cells, whereas medullary T-cell and corticomedullary macrophage infiltration was BP-dependent. Similarly, in a model of diabetic nephropathy, treatment with an ET_A antagonist reduced renal macrophage infiltration and fibrosis.²⁵ In both studies, ET receptor blockade was given systemically and so the specific cell mediating the beneficial effects was unclear.

Figure 3 | Specific deletion of *Ednrb* from myeloid cells promotes an anti-inflammatory renal environment in response to angiotensin II (ANG II). Quantitative reverse transcription polymerase chain reaction analysis of renal cortex and medulla of *Ednrb*^{lox/lox} and LysM-Cre *Ednrb*^{lox/lox} mice before and after 6 weeks of ANG II. Data represent mean ± SEM fold change from baseline; n = 7 mice/group. *P < 0.05 for baseline compared with week 6 within the same genotype; [§]P < 0.05 for one genotype compared with the other at the same time point. Comparisons were made by 2-way analysis of variance with Sidak post-test for planned comparisons.



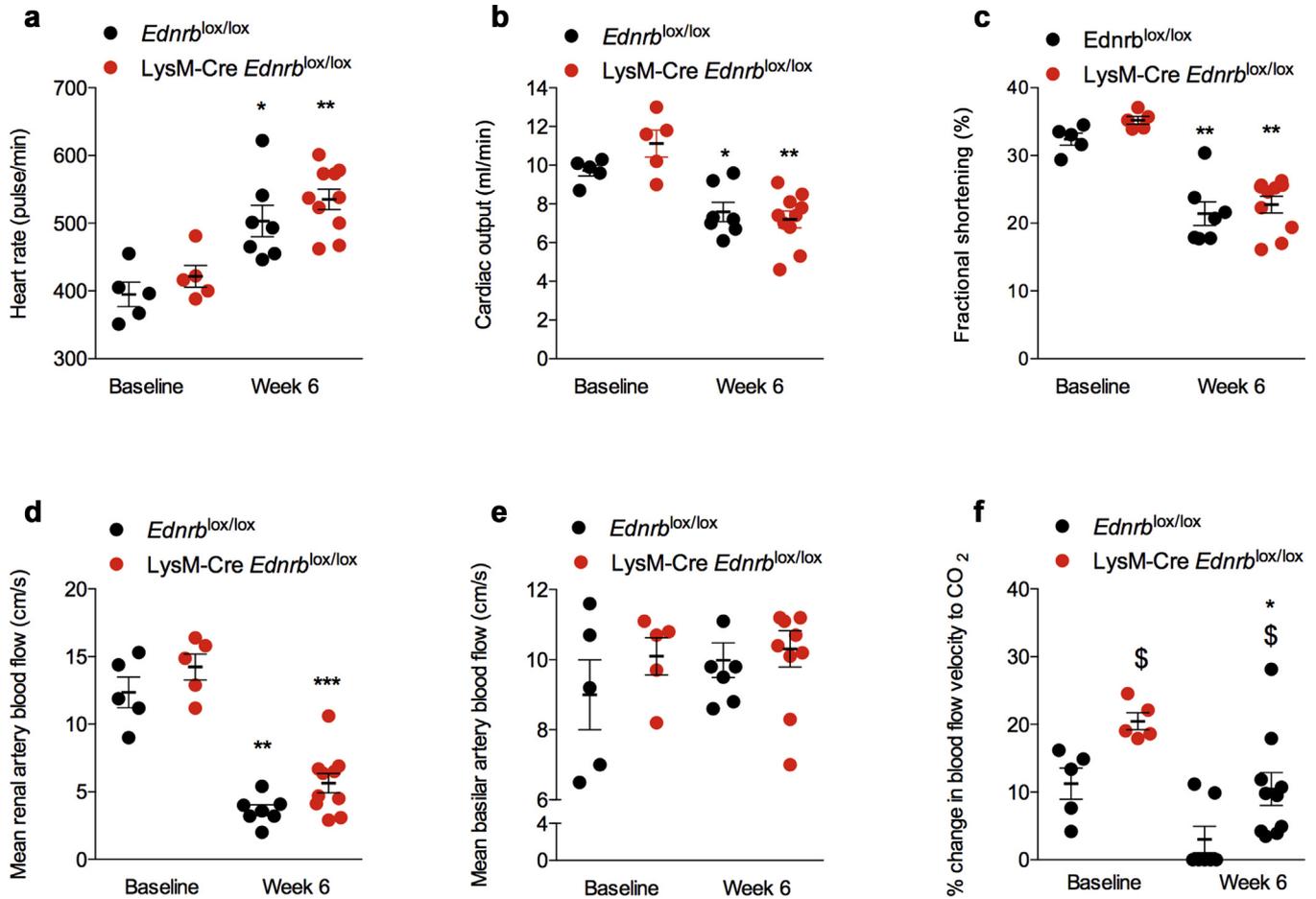


Figure 5 | Specific deletion of *Ednrb* from myeloid cells does not affect angiotensin II (ANG II)–mediated cardiac injury. Effects on (a) heart rate, (b) cardiac output, (c) fractional shortening, (d) renal artery blood flow, (e) basilar artery blood flow, and (f) basilar artery vascular function at baseline and after 6 weeks of ANG II in *Ednrb*^{lox/lox} and LysM-Cre *Ednrb*^{lox/lox} mice. Data represent mean ± SEM of n = 8 mice/group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 for baseline compared with week 6 within the same genotype; §*P* < 0.05 for one genotype compared with the other at the same time point. Comparisons were made by 2-way analysis of variance with Sidak post-test for planned comparisons.

Although no different at baseline, macrophages from myeloid ET_B knockout mice taken after 6 weeks of exposure to ANG II displayed a more M2/anti-inflammatory and reparative phenotype than those from littermate control mice that showed a more M1/inflammatory one. The ability of ANG II to promote an inflammatory macrophage phenotype is well recognized.²⁶⁻²⁸ However, its exact mechanism remains unclear. Our data suggest an interaction between macrophage ANG II receptors and ET_B to promote this phenotypic switch. ANG II-mediated hypertension and end-organ injury are to a large extent mediated by an upregulation of the ET system. Thus, ET receptor

antagonism is able to abrogate these effects.^{6,7} However, previous studies have not explored the type and location of the ET receptor involved. Our novel findings support a key role for macrophage ET_B receptors in mediating these effects.

Few recent studies have explored the effects of ANG II-mediated hypertension on the eye. Retinal hemorrhages and vascular loss are characteristic of hypertensive eye injury and both were seen here. They were reduced with myeloid ET_B deficiency as was retinal inflammation assessed by microglial activation. ANG II contributes to a number of eye disorders including diabetic retinopathy, glaucoma, and retinopathy of

Figure 4 | Specific deletion of *Ednrb* from myeloid cells protects from angiotensin II (ANG II)–mediated retinal injury. All data are after 6 weeks of ANG II. (a) Quantification of retinal hemorrhagic spots in *Ednrb*^{lox/lox} and LysM-Cre *Ednrb*^{lox/lox} mice. (b) Representative images of the inner retina vascular network stained with lectin from *Ednrb*^{lox/lox} and LysM-Cre *Ednrb*^{lox/lox} mice. Vascular network density was calculated from an average of 4 images/retina using the NeuronJ plugin on ImageJ software (National Institutes of Health, Bethesda, MD). (c) Representative images of microglia within the inner retina stained with iba-1 antibody from *Ednrb*^{lox/lox} and LysM-Cre *Ednrb*^{lox/lox} mice. Activated microglia, which have an amoeboid morphology with a large cell body and short neurites (*), were counted from 4 images/retina. Data represent mean ± SEM; n = 5 mice per group. **P* < 0.05 and ***P* < 0.01 for one genotype compared with the other. Between-group comparisons were performed with a Mann-Whitney test. Bars = 50 μm To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

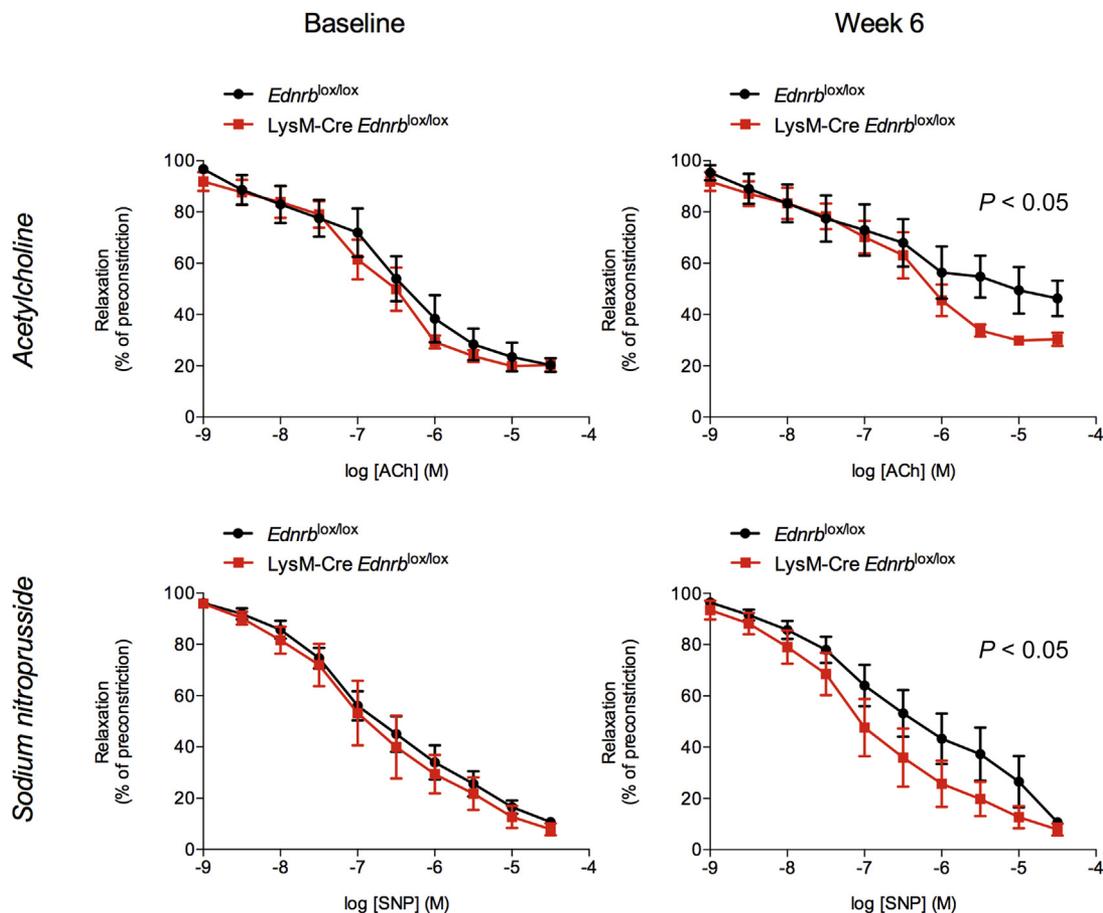


Figure 6 | Specific deletion of *Ednrb* from myeloid cells protects from angiotensin II (ANG II)-mediated endothelial dysfunction.

Endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) vascular function in *Ednrb*^{lox/lox} and LysM-Cre *Ednrb*^{lox/lox} mice at baseline and after 6 weeks of ANG II. Data represent mean \pm SEM of $n = 8$ mice/group. Between-group comparisons were performed with a 2-way analysis of variance.

prematurity.²⁹ In retinopathy of prematurity, blockade of ANG type 1 receptors promotes the re-establishment of normal vessel growth and reduces microglia recruitment.³⁰ These effects are similar to those seen here, and so targeting the myeloid ET system may provide a novel direction for future studies in these areas. Indeed, one study in diabetic mice showed an improvement in the retinal vascular network and reduced pericyte loss with the ET_A antagonist, atrasentan.³¹

As expected, chronic ANG II infusion resulted in both endothelium-dependent and endothelium-independent dysfunction. This injury is a result of damage to both the vascular endothelial cell and the underlying smooth muscle cell. This was ameliorated by myeloid ET_B receptor deficiency. The mechanisms for this protection probably relate in part to the shift in the macrophage phenotype we observed in response to ANG II between the two groups of mice. It is well recognized that inflammatory macrophages contribute to endothelial dysfunction.^{15,32} Phagocytosis also contributes to inflammation resolution through removal of cell debris.³³ Here, phagocytic capacity of knockout macrophages was greater than in controls, and so this may also explain the differential effects seen in the kidneys, eye, and vasculature.

In the current study, the magnitude of the antiproteinuric and antifibrotic effects of effective myeloid ET_B receptor blockade (approximately 40%–60%) is similar to that seen with ET receptor antagonists. As both selective ET_A and mixed ET_{A/B} receptor antagonists are available in the clinic, the addition of our own data to these earlier studies suggests that both approaches may be beneficial in reducing ANG II-mediated renal damage, which is one of the main mechanisms in the development and progression of chronic kidney disease. Consistent with this, the recent SONAR study demonstrated the efficacy of atrasentan, an endothelin-A antagonist, in slowing diabetic chronic kidney disease progression when added to renin-angiotensin system blockade.⁸

In summary, our study has identified a novel role for the myeloid ET system in mediating ANG II-associated injury in the eye, kidney, and vasculature. The long-term protective effects of deleting the myeloid ET_B receptor were only resolved by extending the duration of our experiment, highlighting the importance of “time” as a factor when modeling chronic, progressive human disorders. The protection ultimately conferred by myeloid ET_B receptor depletion may be partly hemodynamic, reflecting the lowering of BP after natriuresis.

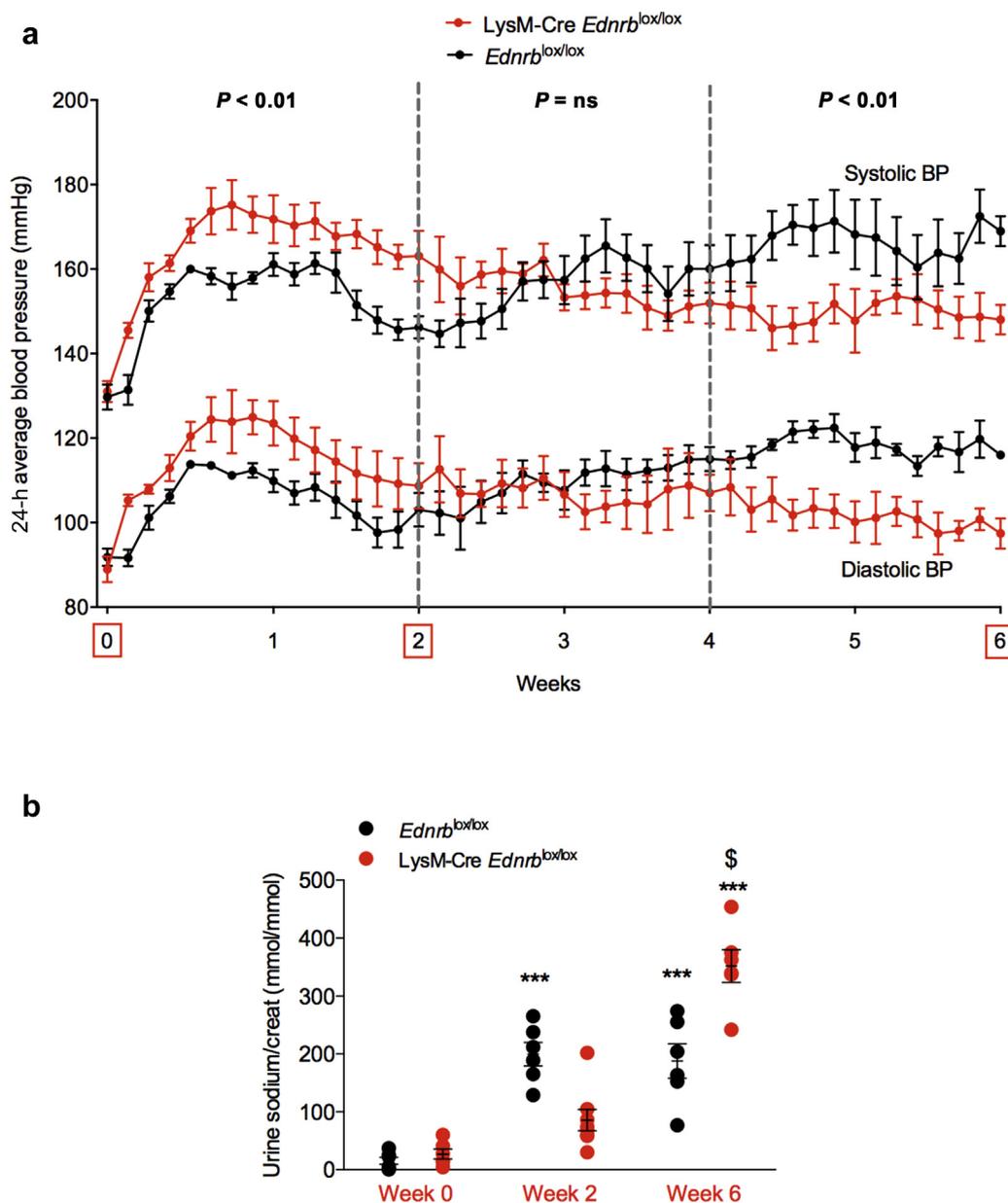


Figure 7 | Specific deletion of *Ednrb* from myeloid cells has a biphasic effect on the blood pressure (BP) response to angiotensin II (ANG II) and promotes long-term natriuresis. (a) Systolic and diastolic BP of *Ednrb^{lox/lox}* and *LysM-Cre Ednrb^{lox/lox}* mice during 6 weeks of ANG II infusion. **(b)** Urinary sodium excretion over this 6-week period. (Continued)

Our data also indicate that reprogramming of macrophage and/or neutrophil phenotype and function is likely to be important. We cannot currently discriminate the relative contributions of macrophages and neutrophils to this protective phenotype, a limitation of the study requiring further work. Moreover, we cannot yet clearly map across to humans the biphasic BP response to myeloid ET_B receptor deletion. We previously reported that cyclophosphamide therapy, depleting circulating monocytes and tissue macrophages (as well as T and B cells), increased BP in patients with vasculitis,¹³ at least initially and a longer study is now needed. This is relevant, because targeting the immune system is recognized as an effective treatment for hypertension in man as seen in studies using interleukin (IL)6 antagonism³⁴ and immunization against ANG II.³⁵ Similarly,

macrophage cell therapy is currently being explored as an anti-inflammatory approach in a number of disease states.³⁶⁻³⁸ Combining these with newer monoclonal antibodies that specifically target ET_A and ET_B³⁹ might allow the current data to be explored in the clinical setting.

METHODS (PLEASE ALSO SEE SUPPLEMENTARY MATERIAL) ANG II-mediated end-organ damage

Mice were infused ANG II (1 µg/kg/min, Sigma Aldrich) subcutaneously using osmotic mini pumps (Alzet, model 2006) for a period of 6 weeks. Animals were fed a high-salt diet (NaCl 3%) and had free access to food and water. BP was measured in conscious, unrestrained mice using a radio-telemetry system (PA-C10 and Dataquest software, Data Sciences International). Recordings were obtained

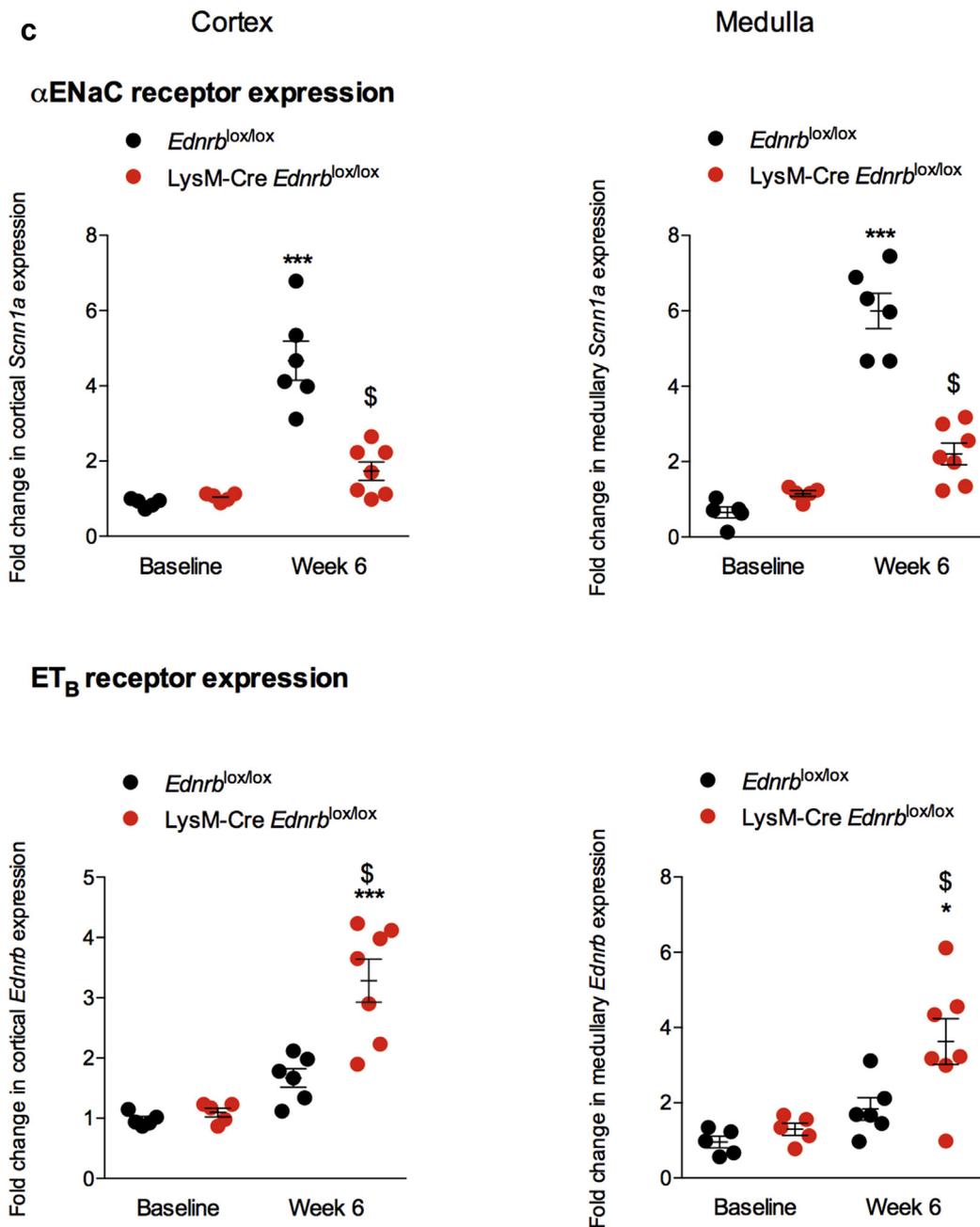


Figure 7 | (Continued) (c) Expression of cortical and medullary α epithelial sodium channel (ENaC) and endothelin-B (ET_B) receptor in *Ednrb*^{lox/lox} and LysM-Cre *Ednrb*^{lox/lox} mice at baseline and after 6 weeks of ANG II. Data represent mean \pm SEM of $n = 5-7$ mice/group. * $P < 0.05$ and *** $P < 0.001$ for baseline compared with week 6 within the same genotype; $^{\$}P < 0.05$ for one genotype compared with the other at the same time point. Between-group comparisons were performed with 2-way analysis of variance.

every 15 minutes for 60 seconds; daytime was defined as 0700–1900 and nighttime as 1900–0700.

Assessment of renal function and albuminuria

Blood urea nitrogen and urinary creatinine concentrations were quantified spectrophotometrically by colorimetric methods. Urinary albumin was measured with a specific enzyme-linked immunosorbent assay (Cusabio, CSB-E13878m).

Renal histopathology and immunohistochemistry

Kidneys were immersed in 10% formalin and embedded in paraffin. Four-millimeter sections were processed for histopathology or

immunohistochemistry. Glomerulosclerosis was defined by focal and segmental consolidation of the tuft by increased extracellular matrix, obliterating the glomerular capillary lumen. For the assessment of glomerulosclerosis, an examiner blinded to the experimental conditions assessed the percentage of glomeruli showing glomerulosclerosis with at least 50 glomeruli examined per field. For immunohistochemistry, paraffin embedded sections were stained with the following primary antibodies: rabbit anti-CD3 (DAKO, A 0452, 1:200), rat anti-F4/80 (AbDSerotec, MCA497, 1:500). CD3 and F4/80 staining were exposed with Histofine reagent (Nichirei Biosciences, 414141F) and color formation stopped by washing in phosphate-buffered saline. Slides were then counterstained with hematoxylin. Photomicrographs

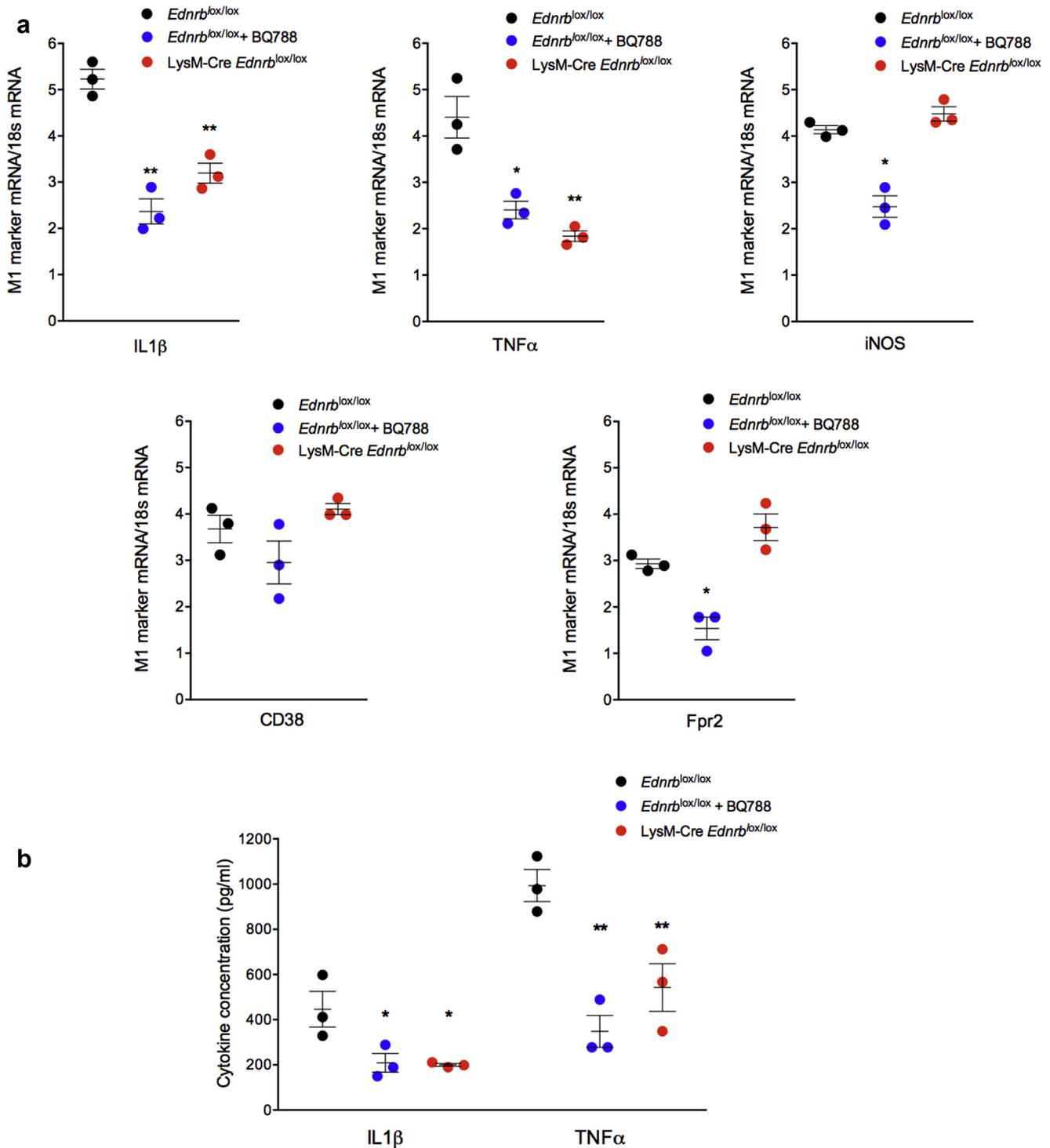


Figure 8 | Specific deletion of *Ednrb* from myeloid cells promotes an anti-inflammatory macrophage phenotype in response to angiotensin II (ANG II). Peritoneal macrophages were harvested from *Ednrb^{lox/lox}* and LysM-Cre *Ednrb^{lox/lox}* mice after 6 weeks of ANG II. (a) A range of M1 macrophage markers and (b) cytokines were assessed after stimulation with lipopolysaccharide/interferon- γ ; (continued)

were taken with an Axiophot Zeiss photomicroscope (Jena, Germany). Quantification of CD3 and F480 staining was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Electron microscopy. Sections of renal cortex were fixed in Trump's solution (EMS, 11750) and embedded in Araldite M (Sigma Aldrich, 10951). Ultrathin sections were counter-stained with uranyl

acetate and lead citrate and examined with a transmission electron microscope (JEM-1011, JEOL, Peabody, MA).

Assessment of retinal injury

After enucleation, eyes were fixed in 4% paraformaldehyde for 30 minutes at room temperature. After washing in phosphate-buffered

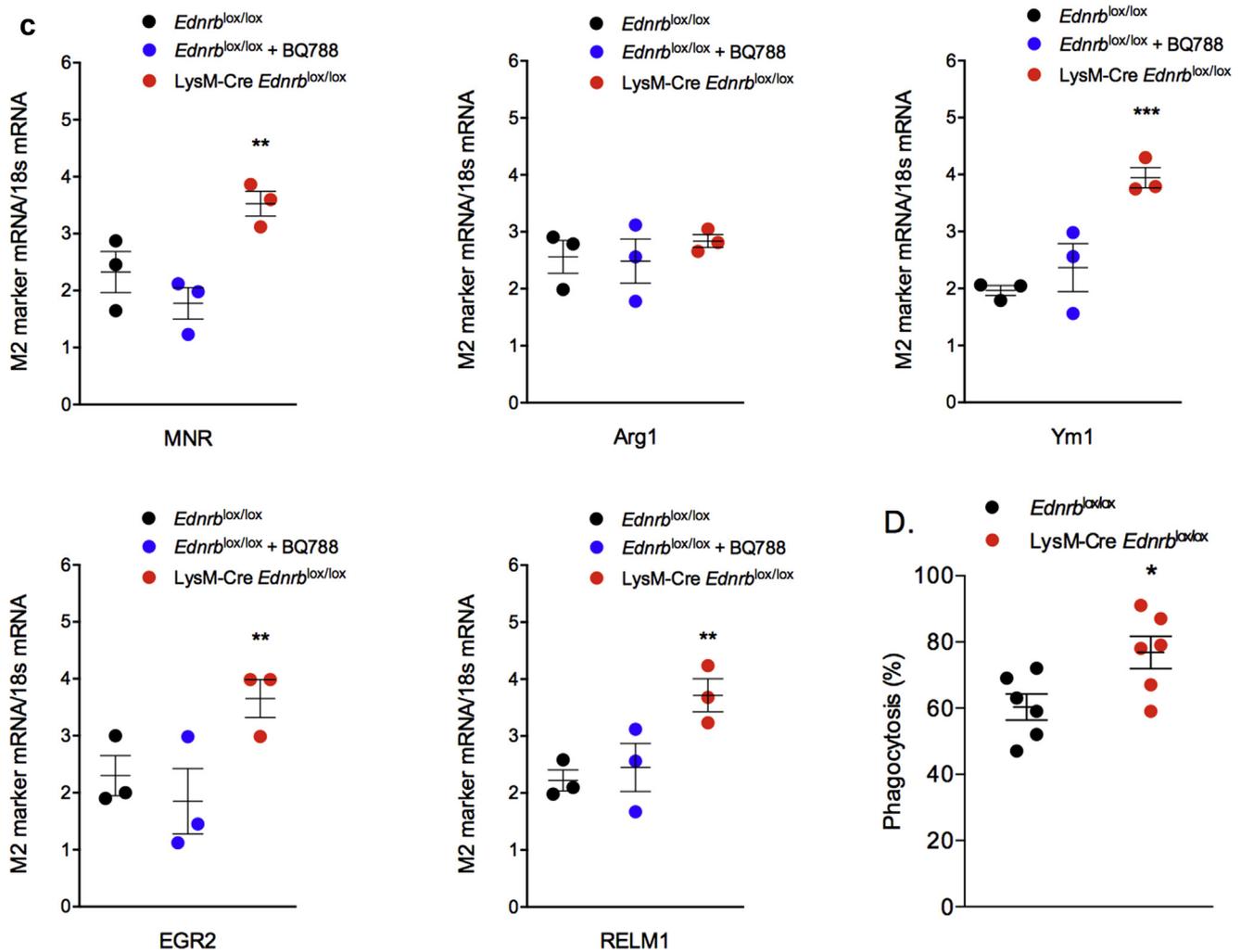


Figure 8 | (continued) (c) M2 markers were assessed after stimulation with interleukin (IL) 4/IL13; (d) phagocytosis. Data are mean ± SEM; n = 6/group. *P < 0.05, **P < 0.01, and ***P < 0.001 for one genotype compared with the other. Between-group comparisons were performed with a 1-way analysis of variance with Sidak post-test for planned comparisons.

saline, and removal of the cornea and lens, the retina was dissected from the retinal pigment epithelium/choroid/sclera. Retinas were incubated overnight with primary antibodies (lectin Alexa conjugated, 1:100, Invitrogen; rabbit anti-Iba1, 1:400, Wako) in phosphate-buffered saline supplemented with 0.5% Triton X-100, followed by incubation with appropriate Alexa-coupled secondary antibodies (Life Technologies). Retinas were flat-mounted and viewed with a fluorescence microscope (DM5500B, Leica, France). Vascular network and activated Iba1+ cell density were calculated from an average of 8 images/animal (4/retina). Vascular network density was obtained using NeuronJ plugin on ImageJ software.

Noninvasive ultrasound assessment of cardiac, renal, and cerebral hemodynamics

Ultrasound examination was carried out under isoflurane anesthesia using an echocardiograph (Acuson S3000; Siemens, Erlangen, Germany) equipped with a 14 MHz linear transducer (14L5 SP). Mice were placed on a heating blanket (38 °C) to avoid hypothermia.

Cardiac parameters and cardiac output. A parasternal long-axis B-mode image was used to measure cardiac output using the mean of 3 successive measurements. Heart dimensions measured, in both systole and diastole, included left ventricular posterior wall

thickness, left ventricular internal diameter, and interventricular septum thickness. Heart rates were obtained with an electrocardiogram.

Renal artery blood flow velocity. A pulsed Doppler sample gate was placed on the longitudinal axis of the right renal artery and the pulsed Doppler spectrum was recorded. Time-averaged mean blood flow velocity was calculated with correction of the angle between the long axis of each vessel and the Doppler beam.

Cerebral vasoreactivity. A horizontal B-mode image at the skull base allowed imaging of the basilar trunk and both internal carotid arteries. Color Doppler mode was activated and the basilar trunk with the circle of Willis was drawn and localized on the screen by their color-coded blood flow. A pulsed Doppler sample gate was placed on the longitudinal axis of the basilar trunk, and the pulsed Doppler spectrum was recorded by a masked operator. Vasoreactivity was quantified as the percentage increase in mean blood flow velocity after 5-minute inhalation of 16% O₂/5% CO₂/79% N₂ compared with mean blood flow velocity on ambient air.⁴⁰

Ex vivo myography

This is described in detail elsewhere.⁴¹ In brief, 2-mm segments of aorta and second-order mesenteric arteries were mounted in a multi-myography system (610 M; Danish Myo Technology, Denmark)

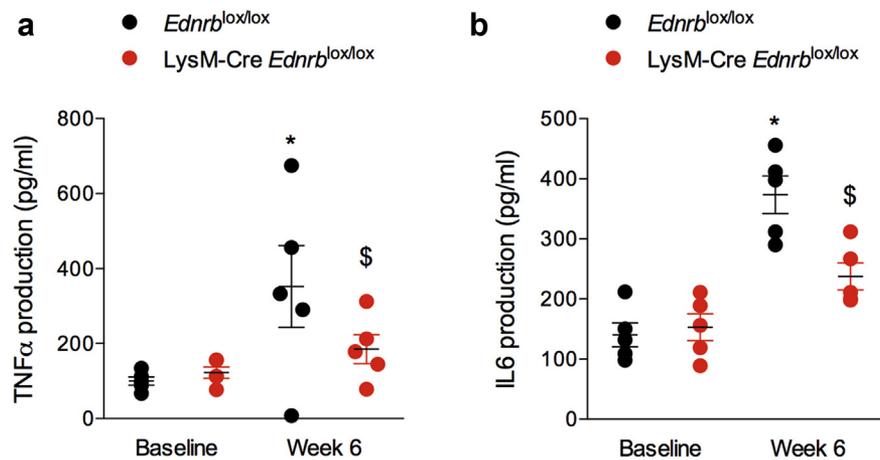


Figure 9 | Specific deletion of *Ednrb* from myeloid cells promotes a less inflammatory neutrophil phenotype in response to angiotensin II (ANG II). Bone marrow–derived neutrophils from *Ednrb*^{lox/lox} and *LysM-Cre Ednrb*^{lox/lox} mice were cultured *in vitro* after 6 weeks of ANG II. (a) Tumor necrosis factor- α (TNF α) and (b) interleukin (IL) 6 production after stimulation with lipopolysaccharide. Data are mean \pm SEM; n = 5/group. *P < 0.05 for baseline compared with week 6 within the same genotype; §P < 0.05 for one genotype compared with the other at the same time point. Comparisons were made by 2-way analysis of variance with Sidak post-test for planned comparisons.

containing a physiological salt solution aerated at 37 °C with 95% O₂/5% CO₂. In all studies, vessel viability was first confirmed by a contractile response on addition of 80 mM KCl, repeated 3 times. Vessel contractility was then assessed by cumulative concentration-response curves to phenylephrine (1 nM to 3 μ M), noradrenalin (1 nM to 3 μ M), and ET-1 (1 pM to 30 nM), and we selected a concentration that produced 80% maximum contraction for each individual vascular ring. To assess vasodilator capacity, vessels were precontracted with phenylephrine and cumulative concentration curves to acetylcholine (1 nM to 3 μ M) and sodium nitroprusside (1 nM to 3 μ M) were obtained. At least 30-minute washout was allowed between drugs. Results are presented as percentage of a maximal response.

In vitro culture of peritoneal macrophages and bone marrow-derived neutrophils

Freshly isolated peritoneal macrophages were plated in 6-well plates at a density of 5 \times 10⁵ cells/3.8 cm² in the Roswell Park Memorial Institute 1640 culture medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Life Technologies, 15140-122) and left to adhere for 24 hours. Cells were then incubated with lipopolysaccharide/interferon- γ (100 ng/ml/10 ng/ml, Sigma Aldrich) or IL4/IL13 (10 ng/ml for both, Invitrogen) for 24 hours. Bone marrow–derived neutrophils were isolated and cultured *in vitro* as previously described.⁴² Cells were then incubated with lipopolysaccharide (100 ng/ml, Sigma Aldrich) for 24 hours.

Cell culture supernatants were removed and frozen immediately at -80 °C until analysis. IL1 β , IL6, and tumor necrosis factor- α were determined using an enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions. Samples were analyzed in duplicate at 450 nm with wavelength correction at 570 nm (Synergy HT BioTEK). Concentrations were calculated from a standard curve using a second-order (quadratic) regression analysis. To assess phagocytic ability, macrophages were incubated with fluorescent beads (10:1 ratio) for 60 minutes. Cells were vigorously washed and detached from the plates before quantification of phagocytosis by flow cytometry.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

P-LT and ND designed the study and provided funding; LG, AC, TEF, VB, PB, AC OL, FS, and CR performed the experiments and carried out analyses; DJW, DCK, MAB, and ND wrote the manuscript; and all authors provided approval for the final manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (Word)

Supplemental Table S1. Differences between baseline and week 6 for macrophages from *Ednrb*^{lox/lox} and *LysM-Cre Ednrb*^{lox/lox} mice in terms of their ability to polarize to an inflammatory (M1) or anti-inflammatory (M2) phenotype, or their phagocytic ability.

Supplemental Methods.

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