ORIGINAL ARTICLE

Angiotensin AT₂ receptor stimulation is anti-inflammatory in lipopolysaccharide-activated THP-1 macrophages via increased interleukin-10 production

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Macrophages have an important role in the pathogenesis of hypertension and associated end-organ damage *via* the activation of the Toll-like receptors, such as Toll-like receptor-4 (TLR4). Accumulating evidence suggests that the angiotensin AT₂ receptor (AT₂R) has a protective role in pathological conditions involving inflammation and tissue injury. We have recently shown that AT₂R stimulation is renoprotective, which occurs in part *via* increased levels of anti-inflammatory interleukin-10 (IL-10) production in renal epithelial cells; however, the role of AT₂R in the inflammatory activity of macrophages is not known. The present study was designed to investigate whether AT₂R activation stimulates an anti-inflammatory response in TLR4-induced inflammation. The effects of the anti-inflammatory mechanisms that occurred following pre-treatment with the AT₂R agonist Compound 21 (C21) (1 μmol ml⁻¹) on the cytokine profiles of THP-1 macrophages after activation by lipopolysaccharide (LPS) (1 μg ml⁻¹) were studied. The AT₂R agonist dose-dependently attenuated LPS-induced tumor necrosis factor-α (TNF-α) and IL-6 production but increased IL-10 production. IL-10 was critical for the anti-inflammatory effects of AT₂R stimulation because the IL-10-neutralizing antibody dose-dependently abolished the AT₂R-mediated decrease in TNF-α levels. Further, enhanced IL-10 levels were associated with a sustained, selective increase in the phosphorylation of extracellular signal-regulated kinase (ERK1/2) but not p38 mitogen-activated protein kinase (MAPK). Blocking the activation of ERK1/2 before C21 pre-treatment completely abrogated this increased IL-10 production in response to the AT₂R agonist C21, while there was a partial reduction in IL-10 levels following the inhibition of p38. We conclude that AT₂R stimulation exerts a novel anti-inflammatory response in THP-1 macrophages *via* enhanced IL-10 production as a result of sustained, selective ERK1/2 phosphorylation, which may have protective roles in hypertension and associated tissue injury.

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INTRODUCTION

Chronic inflammation, which is characterized by elevated cytokine and chemokine expression, has been shown to have a central role in the pathophysiology of hypertension and associated end-organ damage, including renal injury.^{1–4} At the cellular level, chronic inflammation is mediated largely by macrophages. Additionally, macrophage infiltration invariably accompanies hypertensive organ damage, such as that which occurs to blood vessels,⁵ the heart⁶ and the kidneys.⁷ Moreover, circulating monocytes are activated in hypertensive patients and produce increased amounts of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and transforming growth factor-β.⁸ Recent evidence indicates

that rather than being a mere consequence of elevated blood pressure, macrophage activation is actually a causative factor in the development of hypertension.^{9,10}

The renin-angiotensin system is a critical hormonal system that regulates blood pressure, and it is abnormally activated in hypertensive patients. Macrophages also express all major components of the renin-angiotensin system.¹¹ Angiotensin II (Ang II) has been demonstrated to participate in the activation and pro-inflammatory polarization of leukocytes *via* the AT₁ receptor (AT₁R).^{12–14} However, the precise cellular mechanisms underlying these activities are not well defined. One potential inflammatory pathway that has been implicated in hypertension is the activation of innate immune receptors, specifically

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Toll-like receptor-4 (TLR4) signaling,¹⁵ which leads to the production of an array of pro-inflammatory mediators. In fact, peripheral monocyte TLR4 expression is markedly increased in hypertensive patients compared with normotensive controls.¹⁶ Furthermore, Ang II has been shown to upregulate TLR4 expression *via* the AT₁R¹⁷ and exacerbate pro-inflammatory cytokine production in response to TLR4 activation by lipopolysaccharide (LPS) in macrophages.¹⁸ Accumulating evidence suggests that the AT₂ receptor (AT₂R), which is generally considered to be a functional antagonist of the AT₁R, exerts an anti-inflammatory response.^{19–21} We have previously shown that the stimulation of the AT₂R by the selective agonist Compound 21 (C21) attenuates pro-inflammatory signaling in response to the LPS activation of proximal tubule epithelial cells *via* increased IL-10 production.²² However, the abilities of the AT₂R to induce IL-10 production and exert an anti-inflammatory response in LPS-activated macrophages have not been investigated to date.

In macrophages, multiple pathways exist to promote IL-10 production.²³ The activation of mitogen-activated protein kinases (MAPKs), specifically *via* p38 and extracellular signal-regulated kinase (ERK1/2), has been shown to be required to increase IL-10 levels in macrophages, and the inhibition of either of these MAPKs impairs IL-10 production.^{24–28} Moreover, increased ERK1/2 activation correlates well with the extent of IL-10 production in this cell type.²³ Because the AT₂R has been linked to a sustained increase in ERK1/2 phosphorylation, this may be a potential molecular mechanism by which the AT₂R agonist can enhance IL-10 production in macrophages.

The present study was designed to test the hypothesis that the stimulation of the AT₂R attenuates TLR4-mediated pro-inflammatory cytokine production in macrophages *via* increased IL-10 production. We evaluated the effects of C21 on the production of TNF- α , IL-6 and IL-10 in LPS-activated THP-1 macrophages. We demonstrated that pre-treatment with C21 significantly attenuated the levels of pro-inflammatory cytokines. This was found to be dependent upon increased IL-10 production in response to AT₂R activation. Moreover, this upregulation of IL-10 was mediated by a sustained, selective increase in ERK1/2 phosphorylation.

MATERIALS AND METHODS

Cell culture and treatments

The human THP-1 monocytic cell line (ATCC) was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and an antibiotic/antimycotic cocktail (100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and 250 ng ml⁻¹ amphotericin B) at 37 °C in a humidified atmosphere with 5% CO₂. Cell culture media and supplements were all purchased from HyClone (ThermoFisher Scientific, Waltham, MA, USA). To differentiate monocytes from macrophages, 5 $\times$ 10⁵ cells per well were treated with 40 nmol l⁻¹ PMA (phorbol-12-myristate-13-acetate) (Sigma, St Louis, MO, USA) for 48 h in RPMI-1640 containing 5% fetal bovine serum. At the end of the 48-h incubation period, the medium was aspirated, and the cells were washed with RPMI-1640 without fetal bovine serum or antibiotics/antimycotics and were then incubated in this medium for 6–8 h. To eliminate pre-existing cytokine production, the medium was replaced by fresh serum-free medium before the treatments were initiated. LPS (1 μ g ml⁻¹) (*E. coli* O55:B5; Sigma) was used to induce the production of pro-inflammatory cytokines. The cells were pre-treated with C21 (custom synthesized) for 60 min before the addition of LPS, and the drug remained in the medium for the entire duration of the treatment. Treatments with specific inhibitors, including 1 μ mol l⁻¹ candesartan (an AT₁R antagonist; AstraZeneca, Wilmington, DE, USA), 10 μ mol l⁻¹ PD123319 (an AT₁R antagonist; Pfizer, New York, NY, USA), 10 μ mol l⁻¹ SB203580 (a p38 inhibitor; Cell Signaling Technology, Danvers, MA, USA) and 10 μ mol l⁻¹ PD98059 (an MEK (MAPK/ERK kinase) inhibitor; Cell Signaling Technology) were carried out as indicated in the text and figure legends.

Immunoblotting

Macrophages were washed twice with PBS and lysed on a plate using ice-cold cell lysis buffer (Cell Signaling Technology) containing protease (Roche, Indianapolis, IN, USA)- and phosphatase (Sigma)-inhibitor cocktails. Equal amounts of protein in Laemmli buffer were loaded per well (15 μ g per lane for AT₁R, 45 μ g per lane for AT₂R and 30 μ g per lane for ERK1/2 and p38 MAPK) and separated by SDS-PAGE using a Tris-glycine system. Proteins were then transferred onto a PVDF membrane using the wet transfer protocol. The membrane was incubated in 5% non-fat dry milk in PBST for 1 h, followed by incubation overnight with primary antibodies against AT₁R (Biomolecular Integrations, Little Rock, AR, USA), AT₂R (EZ Biolabs, Carmel, IN, USA), p-ERK1/2 (Cell Signaling Technology), p-p38 MAPK (Cell Signaling Technology) at 1:1000 dilutions. An antibody against inducible nitric oxide synthase (iNOS) (Cell Signaling Technology) was used at 1:500, while that against macrophage mannose receptor (MMR; Abcam, Cambridge, MA, USA) was used at a 1:200 dilution. This was followed by a wash with PBST

Figure 1 Expression of angiotensin II receptors in THP-1 macrophages. Protein expression by immunoblotting of angiotensin AT₁ (a) and AT₂ (b) receptors in THP-1 macrophages after 24-h treatments with vehicle (Ctrl), AT₂R agonist C21 (C21; 1 μ mol l⁻¹), LPS (1 μ g ml⁻¹) or both LPS+C21. Cells were pre-treated with C21 (1 μ mol l⁻¹) for 60 min before LPS activation. Data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. control THP-1 macrophages ($n = 5$).

Figure 2 Effects of an AT₂R agonist (C21) and an AT₁R antagonist (candesartan) on cytokine production by LPS-activated THP-1 macrophages. THP-1 macrophages were incubated with either the AT₂R agonist (C21; 1 $\mu\text{mol l}^{-1}$) or the AT₁R antagonist candesartan (Can; 1 $\mu\text{mol l}^{-1}$) for 60 min before activation with LPS (1 $\mu\text{g ml}^{-1}$). To demonstrate the specificity of C21, an additional group of cells were incubated with the AT₂R antagonist PD123319 (PD; 10 $\mu\text{mol l}^{-1}$) for 30 min before C21 pre-treatment. The cytokines TNF- α (a), IL-6 (b) and IL-10 (c) were assessed in the media at 24 h after LPS activation by ELISA. The data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. control, # indicates $P < 0.05$ vs. LPS-treated and \$ indicates $P < 0.05$ vs. LPS+C21-treated THP-1 macrophages ($n = 6$).

Figure 3 Dose-dependent and time-dependent effects of an AT₂R agonist (C21) on cytokine production by LPS-activated THP-1 macrophages. The dose-dependent effects of the AT₂R agonist C21 on TNF- α (a) and IL-10 (b) levels were assessed at 24 h following LPS activation (1 $\mu\text{g ml}^{-1}$). C21 pre-treatment (0.1–10 $\mu\text{mol l}^{-1}$) was administered 60 min before LPS activation, and cytokines in the media were assessed by ELISA. An additional set of cells were incubated with the AT₂R antagonist PD123319 (PD; 10 $\mu\text{mol l}^{-1}$) for 30 min before C21 pre-treatment to demonstrate the receptor specificity of C21. TNF- α (c) and IL-10 (d) production at earlier time points was also determined in the media at the indicated times after LPS activation following C21 (1 $\mu\text{mol ml}^{-1}$) pre-treatment. The data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. respective control ($n = 6$).

and a 1-h incubation with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology). Chemiluminescence was detected by the addition of Luminol HRP substrate (Santa Cruz Biotechnology) and quantified by a software-assisted densitometric analysis (Alpha Innotech, San Leandro, CA, USA). To ensure equal loading, the blots were stripped and re-probed for β -actin (BioVision, Milpitas, CA, USA) for AT₁R and AT₂R and total-p38 and total-ERK1/2 for p-p38 and p-ERK1/2, respectively (Cell Signaling Technology).

Enzyme-linked immunosorbent assay

The cytokines in the medium were assessed by a kit-based enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA).

mRNA expression by RT-PCR

Total RNA from the cells was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized by RT-PCR from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System (Life Technologies, Grand Island, NY, USA). This cDNA was used as a template for the quantitative RT-PCR analysis of the gene expressions of *TNFA*, *IL6* and *IL10* using TaqMan gene expression assays (Applied Biosystems, Grand Island, NY, USA). Relative quantifications were determined using the delta-delta Ct method with GAPDH as a control.

Statistical analysis

The data are presented as the mean $\pm$ s.e.m. Student's *t*-test was used to compare the mean values of two groups. One-way ANOVA with a *post hoc* test

Figure 4 Effects of an AT₂R agonist (C21) on mRNA and protein expression of cytokines by LPS-activated THP-1 macrophages. Macrophages were treated with vehicle (Control), C21 (1 $\mu\text{mol l}^{-1}$), LPS (1 $\mu\text{g ml}^{-1}$) or both LPS and C21 (LPS+C21). C21 (1 $\mu\text{mol l}^{-1}$) pre-treatment was initiated 60 min before LPS activation. The expression of TNF- α , IL-6 and IL-10 at the mRNA level (a–c) was determined by RT-PCR, while TNF- α , IL-6 and IL-10 protein levels were quantified in the media (d–f) by ELISA at 24 h after LPS activation. The data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. control and #indicates $P < 0.05$ vs. LPS-treated THP-1 macrophages ($n = 5–8$).

(Tukey's) for multiple comparisons was used to compare variations between more than two groups. A value of $P < 0.05$ was considered to be statistically significant with $n = 5–8$ experiments per group.

RESULTS

Expression of angiotensin AT₁R and AT₂R in THP-1 macrophages

THP-1 macrophages express both AT₁R and AT₂R. The expressions of both receptor subtypes were not altered by the C21 or LPS treatments (Figures 1a and b); however, the C21 pre-treatment lowered AT₁R expression by $\sim 50\%$ in response to LPS (Figure 1a), which is in agreement with a number of previous reports demonstrating the AT₂R-mediated downregulation of AT₁R in pathophysiological conditions.^{29–31}

Effects of AT₂R agonist (C21) and AT₁R antagonist (candesartan) on cytokine production by LPS-activated THP-1 macrophages

THP-1 macrophages were treated with LPS (1 $\mu\text{g ml}^{-1}$) for 24 h to induce the expression of pro-inflammatory cytokines (TNF- α and IL-6) and the anti-inflammatory cytokine IL-10. Pre-treatment with the AT₂R agonist C21 (1 $\mu\text{mol l}^{-1}$) attenuated the LPS-induced TNF- α and IL-6 production by ~ 33 and 50% , respectively, with a concurrent 75% increase in IL-10 production. This anti-inflammatory response was blocked by the AT₂R antagonist PD123319 (10 $\mu\text{mol l}^{-1}$), suggesting the presence of an AT₂R-mediated effect. However, pre-treatment with the AT₁R antagonist candesartan (1 $\mu\text{mol l}^{-1}$) did not alter the cytokine levels in response to LPS (Figures 2a–c). In an additional set of experiments, C21 (1 $\mu\text{mol l}^{-1}$) was administered 1 h after LPS administration to determine the effects of AT₂R stimulation following the initiation of inflammatory pathways. IL-6, but not TNF- α , was significantly attenuated when the macrophages were treated with C21 post-LPS administration. IL-10 levels appeared to be only modestly increased (Supplementary Figure S1).

The anti-inflammatory effects of an AT₂R agonist (C21) on cytokine production by LPS-activated THP-1 macrophages are mediated by increased IL-10 production

Pre-treatment with the AT₂R agonist C21 dose-dependently (0.1–10 $\mu\text{mol l}^{-1}$) attenuated the production of TNF- α (Figure 3a), while IL-10 production was concurrently enhanced (Figure 3b) in the LPS-activated THP-1 macrophages at 24 h post LPS administration. Pre-treatment with C21 before LPS activation resulted in lower TNF- α levels, which were observed as early as 60 min post LPS administration, and this trend continued for up to 4 h (Figure 3c). The cells pre-treated with C21 for 1 h produced measurable amounts of anti-inflammatory IL-10 at the time of LPS addition, and the IL-10 levels continued to be elevated for up to 4 h compared with the LPS-treated control cells (Figure 3d). Thus, this AT₂R agonist exerts anti-inflammatory effects on early cytokine production in THP-1 macrophages, and this response is dose dependent.

Cytokine production at the levels of mRNA and the protein released into the media was assessed at 24 h post LPS activation to determine the effects of the chronic stimulation of AT₂R. C21 pre-treatment significantly attenuated pro-inflammatory TNF- α and IL-6 mRNA levels in addition to their protein expression levels (Figures 4a, b, d and e). Conversely, the IL-10 level in the media was significantly higher for the C21 pre-treated cells following LPS stimulation (Figure 4f). Interestingly, IL-10 mRNA expression was higher in the C21-treated cells, even in the absence of LPS activation (Figure 4c), although this did not translate into higher IL-10 protein expression in this treatment group (Figure 4f). Furthermore, a neutralizing antibody to IL-10 dose-dependently ameliorated the effects of C21 on LPS-induced TNF- α production (Figure 5). Thus, the increase in IL-10 production in response to C21 in the presence of LPS appears to be critical for attenuating the anti-inflammatory response to LPS.

Involvement of p38 and ERK1/2 MAPKs in the AT₂R-mediated increase in IL-10 production

The activation of MAPKs, specifically p38 and ERK1/2, has been shown to be a key requirement for the regulation of IL-10 production in macrophages.^{25,28,32,33} LPS treatment led to the rapid phosphorylation of p38 and ERK1/2; these activities peaked at 30 min post LPS treatment and returned to basal levels by 2 h post LPS treatment (Figures 6a, b, 7a and b). C21 pre-treatment delayed the peak of p38 phosphorylation to 1 h post LPS treatment (Figures 6a and b).

Figure 5 Dose-dependent inhibition of anti-inflammatory effects of an AT₂R agonist (C21) on TNF- α production by neutralizing interleukin-10 (IL-10) antibody. Cells were incubated with increasing doses of neutralizing IL-10 antibody (0.125–2.5 $\mu\text{g ml}^{-1}$) for 30 min before C21 pre-treatment (1 $\mu\text{mol l}^{-1}$). The cells were activated with LPS (1 $\mu\text{g ml}^{-1}$) 60 min after the addition of C21. Cytokine concentrations in media were measured by ELISA. The data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. LPS-, # indicates $P < 0.05$ vs. LPS+C21-treated THP-1 macrophages ($n = 5$).

Incubation with a p38 inhibitor (SB203580; 10 $\mu\text{mol l}^{-1}$) before C21 pre-treatment partially abolished the C21-mediated increase in IL-10 at 24 h post LPS activation (Figure 6c).

However, C21 pre-treatment resulted in a delayed, sustained increase in ERK1/2 phosphorylation (Figures 7a and b), which persisted for up to 24 h following LPS treatment (Figure 7c). Moreover, pre-incubation of the cells with an MEK inhibitor (PD98059; 10 $\mu\text{mol l}^{-1}$), which prevented ERK1/2 activation, before C21 pre-treatment completely abolished the C21-mediated increase in IL-10 at 24 h post LPS activation (Figure 7d), suggesting that the sustained, selective ERK1/2 phosphorylation associated with the AT₂R agonist is essential for the increase in IL-10 production. C21 treatment alone did not induce ERK1/2 phosphorylation at 24 h (Figure 7c) or at any earlier time points (data not shown), suggesting that LPS-mediated signaling is required for C21 to exert its anti-inflammatory effects.

Effects of AT₂R (C21) agonist pre-treatment on macrophage polarization

Depending on the stimulating factors, macrophages can be polarized to produce predominantly pro-inflammatory or anti-inflammatory mediators. These phenotypes are broadly classified as ‘classically’ activated M1 and ‘alternatively’ activated M2 macrophages, respectively. M1 macrophages express high levels of iNOS, while expression of the macrophage mannose receptor (MMR) is upregulated in M2 macrophages. Consequently, these proteins may be used as phenotypic markers. LPS (1 $\mu\text{g ml}^{-1}$) treatment resulted in an approximate fivefold increase in iNOS expression, which was prevented when the cells were pre-incubated with C21 (1 $\mu\text{g ml}^{-1}$) for 1 h (Figure 8a). The expression of MMR was markedly lower in LPS-treated cells but was ~50% higher in C21-treated macrophages, which was independent of LPS stimulation (Figure 8b).

Figure 6 Effects of pre-treatment with an AT₂R agonist (C21) on the phosphorylation of p38 MAPK. The time course of p38 phosphorylation in response to LPS activation with or without C21 was determined by immunoblotting, (a) and the ratio of phosphorylated to total p38 was quantified (b). C21 pre-treatment (1 $\mu\text{mol l}^{-1}$) was initiated 60 min before the addition of LPS (1 $\mu\text{g ml}^{-1}$). Times indicated are post-LPS addition. The effect of p38 inhibition on IL-10 production was also assessed (c). Macrophages were activated with LPS (1 $\mu\text{g ml}^{-1}$) 60 min after the addition of C21 (1 $\mu\text{mol l}^{-1}$). An additional set of cells were pre-treated with the p38 inhibitor SB203580 (10 $\mu\text{mol l}^{-1}$) for 60 min before C21 was added. Cytokine concentrations in the media were measured by ELISA 24 h after LPS treatment. The data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. control, #indicates $P < 0.05$ vs. LPS-treated and \$indicates $P < 0.05$ vs. LPS+C21-treated THP-1 macrophages ($n = 6$).

Figure 7 Effects of pre-treatment with an AT₂R agonist (C21) on the phosphorylation of ERK1/2. The time course of ERK1/2 phosphorylation in response to LPS-activation with or without C21 was determined by immunoblotting, (a) and the ratio of phosphorylated to total ERK1/2 was quantified (b). C21 pre-treatment ($1 \mu\text{mol l}^{-1}$) was initiated 60 min before the addition of LPS ($1 \mu\text{g ml}^{-1}$). Times indicated are post-LPS addition. To assess the ability of the AT₂R agonist to lead to a sustained increase in ERK1/2 phosphorylation, macrophages were activated with LPS ($1 \mu\text{g ml}^{-1}$) 60 min after the addition of C21 ($1 \mu\text{mol l}^{-1}$), and the cells were collected at 24 h post LPS treatment. Subsequently, the ratio of phosphorylated to total ERK1/2 was quantified by immunoblotting (c). The effect of the inhibition of ERK1/2 phosphorylation on IL-10 production was also assessed (d). Macrophages were activated with LPS ($1 \mu\text{g ml}^{-1}$) 60 min after the addition of C21 ($1 \mu\text{mol l}^{-1}$). An additional set of cells were pre-treated with the MEK inhibitor PD98059 ($10 \mu\text{mol l}^{-1}$) for 60 min before C21 was added. Cytokine concentrations in the media were measured by ELISA 24 h after LPS treatment. The data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. control, #indicates $P < 0.05$ vs. LPS-treated and \$indicates $P < 0.05$ vs. LPS+C21-treated THP-1 macrophages ($n = 6$).

Figure 8 Expression of the M1 marker iNOS and the M2 marker MMR in THP-1 macrophages. Protein expression by immunoblotting of iNOS (a) and MMR (b) in THP-1 macrophages after 24 h of treatment with vehicle (Control), the AT₂R agonist C21 (C21; $1 \mu\text{mol l}^{-1}$), LPS ($1 \mu\text{g ml}^{-1}$) or both LPS+C21. The cells were pre-treated with C21 ($1 \mu\text{mol l}^{-1}$) for 60 min before LPS activation. The data are represented as the mean $\pm$ s.e.m. *Indicates $P < 0.05$ vs. control and #indicates $P < 0.05$ vs. LPS-treated THP-1 macrophages ($n = 6$).

DISCUSSION

Macrophages have an important role in the initiation and progression of hypertension and associated end-organ damage *via* the activation of TLR4-mediated pro-inflammatory signaling. Macrophages express AT₂Rs,¹¹ which have been demonstrated to attenuate TLR-mediated pro-inflammatory cytokine production in proximal tubule epithelial cells.²² In the present study, we have demonstrated that the pre-treatment of THP-1 macrophages with the AT₂R agonist C21 attenuates TNF- α and IL-6 production in response to the activation of TLR4 by LPS. These effects are mainly a result of the increased IL-10 production by macrophages *via* a sustained, selective increase in ERK1/2 phosphorylation.

Ang II has been documented to promote pro-inflammatory cytokine and chemokine production *via* the AT₁R in a manner similar to TLR4-mediated signaling in a number of tissues, including endothelial cells,^{34,35} renal tubular epithelial cells,³⁶ dendritic cells^{37–39} and T lymphocytes.^{40–42} However, its precise role in association with macrophages/monocytes is controversial. The incubation of monocytes with Ang II has been shown to induce the expression of the chemokine monocyte chemoattractant protein-1,⁴³ while this process has not been shown to affect cytokine production.⁴⁴ The renin-angiotensin system is upregulated during monocyte differentiation, and macrophages express relatively higher levels of AT₁R and AT₂ R compared with monocytes.¹¹ The incubation of macrophages with Ang II *via* AT₁R activation has been demonstrated to result in increased IL-6 production without affecting TNF- α .^{18,45} Further, an interaction between Ang II and enhanced TLR4 signaling has been reported.^{17,46–48} However, the interpretation of these findings has been complicated by the fact that the commonly used AT₁R blockers, including losartan and candesartan, possess anti-inflammatory activities independent of the AT₁R.^{18,44}

Conversely, the anti-inflammatory role of the AT₂R, which is generally considered to act as a functional antagonist of the AT₁R, has been demonstrated in a number of *in vitro* and *in vivo* models.^{19–22,49} Here, we report that the AT₂R agonist C21 significantly lowered both TNF- α and IL-6 levels in association with increased production of the anti-inflammatory cytokine IL-10. Some degree of variation was observed in the absolute values of cytokines elicited by LPS in our experiments, which may be attributed to batch-to-batch variations in LPS levels. Nevertheless, in all cases, C21 treatment resulted in an approximate 50% decrease in pro-inflammatory cytokine levels. Because this altered cytokine profile could be abrogated by the AT₂R antagonist PD123319, we conclude that these anti-inflammatory effects were associated with a specific AT₂R-mediated response.

Our preliminary experiments (data not shown) suggested that Ang II did not stimulate the production of cytokines, which is in agreement with the findings by An *et al.*,¹⁸ who have reported that Ang II does not alter cytokine levels in the presence or absence of LPS in THP-1 macrophages. Larrayoz *et al.*⁴⁴ have previously demonstrated that candesartan inhibits LPS-induced pro-inflammatory cytokine production by human monocytes, while in our study, it did not alter the cytokine levels. This might be explained by differences in the cell types and LPS doses used. In the present study, we used a dose of LPS that was 20 times higher than that used by Larrayoz *et al.*,⁴⁴ and it is possible that candesartan may not be effective under conditions of excessive immune activation. Another critical difference between the two studies is the time of sample collection. We collected samples for cytokine estimation at 24 h post LPS administration as opposed to 2 h. Our data simply suggest that candesartan may not possess anti-inflammatory activities in THP-1 macrophages. It is very likely that

in vivo, the blockade of AT₁R results in anti-inflammatory effects due to its collective influences on other cell types, as previously demonstrated.

We found that pre-treatment with C21 in the presence of LPS also attenuated AT₁R expression. The downregulation of AT₁R in response to AT₂R stimulation under pathophysiological conditions has been reported in a number of experimental models.^{29–31} In the present study, however, this observation may be unrelated to the anti-inflammatory response to the AT₂R agonist because the increase in pro-inflammatory cytokine levels did not appear to be mediated by AT₁R activation.

We have previously shown that AT₂R stimulation results in enhanced IL-10 secretion by proximal tubule epithelial cells.²² A similar observation was reported in a specific subset of splenic CD8⁺ AT₂R⁺ T cells, which produced uncharacteristically high levels of IL-10 and AT₂R following stimulation by Ang II as well as by C21, which further augmented IL-10 production.⁵⁰ Here, we report that C21 alone increased IL-10 gene expression levels; however, this did not translate to increased IL-10 protein secretion, except in the presence of TLR4 activation by LPS. This could be a result of post-transcriptional modifications to IL-10 mRNA that have been shown to occur in immune cells as a means of regulating IL-10 production in the absence of inflammatory stimuli.⁵¹

Although there is considerable evidence demonstrating the anti-inflammatory effects of AT₂R stimulation, the signaling pathways involved in mediating this response remain unclear and are still a subject of debate. Moreover, the cell types and experimental conditions used greatly influence the downstream signaling cascades activated by the AT₂R. Typically, AT₂R stimulation results in the activation of phosphatases, including MAP kinase phosphatase-1^{52–54} and SH-2 domain-containing phosphatase-1,^{55–57} which ultimately leads to AT₂R-mediated apoptosis. However, AT₂R stimulation has also been shown to promote cellular differentiation *via* a sustained increase in ERK1/2 phosphorylation,^{58–61} which is independent of cAMP-mediated signaling.⁶² In the present study, AT₂R agonist pre-treatment resulted in a delayed increase in ERK1/2 phosphorylation, which was sustained for up to 24 h post LPS activation; however, treatment with the AT₂R agonist alone did not promote ERK1/2 phosphorylation at any of the time points studied, nor was IL-10 detectable in the medium. Thus, it appears that LPS-mediated signaling pathways are required for the augmented IL-10 production by the AT₂R agonist. On the basis of the protein expressions of the macrophage markers iNOS and MMR, C21 pre-treatment may prime macrophages, so that in the presence of an activating signal, such as LPS, their polarization to the alternatively activated, anti-inflammatory M2 phenotype is favored over the pro-inflammatory, classically activated M1 phenotype. In the present study, we used naïve PMA-differentiated macrophages, which were then stimulated by LPS. However, in the setting of chronic inflammation *in vivo*, a percentage of the macrophages are likely to be polarized to the M1 state. The effects of AT₂R activation in these differentiated macrophages warrant further investigation.

In macrophages, multiple pathways exist that can regulate the production of IL-10, depending upon the activating stimulus.^{28,63–66} Of these, activation of the p38 and ERK1/2 MAPKs has been shown to be critical for the induction of IL-10 synthesis.^{23–28} We report that the inhibition of p38 activation partially abrogated the AT₂R-mediated increase in IL-10, while the inhibition of ERK1/2 activation resulted in a complete lack of IL-10 production in response to AT₂R stimulation, suggesting that p38 MAPK may contribute to, but is not essential for, AT₂R-mediated IL-10 expression. This observation could also be

linked to the altered kinetics of p38 MAPK phosphorylation in response to LPS in the presence or absence of AT₂R agonist pretreatment. However, the precise mechanisms that orchestrate these changes in MAPK phosphorylation over time require further investigation. Overall, our results regarding MAPK activation are in agreement with the *in vivo* study by Kaschina *et al.*,⁶⁷ who have reported that treatment with C21 promotes p38 and ERK1/2 phosphorylation in a model of myocardial infarction.

Over the past decade, AT₂R stimulation has emerged as a potential therapeutic target for the treatment of hypertension and end-organ damage,^{21,22,29,68,69} particularly in the presence of a concomitant AT₁R blockade.^{70,71} Further, AT₂R activation at the level of the kidney has been shown to promote vasodilation and natriuresis, thus conferring renoprotection in the setting of hypertension.^{29,72,73} Although the administration of C21 has been shown to have modest, if any, effects on lowering blood pressure,^{21,69,74–76} its protective effects on inflammation, oxidative stress, fibrosis and vascular remodeling underscore the potential benefit of the addition of an AT₂R agonist to the currently used classical anti-hypertensive drugs to delay the progression of hypertension and end-organ damage. Here, we identify macrophages, which have a central role in the initiation and progression of hypertension-associated target organ damage, as an additional target of AT₂R stimulation.

In conclusion, the present study demonstrated a novel anti-inflammatory role of AT₂R stimulation in macrophages involving the attenuation of TLR4-mediated pro-inflammatory cytokine production. We further demonstrated that the ERK1/2-dependent increase in IL-10 production was a key event involved in mediating this anti-inflammatory response. Thus, stimulation of the AT₂R may be beneficial in the treatment of hypertension due to its protective effects, not only on hemodynamic factors, such as vasodilation and Na⁺ excretion, but also as a consequence of the attenuation of inflammation and associated end-organ injury.

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