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Stimulation of the Angiotensin II AT2 Receptor is Anti-inflammatory in Human Lipopolysaccharide-Activated Monocytic Cells

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Abstract—Recently, AT2 receptors have been discovered on the surface of human immunocompetent cells such as monocytes. Data on regulative properties of this receptor on the cellular immune response are poor. We hypothesized that direct stimulation of the AT2 receptor mediates anti-inflammatory responses in these cells. Human monocytic THP-1 and U937 cells were stimulated with lipopolysaccharide (LPS) and the selective AT2 receptor agonist Compound 21 (C21). Expression of pro- and anti-inflammatory cytokines IL-6, IL-10, tumor necrosis factor-α (TNFα), and IL-1β were analyzed on both the transcriptional and the translational level over course of time. Treatment with C21 attenuated the expression of TNFα, IL-6, and IL-10 after LPS challenge in both cell lines in a time- and dose-dependent manner. We conclude that selective AT2 receptor stimulation acts anti-inflammatory in human monocytes. Modulation of cytokine response by AT2 receptor activation might be a beneficial and novel treatment concept in inflammatory conditions.

KEY WORDS: AT2 receptor; Compound 21 (C21); THP-1 cells; inflammation; monocytes.

INTRODUCTION

The renin-angiotensin system (RAS) figures largely in human physiology and is intricately involved in cardiovascular control, blood pressure regulation, and inflammation [3, 7, 34]. Its biological effects are mediated by at least two receptors, the angiotensin AT1 receptor and the angiotensin AT2 receptor. Many cardiovascular pathologies are characterized by intensified AT1 receptor stimulation, which essentially promotes pro-proliferative and pro-inflammatory responses [6, 17, 25, 26, 41]. In contrast, the AT2 receptor is generally considered to be a functional antagonist of the AT1 receptor and is thought to possess beneficial effects. Although the physiological role of this receptor is still under debate, most of the data available support the concept that the AT2 receptor acts as an endogenous, tissue-protective system involved in cellular repair and anti-inflammation [23, 37]. Recent evidence suggests that selective AT2 receptor activation can counteract the pro-inflammatory and pro-proliferative effects of the AT1 receptor [1, 14, 17, 18, 29, 30, 36].

Recently, AT2 receptors and other components of the renin-angiotensin system have been discovered on the surface of human immunocompetent cells such as monocytes and lymphocytes which both play an important role in initiation and regulation of inflammation [22, 24]. Other studies demonstrate that human monocytes produce angiotensin II, which triggers chemotaxis, adhesion and activation of pro-inflammatory nuclear factor-κβ in these cells [16, 22, 26, 27, 40]. Taken together, the data show that the angiotensinergic system exerts a profound regulatory effect linked to the cellular immune response. However, the

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precise biological functions of AT2 receptors on monocytes are hitherto unknown. Current studies involving the novel non-peptide, specific and selective AT2 receptor agonist Compound 21 (C21) demonstrate inhibition of inflammatory signaling pathways by direct AT2 receptor activation [9, 29]. Toll-like receptor-4 (TLR4) signaling turned out to be one of the major inflammatory pathways the AT2 receptor is involved in [8, 9, 29, 39]. In several animal models C21 could evoke beneficial effects including a marked downregulation of inflammation [13, 28, 36].

Therefore, we hypothesized that direct stimulation of the AT2 receptor on human monocytes can mediate anti-inflammatory responses in these cells. In an *in vitro* approach, we studied whether stimulation of the AT2 receptor with C21 attenuates early TLR4-mediated inflammatory responses by transcriptional and/or translational regulation of pro- and anti-inflammatory cytokines. This study aimed to expand the understanding of regulative properties of the AT2 receptor in inflammation.

MATERIALS AND METHODS

Cell Culture

Human promonocytic THP-1 and human myelomonocytic U937 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. THP-1 cells and U937 cells were cultured in RPMI-1640 medium supplemented with 10 % inactivated fetal bovine serum (FCS) without antibiotics. All cell culture products were obtained from Gibco/Invitrogen, Karlsruhe, Germany, unless otherwise stated. Cells were grown in a humidified incubator at 5 % CO_2 and 37 °C.

Treatment and Stimulation Experiments

For induction of monocyte-macrophage differentiation, THP-1 cells were treated with 0.5 μM phorbol 12-myristate 13-acetate (PMA) in RPMI-1640 medium for 3 h, washed twice with PBS, counted and seeded in 6-well cell culture plates at a density of 1×10^6 cells/well in 2 ml of RPMI-1640 medium supplemented with 10 % FCS. After overnight rest, adherent cells were washed with PBS and subjected to further treatment. U937 cells were used at a density of 5×10^5 cells or 1×10^6 cells/well, respectively. For stimulation experiments, the following pharmacological tools and substances were used: the non-peptide AT2 receptor agonist Compound 21 (C21) at 0.01–10 μM (a kind gift of Vicore Pharma, Gothenborg, Sweden), the competitive AT2 receptor antagonist PD 123319 (10 μM; Tocris Bioscience, Bristol, UK) and lipopolysaccharide (LPS) of the *Escherichia coli* strain 0127:B8 (Sigma, Saint Luis, USA). Cells of indicated lines were incubated in given concentrations of LPS in presence or absence of C21, PD 123319 for 3, 6, and 12 h, respectively. Afterwards, supernatant was collected for cytokine measurement and cells were harvested for extraction of total RNA and protein. Time-matched, vehicle-treated samples served as control. Experiments were performed in biological triplicates and were independently repeated for at least 3 times.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated using the absolutely RNA kit (Stratagene, La Jolla, USA) according to the manufacturer's recommendations including a DNase digest. cDNA synthesis was performed using random hexamer primers and M-MLV reverse transcriptase (Promega, Mannheim, Germany); no template controls and reactions without addition of reverse transcriptase (RT-) served as negative controls. cDNA was quantified by real-time polymerase chain reaction (RT-PCR) Master Mix (Applied Biosystems, Darmstadt, Germany) using FAM-5′/TAMRA-3′ labeled probes for *IL-1β*, *TNF-α*, *IL-10*, *IL-6*, *AT2R*, *β-actin*, and *hypoxanthin-guanine-phosphoribosyltransferase* (*HPRT*) (Metabion, Munich, Germany). Data represent the mean expression level±standard deviation (standardized to *HPRT* expression) calculated according to the 2^-ΔΔCT method of at least three independent measurements per cDNA (technical triplicates). The sequences of primers used are listed in Table 1. cDNA from human placenta served as positive control (a kind gift of S. Endesfelder, Berlin).

Western Blotting

THP-1 and U937 cells were harvested and washed twice with PBS and lyzed in buffer containing 10 mM Tris/HCl, pH 7.5, 300 mM NaCl, 1 % Triton X-100, 2 mM $MgCl_2$, 5 mM ethylene-diaminetetraacetic acid (EDTA), and protease inhibitor cocktail, Complete Mini (Roche, Mannheim, Germany). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electro-transfer to a nitro-cellulose membrane and antibody detection was carried out as described previously [19]. Polyclonal rabbit antihuman AT2 receptor antibody, PathScan® Multiplex

Table 1. Sequences of Oligonucleotides Used for qRT-PCR

Gene	Forward primer 5′-3′ reverse primer 5′-3′	Probe 5′ 6-FAM-TAMRA-3′
AT2R	CTG GCA CCA ATG AGT CCG CC GCA GCT GCC ATC TTC AGG AC	
HPRT	AGT CTG GCT TAT ATC CAA CA CTT CG GAC TTT GCT TTC CTT GGT CAG G	TTT CAC CAG CAA GCT TGC GAC CTT GA
IL-1β	GGC AAT GAG GAT GA CTT GTT C GTA GTG GTG GTC GGA GAT TCG	ATG GCC CTA AAC AGA TGA AGT GCT CCT TCC
IL-6	CCA CTC ACC TCT TCA GAA CGA ATT AGT GCC TCT TTG CTG CTT TCA C	ATG TCT CCT TTC TCA GGG CTG AGA TGC C
IL-10	CAA GTT GTC CAG CTG ATC CTT CAT GGC AAC CTG CCT AAC ATG CTT	AAA GAA AGT CTT CAC TCT GCT GAA GGC ATC TCG
TNFα	TCT CGA ACC CCG AGT GAC AA TCA GCC ACT GGA GCT GCC	TGT AGC CCA TGT TGT AGC AAA CCC TCA AGC
β-actin	CCC TAA GGC CAA CCG TGA AAA GAT G GAA CCG CTC ATT GCC GAT AGT GAT G	

Western Cocktail II containing rabbit anti-elF4E antibody (Cell Signaling, Cambridge, UK) and rabbit antihuman β-actinin antibody (Sigma-Aldrich, Saint Louis, USA) were used. Depicted blots represent a Western blot series ($n=3-5$). Quantification of respective band density was carried out using the image analysis program ImageJ 1.42q (NIH, USA).

Determination of Cytokines

The expression of human pro- and anti-inflammatory cytokines IL-1β, TNF-α, IL-10, and IL-6 were evaluated in the supernatant fraction of treated cells using the cytometric bead array (CBA) inflammation kit and fluorescence activated cell sorting (FACS) analysis according to the manufacturer's instructions (BD Biosciences, San Diego, USA). Fifty microliters of supernatant were used per measurement. The sensitivity of this system was between 1.9 and 7.2 pg per ml for each of the cytokines. Data represent means±SEM of at least three independent experiments.

Cell Viability

Cell viability was determined on the single-cell level by flow cytometry. Cells were seeded and treated as described above. Cells were trypsinized, collected by centrifugation at 300×*g* for 5 min and resuspended in PBS containing 50 μg/mL propidium iodide (PI; Sigma). After incubation for 15 min at 37 °C, cells were subjected to flow cytometric analysis. DNA fragmentation was quantified by determination of hypodiploid DNA content (sub-G1 population), which reflects the percentage of cells with fragmented genomic DNA. Data were collected and analyzed using a FACS-Canto-II flow cytometer equipped with Diva software (BD Biosciences, San Diego, USA). All measurements were performed in technical triplicates, each analyzing at least 10,000 single events.

Statistics

A two-tailed Student's *t* test or a Mann-Whitney *U* test was applied as indicated whenever appropriate. Statistical significance was assumed at $p<0.05$. Vertical lines in histograms indicate standard deviations (SD) or standard error of the mean (SEM). Numbers of independent experiments are noted in the figure legends.

RESULTS

C21 Dose Dependency and LPS Concentration

The AT2 receptor agonist C21 dose dependently attenuated the protein production of IL-6 at 6 h after LPS challenge (Fig. 1a). At C21-concentrations of 1 and 10 μM, IL-6 levels were significantly reduced in comparison to LPS-only treated cells. To investigate whether these AT2 receptor-mediated effects on LPS-induced cytokine release where dependent on LPS concentration, expression of IL-6 was analyzed with 50 ng/ml or 100 ng/ml LPS, respectively. The inhibitory effect of AT2 receptor stimulation with C21 on IL-6 protein expression was significant in both investigated dosages of LPS (Fig. 1b), whereas C21

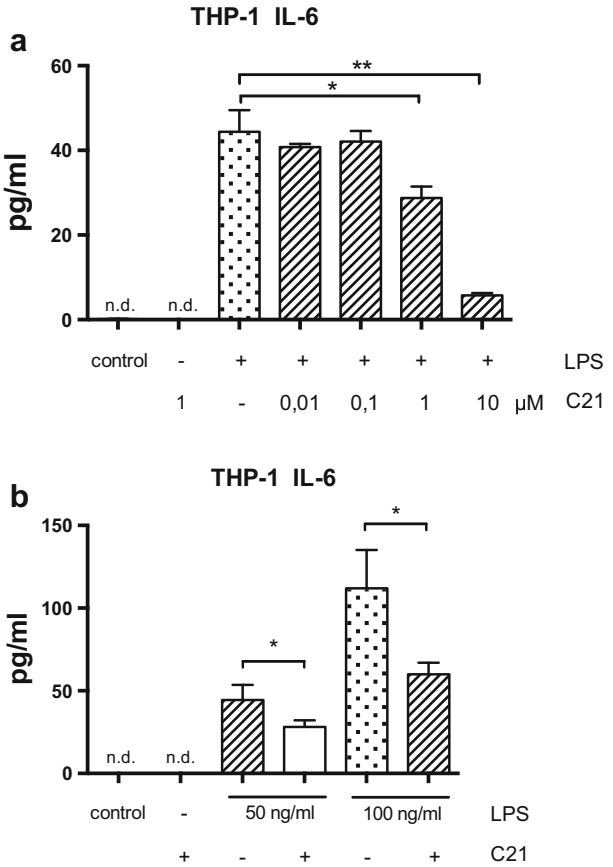

Fig. 1. **a** Dose-dependent effects of AT2 receptor agonist C21 on IL-6 production. THP-1 cells were stimulated with 50 ng/ml LPS and C21 at indicated concentrations for 6 h. Supernatant was collected and protein levels of IL-6 were analyzed by cytometric bead array (CBA). *$p<0.05$; **$p<0.01$ when compared with LPS; data are presented as mean±SEM, Mann-Whitney *U* test ($n=3$); *n.d.* not detectable. **b** AT2 receptor-mediated downregulation of IL-6 is independent from LPS concentration. THP-1 cells were stimulated with 50 ng/ml LPS or 100 ng/ml, with or without 1 µM C21 for 6 h. Supernatant was collected and protein levels of IL-6 were analyzed by cytometric bead array (*CBA*). *$p<0.05$; **$p<0.01$ when compared with LPS; data are presented as mean±SEM, Mann-Whitney *U* test ($n=3$); *n.d.* not detectable.

treatment alone had no effect. Irrespective of the LPS dose administered, we found an approximate 50 % reduction of IL-6 protein release.

Basal Expression of the AT2 Receptor

We confirmed AT2 receptor mRNA expression in THP-1 cells and U937 cells by PCR (Fig. 2a). Human placenta served as positive control. Western blot analysis verified AT2 receptor protein expression in THP-1 and U937 cells (Fig. 2b). The relative expression of the AT2 receptor was not significantly altered by treatment with LPS, C21, PD 123319 or a respective combination of these substances (Fig. 2c).

Effects of AT2 Receptor Activation in LPS-Treated THP-1 Cells

To investigate AT2 receptor-mediated effects on regulation of pro- and anti-inflammatory cytokines, THP-1 cells were treated with LPS and co-stimulated with C21. Expression of IL-6, IL-10, IL-1β, and TNF-α was regulated on both the mRNA and the protein level over time (Fig. 3). LPS significantly enhanced mRNA expression of all investigated cytokines at 3 h of exposure compared to vehicle-treated control. With longer exposure times (*i.e.*, 6 and 12 h) mRNA levels of all studied cytokines decreased rapidly, reaching almost basal mRNA expression levels at 12 h. Concomitant stimulation of the AT2 receptor with C21 significantly decreased *IL-6*, *IL-10*, and *TNFα* mRNA expression at 3 h compared to LPS-treated control. This inhibitory effect was transient and not detectable at 6 and 12 h. LPS-induced expression of *IL-1β* mRNA was not altered by AT2 receptor stimulation in THP-1 cells. On the protein level, LPS challenge significantly induced release of all investigated cytokines. TNFα expression was greatest at 3 h, whereas protein levels of IL-6, IL-10 and IL-1β gradually increased and peaked after 12 h of LPS treatment. Direct stimulation of the AT2 receptor with C21 significantly decreased IL-6 and IL-10 protein expression at 6 and 12 h in comparison to the LPS-treated control. TNFα release was only significantly attenuated by C21 early at LPS exposure. This effect was not detectable at longer stimulation times. In concordance with mRNA expression, release of IL-1β was not influenced by C21 stimulation. In summary, direct stimulation of the AT2 receptor exerted anti-inflammatory effects by inhibiting TLR4-mediated cytokine production in a time-dependent manner.

AT2 Receptor Specificity

To confirm that above effects were specifically depending on the AT2 receptor, we used the selective AT2 receptor antagonist PD 123319. Pharmacological inhibition of the AT2 receptor completely abolished C21-induced downregulation of IL-6 and IL-10 protein expression 6 h after LPS challenge in THP-1 cells (Fig. 4), whereas TNFα and IL-1β protein expression remained unchanged (not shown). This confirms that the observed immune-modulatory effect of C21 specifically depends on

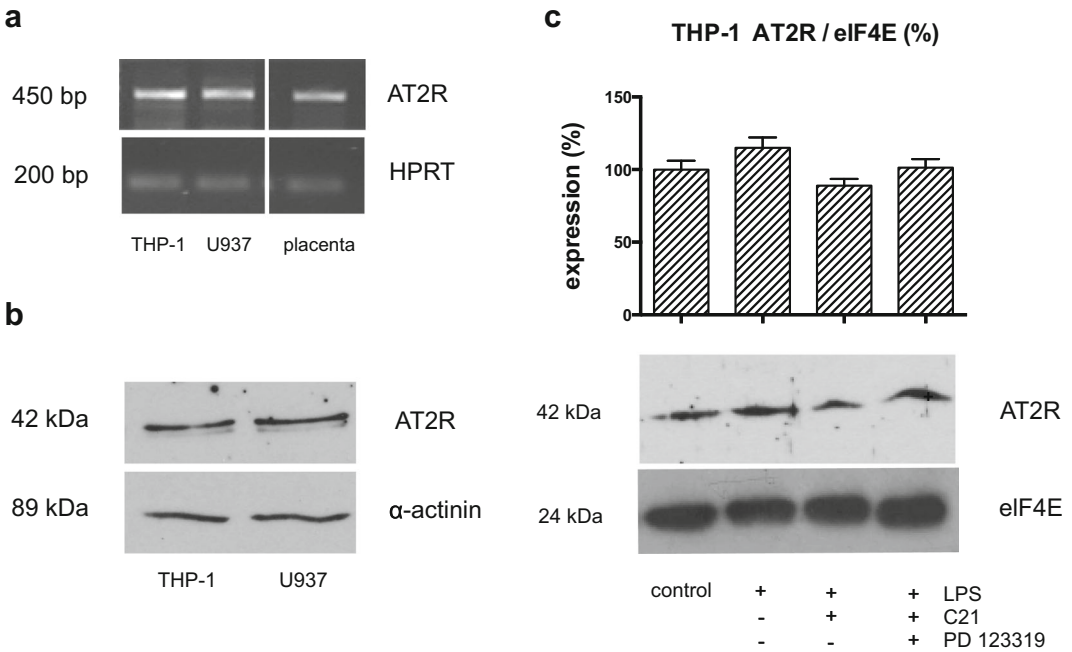

Fig. 2. **a** Expression analysis of the AT2 receptor in THP-1 and U937 cells. mRNA from respective cells and human placenta was reverse transcribed and subjected to PCR with hypoxanthin-guanine-phosphoribosyltransferase (*HPRT*) serving as control. **b** Immuno-blot analysis of basal AT2 receptor protein expression in THP-1 and U937 cells; detection of α-actinin served as loading control. **c** Protein expression by immunoblotting of the AT2 receptor in THP-1 cells after 12-h treatments with vehicle (control), LPS (50 ng/ml), AT2 receptor agonist C21 (1 μM) and/or AT2 receptor antagonist PD 123319 (10 μM), detection of eIF4E served as loading control. Quantification of bands expressed as density ratio AT2R/eIF4E (control set to 100 %); data are presented as mean±SEM, (n=5).

AT2 receptor activation. Treatment with C21 alone had no significant effect on IL-6 and IL-10 production at 6 h.

Effects of AT2 Receptor Activation in LPS-Treated U937 Cells

To test for AT2 receptor-mediated effects on LPS-induced cytokine release in other human monocytic cell lines, analogous experiments as described above were performed with cells of the human monocytic U937 line. Like in THP-1 cells, LPS induced a significant production of pro- and anti-inflammatory cytokines in these cells in a time-dependent manner. Stimulation of the AT2R with C21 had a significant inhibitory effect on protein release of IL-6, IL-1β, and TNFα, but not IL-10 after 6 h of LPS challenge (Fig. 5). Like in THP-1 cells, AT2 receptor stimulation exerted anti-inflammatory effects on early cytokine production in U937 cells. Treatment with C21 alone had no significant effect on cytokine release in U937 cells.

Cell Viability

Finally, we examined the impact of LPS exposition and stimulation of the AT2 receptor on integrity of DNA and viability of THP-1 and U937 cells on the single-cell level by flow cytometric analysis. However, neither treatment with LPS, nor C21 or a combination of both had a significant effect on cell viability in our experiments. Rates of apoptotic cell death were considerably low and ranged from 5 to 7 % in control and treatment groups in both THP-1 and U937 cells (data not shown).

DISCUSSION

Biological functions of the AT2 receptor in inflammation remain to be defined. In the present work we found that selective stimulation of the AT2 receptor with C21 attenuates LPS-induced production of cytokines in human THP-1 and U937 monocytic cells and thereby mediates anti-inflammatory responses in these cells.

The observed effects of AT2 receptor stimulation with C21 were time- and dose- dependent and comprised an inhibition of IL-6 and at least a transient lowering of TNFα on both the transcriptional and translational level. However, on average we detected a 50 % reduction of inflammatory cytokine expression with AT2 receptor activation,

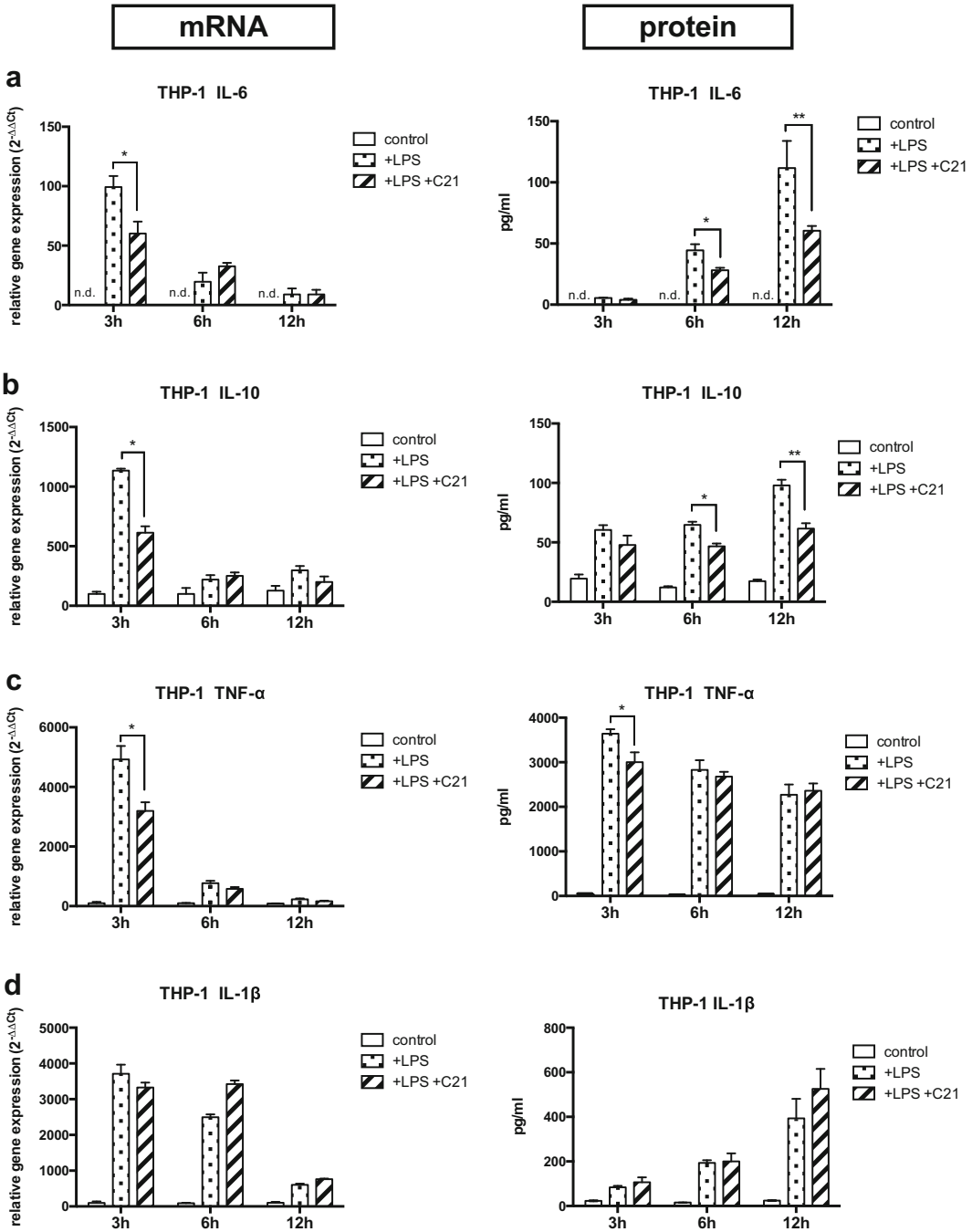

Fig. 3. AT2 receptor activation transcriptionally and translationally modulates pro- and anti-inflammatory cytokines. THP-1 cells were stimulated with 50 ng/ml LPS, with or without 1 μM C21, harvested at indicated time points and mRNA levels of **a** *IL-6* (*left*), **b** *IL-10* (*left*), **c** *TNFα* (*left*) and **d** *IL-1β* (*left*), were analyzed by real-time polymerase chain reaction. *β-actin* served as housekeeping gene, expression of control was set to 100 %. Supernatant of cells was collected at the same time points and protein levels of respective cytokines (**a**–**d**, *right*), were analyzed by cytometric bead array (*CBA*). *$p<0.05$; **$p<0.01$ when compared with vehicle-treated control; data are presented as mean±SEM, Mann-Whitney *U* test ($n=5$); *n.d.* not detectable.

independent from the administered LPS stimulus. This is consistent with previous experimental findings on AT2 receptor-mediated cytokine repression [8, 29]. Co-treatment with the AT2 receptor antagonist PD 123319

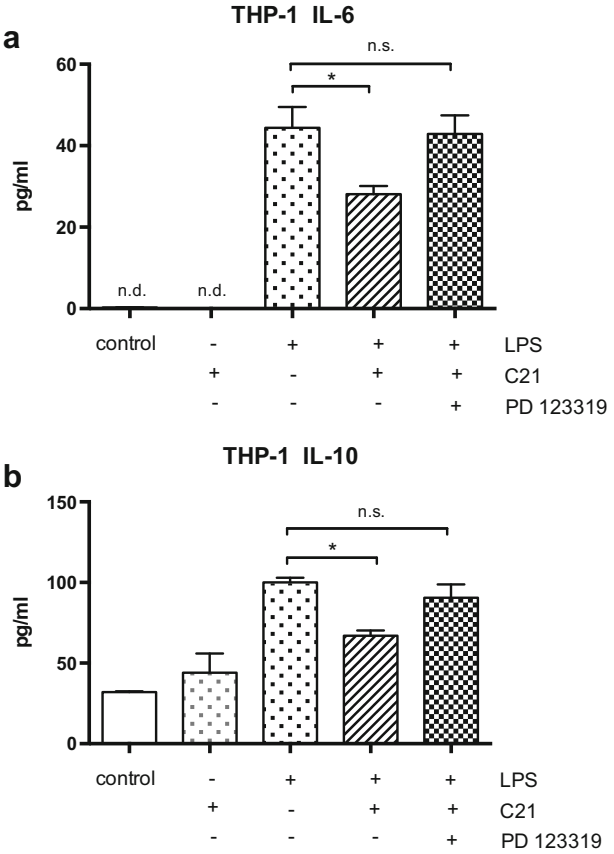

Fig. 4. C21-induced downregulation of IL-6 and IL-10 is depending on AT2 receptor activation. THP-1 cells were stimulated with 50 ng/ml LPS, with or without 1 μM C21 and 10 μM PD 123319 for 6 h. Supernatant was collected and protein levels of **a** IL-6 and **b** IL-10 were analyzed by cytometric bead array (*CBA*). *$p<0.05$; **$p<0.01$ when compared with vehicle-treated control; data are presented as mean±SEM, Mann-Whitney *U* test ($n=3$); *n.d.* not detectable; *n.s.* not significant.

completely abolished the C21-mediated effects indicating that this process is dependent on receptor activation. Furthermore, the low count of apoptotic or necrotic cells in either treatment group proved that the observed effects were regulated by the AT2 receptor. C21 is the most selective AT2 receptor agonist that was discovered so far [21, 38]. Therefore, only low drug concentrations are needed to activate the receptor and adverse cell effects like apoptosis and necrosis should be negligible [38].

We note that gene expression of cytokines ameliorated early in the time course. C21 was shown to stimulate the AT2 receptor dependent MAP kinases within 30 min after application [38]. However, the half-life of C21 is short. C21 levels start to decrease rapidly after application with a half-life after intravenous administration of 0.5–2.5 h [38]. In our study the concomitant protein levels of IL-6 and IL-10 show significant reductions at 6 and 12 h post treatment indicating a sustainable effect on the protein level even when C21 activity has already ceased.

Recently, Dhande and colleagues reported that selective stimulation of AT2 receptor in LPS-activated THP-1 macrophages acted anti-inflammatory by reducing IL-6 and TNF-α but increasing IL-10 production [9]. In our study we could confirm a marked downregulation of IL-6 and TNF-α expression after AT2 receptor activation in two populations of LPS-treated monocytes. However, IL-10 expression patterns in THP-1 cells differ from those observed by Dhande. This might not only be explained by the 10 times higher LPS doses and longer exposure times used by Dhande and coworkers. Most importantly, before LPS stimulation Dhande and colleagues pretreated the cells with C21 for 1 h. *IL-10* mRNA expression in THP-1 macrophages was induced by C21 treatment alone. LPS stimulation did not lead to further IL-10 induction [9]. Taken together, C21 mediated IL-10 induction may not be effective anymore, when C21 and LPS treatment is begun simultaneously, as we did in our experimental setting. Regulation of IL-10 seems to be highly dependent on cell type, stimulus and experimental conditions. Lately, many groups are studying the molecular and transcriptional regulation of IL-10 expression. So far kinetics and quantity of IL-10 expression after stimulation with different stimuli is not explored for many immune cells [31]. In our model of LPS-treated monocytes, the anti-inflammatory effect of C21 obviously is not linked to an increase of IL-10. Whether the TRIF- and MYD88-dependent pathways or impaired type I interferon signaling of which all are absolutely required for IL-10 induction in monocytic and dendritic immune cells are herein involved remains elusive. Thus, further experimental research is necessary to understand the complex regulative properties of the AT2 receptor on IL-10 cytokine expression.

Generalizability of *in vitro* data is often limited. In our experiments there was a different reaction of the investigated cell lines in response to LPS challenge and C21 treatment. Absolute values of cytokine levels in U937 differed in comparison to THP-1 cells, although the LPS stimulus was similar. Moreover, in U937 cells anti-inflammatory effects of AT2 receptor stimulation were even more pronounced. C21 caused a significant inhibition of IL-1β, whereas anti-inflammatory IL-10 remained unchanged. With regard to the different effects observed in C21 stimulated monocytic cells, activation of the AT2 receptor seems to cause various cell specific regulatory

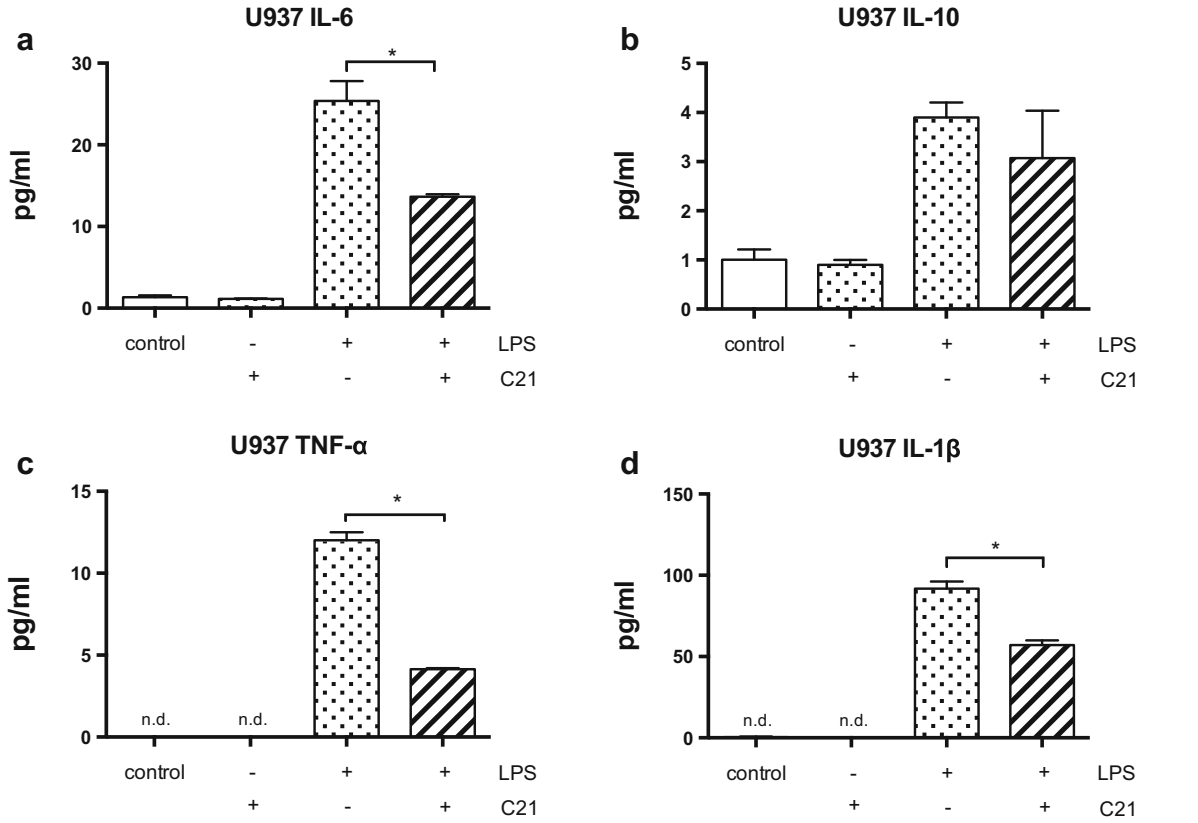

Fig. 5. AT2 receptor activation modulates pro- and anti-inflammatory cytokines in other human monocytic cell lines. U937 cells were stimulated with 50 ng/ml LPS, with or without 1 μM C21 for 6 h. Supernatant of vials was collected and protein levels of **a** IL-6, **b** IL-10, **c** TNFα, and **d** IL-1β were analyzed by cytometric bead array (*CBA*). *$p<0.05$; **$p<0.01$ when compared with LPS control; data are presented as mean±SEM, Mann-Whitney *U* test ($n=3$); *n.d.* not detectable.

effects on the cellular immune response. Whether our results hold true for *in vivo* conditions needs to be assessed in further experiments.

Monocytes and macrophages have an important role in initiation and regulation of inflammation and cardiovascular end-organ damage [5, 12]. In pathologic conditions like infection the number of monocytes is rapidly increasing. Furthermore, monocytes are a key element of the inflammatory cell infiltration in damaged tissues after an ischemic or traumatic injury [11]. As cardiovascular and neuronal injuries are massively affected by inflammation, a modulation of the monocytic cytokine response by specific AT2 receptor stimulation could have significant tissue-protective effects. The beneficial impact on end-organ injury in various animal models supports the concept of C21 mediated AT2 receptor stimulation. Its positive effects may at least in part be exerted by direct interaction with monocytes.

So far, data on C21 in the context of cellular immune responses are poor. Nevertheless, C21 revealed a significant decrease of pro-inflammatory cytokines in plasma and in the peri-infarct zone in a model of myocardial infarction [13]. Moreover, C21 treatment improved systolic and diastolic heart function and reduced the volume of the myocardial scar. Therefore, by effective suppression of the infarction associated inflammatory response, stimulation of the AT2 receptor by C21 is thought to be a novel and promising therapeutic tool to ameliorate scar formation, fibrosis and heart failure [33]. AT2 receptor-mediated actions also play an increasing role in the pathophysiology of neuronal injury. In animal models of ischemic or mechanic neuronal damage a massive overexpression of the AT2 receptor is noted [10, 15]. Inflammation has a major impact on the post-ischemic injury and neuronal damage. In primary rat astrocytes incubation with LPS lead to increased expression of pro-inflammatory IL-6 and TNF-α that was significantly reduced by coadministration of

C21 [32]. *In vivo* treatment with C21 initiated after ischemic brain damage caused by middle cerebral artery occlusion in rats significantly reduced the ischemic area. The expression of pro-inflammatory cytokines was markedly reduced [20].

Taken together, the above studies show that the tissue-protective effect of AT2 receptor activation by C21 is caused to a significant extent by a reduced expression of pro-inflammatory cytokines like IL-6 and TNF-α. Rompe and coworkers were able to prove that these effects were at least in part involving a CYP-dependent epoxidation of arachidonic acid to EETs and an inhibition of NF-κB activity [29]. While the AT1 receptor is known to activate the NF-κB pathway and induces CYP-dependent hydroxylation of arachidonic acid, it is likely that AT2 receptor activation, which often is opposing AT1 receptor-mediated effects, acts *via* these pathways. However, Rompe and colleagues demonstrated that AT2 receptor-mediated inhibition of nuclear factor kappaB (NFκB) also occurs independently from AT1 receptor signaling [29]. With the discovery of the Mas receptor, the AT3 receptor, the AT4 receptor (IRAP) and the MrgD receptor over the past decades, the renin-angiotensin system (RAS) has gained even more complexity [2, 4, 35]. The highly relevant interactions between the several different angiotensin receptor subtypes within the RAS are complex and poorly understood. Alterations of cellular inflammatory responses to angiotensin II and the AT1/AT2 receptor interplay are part of current research.

In conclusion, our study provides evidence that direct stimulation of the AT2 receptor acts anti-inflammatory by attenuating LPS-induced cytokine production in human monocytic cells. Thus, the concept of selective AT2 receptor stimulation with C21 treatment should be translated into pathologies that involve inflammatory activation of monocytes.

Compliance with Ethical Standards. UMS received modest research support from Vicore Pharma (short-term fellowship and free drug supply). All other authors do not have potential conflicts of interest. Research did not involve human participants or animals and strictly followed the rules of good scientific practice.

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