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Losartan prevents the development of the pro-inflammatory monocytes $CD14^+CD16^+$ in haemodialysis patients

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Abstract

Background. The principal cause of mortality in haemodialysis (HD) patients is cardiovascular disease, which is linked to chronic inflammation. Recent studies have demonstrated that angiotensin II receptor AT1 antagonists have anti-inflammatory properties. In this study, we evaluated the effect of losartan on $CD14^+CD16^+$ monocytes in HD patients. In addition, we developed an *in vitro* model to study the mechanisms by which losartan modulates these cells.

Methods. We divided 18 HD patients into two groups, based on anti-hypertensive treatment: 9 patients were treated with losartan (losartan group) and 9 received other anti-hypertensive drugs that did not affect the renin–angiotensin axis (no-losartan group). Losartan was withdrawn in five patients from the losartan group for 2 months. Ten healthy subjects were included as controls. *Invitro*, we studied the differentiation of monocytes from healthy donors on stimulation with interleukin (IL)-10, IL-4 and granulocyte monocytes colony-stimulating factor with or without losartan in the culture medium.

Results. In patients who were taking losartan, the percentage of monocytes that expressed $CD14^+CD16^+$ was lower compared with patients in the no-losartan group. The percentage of $CD14^+CD16^+$ was similar in the losartan group and healthy subjects. When losartan was withdrawn from five patients in the losartan group, the percentage of $CD14^+CD16^+$ monocytes increased compared with before withdrawal. *In vitro*, when we added losartan to the culture medium, $CD14^{++}CD16^-$ monocytes failed to differentiate into $CD14^+CD16^+$ cells.

Conclusion. Losartan acts as an immunomodulator that prevents the development of $CD14^+CD16^+$ pro-inflammatory monocytes in HD patients.

Keywords: $CD14^+CD16^+$ monocytes; haemodialysis; losartan

Introduction

The mortality of haemodialysis (HD) patients has remained high despite significant advances in dialysis

technology and better general care of patients. The chief cause of mortality in these patients is cardiovascular disease, which is linked to chronic inflammation [1–3].

Inflammation has been associated with the activation of mononuclear cells in HD patients [4]. The $CD14^+CD16^+$ subset of monocytes increases in HD patients [5, 6] to a similar extent as in other inflammatory diseases, such as HIV infection and systemic lupus erythematosus [7, 8]. Phenotypically, these cells are more mature than $CD14^+$ $^+CD16^-$ monocytes and produce pro-inflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin (IL)-1β [9].

Our group has reported that increases in $CD14^+CD16^+$ monocytes in HD patients correlate with the generation of endothelial microparticles and endothelial progenitor cell levels [10], suggesting that microinflammation mediates endothelial damage in HD patients [11].

Conversely, angiotensin II (Ang II) modulates certain inflammatory responses. In experimental models of immune complex glomerulonephritis, Ang II has a pro-inflammatory effect, increasing local production of interferon-γ and TNF-α [12]. Human monocytes produce Ang II [13] and express AT1 and AT2 receptors for Ang II [14]. Furthermore, Ang II triggers many responses in monocytes and macrophages, such as chemotaxis, adhesion of endothelial cells and enhancement of phagocytosis [15]. Moreover, the differentiation of dendritic cells (DCs) is regulated by Ang II, and AT1 receptor antagonists, such as losartan, impede this differentiation [16].

Ang II receptor blockers and angiotensin-converting enzyme inhibitors are used widely as anti-hypertensive therapies in HD patients. These drugs have other actions that can be beneficial in these patients: they can reduce cardiac hypertrophy and have anti-apoptotic and anti-fibrotic effects [17–19]. Moreover, some such drugs suppress TNF-α and IL-1 synthesis by human peripheral blood mononuclear cells (PBMCs); thus, they have anti-inflammatory effects [20].

We hypothesize that treatment with losartan, independent of blood pressure control, has other beneficial effects in HD patients, specifically that losartan attenuates the pro-inflammatory state that is associated with monocyte activation in HD patients. In this study, we examined the effects of losartan on $CD14^+CD16^+$ monocytes in HD patients and developed an *in vitro* model to study the mechanisms by which losartan modulates these cells.

Materials and methods

Patients

Twenty stable HD patients who fulfilled the selection criteria were included. All patients had been treated at the dialysis facility of Reina Sofia University Hospital, Cordoba, Spain, for at least 6 months before the study. None of the patients had diabetes mellitus, malignancies, active infections or inflammation or autoimmune disease. All patients were negative for hepatitis B and C virus and HIV. Patients were not on steroids, immunosuppressive medications, non-steroidal anti-inflammatory drugs, statins or vitamin D therapy.

At inclusion, all patients had been on high-flux HD for at least 3 months using helixone membranes (1.8 m^2, FX80; Fresenius Medical Care, Bad Homburg, Germany). Each monthly analysis of the dialysis water system demonstrated the absence of bacteria (<100 colony-forming units/mL) or bacteriological contaminants (endotoxin levels <0.025 endotoxin units). The dialysis characteristics were similar between all patients. Dialysis efficiency was estimated using the e*Kt*/*V* ratio. Blood samples were obtained from all patients immediately prior to the first dialysis session of the week.

Patients were divided into two groups, based on anti-hypertensive treatment: 9 patients were treated with losartan, 100 mg/day (losartan group), and 11 received other anti-hypertensive drugs that did not affect the renin–angiotensin axis (no-losartan group). In the losartan group, patients received losartan in monotherapy (two cases) or associated with amlodipin, carvedilol and/or doxazosin. All patients were on losartan for >2 months before enrolment in the study. In the no-losartan group, patients were treated with amlodipine, carvedilol and/or doxazosin. Two patients from the no-losartan group dropped out before the study: one patient received a renal transplant and the other withdrew consent. Ultimately, the study included 18 patients.

Losartan was halted in five patients in the losartan group for 2 months. During this time, patients were treated with the same anti-hypertensive drugs as patients in the no-losartan group, and blood pressure was well controlled. No clinical or analytical parameters changed after losartan was withdrawn. After 2 months without losartan, the study was repeated.

Ten healthy subjects, matched for age and gender, served as controls. Informed consent was obtained from all patients and healthy subjects after institutional approval.

Cytokine assays

Concentration of IL-1β and TNF-α was assessed using a specific sandwich enzyme-linked immunosorbent assay by R&D Systems (Minneapolis, MN). The samples were measured with a PowerWave XS microplate reader (Biotek, VT) set to 450 nm.

Determination of CD14 and CD16 mononuclear phenotype in HD patients and healthy subjects

A 10-mL sample of peripheral venous blood was drawn from patients and healthy subjects into tubes that contained lithium heparin. To identify $CD14^+CD16^+$ monocytes, blood was incubated with peridinin chlorophyll protein (PerCP)-conjugated monoclonal anti-CD14 (M5E2) and fluorescein isothiocyanate (FITC)-conjugated anti-CD16 (3G8). Both antibodies and the appropriate isotype controls were purchased by Becton Dickinson (San José, CA). Flow cytometry was performed on a FACScalibur (Becton Dickinson). The percentage of $CD14^+CD16^+$ monocytes was calculated by subtracting non-specific staining, as identified in the isotype control histogram.

Apoptosis

PBMCs (10^6 cells/mL) from patients who were not taking losartan and healthy subjects were used to seed 24-well culture plates (Falcon; Becton Dickinson and Company, Paramus, NJ) in complete culture medium [RPMI 1640 supplemented with L-glutamine (2 mM), HEPES (20 M), sodium pyruvate (1 mM), streptomycin (50 ng/mL), penicillin (100 IU/mL) and 10% fetal bovine serum (FBS)] at 37°C in 5% CO_2 (medium and additives were supplied by BioWhittaker, Walkersville, MD).

Cells were cultured with various concentrations of losartan. The active ingredient of losartan was supplied by Merck & Co., Inc. (Rahway, NJ).

Apoptosis in $CD14^+CD16^+$ cells was measured using the Annexin V-phycoerythrin (PE) Apoptosis Detection Kit per the manufacturer's instructions (Bender MedSystem, Vienna, Austria). Flow cytometry was performed on a FACScalibur (Becton Dickinson).

In vitro differentiation of peripheral blood monocytes

PBMCs were isolated from five healthy subjects by density gradient separation using Ficoll (Ficoll/Hypaque; BioWhittaker Inc., Walkersville, MD). The number of experiments performed *in vitro* was 10.

$CD14^+CD16^-$ monocytes were purified from PBMCs using the EasySep human monocyte negative selection enrichment cocktail (StemCell Technologies, Vancouver, Canada). The purity of the monocytes (>90% $CD14^+$) was measured by flow cytometry after staining with PerCP-anti-CD14 and FITC-anti-CD16.

Isolated $CD14^+CD16^-$ monocytes were cultured in complete culture medium that contained RPMI 1640, supplemented with 10% FBS. Granulocyte monocytes colony-stimulating factor (GM-CSF, 800 U/mL/10^6 cells; R&D Systems), IL-4 (500 U/mL/10^6 cells; R&D Systems) and IL-

10 (10 ng/mL; R&D Systems) were added to the culture medium, as described [21]. This combination of cytokines stimulates the development of $CD14^+CD16^+$ cells from human monocytes.

Simultaneously, we added various concentrations of losartan (0, 20, 40, 60 and 100 μM) to the culture medium. Every 2 days, 0.4 mL of medium was removed from each well and replaced with 0.5 mL of fresh medium that contained the appropriate cytokines. On Days 0, 1, 2, 3 and 4, the phenotype was analysed by flow cytometry.

Measurement of intracellular TNF-α in $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes

TNF-α expression was examined in monocytes that were cultured for 6 h in the presence of brefeldin A, with and without lipopolysaccharide from *Escherichia coli* (Sigma Chemical Co., Poole, UK). After incubation, the cells were washed and stained with PerCP-anti-CD14 and FITC-anti-CD16. Then, the cells were washed and permeabilized using FACS Permeabilizing Solution (Becton Dickinson, San José, CA) and incubated with PE-conjugated anti-TNFα (Becton Dickinson, San José, CA) or the corresponding isotype control. After incubation, the cells were washed and re-suspended in 0.5 mL 1% formaldehyde until the flow cytometric analysis.

Statistical analysis

Results are expressed as mean ± SD. Two means were compared by *t*-test for paired or unpaired data. Non-parametric data were analysed by Wilcoxon signed-ranks test or Mann–Whitney *U*-test for paired and unpaired comparisons, respectively. Differences were considered significant at $P < 0.05$.

Results

Table 1 shows demographics and biochemical parameters of HD patients. There was not any statistical difference between the patient groups with the exception of cytokine levels. In patients from the no-losartan group, plasma levels of IL-1β were significantly higher (339 ± 51.7 pg/mL) than in patients from the losartan group (235.5 ± 36.7 pg/mL) and controls (86.3 ± 19.9 pg/mL) ($P = 0.001$). TNF-α plasma levels were also higher in patients from the no-losartan group (291.3 ± 52.1 pg/mL) than in patients from the losartan group (194.1 ± 54.4 pg/mL) and controls (150.1 ± 37.1 pg/mL) ($P = 0.001$). The difference in TNF-α plasma levels between losartan and control was not significant.

Table 1. Characteristics and laboratory values of patients from both groups[a]

Parameter	No-losartan ($n = 9$)	Losartan ($n = 9$)	P-values
Age	57.3 ± 15.3	55.1 ± 13.5	0.89
Sex (male/female)	5/4	6/3	0.58
HD vintage (months)	51.8 ± 34.7	47.55 ± 45	0.41
e*Kt*/*V*	1.84 ± 0.4	1.68 ± 0.2	0.07
MBP (mmHg)	86.1 ± 2	93.5 ± 11.9	0.15
CRP (mg/L)	1.63 ± 0.7	3.3 ± 3.3	0.06
Potassium (mEq/L)	5.04 ± 0.6.	5.3 ± 0.6.	0.21
Albumin (g/dL)	4.3 ± 0.3	4.2 ± 0.3	0.22
Ferritin (ng/mL)	571.7 ± 302.9	529.7 ± 193	0.36
Haemoglobin (g/dL)	11.8 ± 0.6	12.0 ± 1.0	0.31
Leucocytes	7.756 ± 2.222	7.078 ± 2.781	0.28
EPO dose (IU/kg/week)	54.8 ± 35.4	67.7 ± 70.2	0.19
TNF-α (pg/mL)	291.3 ± 52.1	194.1 ± 54.4	0.001
IL-1β (pg/mL)	339 ± 51.7	235.5 ± 36.7	0.001

[a]CRP, C-reactive protein; EPO, erythropoietin; MBP, mean blood pressure. There was no significant difference between the two groups.

Analysis of $CD14^+CD16^+$ monocytes in HD patients and healthy subjects

Absolute number of monocytes was similar in both groups (losartan group: 458 ± 215 cells/mm^3 and no-losartan group: 431 ± 175 cells/mm^3). In patients from the losartan group, 5.8 ± 3.5% of monocytes expressed $CD14^+CD16^+$ versus 10.5 ± 3.5% in the no-losartan group ($P < 0.05$). In healthy subjects, the percentage of $CD14^+CD16^+$ monocytes was 3.9 ± 1.1%, similar to the losartan group and lower than in the no-losartan group ($P < 0.05$) (Figure 1).

To confirm the effect of losartan on $CD14^+CD16^+$ monocytes, it was halted in five patients from the losartan group for 2 months. During this time, patients were treated with the same anti-hypertensive drugs as in patients from the no-losartan group. Blood pressure was controlled well, and no clinical or analytical parameters changed after losartan withdrawal.

The percentage of $CD14^+CD16^+$ monocytes in these patients on losartan was 5.8 ± 3.6%, and after 2 months without it, this percentage increased to 13.1 ± 3.1% ($P < 0.05$) (Figure 2), similar to that in the no-losartan group.

Effects of losartan on apoptosis in $CD14^+CD16^+$ monocytes

We cultured isolated monocytes from HD patients and healthy subjects with various concentrations of losartan and observed that any concentration induced apoptosis (Table 2).

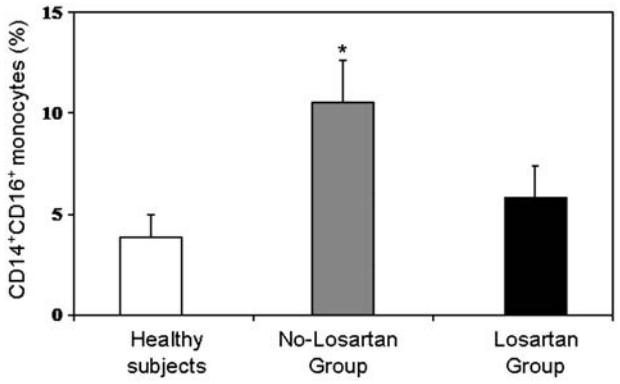

Fig. 1. Percentage of $CD14^+CD16^+$ monocytes in healthy subjects and the no-losartan and losartan groups. *$P < 0.05$ losartan versus no-losartan and healthy subjects.

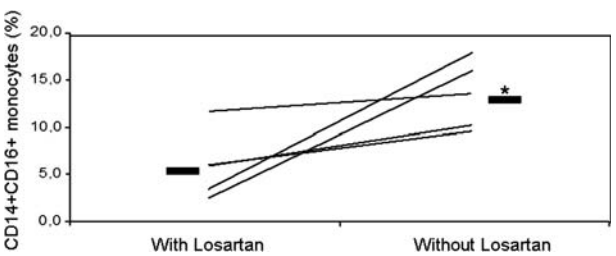

Fig. 2. Percentage of $CD14^+CD16^+$ monocytes in patients in the losartan group before and 2 months after withdrawal of losartan. The gross lines are the mean value of the entire group. *$P < 0.05$.

Table 2. Percentage of apoptotic $CD14^+CD16^+$ cells at baseline and at various concentrations of losartan[a]

	0 μM	5 μM	10 μM	20 μM	40 μM	60 μM	100 μM
No-losartan	4.7 ± 2.26	5.7 ± 1	5 ± 1.7	5.1 ± 1.9	6.2 ± 1.6	5.6 ± 1.9	6.3 ± 1.8
Healthy subjects	3.9 ± 2.2	3.2 ± 1.7	5.6 ± 2.2	3.7 ± 1.2	5.1 ± 2.3	3.9 ± 2.1	5.3 ± 2.2

[a]Data are expressed as mean ± SD.

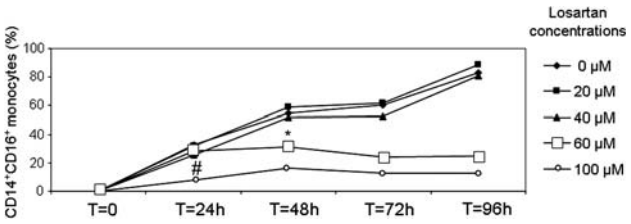

Fig. 3. Kinetics of CD14 and CD16 expression in monocytes cultured in the presence of three cytokines and various concentrations of losartan. *P < 0.05 60 μM versus 0 μM at 48 h. #P < 0.001 100 μM versus 0 μM at 24 h.

In vitro differentiation of peripheral blood monocytes

We cultured freshly isolated monocytes (90–95% with the $CD14^{++}CD16^-$ phenotype) from healthy subjects for 0, 2, 3 or 4 days with cytokines (IL-10, IL-4 and GM-CSF) in the presence or absence of losartan. In the presence of all three cytokines, CD14 was down-regulated from the second day of culture, and CD16 expression increased throughout the culture period (Figure 3). On Day 4, the majority (>90%) of cells expressed the $CD14^+CD16^+$ phenotype.

When we added losartan at 0, 20 and 40 μM, there were no significant differences in the percentage of $CD14^+CD16^+$ cells at any time. At 60 μM losartan, the percentages of $CD14^+CD16^+$ on Days 2, 3 and 4 were significantly lower than without losartan ($P < 0.001$). With 100 μM losartan, the percentage of $CD14^+CD16^+$ was significantly lower from Day 1 to Day 4 ($P < 0.001$, Figure 3). The down-regulation of CD14 at 100 μM losartan was observed from Day 2, but the cells expressed a $CD14^+CD16^-$ phenotype (Figure 4), suggesting that 60 and 100 μM losartan modulates the development on $CD14^+CD16^+$ inflammatory monocytes.

Measurements of intracellular and soluble TNF-α in vitro

To compare the inflammatory activity of $CD14^+CD16^+$ and $CD14^+CD16^-$ populations from the differentiation of pro-inflammatory monocytes, we measured intracellular TNF-α on Day 4. In monocytes that were cultured with losartan, the mean fluorescence channel was 179.8 ± 76.9 versus 464.2 ± 95 in monocytes without losartan ($P = 0.001$). Thus, the $CD14^+CD16^-$ population has less pro-inflammatory activity than the $CD14^+CD16^+$ subset.

We analysed TNF-α levels in the supernatant of cultured monocytes and we did not observe a statistical difference between the $CD14^+CD16^-$ supernatant (54.7 ± 12.1 ng/mL) and $CD14^+CD16^+$ supernatant (63.5 ± 16.8 ng/mL).

Discussion

In this study, we have demonstrated that HD patients who are treated with losartan have a decrease in the percentage of pro-inflammatory $CD14^+CD16^+$ monocytes in peripheral blood compared with patients who are not. The activity of losartan on $CD14^+CD16^+$ monocytes was confirmed by the finding that the percentage of this subset increased in patients after losartan was withdrawn for 2 months. The mechanism by which losartan acts on these cells is linked to its ability to prevent monocyte differentiation toward the $CD14^+CD16^+$ phenotype. *In vitro* studies have demonstrated that losartan does not induce apoptosis in $CD14^+CD16^+$ cells but arrests the differentiation of $CD14^{++}CD16^-$ cells into $CD14^+CD16^+$ monocytes.

In recent years, studies have focussed on the function of renin–angiotensin system blockade in inflammatory responses. Treatment of hypercholesterolaemic monkeys with losartan reduces activated monocytes in peripheral blood [22], and losartan and irbesartan decrease inflammation-associated vascular damage and endothelial dysfunction in a rat model of arthritis [23].

In our study, HD patients who were treated with losartan harboured lower percentages of $CD14^+CD16^+$ monocytes and lower plasma levels of pro-inflammatory cytokine than the no-losartan group. To our knowledge, we demonstrate for the first time that losartan reduces the percentage of pro-inflammatory $CD14^+CD16^+$ monocytes in humans. Patients who are on regular HD have high percentages of $CD14^+CD16^+$ monocytes [6, 24]. These cells maintain the inflammatory state in uraemic patients because they produce pro-inflammatory cytokines, such as TNF-α and IL-1β [9], and they are particularly resistant to apoptosis [25, 26]. The number of activated monocytes must be modulated because these cells and their pro-inflammatory cytokines are directly linked to cardiovascular risk in HD patients [27]. Moreover, our group has reported that the increase in $CD14^+CD16^+$ monocytes in HD patients correlates with endothelial microparticles and endothelial progenitor cells that are endothelial dysfunction markers [10].

We also examined the possible mechanisms by which losartan decreases $CD14^+CD16^+$ monocyte levels. Our first hypothesis was that losartan induced apoptosis in $CD14^+CD16^+$ cells because this form of programmed cell death is the chief process by which homeostasis is maintained, balancing proliferation with cellular demise [28]. Yet, when we cultured $CD14^+CD16^+$ monocytes with various concentrations of losartan, the absence of apoptosis in these cells ruled out this mechanism.

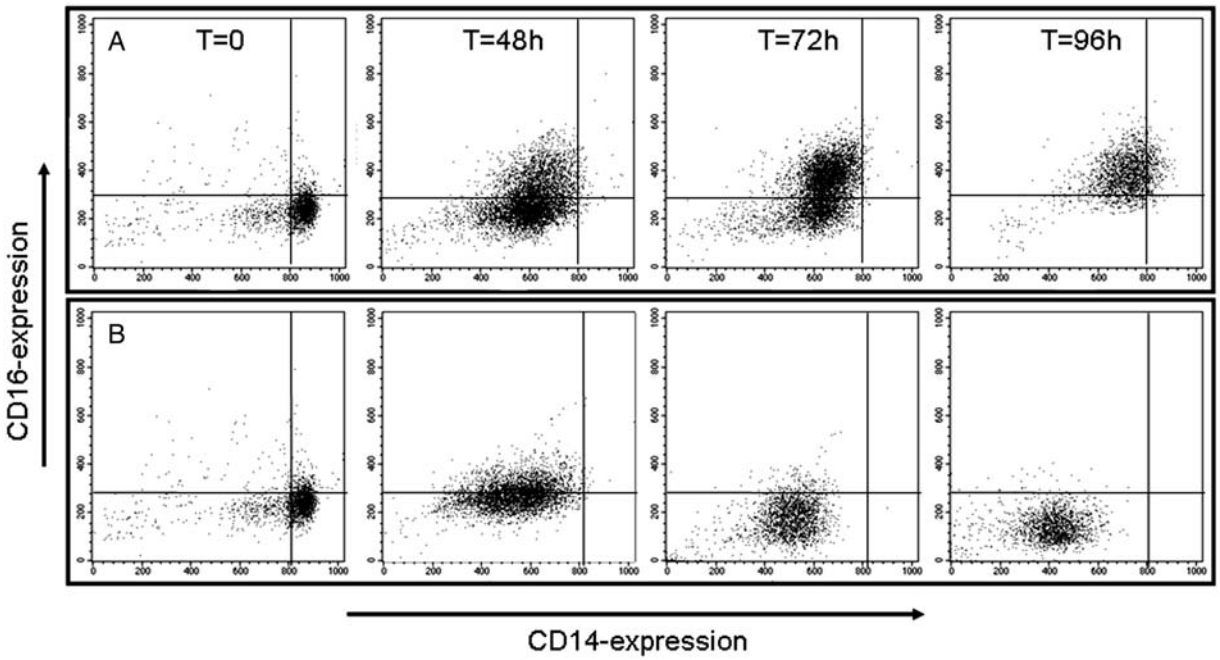

Fig. 4. Kinetics of CD14 and CD16 expression on monocytes cultured in the presence of IL-10, IL-4 and GM-CSF with or without losartan. (**A**) With cytokines. (**B**) With 100 μM losartan.

A second possibility was that losartan acts as an immunomodulator during the differentiation of $CD14^{+}CD16^{+}$ monocytes [16]. To test this hypothesis, we developed a model using a combination of cytokines that stimulate the differentiation of $CD14^{++}CD16^{-}$ monocytes into $CD14^{+}CD16^{+}$ monocytes [21]. When we added ≥60 μM losartan, the percentage of $CD14^{+}CD16^{+}$ cells was significantly lower than in the control, which might explain why patients who are treated with losartan have lower percentages of $CD14^{+}CD16^{+}$ monocytes than those who do not. This finding has implications for inflammatory processes in HD patients. The effect of losartan on inflammation-associated endothelial damage must be studied in these patients.

Notably, the cells that were generated in this model did not express CD16 but were $CD14^{+}CD16^{-}$. These cells did not express intracellular TNF-α; thus, they lacked inflammatory activity, suggesting that under inflammatory conditions, such as those that were reproduced in our experimental model, losartan prevents the differentiation of normal $CD14^{++}CD16^{-}$ monocytes into pro-inflammatory $CD14^{+}CD16^{+}$ monocytes. Additionally, we measured TNF-α levels in the culture medium in the monocyte differentiation assay. We did not observe any statistical difference in TNF-α levels in culture medium between $CD14^{+}CD16^{+}$ monocytes and $CD14^{+}CD16^{-}$ monocytes. This finding was not surprising because previously we had reported that $CD14^{+}CD16^{+}$ monocytes produce pro-inflammatory cytokines, but they need a second stimulus to secrete them [29].

Our results are consistent with Nahmod *et al.* [16], who demonstrated that human monocytes produce Ang II and express AT1 and AT2 receptors for Ang II. They observed that DCs that have differentiated from human monocytes in the presence of losartan have very low pro-inflammatory activity, concluding that the differentiation of DCs is regulated by Ang II. Moreover, this group has recently demonstrated that DCs from AT1-deficient mice produce significantly lower levels of TNF-α [30].

There are several limitations of this report; it was a cross-sectional study of a small sample group, but the strict inclusion/exclusion criteria did not allow us to have a larger number of patients. To correct this deficiency, at least in part, losartan was withdrawn in five patients and we observed that the percentage of $CD14^{+}CD16^{+}$ monocytes increased to a similar level of patients from the no-losartan group. Additionally, to minimize potential biases, measurements for each assay were performed in triplicate in the same laboratory using a single batch of reagents. Furthermore, using mononuclear cells *in vitro*, we are unable to reproduce the environmental conditions that exist in HD patients exactly. However, our *in vitro* experiments might explain the mechanisms by which losartan acts directly on these cells.

The results of this study suggest that losartan acts as an immunomodulator that prevents the development of $CD14^{+}CD16^{+}$ pro-inflammatory monocytes in HD patients and that it has anti-inflammatory effects that are beneficial for these patients.

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Conflict of interest statement. None declared.

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