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Deregulation From CD4+ Memory T Cells to Regulatory Cells in Patients With Chronic Renal Failure: A Pilot Study

Gaosi Xu[1,2†*] and Yingqian Gong[1,3†]

[1]*Department of Nephrology, Second Affiliated Hospital, Nanchang University, Jiangxi, P. R. China*
[2]*Department of Immunology, Shangrao Branch of Jiangxi Medical College, Shangrao, P. R. China*
[3]*Medical Graduate School, Nanchang University, Jiangxi, P. R. China*

Background: The aim of this study was to elucidate whether the CD4+ memory T (Tm) cells differentiation to regulatory T cells (Tregs) play a role in the immunological defects in patients with chronic kidney disease (CKD), and if the oxidized low-density lipoprotein (oxLDL) had affect on on CD4+ Tm cells and Tregs apoptosis in these subjects. *Methods:* CD4+ Tm cells and Tregs were detected by flow cytometry in each group of ten subjects. Apoptosis was measured by flow cytometry and confirmed by Western blotting. *Results:* The oxLDL concentration was significantly higher in CKD stage 4 (CKD4) patients than in controls, particularly in hemodialysis (HD) subjects ($P < 0.001$, respectively). In total, 100 μg/ml oxLDL significantly inhibited the CD4+ Tm cell proliferation. oxLDL-induced Tm generated Tregs apoptosis in controls and CKD4 patients, especially in HD patients ($P < 0.001$, respectively). *Conclusion:* Dysregulation of CD4+ Tm cells converting into Tregs played a role in the immune defects of CKD patients, and oxLDL induced the apoptosis of Tm generating Tregs in these subjects. Larger size of sample should be investigated to confirm the findings in further studies. J. Clin. Lab. Anal. 27:423–426, 2013.

Key words: memory T cell; regulatory T cell; oxidized low-density lipoproteins; apoptosis

Previous studies indicated that a significant percentage of activated CD4+ T cells did not proliferate but undergo apoptosis in patients with chronic renal failure (CRF) and hemodialysis (HD) (1). Clinical demonstrations of immune defects characterized by increased susceptibility to infections and a decreased immune response to T-cell-dependent antigen were observed in patients with CRF (2). Defective CD4+ regulatory T cells (Tregs) numbers and functions had been shown in patients with CRF (3).

The source of Treg cells (usual phenotype is CD4+CD25+Foxp3+) remains unknown. The naturally occurring Tregs can be produced from antigen-induced proliferation of CD4+ T cells in thymus and peripheral tissue (4). Researchers displayed that human CD4+CD25+ Tregs were derived not only from thymus, but also from the central memory T cells (Tm, CD4+CD45RO+CCR7+ phenotype; (5)). Previous research displayed that CD4+CD25+Foxp3+ Tregs expressed low abilities of the antiapoptosis (6). Therefore, once generated, the Tregs were susceptible to apoptosis and had limited replicative potential. The aim of this study is to elucidate that whether the CD4+ central Tm cells differentiating into Tregs played a role in the immunological defects in patients with CRF, and whether oxidized low-density lipoproteins (oxLDLs) has affect on CD4+ Tm cells and Tregs apoptosis in these patients.

The study included ten HD patients (the period of HD range from 13 to 24 months), ten patients with chronic

[†]These two authors contributed equally to this work.

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*Correspondence to: Gaosi Xu, Department of Nephrology, Second Affiliated Hospital, Nanchang University, No. 1 Minde Road, Nanchang 330006, P. R. China. E-mail: gaosi.xu@gmail.com

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kidney disease (CKD) stage 4 (CKD4) (mean ± SD, glomerular filtration rate [GFR] 22.1 ± 4.01 ml/min per 1.73 m^2), and ten healthy volunteers. GFR was estimated according to the modified Modification of Diet in Renal Disease formula (7). Patients with recent (less than 3 months) surgery, myocardial infarction, stroke, diabetes, infectious diseases, malignancy, and the use of immunosuppressive drugs were excluded from this study. All the participants provided informed consents and the study was approved by the Medical Ethics Committee of the Second Affiliated Hospital, Nanchang University.

Serum oxLDL determination was performed by using a mAb-4E6-based ELISA kit (Mercodia, Uppsala, Sweden) as described previously (8). By using a lymphocyte separation medium gradient (Pharmacia Biotech, Uppsala, Sweden), as previously described (9), peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of HD patients, CKD4 patients, and healthy volunteers, respectively, then incubated in a plastic tissue culture plate at 37°C for 1 hr to allow monocytes to adhere. Nonadherent cells were passed through human T cell enrichment columns for negative selection. CD4+ T cells were positively selected as previously described (10) by using CD4 MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). All CD4+ T cells showed purity of 97%. Then CD4+ T cells were incubated for up to 72 hr in RPMI-1640 with or without oxLDL.

CD4+ T cells were then incubated with antibodies against PE-conjugated anti-human CD25 (BD Biosciences Pharmingen, San Diego, CA), phycoerythrin (PE)-conjugated anti-human CCR7 (BD Biosciences Pharmingen), and fluoresceine isothiocyanate (FITC)-conjugated anti-human CD45RO (eBioscience, San Diego, CA, USA) antibodies. APC-conjugated anti-human FoxP3 (eBioscience) for flow-cytometric analysis of Tregs, using a FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences). For phenotypic analysis, purified CD4+ T cells were stained for 30 min. For intracellular staining of FoxP3, cells were fixed and permeabilized before anti-human FoxP3 (clone 101, eBioscience) as described previously (11).

CD4+ T cells (1×10^6 cells/ml) were cultured in vitro in the presence of 10 μg/ml PHA (Sigma, Missouri, USA), with or without 100 μg/ml oxLDL (Sigma). Proliferation was measured by ^{3}H thymidine uptake assay. The mean radioactivity (count per minute, cpm) from triple assay was used for analysis. Apoptosis was evaluated indirectly by Fas (CD95) staining and flow cytometry analysis as described previously (12). Statistical analysis was performed by using SPSS 17.0 for windows. Results were expressed as mean values ± SD unless there was another indication in figure legends. Significance of the difference between the patients and the control group in CD4+ T cells analysis was determined by one-way ANOVA. Students' *t*-test was applied in order to compare the patients group with the control group. Difference was considered significant when *P*-value was less than 0.05.

TABLE 1. Serum Concentrations of Oxidized Low-Density Lipoproteins (oxLDL) in Study Groups

	HD	CKD4	Controls
Concentration (U/l)	90.4 ± 7.12	63.1 ± 7.09	31.2 ± 2.96

HD, hemodialysis; CKD, chronic kidney disease; data in mean ± SD.

The study groups were matched in both age and gender. As shown in Table 1, serum oxLDL concentrations were significantly higher in HD patients than in CKD4 patients ($P < 0.001$). Furthermore, the oxLDL concentration of CKD4 subjects was significantly higher than that of healthy controls ($P < 0.001$). PHA-stimulated CD4+ T cells from HD patients displayed significant decreased proliferating responses (2,889 ± 213 cpm) than that of CKD4 patients (4,156 ± 307 cpm, $P < 0.001$). For determining the role of oxLDL in CD4+ central Tm cell (CD4+CD45RO+CCR7+ phenotypes) proliferation, cells were cultured with PHA and oxLDL. After 72 hr of incubation, 100 μg/ml oxLDL significantly inhibited the cell proliferation.

CD4+CD45RO+CCR7+ central Tm cells can be differentiated into CD4+CD25+Foxp3+ Tregs with PHA stimulation. In total, 100 μg/ml oxLDL significantly induced central Tm-originated Tregs apoptosis in HD patients, CKD4 patients, and controls ($P < 0.001$, respectively). One-way ANOVA analysis indicated a significant difference in Tregs apoptosis in HD patients ($P < 0.001$) compared with CKD4 patients as well as controls. The higher percentage of Fas-expressing CD4+ central Tm cells was found in HD patients than in CKD4 patients ($P < 0.001$), and was confirmed by Western blotting. Taken together, these data strongly suggested that apoptosis of CD4+ central Tm cells were induced by oxLDL exposure.

Researchers displayed impaired CD4+CD25^{high} Foxp3+ Treg cells in end-stage renal disease (ESRD) patients (13). It was suggested that CD4+ T lymphocytes normally expressed Foxp3 in the condition of stimulation and/or proliferation (14). Therefore, dysregulation of CD4+ T cells conversion into Tregs might play a pivotal role in the immunological defects in patients with CRF and regular HD patients. However, the exact mechanism in the conversion dysregulation was not clear.

As shown in Table 1, oxLDL concentrations were significantly higher in HD patients than in both CKD4 patients and healthy controls, suggesting the different degrees of apoB oxidation in patients with ESRD. oxLDL

were found in circulation, where they could bind to T cells (15). Therefore, oxLDL has been suggested to trigger apoptosis of lymphocytes and CD4+ T cells in CRF and HD patients. oxLDL induced a rapid decay of proteasomal proteolysis in derivatization of cell proteins induced by 4-hydroxynonenal. Furthermore, proteasome inhibition might impair cell cycle and altered cell viability through the function involved in the regulation of proteins (15). In this study, higher percentage of Fas-expressing CD4+ central Tm cells were found in HD patients than in CKD4 patients ($P < 0.001$). Coculturing with 100 μg/ml of oxLDL, Fas expressions on CD4+ central Tm cells were significantly higher in HD patients than in CKD4 patients and controls ($P < 0.001$, respectively). Present study showed that oxLDL induce apoptosis of activated CD4+ central Tm cells through a Fas-mediated mechanism.

It was concluded that Foxp3-transducted Treg cells (CD4+CD25+Foxp3+ phenotypes) might have a therapeutic role in protecting against immune injury and disease progression in chronic proteinuric renal disease (16). In vitro, with epitope-specific stimulation, peripheral blood virus-specific Tm cells efficiently induced Foxp3 expression (17). The continuous differentiation of specific CD4+ central Tm cells to induce the generation of Tregs might contribute to the immune defect in the patients with CRF. Researchers indicated that proteasome inhibition by oxLDL lead to CD4+CD25+ Tregs apoptosis, affecting the number and suppressive capability of these Treg cells in chronic HD patients (18). Present results of FACS indicated that oxLDL induced Fas expression on activated CD4+ Treg cells from CKD4 patients and in particular, from HD subjects.

However, the mechanisms involved in Fas expression in response to oxLDL remain to be unclear. Alcouffe et al. showed that oxLDL stimulated Fas expression in PHA-stimulated T cells and their signaling pathways involved reactive oxygen species production, extracellular signal-regulated kinase, and c-Jun *N*-terminal kinase activation (19). oxLDL may increase CD4+ T cells sensitivity to Fas-mediated apoptosis in HD patients, partly as a consequence of their specific dysregulation of IL-2 expression.

There were also some limitations in the study. First, the stimulus and inhibitors used for activation or apoptosis were at the concentration of a single level (PHA at 2.5 μg/ml and oxLDL at 100 μm/ml only). Second, the inhibition induced by oxLDL could be dose and time dependent. Therefore, more detecting points should be observed to elucidate the role of apoptosis induction. Third, the sample size of the study was relatively small. Larger scale of study groups should be included in further investigation.

This study indicates that deregulation from CD4+ Tm cells to regulatory cells may play a role in the cellular immunological imbalance in patients with CRF.

CONFLICT OF INTEREST

There was not any potential conflict of interest in this paper.

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