Oxidized LDL Modulates Apoptosis of Regulatory T Cells in Patients with ESRD

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ABSTRACT

Increased levels of oxidized low-density lipoproteins (oxLDL) contribute to the increased risk for atherosclerosis, which persists even after adjusting for traditional risk factors, among patients with ESRD. Regulatory T cells (CD4+/CD25+ Tregs), which down-regulate T cell responses to foreign and self-antigens, are protective in murine atherogenesis, but whether similar immune regulation occurs in humans with ESRD is unknown. Because cellular defense systems against oxLDL involve proteolytic degradation, the authors investigated the role of oxLDL on proteasome activity of CD4+/CD25+ Tregs in patients with ESRD. CD4+/CD25+ Tregs isolated from uremic patients' peripheral blood, especially that of chronically hemodialyzed patients, failed to suppress cell proliferation, exhibited cell-cycle arrest, and entered apoptosis by altering proteasome activity. Treating CD4+/CD25+ Tregs with oxLDL or uremic serum ex vivo decreased the number and suppressive capacity of CD4+/CD25+ Tregs. In vitro, oxLDL promoted the accumulation of p27Kip1, the cyclin-dependent kinase inhibitor responsible for G1 cell cycle arrest, and increased apoptosis in a time- and concentration-dependent manner. In summary, proteasome inhibition by oxLDL leads to cell cycle arrest and apoptosis, dramatically affecting the number and suppressive capacity of CD4+/CD25+ Tregs in chronically hemodialyzed patients. This response may contribute to the immune dysfunction, microinflammation, and atherogenesis observed in patients with ESRD.


Although impaired kidney function is often accompanied by dyslipidemia, the high prevalence of cardiovascular disease (CVD) in patients with renal disease cannot be explained by the classical risk factors alone, since CVD may progress over time to end-stage renal disease (ESRD). Abnormal lipoprotein metabolism has been implicated as a possible cause of these complications because of its potential role in immune system activation.

Cumulative data based on atherosclerotic experimental models suggest that CD4+ T cells are present within plaques from initial stages of the disease. They are mainly CD4+ T-helper cells with a phenotype characteristic of the Th1 inflammatory Th helper 1 (Th1) subset.

Given the importance of CD4+ T-helper T cells in atherosclerosis (ATS) and the emerging data on regulatory T cells (Tregs) which maintain immunologic self-tolerance and limit responses to foreign antigens, the question of Tregs influence on ATS has become a central theme of research in several laboratories. The best characterized Tregs are the naturally occurring CD4+/CD25+ Tregs (nTregs) that mature in the thymus and comprise 5% to 10% of peripheral CD4+ T cells. Foxp3, a forkhead family transcription factor, is a lineage specification marker for nTregs and plays a crucial role in the suppression of T-cell activation. Although impaired kidney function is often accompanied by dyslipidemia, the high prevalence of cardiovascular disease (CVD) in patients with renal disease cannot be explained by the classical risk factors alone, since CVD may progress over time to end-stage renal disease (ESRD). Abnormal lipoprotein metabolism has been implicated as a possible cause of these complications because of its potential role in immune system activation.
role in their suppressive function. Once specifically activated through their T cell receptor, nTregs can suppress the proliferation and the effector function of other T cells, B cells, and monocytes by contact-dependent or cytokine-mediated mechanisms.

Cellular defense systems against oxidized proteins, such as low-density lipoproteins (oxLDLs), consist of proteolytic degradation through a multicatalytic protease complex also called proteasome, either dependent or independent of the ubiquitin system. The 26S proteasome complex is constituted of a central 20S proteolytic core associated with two regulatory subcomplexes, termed 19S and 11S regulator. The ubiquitin-dependent proteolytic pathway (26S proteasome) is involved in the continuous turnover of regulatory proteins and in the selective degradation of misfolded and denatured proteins. It is thought to play a major role in regulating numerous cell processes (signal transduction, cell cycle progression, endocytosis, apoptosis) and “detoxifying” altered proteins. Because we have recently shown, oxLDLs induce apoptosis of activated CD4+ T cells through a Fas-mediated mechanism, we hypothesize in the current study that the toxic effects of oxLDL on CD4+ CD25+ T cells and more specifically, on the nTregs subpopulation from uremic patients may be at least one mechanistic explanation of the cellular immune dysfunction and the accelerated ATS encountered in this population. Thus, the study was aimed at investigating the role played by oxLDL on CD4+/CD25+ Tregs suppression capacity, and on the accumulation of p27Kip1 and Bax proteins in parallel with their inhibition of the 20 S and 26 S proteasomes corresponding to T cell cycle arrest and CD4+ CD25+ Tregs apoptosis.

Because little is known about effects of oxLDL on CD4+ CD25+ Tregs, we performed ex vivo and in vitro culture experiments on PHA-stimulated CD4+ CD25+ Tregs from uremic patients and healthy subjects under various conditions.

RESULTS

Baseline Characteristics

Demographic, clinical and biologic characteristics of the patients and the control subjects are listed in Table 1. Highly sensitive

| Table 1. Baseline characteristics of the study groups of patients and control subjects |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | HD patients     | PD patients     | CKD patients    | Control subjects |
| Age, years      | 48.3 ± 3        | 49.1 ± 6        | 47.8 ± 3        | 49.3 ± 5        |
| Men, n (%)      | 8 (53)          | 8 (53)          | 8 (53)          | 8 (53)          |
| BMI (kg/m²)     | 27.7 ± 5.1      | 24.8 ± 3.6      | 24.6 ± 5.8      | 23.2 ± 4.1      |
| GFR, ml/min/1.73 m² | 3.8 ± 2.1      | 10.3 ± 3.1      | 16.7 ± 3.5      | 98.8 ± 3.4      |
| hsCRP, mg/L     | 10 ± 4          | 10 ± 3          | 8 ± 4           | 4 ± 2           |
| Fibrinogen, g/L | 3.41 ± 0.31     | 2.19 ± 0.23     | 3.52 ± 0.37     | 2.75 ± 0.19     |
| Albumin, g/L    | 40 ± 7          | 38 ± 7          | 39 ± 4          | 40 ± 5          |
| Hb, g/L         | 120 ± 4         | 119 ± 3         | 120 ± 5         | 148 ± 4         |
| Ferritin level, ng/ml | 265 ± 36       | 253 ± 49        | 273 ± 31        | 294 ± 58        |
| Transferrin saturation, % | 21 ± 2         | 22 ± 5          | 21 ± 3          | 28 ± 5          |
| Uric acid, µmol/L | 385 ± 46       | 495 ± 53        | 498 ± 51        | 382 ± 35        |
| iPTH (ng/L)     | 193 ± 42        | 207 ± 49        | 132 ± 36        | 42 ± 12         |
| Homocysteine (µmol/L) | 18 ± 3         | 13 ± 5          | 12 ± 3          | 8 ± 2           |
| rHuEPO dosage (IU/kg/wk) | 135 ± 15      | 122 ± 10        | 62 ± 11         |                 |
| Statin          | 15 (100)        | 15 (100)        | 15 (100)        |                 |
| Other treatments |                 |                 |                 |                 |
| ACE inhibitor   | 12 (80)         | 8 (60)          | 7 (47)          |                 |
| ARB             | 5 (33)          | 5 (33)          | 2 (13)          |                 |
| β-blocker       | 7 (47)          | 8 (53)          | 12 (80)         |                 |
| Calcium anticholesterol | 7 (47)      | 6 (40)          | 10 (67)         |                 |
| Aspirin         | 15 (100)        | 15 (100)        | 15 (100)        |                 |
| Duration of HD (months) | 28 [18 to 35] | 23 [16 to 28] |                 |                 |
| eKtV/urea        | 1.42 ± 0.07     | 2.63 ± 0.13     |                 |                 |
| nPNA (g/kg/d)   | 1.38 ± 0.05     | 1.41 ± 0.16     |                 |                 |

*Data are expressed as mean ± SD, number (percentage), or median [range]. HD, hemodialysis; PD, peritoneal dialysis; CKD, chronic kidney disease; BMI, body mass index (calculated as weight in kilograms divided by the square of height in meters); GFR, glomerular filtration rate; hsCRP, highly sensitive C-reactive protein determined by the immunonephelometric method; Hb, hemoglobin; iPTH, intact parathyroid hormone determined by immunoassay; rHuEPO, recombinant human erythropoietin.

**No medication.

See Concise Methods section for details.

**Weekly KtV

#0.001, $P < 0.001; ^{*} P = 0.01$, compared with control subjects; $^{*} P < 0.01$, compared with CKD patients.
C-reactive protein and fibrinogen levels were significantly higher in all patients’ groups compared with control subjects, but did not reach statistical significance when compared with those of CKD patients \((P = 0.12)\). In the patients’ groups, the hemoglobin and the ferritin levels, as well as the transferrin saturation, were between the usually accepted targets in dialysis and nondialysis patients with CKD receiving EPO therapy.\(^{18}\) As expected, the HD treatment did not affect the serum uric acid level. However, it significantly increased the serum homocysteine concentration, as did PD treatment \((18 \pm 3 \mu M/L \text{ and } 13 \pm 5 \mu M/L, \text{ respectively}; P < 0.01 \text{ versus control subjects})\). Not unexpectedly, despite the fact that all dialysis patients received folic acid supplementation, this decreased but did not normalize homocysteine levels. The effect of supplementation was manifest in both HD and PD patients (data not shown). The HD and PD patients had been undergoing dialysis treatment for a median of 28 mo \((\text{range: } 18 \text{ to } 35 \text{ mo})\) and 23 mo \((\text{range: } 16 \text{ to } 28 \text{ mo})\), respectively. Dialysis dose \((\text{eKt/V urea})\) and nutritional state \((\text{nPNA})\) reached the K/DOQI guidelines, and all participants were considered well nourished as evaluated by the Subjective Global Nutritional Assessment and the albumin level.\(^{27}\)

**Plasma oxLDL Concentrations**

Figure 1 shows that the oxLDL concentrations were significantly higher in HD patients than in the other patients’ groups and the control subjects \((P = 0.01)\), even though the HD patients were chronically taking an HMG CoA reductase inhibitor, an ACE inhibitor and/or an ARB, vitamin B complex, and C supplements. Slow intravenous iron administration \((\text{over } 3 \text{ min})\) during the HD session did not produce changes in oxLDL levels and inflammation \((\text{hsCRP and fibrinogen})\) above those induced by the HD procedure itself \((\text{data not shown})\). Furthermore, recombinant human erythropoietin administration had no influence on the oxLDL concentrations irrespective of degree of renal anemia.

**Phenotypic Analysis of Circulating CD4\(^+\)/CD25\(^+\) T Cells**

Freshly isolated PBMC from patients and control subjects were labeled with CD4 and CD25 markers to characterize CD4\(^+\)/CD25\(^+\) T cells. As indicated in Figure 2A, resting CD4\(^+\) T cells from HD patients showed significantly lower expression of CD4\(^+\)/CD25\(^{\text{high}}\) \((\text{fluorescence intensity of } \text{CD25 })\text{ percentiles})\). Whiskers extend on either side of the median (50th percentile). The bottom and the top of a box represent the 25th and 75th percentiles, and the line in the box shows the median \((50\%)\) percentile. Whiskers extend from either side of the box to the farthest observation, but they never exceed 1.5 times the height of the box \((\text{i.e., the interquartile range})\). Any values outside the whiskers are plotted individually and are considered possible outliers. On a normal distribution, the box and whiskers cover roughly 99% of the population. \(*P < 0.01, \text{ vs. control subjects}\)
Figure 2. Frequency of circulating CD4/CD25+ T cells in the study groups of patients and control subjects ex vivo, and after phytohemagglutinin (PHA) stimulation in vitro. (A) CD4+ T cells were separated into CD25high, CD25int, and CD25- T cell subsets, defined by the fluorescence intensity of CD25 obtained using flow cytometry as described (see Concise Methods for details). Flow cytometry analysis from a hemodialyzed (HD) patient (I.), a peritoneal dialysis (PD) patient (II.), a chronic kidney disease (CKD) patient (III.), and a control subject (IV.), all representative. PE, phycoerythrin. (B) Frequency of CD4+/CD25high population as percentage of CD4+ T cells in hemodialyzed (HD) patients (n=15), peritoneal dialysis (PD) patients (n=15), chronic kidney disease (CKD) patients (n=15), and in control subjects (n=15). CD4+ T cells were cultured in vitro after 24-h PHA stimulation. *P<0.01 (ANOVA). Data are expressed as mean ± SEM. (C) The frequency of CD4+/CD25high T cells from control subjects (n=5) was examined after incubation in 10% human uremic serum (three sera from HD patients [C + 10% HD]), three from PD patients [C + 10% PD], or three from CKD patients [C + 10% CKD] after 24-h PHA stimulation. Data are expressed as box-and-whisker plots. The bottom and the top of a box represent the 25th and 75th percentiles, and the line in the box shows the median (50th percentile). Whiskers extend on either side of the box and aim to cover all observations, but they never exceed 1.5 times the height of the box (i.e., the interquartile range). On a normal distribution, the box and whiskers cover roughly 99% of the population. *(ANOVA). AU, arbitrary unit.

Figure 3. Forkhead family transcription factor 3 (FOXP3) expression in stimulated CD4+CD25+ T cells. (A) FOXP3 mRNA levels in phytohemagglutinin (PHA)-stimulated CD4+CD25+ T cells from the study groups of patients and control subjects are presented as the cycle threshold value ratio of FOXP3 to β-actin, in hemodialyzed (HD) patients (n=15), peritoneal dialysis (PD) patients (n=15), chronic kidney disease (CKD) patients (n=15), and control subjects (n=15). *P<0.01 (ANOVA). Data are expressed as mean ± SEM. (B) The frequency of CD4+, CD25+, and FOXP3+ T cells was determined by flow cytometry in hemodialyzed (HD) patients (n=15), peritoneal dialysis (PD) patients (n=15), chronic kidney disease (CKD) patients (n=15), and control subjects (n=15). Percentage of FOXP3+ cells within the CD4+/CD25high, CD4+/CD25int, and CD4-/CD25+/CD25int cell populations after PHA stimulation. *ANOVA. Data are expressed as mean ± SEM. (C) Mean fluorescence intensity (MFI) of FOXP3 from PHA-stimulated CD4+/CD25high T cells in patients and controls. HD patients (n=15), PD patients (n=15), CKD patients (n=15), and control subjects (n=15). Indicated values (shown as box and whiskers) represent MFI signals obtained from FOXP3 stainings. Mean fluorescence intensity values of cells stained by isotype-matched control antibody (background MFI) were subtracted from FOXP3 MFI values. *P<0.01 (ANOVA). AU, arbitrary unit.

CD25+ T cells were 10.2-fold, 10.9-fold, 12.3-fold, and 21.4-fold higher than in the CD25- T cell subset in HD patients, PD patients, CKD patients, and control subjects, respectively. As expected, the FOXP3 expression was correlated with the CD25 expression on CD4+ T cells; that is, the CD4+/CD25high T cells showed the highest expression of FOXP3. A fraction of CD4+/CD25low T cells also expressed FOXP3, whereas CD4+/CD25- T cells were mostly FOXP3 negative (Figure 3B). Despite lim-
iting numbers of CD4\(^+\)/CD25\(^+\) T cells available, the freshly isolated CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) T cell population (CD4\(^+\)/CD25\(^+\) Tregs) from patients and control subjects was CD127 negative (data not shown).

To obtain information about FOX3 expression levels per cell, we determined the FOX3 MFI of PHA-stimulated CD4\(^+\)/CD25\(^{high}\) T cells in patients and control subjects. A decreased level of FOX3 was seen in HD patients (MFI: 16.2 ± 4.3) as compared with PD patients (22.8 ± 5.3; P < 0.01), CKD patients (25.1 ± 5.4; P < 0.01), and control subjects (38.2 ± 4.8; P < 0.001) (Figure 3C). A less significant difference was seen in FOX3 MFI between PD patients and CKD patients. Overall, there was a positive correlation between the percentage of FOXP3\(^+\) cells within the CD4\(^+\)/CD25\(^{high}\) population and the FOX3 MFI of CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) T cells (total population: r = 0.62, P < 0.001; HD patients: r = 0.53, P < 0.001; PD patients: r = 0.55, P < 0.01; CKD patients: r = 0.68, P < 0.001; control subjects: r = 0.72, P < 0.001). In conclusion, HD patients showed a reduced number of blood circulating CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) regulatory T cells (CD4\(^+\)/CD25\(^+\) Tregs) and decreased FOX3 expression level per cell.

**In Vitro Influence of oxLDL on FOX3 Synthesis**

As shown in Figure 2A, CD4\(^+\)/CD25\(^+\) T cell percentages from each patients’ group and control subjects did not differ significantly (86% ± 6%, P = 0.34). To pursue the question of whether uremia or oxLDL specifically affect CD4\(^+\)/CD25\(^+\) Tregs in vitro, we also compared these effects on CD4\(^+\)/CD25\(^+\) T cell population. Incubation of CD4\(^+\) T cells from healthy control subjects with oxLDL (200 \(\mu\)g/ml) resulted in a non-significant lowering of the numbers of CD4\(^+\)/CD25\(^+\) T cells (Figure 4A). When the assay was repeated in the presence of 100 \(\mu\)g/ml uremic serum from HD patients and with CD4\(^+\)/CD25\(^+\) T cells from PD patients in culture medium, the CD4\(^+\)/CD25\(^+\) T cell percentage remained unchanged.

As shown in Figure 4B, the percentage of CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) T cells decreased as the oxLDL concentration increased when the same concentration of native LDL had minor effect on the expression of FOX3 in CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) T cells. Indeed, CD4\(^+\)/CD25\(^{high}\) T cells were significantly more sensitive to oxLDL than to native LDL as the mRNA expression of FOX3 was significantly reduced by 60% ± 8% in comparison to a negligible effect on native LDL (6% ± 2%; P < 0.001) (Figures 4C). When the assay was repeated in the presence of 100 \(\mu\)g/ml uremic serum from HD patients, the mRNA expression of FOX3 of CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) T cells from control subjects was also significantly reduced (56% ± 8%; P < 0.001). This was correlated with FOX3 protein expression as seen in Figure 4D. To further investigate the role of oxLDL on FOX3 expression, we cultured HA-stimulated CD4\(^+\)/CD25\(^+\)/FOX3\(^+\) T cells from control subjects in the presence of various oxLDL concentrations (100 \(\mu\)g/ml and 200 \(\mu\)g/ml). In our experimental conditions, first signs of oxLDL toxicity were evident in the PHA-stimulated CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) T cells culture at 100 \(\mu\)g/ml ox-

LDL, culminating in a 0.477 value relative to the controls at 200 \(\mu\)g/ml. This value was comparable to that of FOX3 expression in CD4\(^+\)/CD25\(^{high}\)/FOX3\(^+\) T cells from HD patients as shown in Figure 4I.

We could conclude that oxLDL, known to trigger lymphocyte apoptosis, would exert a profound depleting effect on CD4\(^+\)/CD25\(^+\)/FOX3\(^+\) T cells in HD patients. Furthermore, the nucleation of CD4\(^+\)/CD25\(^+\)/FOX3\(^+\) T cells from HD patients, as well as the mRNA expression of FOX3 with oxLDL (100 \(\mu\)g/ml) resulted in a significant (59%) relative reduction in the CD4\(^+\)/CD25\(^+\)/FOX3\(^+\) T cell population, compared with a nonsignificant lowering of the number of effector CD4\(^+\)/CD25\(^+\)/FOX3\(^+\) T cells (data not shown). We reasoned that the effect of oxLDL on CD4\(^+\)/CD25\(^+\)/FOX3\(^+\) T cells could be mediated by the effect on FOX3. Thus, suggests that in vivo, oxLDL sensitizes CD4\(^+\)/CD25\(^+\)/FOX3\(^+\) Tregs, which once stimulated, may lose capacity to express the key transcriptional regulator FOX3.

**In Vitro CD4\(^+\)/CD25\(^+\) Tregs Suppression Capacity**

As demonstrated in Figure 3A, the FOX3 expression correlated with the CD25 expression of CD4\(^+\) T cells. After CD4\(^+\)/CD25\(^+\) Tregs were selected, they were resuspended and stimulated.

These cells were found to be hyporesponsive to stimulation with PHA in the presence of APCs in all groups of patients and control subjects (Figure 5A). However, the addition of 100 \(\mu\)g/ml exogenous IL-2 to the cultures overcame the proliferative effect of the CD4\(^+\)/CD25\(^+\) Tregs, with a statistically better response in HD patients and control subjects (data not shown). This suggests, first, that CD4\(^+\)/CD25\(^+\) Tregs express high-affinity IL-2 receptors and, second, that this expression decreases in uremic patients, especially those on dialysis.\(^{28}\)

CD4\(^+\)/CD25\(^+\) Tregs from HD patients did not efficiently suppress the proliferation of the cocultured CD4\(^+\)/CD25\(^+\) T cells (1:1; 21,153 ± 2045 cp/m) as shown in Figure 5A. In striking contrast, CD4\(^+\)/CD25\(^+\) Tregs from control subjects were able to strongly inhibit the proliferation of CD4\(^+\)/CD25\(^+\) responder T cells (1:1; 4172 ± 382 c/min). When cultured with 10% uremic serum from HD patients, CD4\(^+\)/CD25\(^+\) Tregs from control subjects showed significantly less capacity to suppress CD4\(^+\)/CD25\(^+\) T cell proliferation (1:1; 20,695 ± 2053 c/min). However, CD4\(^+\)/CD25\(^+\) Tregs from control subjects cultured with 10% uremic serum from CKD patients showed comparable suppression capacity to control cells cultured in 10% heat-inactivated fetal calf serum (1:1; 4789 ± 438 c/min and 4173 ± 419 c/min, respectively; P = 0.08). CD4\(^+\)/CD25\(^+\) Tregs from control subjects showed the same low capacity to suppress the proliferation of the cocultured CD4\(^+\)/CD25\(^+\) T cells (1:1; 19.736 ± 1759 c/min), as did CD4\(^+\)/CD25\(^+\) Tregs from HD patients (P = 0.78). However, in culture with native LDL, CD4\(^+\)/CD25\(^+\) Tregs from control subjects were able to suppress the proliferation of CD4\(^+\)/CD25\(^+\) T cells (1:1; 4326 ± 305 c/min). This inhibition was highly significant because the CD4\(^+\)/CD25\(^+\) Tregs reproducibly reduced proliferation of CD4\(^+\) T cells by 65% at day 3 and >97% by day 5, compared with the response of the CD4\(^+\)/CD25\(^+\) T cells from HD patients.
RT-PCR products in 1.5% agarose gel after T cells. (A) Percentage of phytohemagglutinin (PHA)-stimulated CD4+ mRNAs ratio. Total mRNA was isolated from purified CD4+ T cells from one HD patient were cultured in culture medium (HD medium). Values were expressed relative to the control values. Expression of FOXP3#T cells of control subjects (n = 15) observed by flow cytometry after 48 h in presence of native LDL or oxidized LDL (oxLDL). Data are expressed as mean ± SEM. *P < 0.001, compared with native LDL-treated T cells for the same concentration of lipoproteins; **P < 0.01, compared with native LDL-treated T cells for the same concentration of lipoproteins; ***P < 0.0001, compared with native LDL-treated T cells for the same concentration of lipoproteins. Data are expressed as mean ± SEM of three independent experiments. (B) Expression of FOXP3 mRNA in PHA-stimulated CD4+ T cells (CD4+ CD25+ Tregs from patients and control subjects. HD patients and from HD patients in culture medium (HD medium; n = 15) observed by flow cytometry after 48 h in presence of native LDL or oxidized LDL (oxLDL). Data are expressed as mean ± SEM of three independent experiments. (C) Expression of FOXP3 mRNA in CD4+ CD25+ T cells from control subjects (n = 5) and in presence of 10% uremic serum from hemodialyzed (HD) patients and from HD patients in culture medium (HD medium). Data are expressed as mean ± SEM. *P < 0.01, compared with oxLDL-treated T cells (200 ng/ml). Data are expressed as mean ± SEM of three independent experiments. (D) Western blot analyses of CD4+ CD25+ Tregs from patients and control subjects. HD patients and from HD patients in culture medium (HD medium; n = 15) observed by flow cytometry after 48 h in presence of native LDL or oxidized LDL (oxLDL). The bands corresponding to FOXP3 from control subjects (n = 5) were also analyzed for comparison. (A) Western blot analyses (as described in the Concise Methods section) of CD25 high RA and CD25 low RA T cells, with Western blot analyses (as described in the Concise Methods section) of CD25 expression in stimulated CD4+ CD25+ T cells from HD patients and control subjects. HD patients and oxLDL-treated T cells (200 ng/ml). Data are expressed as mean ± SEM. *P < 0.01; **P < 0.001; ***P < 0.0001, compared with oxLDL-treated T cells (200 ng/ml). Data are expressed as mean ± SEM of three independent experiments.
showed a significantly decreased proteasome proteolytic activity as compared with the other patient groups and control subjects (20 S, P = 0.003; 26 S, P = 0.001) (Figure 6A). 20 S and 26 S proteasome activity in PHA-stimulated CD4+ CD25+ Tregs from HD patients represented 51% and 49% of the 20 S and 26 S proteasome activity seen in PHA-stimulated CD4+ CD25+ Tregs from control subjects, respectively.

To determine whether oxLDL is capable of penetrating inside CD4+ CD25+ Tregs and subsequently inhibiting chymotrypsin-like activity of the 26 S proteasome in vivo, we incubated intact but PHA-stimulated CD4+ CD25+ Tregs from control subjects with native LDL or oxLDL at various concentrations for 48 h, followed by an additional 2 h incubation with the specific fluorogenic substrate for chymotrypsin-like activity. The catalytic activity of chymotrypsin-like proteasome was measured by monitoring the release of the fluorophore, AMCs, hydrolyzed from specific substrates. Figure 6B shows that oxLDL inhibited chymotrypsin-like activity in a dose-related manner in intact CD4+ CD25+ Tregs, but the inhibitory potency of chymotrypsin-like activity was lower when compared with inhibition of the purified 20 S proteasome and in whole cell lysates (data not shown). Consistent with our prediction, the cell-permeable proteasome inhibitor lactacystin inhibited chymotrypsin-like activity with much higher concentrations in intact CD4+ CD25+ Tregs than in CD4+ CD25+ Tregs lysates (data not shown). Taken together, these findings clearly indicate the cell permeability of oxLDL and the inhibition by oxLDL of chymotrypsin-like activity of the 26 S proteasome (and for a smaller part of the 20 S proteasome) in intact CD4+ CD25+ Tregs.

Accumulation of p27Kip1 Protein and Corresponding G1, Cell Cycle Arrest of Stimulated CD4+ CD25+ Tregs Ex Vivo and In Vitro

PHA-stimulated CD4+ CD25+ Tregs from patients and control subjects were divided into two portions: one for cell cycle analysis and the other for total protein extraction and Western immunoblot analysis. As shown in Figure 7A, CD4+ CD25+ Tregs from HD patients showed a dramatic increase in the CD25+ T cell fraction of cells in the G1 phase. Of note, G1 arrest seen in CD4+ CD25+ Tregs from HD patients was also encountered in CD4+ CD25+ Tregs from PD patients and in CD4+ CD25+ Tregs from CKD patients, but...
Figure 6. 20 S and 26 S proteasome activity in stimulated CD4+/CD25+ Tregs in vivo and in vitro. (A) Phytohemagglutinin (PHA)-stimulated CD4+/CD25+ Tregs from healthy dialysis (HD) patients (n = 5), peritoneal dialysis (PD) patients (n = 5), chronic kidney disease (CKD) patients (n = 5), and control subjects (n = 5) were analyzed for proteasome activity. Purified 20 S and 26 S proteasome were incubated with fluorogenic peptide substrates for chymotrypsin-like activity. After incubation, the fluorescence intensity of AMCs was determined as described in the Concise Methods section. P = 0.003 (20 S proteasome activity); *P = 0.0002 (26 S proteasome activity). Data are expressed as mean ± SEM. (B) Inhibitory effect of oxLDL on chymotrypsin-like activity of the 26 S proteasome in intact but PHA-stimulated CD4+/CD25+ Tregs. CD4+/CD25+ Tregs from control subjects (n = 5) were incubated with various concentrations of native LDL ( ), oxidized LDL (oxLDL) ( ) for 2 h or lactacystin for 12 h, and then PHA-stimulated and coincubated for 2 h with fluorogenic peptide substrate for chymotrypsin-like proteasome activity. After incubation, the medium was collected and subsequently subjected to a proteasome activity assay as described in the Concise Methods section. The chymotrypsin-like activity was expressed as the percentage of the control (defined as 100%). LDL and oxLDL concentrations were in μg/ml, and lactacystin concentrations were in μM. *P = non significant. **P < 0.001 compared with LDL-treated T cells for the same concentration of lipoproteins or lactacystin as mentioned. Data are expressed as mean ± SEM.

This was significantly less important. Importantly, in CD4+/CD25+ Tregs from all patients and control subjects, G1 arrest correlated closely with the induction of p27Kip1, as shown in Figure 6A. Very low p27Kip1 induction was observed in CD4+/CD25+ Tregs from control subjects, which correlated well with very low G1 arrest. These results were further confirmed in culture with oxLDL after PHA stimulation (see below).

p27Kip1 is known as a substrate of the 26 S proteasome, and the proteasomal degradation of p27Kip1 is responsible for cell cycle progression. Thus, we suggested that uremic and oxLDL might increase the accumulation of p27Kip1 proteins in parallel with an inhibition of the 26 S proteasome in PHA-stimulated CD4+/CD25+ Tregs from uremic patients. To examine this possibility, PHA-stimulated CD4+/CD25+ Tregs were analyzed in various conditions, as shown in Figure 7C. Serum from HD patients and oxLDL substantially upregulated levels of p27Kip1 in the presence of so called uremic toxins or oxLDL, as due to proteasome inhibition, we examined the ability of proteasome inhibitors lactacystin to induce up-regulation of p27Kip1 in PHA-stimulated CD4+/CD25+ Tregs from control subjects. Consistent with uremic serum (i.e., from HD patients) treatment, exposure of PHA-stimulated CD4+/CD25+ Tregs from control subjects to proteasome inhibitor lactacystin for 12 h caused a dose-dependent increase in p27Kip1.

oxLDLs Induce Concentration-Dependent Stimulated CD4+/CD25+ Tregs Growth Inhibition and Corresponding Accumulation of p27Kip1 Protein

To investigate whether the level of p27Kip1 protein induced by oxLDL correlates the magnitude of growth inhibition, we analyzed the effects of oxLDL and native LDL on PHA-stimulated CD4+/CD25+ Tregs survival in vitro. CD4+/CD25+ Tregs from a control subject and a microplate growth assay were used to test the correlation between p27Kip1 protein and anchorage-dependent growth (see Concise Methods for details). For p27Kip1 protein detection, CD4+/CD25+ Tregs at similar cell density to the microplate growth assay were plated into 100-mm culture dishes and treated for 48 h with oxLDL and native LDL at different concentrations. As shown in Figure 8A, oxLDL induced a concentration-dependent and anchorage-dependent growth inhibition and a concentration-dependent p27Kip1 accumulation in CD4+/CD25+ Tregs. Under the same conditions, native LDL did not induce p27Kip1 protein at the highest concentration (200 μg/ml), or any growth inhibition. Furthermore, oxLDL, but not native LDL, significantly increased the half-life of p27Kip1 protein (Figure...
8B). Indeed, to confirm that oxLDL-induced p27Kip1 protein is a post-translational event, we measured the half-life of p27Kip1 protein. CD4+CD25+ Tregs from a control subject were analyzed in vitro (C). (A) Cell cycle analysis of phytohemagglutinin (PHA)-stimulated CD4+CD25+ Tregs was done by flow cytometry for the indicated times. Data shown are expressed as one representative hemodialysis (HD) patient and one control subject out of five analyzed. (B) PHA-stimulated CD4+CD25+ Tregs from HD patients (n = 5), peritoneal dialysis (PD) patients (n = 5), chronic kidney disease (CKD) patients (n = 5) and control subjects (n = 5) were analyzed for Western blotting analysis of p27Kip1. Simultaneous immunoblotting of β-actin was used as an internal control for equivalent protein loading. Data are expressed as mean ± SEM. (C) PHA-stimulated CD4+CD25+ Tregs from one HD patient in culture medium were analyzed for proteasomal degradation-related protein p27Kip1. Simultaneous immunoblotting of β-actin was used as an internal control for equivalent protein loading. *P = non significant. **P < 0.001, compared with oxLDL-treated T cells (200 μg/ml). Data are expressed as mean ± SEM from three separate experiments.

Therefore, these results illustrate that oxLDL significantly increases the half-life of p27Kip1 protein and suggest the accumulation of p27Kip1 protein induced by oxLDL results from post-translational mechanisms. We obtained the same results when PHA-stimulated CD4+CD25+ Tregs from control subjects were cultured with 10% uremic serum from HD patients (Figure 8C).

To further confirm that the enhanced stabilities of proteasome-related substrates such as p27Kip1 in the presence of oxLDL (10 to 200 μg/ml) was due to proteasome inhibition, we...
Correlation of growth inhibition induced by oxidized LDL (data not shown).

In PHA-stimulated CD4+ Tregs, p27Kip1 expression is represented. Results were normalized according to the level of β-actin.

Figure 8. Correlation of growth inhibition induced by oxidized LDL (oxLDL), corresponding high level and half-life increase of p27Kip1 protein. (A) Phytohemagglutinin (PHA)-stimulated CD4+ Tregs from a control subject were treated with oxLDL or native LDL at the concentrations indicated. Anchorage-dependent growth as determined by MTT assay was performed in 96-well microtiter plates. The absorbance at 570 nm was measured for 24 h after cells were cultured with 10% normal serum from control subjects. Further cell growth inhibition was induced by serum from HD patients and oxLDL (Figure 9A). Indeed, exposure of PHA-stimulated CD4+ Tregs from control subjects to proteasome inhibitor lactacystin for 48 h caused a dose-dependent increase in p27Kip1 (data not shown).

Effects of oxLDL on the Accumulation of Proteasomal Degradation-Related Proteins Bax and Bcl-xL on the Apoptosis Marker Fas and on DNA Fragmentation in Stimulated CD4+/CD25+ Tregs

Indeed, exposure of PHA-stimulated CD4+/CD25+ Tregs from control subjects to proteasome inhibitor lactacystin for 48 h caused a dose-dependent increase in p27Kip1 (data not shown).

Effects of oxLDL on the Accumulation of Proteasomal Degradation-Related Proteins Bax and Bcl-xL on the Apoptosis Marker Fas and on DNA Fragmentation in Stimulated CD4+/CD25+ Tregs

The pro-apoptotic protein Bax has a pivotal role in controlling programmed cell death (apoptosis) by regulating mitochondrial integrity and mitochondrial-initiated caspase activation, which is known to be regulated by proteasomal degradation. To determine whether oxLDL can favor the expression of Bax, we evaluated the effect of serum from HD patients or control subjects on PHA-stimulated CD4+ Tregs from control subjects or native LDL, and observed that the expression of Bax was increased in response to proteasomal inhibition (Figure 9A). Indeed, exposure of PHA-stimulated CD4+ Tregs from control subjects to proteasome inhibitor lactacystin for 48 h caused a dose-dependent increase in p27Kip1 protein. These results were in agreement with our expectation that Bax expression is increased in response to proteasomal inhibition by serum from HD patients and oxLDL (Figure 9A). Indeed, exposure of PHA-stimulated CD4+ Tregs from control subjects to oxLDL induced a significant increase of Bax expression in PHA-stimulated CD4+ Tregs compared to native LDL (P = 0.001). These changes were comparable to those encountered when PHA-stimulated CD4+ Tregs from HD patients or control subjects exposed to oxLDL expressed significantly higher Bax levels than native LDL (P = 0.001). These expressions were comparable to those encountered when PHA-stimulated CD4+ Tregs from HD patients or control subjects were analyzed in culture medium. The same results were obtained with the other patients’ groups (data not shown).

Incubation with incremental doses of oxLDL induced a significant increase of Bax expression in PHA-stimulated CD4+ Tregs from control subjects (data not shown). Copper-oxidized LDL, characterized by a mild oxidation level, indicates that the accumulation of Bax expression was specific to activated CD4+ Tregs from HD patients. Thus, we suggested that oxLDL, but not native LDL, might increase the accumulation of Bax, but not the anti-apoptotic protein Bcl-xL, in parallel with the inhibition of the 20 S and 26 S proteasome in PHA-stimulated CD4+ Tregs. To examine this possibility, we treated PHA-stimulated CD4+ Tregs from control subjects with various concentrations of oxLDL and native LDL for 48 h, and determined the levels of Bax expression.
and Bcl-xL by Western blotting. Oxidized LDL substantially upregulated levels of Bax but not Bcl-xL in a concentration-dependent manner (data not shown).

To further confirm that the enhanced stabilities of proteasome-related substrates such as p27Kip1 or Bax in the presence of oxLDL were due to proteasome inhibition, we examined the ability of proteasome inhibitor lactacystin to induce up-regulation of Bax in PHA-stimulated CD4+/CD25+ Tregs from control subjects. Consistent with oxLDL treatment, expression of PHA-stimulated CD4+/CD25+ Tregs from control subjects to proteasome inhibitor lactacystin for 12 h caused a dose-dependent increase in Bax but not Bcl-xL (data not shown).

Consistent with control subjects, oxLDL caused a significant and concentration-dependent increase in the percentage of PHA-stimulated CD4+/CD25+/Fas+ Tregs (Figure 9B). In contrast, the same concentration of native LDL had only a minor effect (P < 0.001). This was confirmed by Western blot for Fas protein determination. For comparison, a significantly higher percentage of PHA-stimulated CD4+/CD25+ Tregs from HD patients expressed Fas (HD medium: 4.2% ± 0.7%) compared with CD4+/CD25+ Tregs from control subjects (P < 0.01). The increased state of CD4+/CD25+ Tregs apoptosis suggested by Fas markers was confirmed by Western blot and by DNA analysis after culture and PHA stimulation (Figure 9C). In all experimental conditions, the apoptotic DNA fragmentation significantly increased in FAS-sorted CD4+/CD25+ Tregs from HD patients compared with PD and CKD patients (P < 0.005) and control subjects (P = 0.001), in accordance with the decline in cell viability. These data suggest, first, that in chronic

Figure 9. (A) Accumulation of proteasomal degradation-related protein Bax but not Bcl-xL in stimulated CD4+/CD25+ Tregs. Western blot and corresponding mean fluorescence intensity (MFI) were calculated for Bax and Bcl-xL, in hemagglutinin (PHA)-stimulated CD4+/CD25+ Tregs from hemodialyzed (HD) patients (n = 5) in culture medium (HD medium), in presence of 10% human normal serum (C) (HD + 10% C), in PHA-stimulated CD4+/CD25+ Tregs from control subjects (n = 5), in culture medium (C medium), and after incubation of 10% human uremic serum (HD patients) (C + 10% HD). Furthermore, PHA-stimulated CD4+/CD25+ Tregs from control subjects (n = 5) were cultured in the presence of oxidized LDL (200 µg/ml) (C+oxLDL 200) and native LDL at the same concentration (C + LDL 200) for 48 h. Then, the cells were analyzed as described above for proteasomal degradation-related protein Bax and Bcl-xL. Box-and-whisker plots are used to represent the distributions. The bottom and the top of a box represent the 25th and 75th percentiles, and the line in the box shows the median (50th percentile). Whiskers extend on either side of the box and aim to cover all observations, but they never exceed 1.5 times the height of the box (i.e., the interquartile range). Any values outside the whiskers are plotted individually and are considered possible outliers. On a normal distribution, the box and whiskers cover roughly 99% of the population. (B) Fas expression in PHA-stimulated CD4+/CD25+ Tregs in vitro. PHA-stimulated CD4+/CD25+ Tregs from control subjects (n = 5) were cultured for 48 h in presence of native LDL or oxLDL at various concentrations. The cells were then Western blotted for Fas protein determination. Simultaneous immunoblotting of α-tubulin was used as an internal control for equivalent protein loading. Fas protein determination in PHA-stimulated CD4+/CD25+ Tregs from control subjects (n = 5) in presence of 10% uremic serum from hemodialyzed (HD) patients (C + 10% HD) and from HD patients in culture medium (n = 5) (HD medium) was also analyzed for comparison. *P = 0.01; **P < 0.001, compared with LDL-treated T cells for the same concentration of lipoproteins; ***P < 0.01; "P = nonsignificant, compared with oxLDL-treated T cells (200 µg/ml). Data are expressed as mean ± SEM. (C) Agarose gel analysis of cell DNA fragmentation in stimulated CD4+/CD25+ Tregs. DNA isolated from a control subject cultured with native LDL or oxLDL as mentioned. DNA fragmentation was also determined in PHA-stimulated CD4+/CD25+ Tregs from control subjects (n = 5) in presence of 10% uremic serum (HD patients) and from HD patients in culture medium (n = 5) for comparison.
HD patients, oxLDLs participate in CD4+/CD25+ Tregs apoptosis by two different pathways (cell cycle arrest and Fas engagement, respectively) and, second, that oxLDLs potentiate this physiologic process through p27kip1 and Bax accumulation.

**DISCUSSION**

There are sufficient data to support the presence and role of oxLDL in the development of the atherosclerotic plaque.\(^3,4,29\) In this study, we have shown that HD patients exhibited a reduction in the number of peripheral CD4+/CD25+ Tregs, which showed a reduced suppressor function. Considering the oxLDL effects, the relative percentage of CD4+/CD25+ Tregs of the total CD4 population was significantly reduced by incubation with oxLDL compared with a nonsignificant depleting effect on CD4+/CD25- T cells. Indeed, the effect of uremia and oxLDL played no significant role on this latter T cell population in vitro. Thus, we may suggest that oxLDLs have a specific role on CD4+/CD25+ Tregs.

More interestingly, CD4+/CD25+ Tregs from HD patients exhibited early cell cycle arrest and became apoptotic. These phenomena were the consequence of the oxLDL-inhibited proteasome proteolytic activity of p27kip1 and Bax proteins, both of which accumulated in PHA-stimulated CD4+/CD25+ Tregs in vitro. Moreover, the compromising effect of oxLDL on CD4+/CD25+ Tregs from control subjects appeared to be equal to that of uremic serum from HD patients in vitro. The current study design, however, did not allow us to rule out the possibility that other uremic toxins by themselves were causally associated with the differential effect of oxLDL. Because some degree of LDL oxidation has been noted in the plasma of subjects with chronic renal impairment, we speculated that the CD4+/CD25+ Tregs pool might contact modified LDL in the circulation and not just within the atheroma.\(^4\) Thus, plasma oxLDL in uremic patients might be the trigger of CD4+/CD25+ Tregs cycle arrest and apoptosis through proteasome alteration.

**FOXP3** has been shown to govern the development and function of CD4+/CD25+ Tregs.\(^14,30\) We thus explored whether the suppressive function of oxLDL was associated with its effect on FOXP3. We found that both uraemic serum from HD patients and oxLDL triggered a time- and concentration-dependent down-regulatory effect on the expression of FOXP3. This means the circulating CD4+/CD25+ Tregs in HD patients in addition to being reduced in number, also expressed low levels of FOXP3, per cell, and failed to suppress the proliferation of effector cells. Proteasome-mediated degradation of cell proteins plays a pivotal role in the regulation of several basic cellular processes, including differentiation, proliferation, cell cycling, apoptosis, gene expression, and signal transduction. Imbalances in proteasome-mediated protein degradation contribute to various human diseases such as cancer, neurodegenerative diseases, and ATS.\(^31–34\) Although we did not address the question on the ubiquitin conjugates (i.e., p27kip1, Bax, and Bcl-xL), our results clearly demonstrated that oxLDL inhibited proteasome enzymatic activity of the cell-dependent kinase inhibitor (CKI) p27kip1 and the protoapoptotic molecule Bax. The consequences resulted in the increased accumulation of these key regulatory proteins in CD4+/CD25+ Tregs from HD patients. The mechanisms by which oxLDLs modify CD4+/CD25+ Tregs proteasome activity in uremic patients remain poorly understood. However, besides enhancing the oxidative damage of proteins such as p27kip1 and Bax, oxLDL led to accumulation of ubiquitinated proteins via inhibition of proteasome enzymatic activity.\(^35,36\) This was confirmed by the use of a potent proteasome inhibitor, lactacystin, which inhibited chymotrypsin-like activity. Our findings clearly indicated the cell permeability of oxLDL and the inhibition by oxLDL of chymotrypsin-like activity of the 26 S proteasome, and a lesser part of the 20 S proteasome on the inside of intact CD4+/CD25+ Tregs from HD patients. It can be speculated that the oxLDL-related protein damage is responsible for the CD4+/CD25+ Tregs cycle arrest at G1 phase and their apoptosis.\(^35,36\) Furthermore, using oxLDL as a trigger, our results are substantiated by those of others showing that oxLDL inhibit the proliferation of CD4+/CD25+ Tregs in vitro and in vivo, in part through modulation of CKI pathway.\(^37\)

Our results are in agreement with the hypothesis that oxLDLs produce a rapid decay of proteasomal proteinolysis in inducing derivatization of cell proteins by 4-hydroxyonenal (4-HNE), resulting in an inhibition of the 19S.\(^38\) This is because 4-HNE cross-linked proteins are resistant to proteolysis and are able to inhibit the 26S proteasome, and because 26S proteasomes are less resistant to H2O2-induced oxidative stress than the 20S proteolytic core.\(^39,40\) The second step (i.e., inhibition of the 20S core) may be a result of the progressing intracellular oxidative stress induced by oxLDL. At this stage, when the proteasome is completely inhibited, CD4+/CD25+ Tregs are rapidly dying. Moreover, proteasome inhibition may also impair cell cycle regulation and alter cell viability through the defect of its other housekeeping functions involved in the regulation of proteins that play a role in the balance between cell death and survival (i.e., Bax and Bcl-xL, respectively).\(^41\)

However, cell cycle status is not the sole determinant of the cell response to proteasome inhibition induced by oxLDL. In the current study, we found that the accumulation of Bax induced by oxLDL was simultaneously accompanied by that of p27kip1. Because Bax is a member of the Bcl-2 family and an abnormal accumulation of Bax, which binds to the voltage-dependent anion channel in the outer mitochondrial membrane, may lead to mitochondrial perturbation and allow cytochrome c release, we inferred that proteasome-mediated apoptosis driven by oxLDL resulted partly from the accumulation of Bax.\(^42\) However, we cannot rule out the possibility that the accumulation of other proapoptotic molecules such as BIK and TBID may be implicated in CD4+/CD25+ Tregs death. On the other hand, we demonstrated that oxLDL affected the antiapoptotic protein Bcl-xL degradation by increasing its re-
movation in parallel with the activation of the 20 S and 26 S proteasome in PHA-stimulated CD4⁺/CD25⁺ Tregs from control subjects treated with various concentrations of oxLDL or cultured with uremic serum from HD patients.

A key feature of Fas signaling is the activation of apoptosis by the proapoptotic protein Bax, which is known to be regulated by proteasomal degradation, and the inhibition of apoptosis by Bcl-2 or Bcl-xL. Use of this alternative pathway in vivo is debated, but it may be present in lymphocytes, as peripheral T cells have been suggested to be able to switch between a Bcl-xL-sensitive and -insensitive phenotype during Fas-induced cell death. Although CD4⁺ T cells are resistant to Fas-mediated apoptosis, oxLDLs sensitize stimulated CD4⁺ T cells to Fas-mediated apoptosis through up-regulation of Bax and down-regulation of Bcl-2. Importantly, the present results showed that CD4⁺/CD25⁺ Tregs from control subjects were usually resistant to Fas-mediated apoptosis. These cells, however, once PHA stimulated, were sensitized to Fas-mediated apoptosis in the presence of oxLDL or in culture with uremic serum. This suggests that Fas may be able to induce CD4⁺/CD25⁺ Tregs apoptosis in uremic patients in patients with high plasma oxLDL concentrations (i.e., hyperlipidemic patients) when Fas expression is up-regulated in circulating CD4⁺/CD25⁺ Tregs. The fact was further confirmed by DNA fragmentation analysis.

Thus, our findings suggest that the decreased CD4⁺/CD25⁺ Tregs number in chronic HD patients were the consequences of the p27^Kip1^ and Bax but not Bcl-xLaccumulation in these CD4⁺ T cell subsets, which was due to oxLDL proteasome activity alteration. This was further accompanied by the activation of the apoptosis marker Fas and finally the DNA fragmentation. Hence, oxLDL induction of G₁ cell cycle arrest and apoptosis by proteasome inhibition can dramatically affect CD4⁺/CD25⁺ Tregs in patients with ESRD chronically hemodialyzed. A fact which may have important implications in clinics, since this response could contribute to the CD4⁺ T cell immune dysfunction in patients with ESRD including microinflammation and atherogenesis.

**CONCISE METHODS**

**Patients and Control Subjects**

Investigations were carried out in 30 patients with ESRD undergoing either chronic HD (n = 15), or continuous ambulatory peritoneal dialysis (CAPD; n = 15) or at least 6 mo before the study, in 15 patients with chronic kidney disease (CKD) stages to 5, and in 15 normotensive healthy subjects with normal kidney function. The control group did not receive BP lowering agent, lipid lowering agent, or aspirin. They were known to have cardiovascular disease. No modification in dialysis procedure was undertaken in dialyzed patients. Chronic HD and CAPD patients had been dialyzed as described previously. The dose of dialysis regimen (equilibrated Kt/Vurea [Kt/Vurea]) and the normalized protein equivalent of nitrogen appearance (NPN) in g/kg/d remained constant before and during the study.

**Enzyme-Linked Immunosorbent Assay (ELISA) for Plasma oxLDL Determination**

Plasma oxLDL concentrations were measured using an mAb-4E6–based enzyme-linked immunosorbent assay (product no. 10-1158-01; Immunotech, Uppsala, Sweden). This antibody (Ab) is directed against a conformational epitope in the apoB-100 moiety of LDL that is generated as a consequence of substitution of at least 60 lysine residues of apoB-100 with aldehydes. Venous blood samples from all patients and control subjects were obtained. The LDL fraction was separated from blood plasma before the ELISA procedure to minimize potential interferences with other plasma constituents, such as oxVLDL, anti-oxLDL autoAbs, and anti-phospholipid Abs. Oxidized LDL were measured in ELISA as described previously. In each ELISA plate, various concentrations of standard oxLDL, which was prepared by incubating LDL with 5 μmol/L CuSO₄ at 37 °C for 3 h, were run simultaneously to determine a standard curve.

**Cell Phenotypic Analysis**

Peripheral blood mononuclear cell (PBMC) analysis from patients and control subjects was performed using the EPICS XL-MCL flow cytometer (Coulter Instrumentation Laboratory, Lausanne, Switzerland). To determine the frequencies and phenotypes of CD4⁺/CD25⁺ Tregs, which distinctly discriminates between nTregs and effector cell subsets in humans, we performed multicolor flow cytometry using the following Abs: anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CD127 (Immunotech, Berkeley, CA), and anti-forkhead family transcription factor 3 (FOXP3) (eBioscience, San Diego, CA). On the basis of their degree of CD25 expression, CD4⁺/
CD25\textsuperscript{high} T cells (CD4\textsuperscript{+}/CD25\textsuperscript{+} Tregs, top 2\% cells), CD4\textsuperscript{+}/CD25\textsuperscript{low} T cells (activated cells), and CD4\textsuperscript{+}/CD25\textsuperscript{−} T cells (effector cells) were isolated as previously reported.\textsuperscript{21} For intracellular staining of FOXP3, cell surface staining was first completed and intracellular FOXP3 labeling was completed using the Ready-SET-Go! FOXP3 labeling kit (eBioscience), and all samples were resuspended in 1% formaldehyde (Sigma, St. Louis, MO) in 1x Dulbecco’s phosphate-buffered saline (PBS) (Mediatech, Manassas, VA). The percentage of positive cells and the mean fluorescence intensity (MFI) in arbitrary units for a specific marker were calculated. The flow cytometer was calibrated with flow-count beads, and results were analyzed with System II software (all from Beckman-Coulter Instrumental, Nyon, Switzerland). Lymphocyte gating was in general performed by the software based on forward scatter, side scatter, and CD4 staining characteristics, but also manually where indicated. Analysis was performed on a minimum of 1 × 10\textsuperscript{6} cells and expressed as a percentage of CD4\textsuperscript{+} T cells.

**Real-Time PCR Analysis of FOXP3 Expression**

Total RNA was isolated from freshly purified CD4\textsuperscript{+}/CD25\textsuperscript{+} T cells of a sufficient PBMC sample could be obtained, according to the protocol for Trizol Reagent (Invitrogen, Carlsbad, CA). cDNA was generated by Moloney murine leukemia virus reverse transcriptase (BBI, Markham, Ontario, Canada) using an oligo(dT)\textsubscript{17} primer (Invitrogen/Life Technologies- BRL, Carlsbad, CA) according to the manufacturer’s instructions. Relative quantitative real-time PCR was performed using SYBR-green I Premix Ex Taq on the ABI Prism 7000 (Takara Bio Inc., Foster, CA) following the manufacturer’s instructions. The primers for β-actin were available commercially, and the primers for FOXP3 were synthesized by Vigenotech (sense: 5′-GGCACCTCTCCAGGACAG-3′; antisense: 5′-GCTGTCATG-GCTGTTGGCTC-3′). Thermal cycle parameters were 30 s at 95°, and 40 cycles of denaturation at 95° for 10 s, followed by annealing at 60° for 15 s and extension at 72° for 40 s. PCR assays were performed in triplicate, and data were analyzed with the ABI Prism Detection system using the comparative threshold cycle method as described previously.\textsuperscript{22} Standard curves were generated and indicated excellent amplification efficiency (90% – 100%).

**LDL Isolation and Oxidation**

LDLs (density 1.019 to 1.063) were isolated from pooled fresh human sera by sequential ultracentrifugation as described previously.\textsuperscript{23} Ultracentrifuged LDLs were diazoyzed in PBS containing 10 mmol/L EDTA-2Na for 24 h, and stored in a 0°C place (4°C). After dialysis in PBS without 1 mmol/L EDTA-2Na for 24 h, LDLs were oxidized with 5 μmol/L of 15,15′-dibromoperoxide, at 37°C for 24 h. The reaction was stopped by the addition of 1 mmol/L EDTA-2Na, and cells were then used after 24 h of dialysis in PBS. The level of oxidation was measured by two methods: (1) increased mobility in agarose gel (1.5-fold higher R\textsubscript{p} versus native LDL) and (2) the thiobarbituric acid-reactive substances (TBARS) method. Under the standard conditions, oxLDL contained 5.2 ± 0.7 nmol TBARS per milligram of apoB (versus 0.5 ± 0.1 nmol for native LDL). When native LDLs were incubated with activated CD4\textsuperscript{+} T cells for 24 h, the oxidation level increased from 0.6 ± 0.2 nmol of TBARS per milligram of apoB at t = 0 to 1.3 ± 0.2 at t = 72 h. The change observed with oxLDL was less at the same time.

The extent of lipid peroxidation was estimated as malondialdehyde (MDA) or 4-hydroxy-alkenals (4-HNE) content by a colorimetric commercial kit (LPO 586, Bioxytech, Bonneuil sur Marne, France) resulting in a mean value of 18.2 ± 5.3 nmol, 30.6 ± 6.2 nmol and 32.2 ± 7.4 nmol of MDA-4-HNE per milligram of LDL protein in HD patients, CKD patients, and control subjects, respectively. The oxLDL concentration used for our experiments (200 μmol/L) was equivalent to that reported in human plasma from HD patients, PD patients, CKD patients, and healthy subjects.

**CD4\textsuperscript{+} T Cell Preparation, Stimulation, and Culture Conditions**

Peripheral blood mononuclear cells were isolated from patients and control subjects (50 ml freshly drawn blood) by standard techniques using density gradient through a ficoll-hypaque gradient (density = 1.077 g/ml, 300 × g) (Pharmacia LKB, Uppsala, Sweden). Peripheral blood mononuclear cells were incubated on plastic tissue culture plates at 37°C for 2 h to allow monocytes to adhere. Nonadherent cells were aspirated. T lymphocytes were then passed over human T cell enrichment columns (HIV systems, Minneapolis, MN). The negatively selected cells contained more than 98% CD3\textsuperscript{+} T cells, as assessed by flow cytometry.

Cells were cultured in triplicate in U-bottom 96-well plates in RPMI supplemented with 10% heat-inactivated FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 25 μg/ml of mercaptoethanol. During experiments, CD4\textsuperscript{+} /CD25\textsuperscript{−} Tregs were incubated for up to 72 h in RPMI alone with or without native LDL or oxLDL before being washed in warm sterile PBS to remove the oxidant before analysis. To analyze the potential effect of culture medium, we also examined CD4\textsuperscript{+}/CD25\textsuperscript{−} Tregs in vitro after incubation in RPMI containing 10% human uremic (HD, PD, or CKD patients where mentioned) or normal (control subjects) serum and stimulated with phytomenagglutinin (PHA) during 24 h in the presence of antigen-presenting cells (APCs) except where otherwise specified. Cells were counted by flow cytometry and resuspended in culture medium.

CD4\textsuperscript{+} T cells stimulation was also analyzed in the presence of various concentrations of native LDL or oxLDL (concentrations expressed as μg of apoB/ml), and co-incubated with PHA. The effect of oxLDL was not due to a direct effect on PHA, since oxLDL did not inhibit the binding (at 4°C and 37°C) of FITC-PHA to CD4\textsuperscript{+} T cells. We also examined the possibility that PHA-stimulated CD4\textsuperscript{+}/CD25\textsuperscript{−} Tregs may oxidize LDL or increase the oxidation level of oxLDL during cell culture. In the experimental conditions, PHA-stimulated CD4\textsuperscript{+} /CD25\textsuperscript{−} Tregs have insignificant oxidative power on LDL (data not shown).

**Coculture Experiments**

CD4\textsuperscript{+} /CD25\textsuperscript{−} Tregs from patients’ groups and control subjects were analyzed in coculture to address their suppressive capacity. CD4\textsuperscript{+} /CD25\textsuperscript{−} Tregs, CD4\textsuperscript{+} /CD25\textsuperscript{−} T cells, or a 1:1 mixture (cocultures) were stimulated with 2.5 μg/ml PHA (Murex, HA 16, Wellcome, Dartford, UK) in presence of APCs (1 × 10\textsuperscript{5} cells) and monitored for proliferation by [\textsuperscript{3}H] thymidine incorporation (Amersham Life Sciences, Arlington Heights, IL). Briefly, at 72 h of culture, the cells were pulsed with 0.5 μCi [\textsuperscript{3}H] thymidine and cultured a further 18 h before
et al. was investigated in PHA-stimulated CD4+ Tregs for 10 min), the radioactivity of TCA-soluble and TCA-precipitable fractions (precipitate dissolved in 50 μl of NaOH 1N) were counted by liquid scintillation counting (AquaSafe, Packard Tricarb 4530, Downers Grove, IL).

In Situ Proteolysis Measurements and In Vitro Determination of Proteasome Activity

The degradation of cellular proteins was determined under the conditions described by Grüne et al. Briefly, CD4+/CD25+ Tregs proteins were precipitated by 10% TCA for 30 min at 4°C and after centrifugation (15,000 g for 10 min), the radioactivity of TCA-soluble and TCA-precipitable fractions (precipitate dissolved in 50 μl of NaOH 1N) was counted by liquid scintillation counting (AquaSafe, Packard Tricarb 4530, Downers Grove, IL).

Chymotrypsin-like activity of the 20S and 26S proteasome was determined using a commercially available fluorometric kit (Chemicon International, Temecula, CA), following company instructions. Briefly, chymotrypsin-like activities were assessed with the substrates Suc-LLL-Y-7-amido-4-methylcoumarin (AMC) or L-ZZE-AMC, at final concentrations of 18 and 45 μmol/L, respectively. Trypsin-like activity was not routinely determined because of high background nonproteasome-mediated cleavage of substrates. The reaction was run at 37°C for 30 min and then quenched by adding 30 μl of cold ethanol followed in 10 min by 1 ml H2O. Cleavage/loss of proteasomal subunits was determined by Western blot analysis under our experimental conditions, cleavage of substrates was linear over this time period. The samples were read at excitation wavelength, 385 nm, and emission wavelength, 440 nm. AMC fluorescence was compared with an AMC standard curve (10 nM AMC to 1 μM AMC). The proteasome inhibitor, lactacystin (Sigma) was used as a control for proteasome activity.

Protein Extraction and Western Blot Analysis

Proteins were extracted from CD4+ Tregs cultures with RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 200 mmol/L PMSF, and protease inhibitor cocktail tablets). The solubilized proteins were resolved on 12% SDS-PAGE gels and subsequently transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST (50 mmol/L Tris-HCl/0.1% Tween 20, pH 7.4) buffer for 1 h and incubated with various Abs according to manufacturer's instructions (see below). Protein concentrations were determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) in which protein absorbance is measured using spectrophotometry at 595 nm. Immunodetection was performed by enhanced chemiluminescence (ECL) (PerkinElmer Life Sciences, Boston, MA) using Hyperform ECL (American Life Sciences, Arlington Heights, IL). Densitometric analysis was performed using an IS-1000 Digital Imaging System and Alpha Innotech Software (Alpha Innotech Corporation, San Leandro, CA). The net intensity of each band of interest was measured in each of three separate Western blots. The ratio of the intensity of the detected band to β-actin, β-tubulin, or α-tubulin (Neo Markers, Fremont, CA) was used to determine equal loading. Abs that were used in these studies were obtained from commercial sources and included Abs that were directed against BAX (R&D Systems), gp120 (CD45) (all from Santa Cruz Biotechnology, Santa Cruz, CA).

Cell-Cycle Analysis

Cell cycle analysis was performed by PI staining with propidium iodine (PI). Cells were washed twice in cold PBS and then resuspended in 70% ethanol at 4°C for at least 6 h. The samples were rehydrated in cold PBS, treated with 50 μg/ml RNase A, and stained with 50 μg/ml PI. DNA content was measured using a flow cytometry (Coulter, Coulter/AL-MCL; Coulter Instrumentation Laboratory, Miami, Switzerland; G1 (2N), S (2N to 4N) and G2/M fraction (4N) were determined with MultiCycle Cell Cycle Analysis software (Phoenix Flow Systems, San Diego, CA).

Bax and Bcl-xL Determination

Expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-xL was investigated in PHA-stimulated CD4+/CD25+ Tregs from the patients' groups and the control subjects using flow cytometry. Staining for intracellular proteins was performed as follows. CD4+/CD25+ Tregs were fixed with 1% (v/v) parformaldehyde in PBS for 30 min at room temperature and then permeabilized with 0.1% (v/v) saponin (Sigma, St. Louis, MO) in PBS for 5 min at 4°C. Cells were then stained with FITC-conjugated anti-human BAX or anti-Bcl-xL Abs (conformationally active form of the protein) for 30 min at 4°C. FITC-conjugated mouse IgG1 and IgG2b were used as isotype controls. After several washes with 0.1% saponin solution, cells were analyzed by flow cytometry.

The MFI was calculated for Bax and Bcl-xL in CD4+/CD25+ Tregs. Fluorescence intensity was measured using a quantitative FITC standard, Quantum 26 (Bangs Laboratories, Fishers, IN), consisting of five FITC-labeled microbead populations calibrated to specific fluorescence intensities that are expressed in molecules of equivalent soluble fluorochromes units. The MFI determined for each intracellular protein was converted to molecules of equivalent soluble fluorochromes units based on a standard curve prepared and run daily in parallel with experimental samples. QuickCal v2.1 software (Bangs Laboratories) was used for these calculations.

Apoptosis Evaluation

Apoptosis was indirectly assessed by Fas (CD95) staining and flow cytometry. Once isolated, CD4+/CD25+ Tregs were adjusted to 1 × 10^6 cells/ml and resuspended in binding buffer (10 mM Hapes/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl2, filtered through a 0.2-μm filter); 5 μl anti-Fas (anti-CD95) mAb FITC was added to 195 μl of cell suspension. After incubation, cells were washed and resuspended in 190 μl of binding buffer and 10 μl of PI stock solution (20 μg/ml) (Pharmingen, Becton Dickinson, Basel, Switzerland). Live cells were considered to be those cells that were negative for both dyes; dead cells were positive for both fluorochromes, and apoptotic cells were positive only for anti-Fas (anti-CD95) mAb FITC and negative for PI.

DNA Fragmentation Was Assessed in CD4+/CD25+ Tregs that Expressed Fas (CD95)

Briefly, quantification of DNA fragmentation was performed by determination of fractional solubilized DNA by diphthalamine dye and
spectrofluorometric assay. Colored solution was transferred to a well of a 96-well flat-bottom ELISA plate, and OD was determined by a spectrophotometer at a wavelength of 620 nm. Percentage DNA fragmentation was calculated as the ratio of diphenylamine fluorescence in the supernatant divided by the total fluorescence in the supernatant plus the pellet multiplied by 100. Qualitative DNA fragmentation analysis into nucleosomal bands was detected by agarose gel electrophoresis as described previously.26

Statistical Analysis
Unpaired data were analyzed nonparametrically with the Mann-Whitney U-test. The Wilcoxon rank-sum test was used for paired data. Significance of the differences in CD4+ T cell analyses between the patients’ groups and the control subjects was calculated by ANOVA and Bonferroni’s multiple-comparison test. Linear regression analysis was used to establish correlations between variables. Results are expressed as mean ± SD (or mean ± SEM where mentioned), as the median in the case of skewed distribution, and range as specified. Statistical significance was defined as \( P < 0.05 \).

NOTE ADDED IN PROOF
While this paper was in press, an article appeared confirming our data showing that the absolute number and the function of CD4+CD25bright+ T-cells from patients with ESRD are low (Hendrikx TK, van Gurp EA, Mol WM, Schooijk W, Leweek bind VD, Ijermans JN, Weimar W, Baan CC. End-stage renal failure and regulatory activities of CD4+CD25bright+Fox3+ T-cells. Nephrol Dial Transplant 2009 Feb 4 [epub ahead of print]).

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DISCLOSURES
None.

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