Naïve and central memory T-cell lymphopenia in end-stage renal disease

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End-stage renal disease (ESRD) is associated with increased propensity to infections, diminished response to vaccination, impaired cell-mediated immunity, and reduced CD4+/CD8+ T-lymphocyte ratio. Four subsets of CD4+ and CD8+ T cells have been recently identified: naïve cells (as yet uncommitted), central memory (CM) cells (previously programmed), and CD45RA-positive and CD45RA-negative effector memory (EM) cells (programmed to perform specific effector functions). The effect of ESRD on subpopulations of T lymphocytes is unclear and was studied here. Twenty-one hemodialysis patients and 21 age-matched controls were studied. Pre- and post-dialysis blood samples were obtained and analyzed by three-color flow cytometry. CD4+/CD8+ ratio and the numbers of the naïve and CM CD4+ and CD8+ T cells were significantly reduced, whereas the numbers of EM CD4+ and CD8+ T cells were unchanged in the ESRD group. The reduction of the naïve and CM T-cell counts in the ESRD group was associated with increased apoptosis of these cells. Negative correlations were found between severity of azotemia, oxidative stress, and hyperphosphatemia with the number of naïve T cells. Comparison of diabetic with non-diabetic ESRD patients revealed higher numbers of total CD8+ cells and EM CD8+ T cells in the diabetic group. Dialysis did not significantly change the naïve and CM CD4+ or CD8+ cell counts, but significantly lowered CD8+ EM cell count. Thus, ESRD results in increased apoptosis and diminished populations of naïve and CM T lymphocytes. This phenomenon may, in part, contribute to the impaired immune response in this population.

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KEYWORDS: chronic renal failure; dialysis; immune disorder; infection; T-lymphocyte subsets; oxidative stress; apoptosis

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Although bacterial infections have diminished as a cause of death in the general population, they constitute the second most common cause of death in the end-stage renal disease (ESRD) population.[1–3] This is thought to be largely owing to the impaired host immune response in uremia.[1,4,5] The reported immunological abnormalities in ESRD patients include decreased granulocyte and monocyte/macrophage phagocytic function,[4,6,7] defective antigen presentation by monocyte/macrophages,[4,8,9] reduced antibody production by B lymphocytes,[4,10,11] and impaired T-cell-mediated immunity.[4,12,13] The exact mechanisms responsible for these derangements are not fully understood.

T lymphocytes play a central role in generation of the adaptive immune response. In the presence of infection, naïve (pre-immune) T cells recognize epitopes of the structural molecules expressed by the invading microbe. This leads to activation, massive expansion, and differentiation of these cells into two types of antigen-experienced lymphocyte subsets, short-lived effector T cells and long-lived memory T cells.[14] Once, re-challenged with the same antigen, the long-lived memory T cells can elicit the full immunologic response rapidly.[14,15] Recently, several studies have identified two major subsets within the memory cell population, namely central memory (CM) and effector memory (EM) T lymphocytes.[14,15] The EM T cells have been further divided into two subpopulations, the CD45RA-negative effector memory (TEM) and CD45RA-positive effector memory (TEMRA) T cells[14,15] (Figure 1). The EM cells exert effector functions at the sites of inflammation, whereas the naïve and CM cells express homing receptors that allow them to lodge in the secondary lymphoid organs.[14–16] A limited number of studies have examined the distribution and function of T lymphocytes in the ESRD patients.[17–19] However, little information is available on the effect of ESRD on the naïve and memory T-lymphocyte subsets. Therefore, we evaluated T-lymphocyte subsets pre- and post-hemodialysis in a group of ESRD patients and compared the results with those obtained in a group of age-matched control individuals.

RESULTS

General data

Data are summarized in Tables 1 and 2. The underlying causes of ESRD were diabetic nephropathy in 12, hypertension

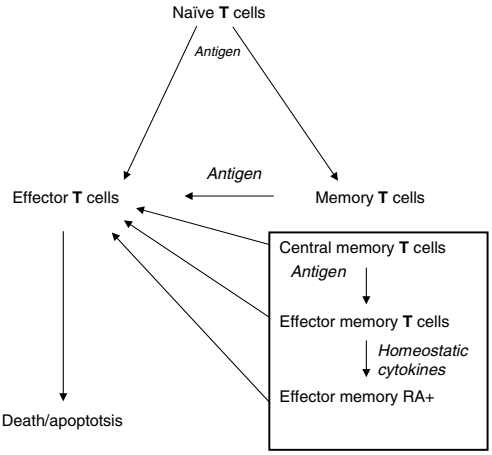

Figure 1 | A linear model of central and effector T-cell memory generation in primary response to infection.

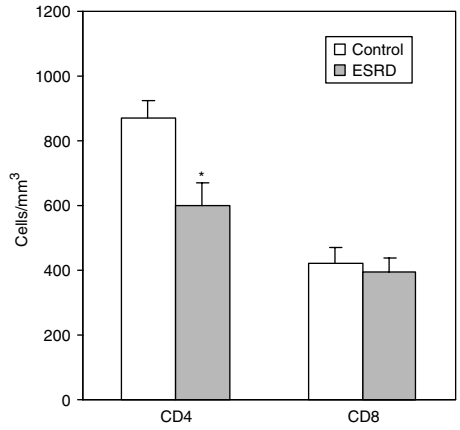

Figure 2 | Distribution of CD4+ and CD8+ T cells in control and ESRD groups. *$P<0.05$.

Table 1 | Clinical and biochemical parameters in normal control and ESRD groups

	Control (n=21)	ESRD (n=21)	P-value
Age (years)	46±1.8	49±3.4	NS
BUN (mg/dl)	13.33±1.02	69.28±4.66	<0.001
Creatinine (mg/dl)	0.85±0.18	10.45±0.63	<0.001
Calcium (mg/dl)	9.14±0.24	9.10±0.20	NS
Phosphorus (mg/dl)	3.20±0.16	5.43±0.30	<0.001
iPTH (pg/ml)	40.00±3.84	293.95±57.45	<0.001
Hemoglobin (g/dl)	14.03±0.38	11.72±0.23	<0.001
Ferritin (ng/ml)	32.63±6.26	209.90±23.79	<0.001
Transferin saturation (%)	18.66±2.12	26.80±2.15	0.012
Albumin (g/dl)	3.8±0.14	3.97±0.43	NS
Cholesterol (mg/dl)	159.50±5.47	139.09±7.09	0.031
Triglyceride (mg/dl)	100.16±15.51	146.71±16.80	0.052
MDA (μmol/l)	1.72±0.08	2.01±0.06	<0.001
K_t/V	—	1.53±0.16	—

BUN, blood urea nitrogen; ESRD, end-stage renal disease; iPTH, immunoreactive parathyroid hormone; MDA, malondialdehyde; NS, nonsignificant.

Table 2 | Leukocyte and differential counts in normal control and ESRD groups

Test	Controls	Pre-dialysis	Post-dialysis
Leukocytes (cells/mm^3)	5852±187	6803±499[a]	6014±454[b]
Neutrophils (cells/mm^3)	3433±169	4490±435[a]	4052±389[b]
Monocyte (cells/mm^3)	415±122	523±192[a]	347±147[b]
Lymphocyte (cells/mm^3)	1795±108	1557±118	1367±118[a,b]
CD4+/CD8+ (ratio)	2.63±0.34	1.81±0.21[a]	2.42±0.27[b]

ESRD, end-stage renal disease.
[a]$P<0.05$ compared to controls.
[b]$P<0.05$ compared to pre-dialysis.

in three, chronic glomerulonephritis in five patients, and polycystic kidney disease in one. The types of vascular access included A-V fistulas in 14 and A-V grafts in seven patients. As expected, serum creatinine and blood urea nitrogen concentrations were significantly higher in the ESRD patients compared to the control group. Likewise, serum concentrations of phosphorus, triglylcerides, and lipid peroxidation product, malondialdehyde (MDA), were significantly elevated in the ESRD group. Blood hemoglobin concentration was significantly lower, whereas serum ferritin level and transferin saturation were higher in ESRD patients than the corresponding values found in the control group. However, serum cholesterol, albumin, and calcium concentrations in the ESRD patients were not significantly different from those observed in the control group. The mean K_t/V value in the ESRD patients was greater than 1.5, reflecting adequacy of dialysis regimen in the study participants. In the pre-dialysis samples, total white blood cell counts and the number of granulocytes and monocytes were significantly higher in the ESRD patients as compared to the corresponding values found in the control group. However, the total lymphocyte count and CD8+ T-cell count in the pre-dialysis blood samples were not significantly different from the corresponding values in the control group. As expected, the number of CD4+ cells and the ratio of CD4+ to CD8+ cells were significantly reduced in the ESRD group.

T-lymphocyte subset data

Data are shown in Figures 2–8. The ESRD patients exhibited a marked reduction of the naïve subsets of CD4+ and CD8+ T cells as compared to the corresponding values in the normal control group (Figure 2). Likewise, the CM subsets of both CD4+ and CD8+ T cells were reduced in the ESRD group (Figures 3 and 4). However, EM subsets (TEM and TEMRA) of the CD4+ and CD8+ T cells in the ESRD group were not significantly different from those observed in the control group (Figures 3 and 4).

Comparison of data obtained in the diabetic ($n=12$) and non-diabetic ($n=9$) ESRD patients revealed higher numbers of CD8+ T cells and EM T-cell subsets (TEM and TEMRA) in the diabetic ESRD patients (Figures 5 and 6). However, the number of naïve and CM cells did not significantly differ among the diabetic and non-diabetic subgroups. Comparison of data obtained before and after a 3-h hemodialysis treatment revealed significant reductions in total white blood cell count, monocytes, lymphocyte, and CD8+ T cells after

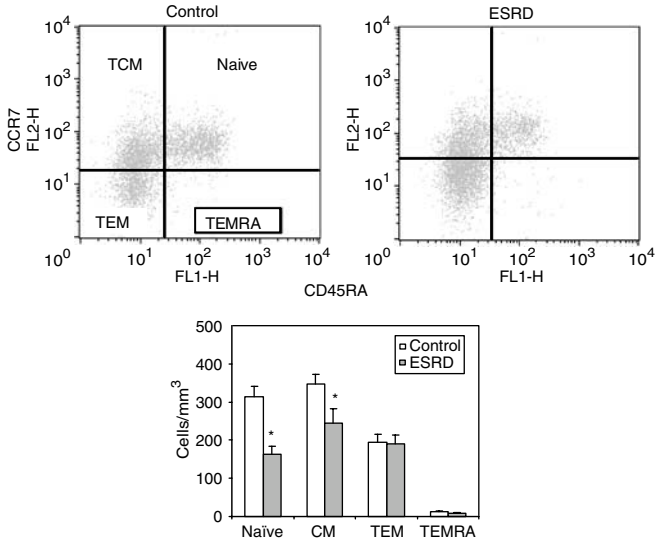

Figure 3 | Distribution of naïve, CM, TEM, and TEMRA subpopulations of CD4+ T lymphocytes in the control and ESRD groups. *$P<0.05$.

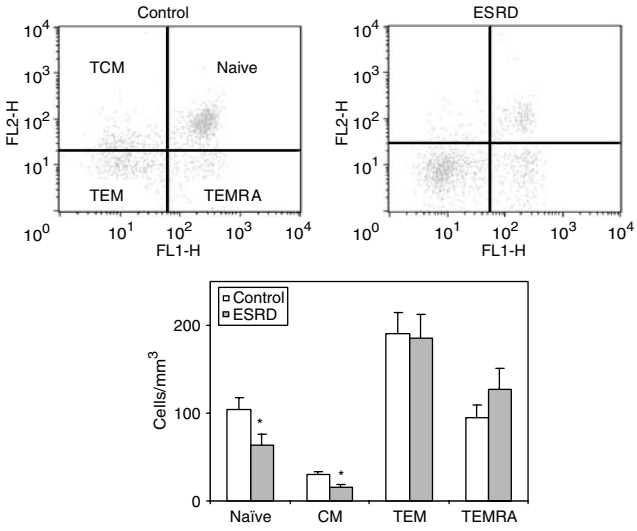

Figure 4 | Distribution of naïve, CM, TEM, and TEMRA subpopulations of CD8+ T lymphocytes in the control and ESRD groups. *$P<0.05$.

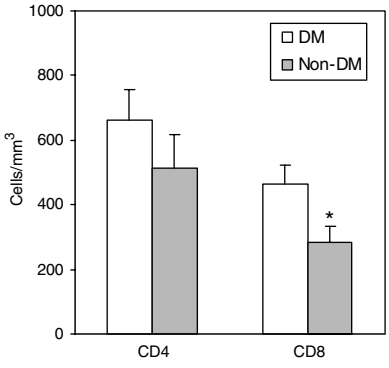

Figure 5 | Distribution of CD4+ and CD8+ T cells in diabetic (DM, $n=12$) and non-diabetic ($n=9$) ESRD patients. *$P<0.05$.

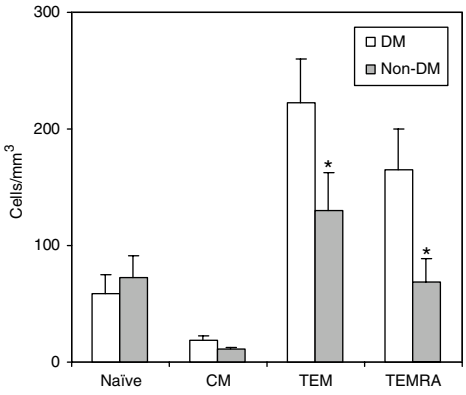

Figure 6 | Distribution of naïve, CM, TEM, and TEMRA subpopulations of CD8+ T lymphocytes in diabetic (DM) and non-diabetic ESRD patients. *$P<0.05$.

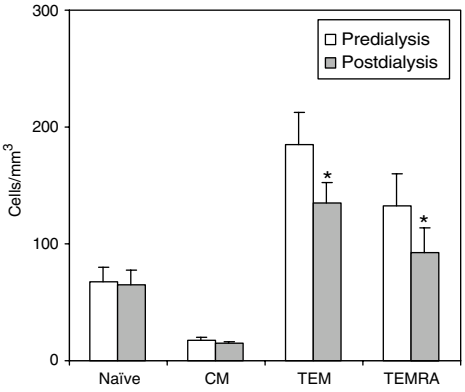

Figure 7 | Effect of hemodialysis on naïve, CM, TEM, and TEMRA subpopulations of CD8+ T lymphocytes. *$P<0.05$ vs pre-dialysis.

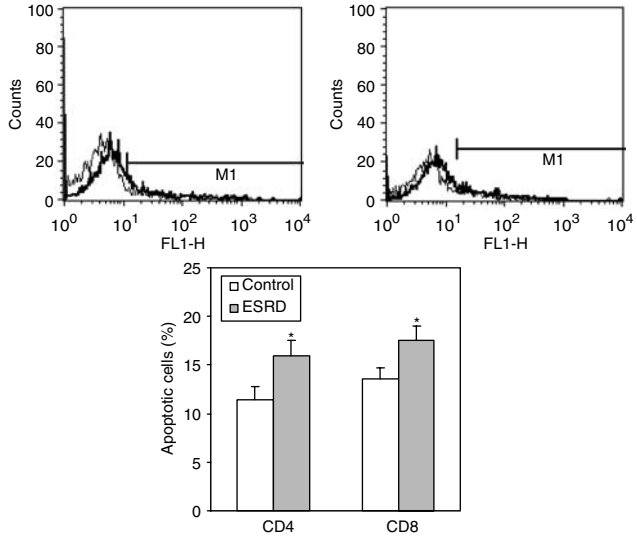

Figure 8 | Homing receptor (CCR7)-positive T-cell apoptosis, assessed by Annexin V staining in ESRD and control groups. *$P<0.05$.

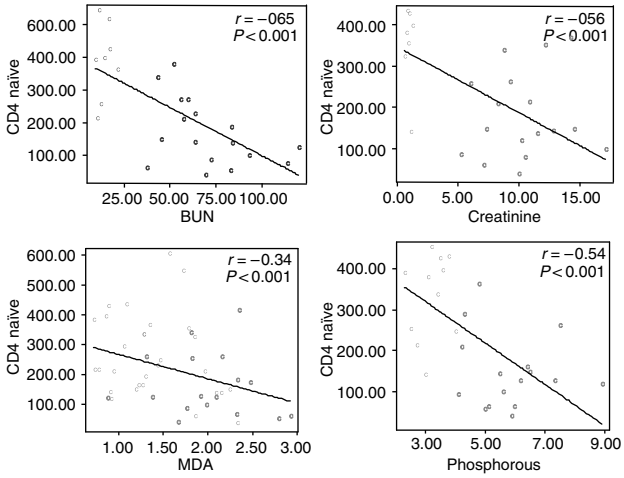

Figure 9 | Correlations between serum concentrations of urea nitrogen, creatinine, MDA, and phosphorous with the number of CD4+ naïve T lymphocytes in ESRD and control groups.

dialysis. Hemodialysis did not change the distribution of subsets of CD4+ cells but slightly lowered the numbers of EM subsets (TEM and TEMRA) of the CD8+ cells (Figure 7).

Apoptosis data

Compared to the controls, the ESRD patients exhibited a significant increase in apoptosis of both CCR7+ CD4+ and CCR7+ CD8+ T cells (Figure 8). CCR7+ cells include naïve and CM T cells.

Correlations

A significant inverse correlation was found between severity of azotemia (blood urea nitrogen and creatinine concentrations) and the number of naïve and CM subsets of T lymphocytes ($r = -065$, $P < 0.001$ and $r = -0.54$, $P < 0.001$, respectively). Similarly, significant negative correlations were noted between number of naïve CD4+ T lymphocytes and plasma MDA (a marker of oxidative stress) ($r = -0.34$, $P < 0.01$), serum phosphorus levels ($r = -0.54$, $P < 0.001$), and neutrophil counts ($r = -0.305$, $P = 0.017$). Similar negative correlations were noted between the above parameters and the numbers of CD4+ CM cells ($r = -0.307$, $P = 0.016$) (Figure 9). In contrast, neutrophil and leukocyte counts were directly correlated with the TEMRA+ CD8+ cell counts ($r = 0.516$, $P = 0.000$ and $r = 0.589$, $P = 0.000$, respectively) .

DISCUSSION

The results presented here demonstrate that the numbers of naïve and CM CD4+ and CD8+T cells are significantly reduced in the peripheral blood of ESRD patients as compared to those found in the normal control group. The observed reduction in the number and proportion of naïve and CM subsets was not associated with a significant change in the proportion of the EM T-cell subsets.

In the initial stages of adaptive immune response, exposure to antigen leads to clonal expansion and differentiation of antigen-specific naïve T cells, resulting in generation of the memory T cells and effector T cells.[14] Effector T cells perform their effector function via secretion of cytokines and destruction of target cells. At the conclusion of an immune response, effector T-cell populations contract and only a small number of the given memory T cells are maintained.[14,15] According to the linear model of T-cell differentiation, naïve and CM T cells give rise to TEM and TEMRA cells.[14,15] Naïve and CM subsets of T cells are essential for generation of a robust immune response.[14,15] Therefore, the observed reduction of the naïve and CM T cells in ESRD patients may, in part, contribute to diminished response to vaccination and increased predisposition to infections in the ESRD population.

The underlying mechanism responsible for the selective reductions of the naïve and CM T cells in the peripheral blood of ESRD patients is not known. The reduction of the given T-cell subpopulations in peripheral blood may be due either to increased apoptosis or accelerated activation and differentiation of T cells into EM T-cell subsets.[5,13,20] The latter is plausible as dialysis-dependent patients are commonly subject to repetitive exposure to microbial products and other antigenic stimulations that may lead to accelerated turn over and exhaustion of the naïve and CM T cells. In addition, accelerated apoptosis found in the present study contributes to the observed reductions of naïve and CM T cells in the ESRD patients. It is of note that in a study of ESRD patients, Matsumoto *et al.*[12] found accelerated T-cell apoptosis via death receptor Fas (CD95) and its ligand FAS L (CD95L), which are members of the tumor necrosis factor receptor/ligand superfamily. Similarly, Meier *et al.*[5] found accelerated apoptosis of the activated T cells (CD69+ T cells) in as yet untreated and hemodialysis-treated uremic patients. Alvarez-Lara *et al.*[21] reported that in uremia, Th1 lymphocytes are more prone to apoptosis than Th2 cells. In addition, Moser *et al.*[13] demonstrated increased T-lymphocyte apoptotic cell death in ESRD patients. They further showed that CD45RO+ memory cells, which include CM cells, are especially susceptible to apoptotic cell death.

Taken together, the above observations suggest that the reduction in the numbers of naïve and CM T cells in uremic patients found here may be due to heightened susceptibility of these cells to apoptosis. It is not entirely clear as to whether chronic uremia, *per se*, or the maintenance hemodialysis procedure is responsible for the observed increased apoptosis and diminished numbers of naïve and CM T cells in this population. However, increased T-cell apoptosis found in as yet undialyzed ESRD patients points to the role of uremia, *per se*, in this process.[5] Potential factors that may contribute to increased apoptosis and altered distribution of lymphocyte subsets in ESRD patients include uremic toxins,[4,11] stress hormones,[11,22] and the treatment modalities among others.[1,18] In this context, the numbers of naïve subsets of T cells were negatively related to severity of azotemia in our

ESRD patients. This observation points to the contribution of uremic toxicity to the pathogenesis of naïve T-cell lymphopenia. In addition, the inverse correlation between MDA, a marker of oxidative stress, and the number of naïve T-cell subset suggests the possible role of reactive oxygen species in this process. This presumption is of particular relevance, as the naïve and CM T-cell subsets are exquisitely vulnerable to oxidative stress.[23] As oxidative stress is a constant feature of ESRD,[24] accumulation of uremic toxins and presence of oxidative stress may work in concert to lower the numbers of these important subsets of T lymphocytes. Phosphate retention and hyperphosphatemia, which are common consequences of renal failure, result in a wide array of disorders including secondary hyperparathyroidism and dysregulation of cytosolic Ca^{2+} concentration. In an earlier study, Alexiewicz *et al.*[6] showed that impaired phagocytosis by neutrophilic granulocytes in ESRD patients is associated with, and is in part due to, elevation of resting cytosolic Ca^{2+} concentration, an event that is driven by hyperparathyroidism. The present study revealed a negative correlation between severity of hyperphosphatemia (a main cause of ESRD-induced secondary hyperparathyroidism) and the number of naïve subsets of T lymphocytes. This observation suggests that hyperphosphatemia and secondary hyperparathyroidism may be involved in this process. Further studies are needed to explore this possibility.

The numbers of EM subsets of CD8+ cells declined significantly after dialysis compared to the baseline values. The reason for the observed reduction of CD8+ EM cell subset following hemodialysis is not known and requires further investigation. Comparison of diabetic and non-diabetic patients revealed significantly higher total CD8+, TEM, and TEMRA+ cell counts in the former group. The reason for the observed difference is not clear. However, this may be due to a greater immunological stimulation in response to advanced glycation end products, repetitive injections (i.e. insulin) among other factors.

In conclusion, ESRD patients exhibit a marked reduction of the naïve and CM T-cell subsets. This is associated with, and is at least in part due to, increased apoptosis of these cells. Given the central role of these subsets of T lymphocytes in orchestrating the immune response to the *de novo* exposure or re-exposure to pathogenic organisms, the defects identified here may, in part, contribute to increased susceptibility of ESRD population to various infections.

MATERIALS AND METHODS

Patients

The study protocol was approved by Human Subjects Institutional Review Board of the University of California Irvine and was completed with the assistance of the University of California General Clinical Research Center.

Twenty-one patients with ESRD maintained on hemodialysis for a minimum of 3 months were recruited for the study. Individuals with evidence of acute or chronic infection, acute intercurrent illnesses, and those receiving immunosuppressive drugs were excluded. Medical history, systolic and diastolic blood pressures (pre- and post-dialysis), body weight, interdialytic weight change, routine monthly laboratory data, and dialysis prescription including dialyzer type and medications were recorded. Pre- and post-dialysis blood samples were obtained.

A group of 21 normal age-matched control subjects were also studied. Control subjects exhibiting acute or chronic infection, acute intercurrent illnesses, and chronic illnesses such as hypertension, diabetes, malignancy, psychiatric disorders, or those requiring chronic medications were excluded. A brief medical history was obtained after informed consent was signed. Blood was obtained by venipuncture. All participants provided informed consent before enrollment in the study.

Blood collection

In all ESRD patients, whole blood was collected after canulation of the vascular access was completed but before the initiation of dialysis. Post-dialysis samples were obtained form the venous dialysis tubing. The blood samples were obtained using a needle and syringe, applying gentle aspiration to minimize shear stress. Blood samples form the control individuals were collected from a peripheral vein in the same manner. Total leukocyte and differential counts were obtained.

Mononuclear cell preparation

Peripheral blood mononuclear cells were isolated from heparinized blood by gradient centrifugation method using lymphocyte separation medium (Mediatech Inc., Herndon, VA, USA). A 6 ml volume of heparinized blood was added to 6 ml of calcium- and magnesium-free 1 × Hank's balanced salt solution (Irvine Scientific, Santa Ana, CA, USA). The mixture was slowly added to 12 ml of lymphocyte separation medium and centrifuged at 5G (22°C) for 30 min. Peripheral blood mononuclear cell layer was collected, washed twice with phosphate-buffered saline (PBS), and resuspended in 1 × Hank's balanced salt solution.

Immunostaining

T-lymphocyte subsets were analyzed by three-color flow cytometry using peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) (6 μg/ml PBS; Becton-Dickinson, San Jose, CA, USA), fluorescein isothiocyanate-conjugated anti-CD45RA mAb (50 μg/ml PBS; Becton-Dickinson, San Jose, CA, USA), and phycoerythrin-conjugated anti-CCR7 antibodies (25 μg/ml PBS; R & D System, Minneapolis, MN, USA). A 200 μl volume of whole blood drawn from the peripheral vein was incubated with 20 μl of each of the above antibodies for 20 min at room temperature in dark. The cells were then incubated in 2 ml of fluorescence-activated cell sorting lysing solution (Becton-Dickinson, San Jose, CA, USA) for 15 min to lyse red blood cells. The cells were then washed twice with PBS to remove red blood cell remnants and leukocytes were resuspended in 0.5 ml of 1% paraformaldehyde and used for flow cytometry.

Flow cytometry

Lymphocyte phenotyping was carried out by three-color analysis using a FACSort flow cytometer and CellQuest software (Becton-Dickinson). For each sample, data from 10 000 cells were collected and analyzed. Forward and side scatters were used to gate and exclude cellular debris and FL3 channels were used to gate CD4+ and CD8+ T cells. During analysis, an electronic gate was placed on CD4+ or CD8+ T cells. The associated expression of CD45RA vs

CCR7 was then used to obtain the percentages of cells uniquely identifying naïve (CD45RA+, CCR7+), TCM (CD45RA−, CCR7+), TEM (CD45RA−, CCR7−), and TEMRA (CD45RA+, CCR7−) subsets of CD 4+ or CD8+ T cells.

Detection of apoptosis

Apoptosis was measured by Annexin V-fluorescein isothiocyanate binding assay according to the manufacturer's instructions (CalTag, Burlingame, CA, USA). Briefly, peripheral blood mononuclear cells (0.5×10^6) were stained with 10 μl of PerCP-conjugated anti-CD4/anti-CD8 and phycoerythrin-conjugated anti-CCR7 mAb, washed with 2 ml of PBS, and resuspended in 100 μl of Annexin V-conjugate binding buffer) to which 5 μl of fluorescein isothiocyanate-conjugated Annexin V was added. The mixture was incubated in dark at room temperature for 15 min, after which 400 μl of binding buffer was added and 5000 cells were acquired and analyzed by fluorescence-activated cell sorting. FL3 channels were used to gate CD4+ and CD8+ T cells. An electronic gate was placed on CCR7 PE+ T cells (which includes naïve and CM cells) and population of cells positive for Annexin V-fluorescein isothiocyanate was analyzed using green fluorescence (FL1) on the x axis against cell numbers on the y axis.

Malondialdehyde assay

Plasma concentration of MDA was measured by high-performance liquid chromatography as described in our previous studies.[25]

Data analysis

Quadrant statistics and histogram statistics were used in dot plot of CellQuest and histogram plot software. Relative proportion and calculated absolute numbers of CD4+ and CD8+ T lymphocytes and their subsets of controls, pre-dialysis, and post-dialysis groups were analyzed with independent and paired sample t-tests in SPSS. Likewise, Annexin V data were analyzed using the independent t-test. Data were expressed as mean $\pm$ s.e. P-values less than 0.05 were considered significant.

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