Premature T Cell Senescence in Pediatric CKD

Roshan P. George,*† Anesh K. Mehta,† Sebastian D. Perez,† Pamela Winterberg,*† Jennifer Cheeseman,‡ Brandi Johnson,† Jean Kwun,‡ Stephanie Monday,‡ Linda Stempora,‡ Barry Warshaw,*† and Allan D. Kirk*†‡

*Division of Pediatric Nephrology, Department of Pediatrics, Children’s Healthcare of Atlanta, Atlanta, Georgia; †Division of Pediatric Nephrology, Department of Pediatrics, Emory Transplant Center, Emory University, Atlanta, Georgia; and ‡Division of Surgery, Duke University, Durham, North Carolina

ABSTRACT

An individual’s immune function, susceptibility to infection, and response to immunosuppressive therapy are influenced in part by his/her T cell maturation state. Although childhood is the most dynamic period of immune maturation, scant information regarding the variability of T cell maturation in children with renal disease is available. In this study, we compared the T cell phenotype in children with renal failure (n=80) with that in healthy children (n=20) using multiparameter flow cytometry to detect markers of T cell maturation, exhaustion, and senescence known to influence immune function. We correlated data with the degree of renal failure (dialysis or nondialysis), prior immunosuppression use, and markers of inflammation (C-reactive protein and inflammatory cytokines) to assess the influence of these factors on T cell phenotype. Children with renal disease had highly variable and often markedly skewed maturation phenotypes, including CD4/CD8 ratio reversal, increased terminal effector differentiation in CD8+ T cells, reduction in the proportion of naïve T cells, evidence of T cell exhaustion and senescence, and variable loss of T cell CD28 expression. These findings were most significant in patients who had experienced major immune insults, particularly prior immunosuppressive drug exposure. In conclusion, children with renal disease have exceptional heterogeneity in the T cell repertoire. Cognizance of this heterogeneity might inform risk stratification with regard to the balance between infectious risk and response to immunosuppressive therapy, such as that required for autoimmune disease and transplantation.


Children are born with a naïve T cell repertoire that is shaped throughout life by antigenic exposure. Indeed, no time is more dynamic with regard to new antigen encounter and immune stimulation than childhood. T cells respond to these antigenic threats by undergoing clonal maturation and proliferation, mediating and regulating a cascade of immunologic events. The net effect of the ebb and flow of each immune response determines one’s protective immunity at any given time. The size of the T cell compartment is highly regulated and is determined by thymopoiesis, proliferation of post-thymic T cells, antigen-induced expansion, maturation, exhaustion, senescence, and apoptosis. Importantly, cells in each of these phenotypic states have markedly different functional capabilities. As a result, the T cell repertoire assumes a unique, antigen-specific mosaic of cells in varying states of functional maturation.

Pediatric patients have ongoing thymic output of naïve T cells which fosters an enormously diverse antigen-recognition repertoire. However, with age, there is a gradual decline in the thymic output, a consequential reduction in naïve lymphocytes, and a resultant decrease in the diversity of the antigen-recognition repertoire. At the elderly extreme of age, an individual’s T cell repertoire is predominated by a markedly decreased population of...
naïve lymphocytes, an oligoclonally expanded population of antigen–experienced lymphocytes, reduced antigen receptor diversity, and enhanced proinflammatory cytokine activity. This constellation has been termed immune senescence.\(^6\) Although age is generally considered a surrogate for immune maturation, unique circumstances such as chronic inflammation could lead to premature immune dysfunction, regardless of one’s chronologic age. Nonphysiologic stimulation of T cells occurs in chronic inflammatory states like rheumatoid arthritis,\(^7,8\) and latent infections such as cytomegalovirus.\(^9\)

CKD or ESRD, especially maintenance dialysis, are also known to create a state of chronic inflammation, even in children, secondary to multiple causes, such as uremia, exposure to artificial dialysis membranes, and infectious complications.\(^10,11\) In the presence of such sustained stimulation, T cell replicative capacity is impaired, resulting in functional exhaustion and replicative senescence.\(^12\) Such T cell dysfunction is known to impair response to infections, complicate post-transplant immunosuppressive therapies, and heighten susceptibility to viral reactivation.\(^13,14\) ESRD patients display inadequate response to vaccinations and increased incidence of malignancies.\(^15,16\) Patients with CKD have increased levels of inflammatory serum biomarkers like C-reactive protein.\(^17–19\) An elevated serum C-reactive protein is also one of the most commonly used markers to stratify cardiovascular risk.\(^20,21\) Recent studies suggest that there is an association between cardiovascular disease (atherosclerosis, acute coronary events, hypertension) and T cell exhaustion or senescence.\(^22–26\) We hypothesized that children on dialysis, through persistent inflammation, the effects of the index renal disease, and environmental antigenic exposure, are forced into a premature state of immune senescence. This accelerated immune aging will have an effect on their future medical care, especially on decisions surrounding immune therapy and kidney transplantation in children.\(^27\)

The maturational state of the immune system is becoming increasingly quantifiable, offering the potential to objectively test our hypothesis. In a healthy population, CD4/CD8 ratio is normally >1.0.\(^28,29\) The CD4\(^+\) and CD8\(^+\) T cells each have four subtypes – naïve cells (not yet programmed, but poised to respond to novel antigens and initiate immune responses), central memory cells (programmed previously and on re-encountering antigen, capable of rapid proliferation, expansion, and differentiation to effector memory cells), and CD45RA\(^+\) and CD45RA\(^-\) effector memory cells (programmed for specific effector functions).\(^30–32\) The proportion of naïve T cells decreases with advancing age.\(^33\) The progression from naïveté to effector status is typically assessed by studying the acquisition or loss of surface markers such as CCR7 and CD45 isoforms.\(^34\) Similarly, two major phenotypes have been used to identify age-related immune deterioration – immune senescence and immune exhaustion. Senescence is characterized by the loss of proliferative capacity, resistance to apoptotic cell death, and production of inflammatory cytokines.\(^35\) T cell senescence during ex vivo proliferation is accompanied by the loss of CD28 or CCR7 expression and reciprocal expression of CD57.\(^36,37\)

T cell exhaustion is defined as the loss of essential functional activity, particularly affecting many antiviral properties of CD8\(^+\) T cells, and is often accompanied by the expression of programmed death 1 (PD1) on the cell surface.\(^38\) Finally, highly differentiated T cells have short telomeres and lose telomerase activity, indicating replicative senescence.\(^39,40\)

At present, there is scant data to aid the clinician in objectively assessing the overall immune competence of a child with CKD, and in turn to assist in making rational decisions regarding risk of immune manipulation before and after transplantation, or for treating autoimmune disease. Herein, we have studied children with CKD with regard to their T cell maturation, exhaustion, and senescence. We find that premature immune maturation is common, and at times advanced.

## RESULTS

We recruited a total of 100 subjects. These patients were divided into two groups: (1) pediatric patients with CKD (ages ranging from 1 to 21 years), \(n=80\) and (2) healthy controls (children, 1–17 years old), \(n=20\). Patients with CKD could be further categorized into patients on dialysis (\(n=45\); hemodialysis, \(n=23\); peritoneal dialysis, \(n=22\)) and patients with CKD who were not receiving dialysis (\(n=35\)). Among the CKD patients, 19 had undergone prior renal or nonrenal solid organ transplantation, now presenting with renal insufficiency. Out of these 19 patients, ten patients (eight with history of failed renal transplant and two with history of cardiac transplant) were on dialysis (two on hemodialysis, eight on peritoneal dialysis) and nine patients (seven with history of cardiac transplant and two with failing kidney transplant, being evaluated for a second kidney transplant) were in the CKD, not on dialysis group. Out of the ten patient with a history of prior transplant, in the dialysis group, only two (approximately 4% of the total dialysis patients) also had a history of primary immune mediated disease before transplant. Out of 35 patients with CKD, not on dialysis, nine had a history of prior transplant, but out of these nine patients, none had a history of primary immune mediated disease. Patient characteristics are presented in Table 1.

Multiparameter flow cytometry was used to assess markers of T cell maturation, exhaustion, and senescence (Figure 1). The following four characteristics were analyzed to assess immune senescence: (1) inversion of CD4/CD8 ratio, (2) subpopulation shifts in T cells (naïve [CD45RA\(^+\), CCR7\(^+\)], central memory [CD45RA\(^-\), CCR7\(^+\)], effector memory [CD45RA\(^-\), CCR7\(^-\)], and effector memory RA [CD45RA\(^-\), CCR7\(^-\)] frequencies), (3) increase in senescent (CD57\(^+\)) and exhausted (PD1\(^+\)) populations, and (4) downregulation of CD28, a critical costimulatory molecule on the surface of T cells known to be lost in terminal phases of T cell differentiation. These markers provide a general view of immune maturation and are a surrogate for prior immune experience.
Children with CKD Have T Cell Frequencies Skewed toward Advanced Differentiation

Analysis of the T cell populations showed significant skewing toward advanced differentiation phenotypes in both the CD8 and CD4 subsets in children with CKD (Table 2). CD4+ T cell subset comparison between healthy control and CKD groups showed an increase in central memory (\(P=0.01\)) and effector memory (\(P=0.02\)) T cell populations in the CKD cohort. In the CD8+ T cell subset, patients with CKD also showed an increase in central memory cells (ANOVA \(P=0.05\)) compared with healthy controls.

On analyzing T cell maturation subsets in patients by their dialysis status, there was a significant difference in the CD4+ T cell compartment (\(P=0.04\)) which skewed toward maturation. However, a similar variation by dialysis was not evident within the CD8+ compartment.

CKD Increases Expression of Senescence and Exhaustion Markers on CD8+ T Cells

Children with CKD receiving dialysis had significantly more CD57+ CD8+ T cells (\(P=0.004\)). CD28- CD8+ T cells in CKD patients was also elevated (\(P=0.01\)) compared with healthy controls (Figure 2).

PD1+ CD8+ T cells (\(P=0.03\)), were higher in CKD when compared with controls. Telomere shortening (Figure 3) showed a weak r-square value of 0.0478 but was significantly associated with CD28- CD8+ T cells (\(P=0.04\)).

We were unable to find a correlation between the markers of maturation, senescence, or exhaustion with the duration of dialysis.

Prior Immunosuppression Exposure Contributes to a More Significant Immune Senescence Phenotype in Children with CKD

Although 22% (18 out of 80) with CKD had CD4/CD8 reversal compared with 10% (two out of 20) of healthy controls, this did not reach statistical significance (Fisher test \(P=0.35\)). CD4/CD8 reversal was significantly associated with prior
exposure to immunosuppression. Data were incomplete on one patient, who was excluded from analysis. Forty-one percent of patients with prior immunosuppression and only 7% of patients without prior immunosuppression had CD4/CD8 reversal (P<0.001). There was no clear statistical difference segregating patients’ CD4/CD8 ratios by dialysis.

Patients who received prior immunosuppression showed a significant difference within the T cell subset groups in both the CD8 and CD4 compartments (Table 3). To understand if the effect of prior immunosuppression was being confounded by the effect of dialysis, a two-way multivariate analysis of variance (MANOVA) was conducted. Prior immunosuppression influenced both CD4 and CD8, whereas dialysis still had an influence on CD4. As such, both prior immunosuppression and renal failure contributed to the effects seen.

In patients who received prior immunosuppression, there was a higher percentage of CD57+ CD8+ T cells (P<0.001) and of PD1+ CD8+ T cells (P<0.001) when compared with patients who had not received immunosuppressive therapy (Table 4). An increase in CD28− CD8+ T cells was also present in both the CD8+ and CD4+ T cell compartments in the prior immunosuppressive therapy group.

The Role of Inflammation
Analysis of inflammatory cytokines in serum samples showed no significant difference in the inflammatory cytokines between the groups, albeit there were trends toward lower IL-5 and increased Interferon γ-induced protein 10 (IP-10) in those with CKD, compared with normal controls (Table 5). Patients with CKD on dialysis did have a higher percentage of elevated (>3 mg/L) high sensitivity C-reactive protein (hsCRP) compared with CKD patients not receiving dialysis (Table 6).

DISCUSSION
Although activation and recrudescence of T cell populations is an essential part of physiologic immunity, chronic immune activation, such as that seen in persistent inflammatory states, can be counterproductive, leading to premature immune aging. As such, children enduring the effects of renal disease or the treatments associated with renal failure could have impaired clinical immune competence more characteristic of older adults. Infection and cardiovascular disease remain the leading causes of mortality in ESRD with sepsis accounting for the majority of deaths in this population. An acquired cellular immunodeficiency has been described in patients receiving dialysis therapy and has been attributed to the bio-materials used in dialysis or to uremia itself. In our study, we find that children with CKD have generalized features of advanced immune exhaustion and senescence, and that both renal failure and prior immunosuppression accentuates this phenotype. A combination of findings, namely, reversal of CD4/CD8 ratio, decrease in naïve T cells, increase in CD57+ and CD28− T cells, and elevated hsCRP, comprise a cluster of immune measures pointing to an ‘immune risk phenotype’ that could potentially lead to increased risk for infections, malignancies, bone disease, and cardiovascular comorbidities.

The ratio of CD4/CD8 T cells has been used as an indicator to evaluate an individual’s immune function. In general, the CD4/CD8 ratio in healthy people is >1 and an inverted CD4/CD8 ratio is associated with increased morbidity and mortality. In our study, this ratio appears to be altered in children with CKD, especially in those with prior exposure to immunosuppression.

As T cells mature, there is a clonal increase in central and effector memory T cells. In children this is generally balanced by a diverse naïve T cell compartment supplied by robust thymopoiesis. Reduction in diversity impairs functional efficiency in responding to infectious and oncogetic antigens. In our CKD patients, there was a reduction in the naïve cell compartment indicating a contraction of the T cell repertoire. We did not measure thymic output directly, but these data support impaired thymopoiesis relative to the demands of chronic inflammation related to CKD or associated conditions.

As T cells differentiate, they increasingly express CD57, which is often used as a marker for senescence along with loss of CD28. The costimulatory molecule CD28 plays a critical role in...
several T cell functions, including IL-2 gene transcription, lipid raft formation, cytokine mRNA stabilization, and cell adhesion. T cells lacking CD28 will hence show profound functional alterations and deficiencies. Both of these markers (CD57+ and CD28−) were significantly elevated in our CKD patients when compared with normal controls, suggesting premature senescence in the pretransplant population.

Children on dialysis showed an increase in hsCRP (a value of >3.0 mg/L was considered as ‘high’ hsCRP), which is a non-specific marker of inflammation and can be used to stratify risk for cardiovascular disease. Repeated T cell stimulation from chronic inflammation may give rise to effector memory T cells. Activated T cells also secrete cytokines and further studies would need to analyze if these senescent T cells have enhanced cytokine production. There was no statistically significant increase in systemic levels of proinflammatory cytokines in our CKD group; however, IP-10, which is a proinflammatory chemokine and plays a role in renal endothelial microvascular injury, showed a trend toward significance.

Aging CD8+ T cells undergo telomere shortening, which may limit their replicative capacity. Telomeres are found at the ends of chromosomes and are repeating hexameric units of nucleotide sequences that stabilize the structure of the chromosomes. With each cell division, there is a loss of 50–100 base pairs of telomeric DNA. Critically short telomeres trigger cell cycle arrest and replicative senescence. In our study, there was a weak but significant association of telomere length with CD28−CD8+ T cells but there was no correlation to other markers of maturation or senescence. Moreover, there was no difference in telomere lengths when segregating by dialysis status. It is likely that in pediatric patients, despite chronic inflammation and excessive proliferation, there may be a more efficient upregulation of the enzyme telomerase, which adds telomeric end repeats. Signaling through CD28 has been, however, implied in telomere activation.

No dominant pattern was seen in these children at the extreme ends of immune maturation, except for the observation that the use of prior immunosuppression, either from prior transplant or treatment of an immune-based index disease, markedly accentuates the phenotypic changes seen in the pediatric CKD population.

Immune management is of critical importance in organ transplantation, especially in pediatric patients. Currently, all patients, post transplantation, are placed on similar immunosuppression protocols with varying outcomes in terms of infections, medication related toxicities, and malignancies. Prior studies show long-term phenotypic changes, especially
increased counts of CD8+ T cells coexpressing CD57 in pediatric renal transplant patients after T cell depletion.\textsuperscript{53} An association between the expansion of the CD8+CD57+ cells and long-term renal allograft function has been observed and a potential regulatory or suppressor function of this subset has been considered.\textsuperscript{54,55} Donor-specific CD8+ CD28- T cells are detectable in patients with stable function of renal, heart, and liver allograft, and are absent in patients undergoing acute rejection.\textsuperscript{56} Furthermore, as described above, senescent T cells have CD28- phenotype and this may render these cells resistant to some of the upcoming therapies on the basis of costimulation blockade while remaining sensitive to traditional regimens utilizing calcineurin inhibitors.\textsuperscript{57} This may also significantly affect post-transplant therapeutic decisions in these patients. These data show that children with CKD, specifically those presenting for transplantation and preparing for considerable immunosuppressive manipulation, cannot be considered as a homogeneous population. Although immune maturation has typically been shown to predictably increase with age, in the case of ill children and in particular those with CKD, accelerated maturation and even immune exhaustion or senescence can be seen very early in life. Given the known correlates between T cell phenotype and immune outcome, cognizance of this potential immunologic skewing may be useful when choosing immunebased therapies for transplant recipients. We do not anticipate that these changes are unique to CKD, but rather suspect that they are general indicators of immune perturbation. As such, we anticipate that similar assessment of other chronic diseases, particularly those with inflammatory or immune etiologies, will show that the immune repertoire of children can be markedly and prematurely altered. Similarly, we have not attempted to draw cause and effect relationships between any single agent and a particular immune profile, but only to draw enhanced attention to the breadth and depth of immune repertoire disturbances seen in children with CKD.

### Table 3. Effects of prior immunosuppression on T cell maturation in the CD4 and CD8 compartments in patients

<table>
<thead>
<tr>
<th>T Cell Subsets</th>
<th>No Prior IS (n=40)</th>
<th>Prior IS (n=39)</th>
<th>Univariate ANOVA P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T Cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive CD4, %</td>
<td>50.8 (22.1)</td>
<td>34.6 (20.5)</td>
<td>ref</td>
</tr>
<tr>
<td>CM CD4, %</td>
<td>34.9 (21.5)</td>
<td>41.7 (20.4)</td>
<td>0.07</td>
</tr>
<tr>
<td>EM CD4, %</td>
<td>11.0 (9.8)</td>
<td>19.2 (17.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Temra CD4, %</td>
<td>3.4 (2.9)</td>
<td>4.9 (8.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>MANOVA <strong>Pillai</strong> test P value=0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8+ T Cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naive CD8, %</td>
<td>55.2 (23.9)</td>
<td>45.5 (23.9)</td>
<td>ref</td>
</tr>
<tr>
<td>CM CD8, %</td>
<td>19.2 (21.5)</td>
<td>17.7 (15.0)</td>
<td>0.75</td>
</tr>
<tr>
<td>EM CD8, %</td>
<td>14.6 (13.9)</td>
<td>15.5 (10.7)</td>
<td>0.37</td>
</tr>
<tr>
<td>Temra CD8, %</td>
<td>12.5 (12.5)</td>
<td>21.1 (19.2)</td>
<td>0.004</td>
</tr>
<tr>
<td>MANOVA <strong>Pillai</strong> test P value=0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as mean (±SD). Data on one patient missing. The percentage of naïve T cells were reduced in both CD4+ and CD8+ T cell populations in patients who received immunosuppression. There was a significant increase in EM and Temra T cells in the CD4+ T cell compartment and in the Temra cells in the CD8+ T cell compartment in the patients who received immunosuppression as compared with those who did not IS, immunosuppression; CM, central memory T cells; EM, effector memory T cells; Temra, effector memory RA T cells.

**Concise Methods**

### Study Population

Children with CKD being managed in Emory Children’s Center and Children’s Healthcare of Atlanta were recruited in our cross-sectional study under an Institutional Review Board approved tissue acquisition protocol (Immune Monitoring Protocol, Institutional Review Board #6248; Clinicaltrials.gov Identifier: NCT01283295). Consent was obtained from parents or legal guardians and assent from patients older than 6 years (verbal assent from children older than 6 years and a written assent from patients older than 11 years). Patients with active oncologic disease, systemic infection, or those receiving ongoing therapy for autoimmune disease or transplant rejection, were excluded. Children younger than 1 year of age were also excluded. Patients 1–21 years old were included. Patients who either had a failed renal allograft, or patients with cardiac transplant who were being evaluated for renal replacement therapy or requiring dialysis, were included as we felt that this is a significant subpopulation of patients presenting to the pediatric nephrologist. Out of the 19 patients with a history of solid organ transplant, nine were on small doses of standard maintenance immunosuppressive medications (such as prednisone, azathioprine, or mycophenolate mofetil). Patients with immune-mediated diseases such as lupus nephritis or other GN had completed their therapy and were in a period of disease quiescence at the time of enrollment. We used IL-2 inhibitor basiliximab for induction and a steroid-based immunosuppressive regimen as part of our transplant protocol.

### Table 4. Effects of prior immunosuppression on T cell senescence and exhaustion

<table>
<thead>
<tr>
<th>T Cell</th>
<th>No Prior IS (n=40)</th>
<th>Prior IS (n=39)</th>
<th>t Test</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD57+,CD8, %</td>
<td>19.0 (15.4)</td>
<td>34.9 (23.8)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>PD1+,CD8, %</td>
<td>17.4 (8.3)</td>
<td>29.5 (15.9)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>CD28-,CD8, %</td>
<td>22.2 (16.8)</td>
<td>34.2 (24.1)</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>CD28+,CD4, %</td>
<td>4.5 (5.5)</td>
<td>7.6 (9.3)</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as mean (±SD). Data on one patient missing. Significant increase in CD57+CD8+ and PD1+CD8+ T cells was noted. CD28- T cells were also increased in both CD8 and CD4 T cell compartments. IS, immunosuppression.
Table 5. Comparing cytokine levels in patients with CKD and healthy controls

<table>
<thead>
<tr>
<th>Cytokines/Chemokines</th>
<th>Healthy Controls (n=20)</th>
<th>CKD (n=80)</th>
<th>t Test P Value (log values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5</td>
<td>99.1 (29.8)</td>
<td>83.3 (52.6)</td>
<td>0.06</td>
</tr>
<tr>
<td>IL-6</td>
<td>76.8 (18.1)</td>
<td>116.4 (196.5)</td>
<td>0.20</td>
</tr>
<tr>
<td>IL-9</td>
<td>125.8 (73.8)</td>
<td>117.6 (54.8)</td>
<td>0.73</td>
</tr>
<tr>
<td>IL-10</td>
<td>82.4 (13.1)</td>
<td>98.7 (78.7)</td>
<td>0.55</td>
</tr>
<tr>
<td>TNFα</td>
<td>56.1 (10.5)</td>
<td>72.3 (124.2)</td>
<td>0.73</td>
</tr>
<tr>
<td>IP-10</td>
<td>597.5 (361.8)</td>
<td>1116.7 (1147.5)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Data is expressed as mean (±SD). No statistically significant difference was seen in the cytokines when comparing healthy controls to patients with CKD. TNFα, tumor necrosis factor alpha.

Demographic data such as age, gender, race/ethnicity, and primary diagnosis were collected from the medical records. A single collection of whole blood, PBMC, serum, and plasma aliquots from each patient were stored in our biorepository.

Laboratory Measurements

Flow Cytometry

Whole blood was collected in Cyto-Chex BCT tubes (Streck, Omaha, NE). Samples were analyzed by polychromatic flow cytometry for memory status (CD45RA, CCR7), differentiation (CD27, CD28), exhaustion (PD-1), and senescence (CD57), as previously described. Briefly, we used a broad lymphocyte gate to separate the lymphocyte population by forward light scatter and side scatter properties, a singlet cell isolation gate, and dump gates to separate the lymphocyte population by forward light scatter and side scatter properties, a singlet cell isolation gate, and dump gates to separate CD3 T cells that were then differentiated into CD8 and CD4 subpopulations, and thereafter by maturation parameters (Figure 1). Analysis of flow cytometry data was performed with FlowJo (TreeStar, Inc., Ashland, OR).

Telomere Assay

Genomic DNA was isolated using the QIAcube (Qiagen, Germantown, MD) from PBMC and quantified with a spectrophotometer. Real-time PCR–based quantification of telomere length was performed in triplicate using the SYBR green method (Invitrogen, Carlsbad, CA) and 30 ng of DNA. The ratio of telomere signal/single copy gene signal is proportional to telomere length and was analyzed to derive the telomere length.59

Assays of Systemic Inflammation

A multiplex luminex assay was used to capture major inflammatory cytokines in patient serum samples (Bio-Plex Pro Human Cytokine 27-plex Assay; Bio-Rad, Hercules, CA), following the manufacturer’s instructions. For this study we focused on quantitative measurements of inflammatory cytokines IL1α, IL1β, IL2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, basic fibroblast growth factor, IP-10, monocyte chemottractant protein IFNγ, macrophage inflammatory protein 1α (MIP1α), MIP1β, TNFα, and vascular endothelial growth factor. HsCRP and ferritin levels were also analyzed as a marker of systemic inflammation. HsCRP was measured at Associated Regional and University Pathologists, Inc. laboratories and a reference range of <3.0 mg/L was assigned as low risk.

Statistical Analyses

All statistical analyses were performed using project 3.1.1 (http://www.R-project.org/). Statistical significance was assigned for a significance level of 0.05 or lower. The primary analysis, testing whether T cell maturation, exhaustion, and/or senescence differ between groups, was tested with MANOVA. This was used initially to determine if there were any differences between the groups. This multivariate test is able to determine whether there are differences between groups in multiple dependent variables at the same time, accounting for correlations between these and thereby being more efficient than multiple univariate tests and avoiding multiple testing problems. The ‘Pillai’ test statistic was used to calculate P values because it tends to be more robust to deviations from test assumptions.50–62 In cases where the MANOVA tests showed a difference between the groups (significant at the 0.10 α level), univariate one-way ANOVA was used to determine if individual cell types differed between groups. Because the naïve, central memory, effector memory, and Temra T cells were all recorded as proportions of CD4 or CD8 cell populations (i.e., the four measures are dependent because their sum totals to 100%), these measures were transformed for analysis with an additive log-ratio transformation for compositional data, taking the naïve cell percentages as the reference group. This transformation measures the levels of each cell type as a ratio of the naïve cell percentages.63 Other differences between groups were tested with ANOVA tests or two-sample t tests after log transformations (data were non-normally distributed). Finally, a simple linear regression model was used to test the correlation between telomere and CD28 CD8+ T cells.

Table 6. Comparing hsCRP in two groups of patients with renal failure

<table>
<thead>
<tr>
<th>hsCRP Group</th>
<th>Dialysis (n=45)</th>
<th>CKD, No Dialysis (n=35)</th>
<th>Chi-Squared P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low, n (%)</td>
<td>20 (45.5)</td>
<td>25 (75.8)</td>
<td>0.01</td>
</tr>
<tr>
<td>High (&gt;3.0 mg/L, n (%))</td>
<td>24 (54.5)</td>
<td>8 (24.2)</td>
<td></td>
</tr>
</tbody>
</table>

Patients on dialysis had elevated levels of hsCRP. Data on hsCRP were available in 44 out of 45 dialysis patients and similarly in 33 out of 35 patients with CKD, no dialysis.

ACKNOWLEDGMENTS

This work was supported by the Children’s Healthcare of Atlanta ‘Friends’ Grant and National Institutes of Health grant AI097423 (to A.D.K.). The funders had no role in the study design, data

www.jasn.org
collection, analysis, decision to publish, or manuscript preparation. Early parts of this work were previously presented at the American Society of Nephrology Kidney Week 2013 Annual Meeting in Atlanta (November of 2013) and at the American Transplant Congress 2013 (May of 2013), Seattle, WA and published in abstract form.

DISCLOSURES

None.

REFERENCES


