Invariant natural killer T cells are depleted in renal impairment and recover after kidney transplantation

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ABSTRACT

Background. Altered immune function in patients with renal failure results in both susceptibility to infection and increased inflammatory response. Invariant natural killer T (iNKT) cells are a conserved, immunoregulatory T lymphocyte subset that responds to lipid antigens with near-immediate cytokine production and cytotoxicity. iNKT cells are required for the antibacterial host response. Whether renal failure and renal replacement therapy alter iNKT cell abundance or phenotype has not been investigated.

Methods. iNKT cells were studied by flow cytometry in the peripheral blood of patients with acute renal failure, chronic haemo- and peritoneal dialysis (PD), chronic kidney disease, and after transplantation.

Results. A very marked reduction in iNKT lymphocytes was found in acute renal failure before the first haemodialysis (HD) session. iNKT cells were depleted in end-stage renal disease patients receiving either HD or PD. iNKT cell depletion was accentuated after an HD session. Lesser degrees were observed in patients with non-dialysis-dependent chronic kidney disease. CD56 and CD161 NK cell marker expression was decreased in renal impairment. CD56+ and CD161+ iNKT cells produced more interferon-γ than negative cells of the same donor. Within the first year after kidney transplantation, the decrease in iNKT cells and their NK cell markers was reverted.

Conclusions. We describe for the first time that iNKT lymphocytes are reduced in end-stage renal disease and further depleted by HD. iNKT cells are important for early host response including activation of other immune cells and their depletion may contribute to immune dysfunction in renal disease.

Keywords: iNKT, dialysis, end-stage renal disease, natural killer T cells, specific immunity

INTRODUCTION

End-stage renal disease is associated with profound immune system alterations. Clinically, high rates of infection in the presence of elevated levels of systemic inflammatory markers [1] suggest an altered, and less efficient host response and at the same time detrimental inflammation, e.g. in atherosclerosis [2, 3]. Among peripheral blood leucocytes in renal impairment, neutrophilic granulocytes were increased, monocyte counts and subsets altered [4] and lymphocyte counts decreased [5, 6]. Peripheral blood neutrophilia and lymphopenia correlated with increased mortality in the general population and also in end-stage renal disease [7]. This was shown in several independent studies in patients receiving both haemodialysis (HD) [6, 8–10] and peritoneal dialysis (PD) [11] for renal replacement. Lymphopaenia was already apparent in patients with chronic kidney disease (CKD) before renal replacement therapy was required [12]. Decreases in T-cell number and function [13] were observed with the current HD regimens in the majority [14, 15] albeit not in all cohorts [16]. A study comparing patients with end-stage renal disease before and after the first HD treatment found no significant difference [17] suggesting T-cell depletion to be due to uraemia rather than a specific treatment modality. Among T-cells, CD4+ T-cell concentrations were decreased in patients receiving HD [14–18]. However, this was not observed in PD patients or patients before the start of renal replacement therapy [15]. Naïve CD4+ T cells [16–18] and also CD4+CD25+ and Foxp3+ regulatory T cells were depleted [19, 20]. Antigen-specific classical T cell responses were only partially impaired to HBsAg, the frequency of CMV reactive CD4+CD28− T cells was even increased in aged HD patients [21].
Invariant natural killer T (iNKT) cells are a T lymphocyte population that is defined by expression of a semi-invariant T-cell receptor composed of an invariant Vα24 rearrangement (Vα24i) and a more variable Vβ11 in humans. This TCR recognizes glycolipids contained in the human body and bacterial cell walls [22]. iNKT cells exert their function by both direct cytotoxicity and cytokine production, activating other immune cells [22]. Gram-negative bacteria and also Gram-positive streptococci and staphylococci activate iNKT cells that play an important role in the host response to these pathogens [23]. In kidney disease, iNKT cells can have a pro-inflammatory role in ischaemia reperfusion [24] but also an immunoregulatory, protective function, e.g. in animal models of crescentic glomerulonephritis [25, 26]. Human iNKT cells exist as CD4+, CD8+ and double-negative (CD4−CD8−) cells. Extrathymic differentiation and proliferation of human iNKT cells has been investigated by comparing iNKT cells from the blood and thymus [27, 28]. CD4+ iNKT cells appear to be more recent thymic emigrants [27]. The NK cell markers CD161 and CD56 are expressed on subpopulations of human iNKT cells. The proportion of CD56+ iNKT cells was similar in the thymus and peripheral blood at ≈20% [27]. The proportion of CD161+ iNKT cells rose from the thymus to peripheral blood from 40 to 80%, suggesting extrathymic differentiation [27, 28]. CD161 is the receptor of lectin-like transcript I [29, 30] that can induce iNKT cell cytokine production. iNKT cells can produce canonical T-cell cytokines such as interferon (IFN)γ and interleukin (IL)-4 upon activation. Little data exist on cytokine production of these subsets. In vivo during recovery from allogenic stem cell transplantation, IFNγ was made exclusively by CD161+ iNKT cells [31], and the IL-17 production of cultured human iNKT cells was associated with CD161 expression [32]. However, to date it is unknown whether, at steady state and in vivo, human CD56+ and CD161+ iNKT cells also represent a functional subgroup regarding specific cytokine production [22].

Earlier studies of human iNKT cells were limited by the reagents available for specific detection [22, 33]. In addition, studies of disease association also have to account for the large normal range of the human iNKT cell counts that appears to have a strong genetic basis [34]. Using specific Vα24i staining, the iNKT cell frequency was decreased in patients with multiple sclerosis [35], atherosclerosis [36], celiac disease [37], and very recently also with sarcoidosis [38] compared with healthy controls. The iNKT cell frequency was negatively correlated with mortality after allogenic stem cell transplantation in humans [39]. iNKT cells expanded in vitro can successfully be activated in humans in vivo [40]. Current experimental therapeutic approaches aim at replenishing iNKT cells in humans with metastatic malignancies with a good safety profile [41, 42]. Clinical response was observed in some patients [41, 42]. iNKT cells have not been characterized in patients with end-stage renal disease. We observed very low numbers of iNKT cells in a small cohort of uraemic patients before the first emergency HD session and therefore further examined iNKT cell number and phenotype in patients with renal impairment on renal replacement therapy and after kidney transplantation.

### RESULTS

#### iNKT lymphocytes are diminished in renal impairment

iNKT cells in the peripheral blood were investigated using an antibody specific for the invariant TCR alpha-chain (6B11) as described [43] (Figure 1A). In a group of patients (Table 1) before their first course of emergency HD in the acute care setting, iNKT cell concentrations were reduced to close to detection limit among both CD3+ and CD4+ T cells (Figure 1).

To test whether this was a general feature of renal failure rather than due to other factors leading to hospital admission, we enumerated iNKT cells among the peripheral blood CD3+ and CD4+ T cells in stable outpatients with end-stage renal disease (Tables 1 and 2). Numbers were significantly reduced in patients receiving HD (Figure 1). HD involves contact of blood with foreign surfaces that might impact on iNKT cells. We therefore next investigated patients treated with PD with very similar results (Figure 1). iNKT cells were also measured...
in patients with stable chronic kidney disease (CKD stages III–V) (Tables 1 and 2). iNKT cell counts showed a trend towards reduction; however, this was not significant compared with healthy controls (Figure 1).

iNKT cell counts were assessed in consecutive outpatients and in prevalent HD and PD patients. Healthy controls were age matched to end-stage renal disease patients; however, CKD patients were significantly older. An iNKT cell decrease in blood with age has been described by some [44] but not by other authors [39], and a decrease was not replicated in aged mice [45]. In our cohort, using the V24i-specific 6B11 antibody, no significant correlation of iNKT cell count with age was observed in either healthy controls or in HD, PD or CKD populations (Supplementary data, Figure S1A–D). Similar to what was observed by others [46], there were no significant differences between males and females (Supplementary data, Figure S1G–H).

We further analysed the association of iNKT cell numbers with other clinical characteristics, clinical chemistry and inflammation markers in the HD cohort (Table 2, Supplementary data, Figure S2). No association with HD duration, haematocrit, total leucocytes, cholesterol, calcium or C-reactive protein was detected (Supplementary data, Figure S2). In neither HD nor PD patients, there was a significant difference in patients with primary immune underlying renal disease compared with others (data not shown).

Our data show decreased peripheral blood iNKT cell in separate patient populations with decreased renal function independent of treatment modalities.

iNKT cell counts are further decreased by HD

iNKT cell counts were decreased in all patients with end-stage renal disease irrespective of the dialysis modality and even before the first renal replacement session. Given a possible additional impact of foreign surfaces, we investigated the influence of an HD treatment session on iNKT cells in the peripheral blood. Blood counts and flow cytometry for iNKT cells were performed after the long interval and after the following HD session (Figure 2). HD treatment significantly increased haematocrit, but not leucocyte counts and did not alter the proportion of lymphocytes in the peripheral blood (Figure 2A–C). However, iNKT cells among T cells were further reduced (Figure 2D). The same result was found if iNKT cell concentrations were compared (pre: 1.9 ± 0.1/μL, post: 0.04 ± 0.01/μL, P < 0.05). Levels of invariant T-cell receptor on iNKT cells were unaffected by HD, arguing against unspecific loss of the marker (Figure 2E). The iNKT cell reduction was most marked in patients with higher starting counts (Figure 2F). These data suggest that iNKT cells are preferentially affected by extracorporeal treatment compared with other CD3+ T cells.

iNKT cell CD56 and CD161 expression is decreased in renal failure

iNKT cells differentially express T helper and NK cell surface markers and increase CD161 expression during peripheral maturation [27, 28]. A relative decrease in total CD4+ T cells in HD patients as described [14–18] was also observed in our cohort of HD patients (59 ± 2%) compared with both CKD patients (66 ± 2%) and healthy controls (70 ± 2%) (Bonferroni after ANOVA). However, in patients with both chronic kidney and end-stage renal disease, the proportion of CD4+/iNKT cells was unaltered (Figure 3A and B).

NK cell prevalence was unaltered in HD and CKD patients (6.2 ± 1% CD56+ of PBMC in HD, 6.8 ± 1% in CKD, 5.9 ± 1% in controls, Bonferroni after ANOVA). In contrast, CD56
expression on iNKT cells from HD patients was significantly lower than healthy controls (Figure 3C). CD161 expression was significantly decreased in both CKD and HD patients. This was found on both iNKT cells (Figure 3C) and NK cells (77 ± 1% in HD, 80 ± 1% in CKD, 87 ± 1% in controls, Bonferroni after ANOVA).

These data demonstrate altered iNKT cell subset homeostasis in renal impairment.

**CD56**<sup>+</sup> and CD161<sup>+</sup> iNKT cells produce increased levels of IFNγ

In human iNKT cells at steady state, it has not been investigated whether function differs according to CD56 and CD161 surface marker expression. We therefore performed intracellular cytokine staining after *ex vivo* stimulation of freshly isolated cells. Figure 4A–D shows that both CD56 and CD161<sup>+</sup> produced significantly more IFNγ, and CD161<sup>+</sup> cells also IL-4 than CD56<sup>+</sup> or CD161<sup>−</sup> iNKT cells from the same healthy donor. We also tested cytokine production according to CD56 and CD161 expression in iNKT cells from HD patients. Very similar to healthy controls, cytokine production was higher in CD56- and CD161-expressing cells (Figure 4E).

These results are, to our knowledge, the first to show that human iNKT cell cytokine expression is differentially regulated according to NK marker expression under steady-state conditions *in vivo*.

**iNKT cell counts revert after renal transplantation**

Whether or not iNKT cell counts recover after renal transplantation was tested in patients at routine control visits after kidney transplantation (Figure 5, patients are characterized in Tables 1 and 2). Patients were included if renal function and immunosuppressive regimen were stable and there was no clinical evidence of rejection. There was a non-significant trend toward less recovery in patients with primary immune renal disease (0.11 ± 0 versus 0.04 ± 0% of CD3<sup>+</sup> T cells, P = 0.14). iNKT cell numbers were independent of age or sex (Supplementary data, Figure S3).

After renal transplantation, iNKT cell concentrations were equal to healthy controls among both CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells (Figure 5A and B). Also, the proportion of both CD56<sup>+</sup> and CD161<sup>+</sup> iNKT cells was normalized (Figure 5C and D).

We further analysed the impact of immunosuppressive therapy. All patients received prednisolone 5 mg o.d. and all

### Table 1. Characterization of the patient and proband groups

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<tr>
<th></th>
<th>ctrl</th>
<th>ARF</th>
<th>HD</th>
<th>PD</th>
<th>CKD</th>
<th>Ntx</th>
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<tr>
<td>n</td>
<td>73</td>
<td>6</td>
<td>38</td>
<td>11</td>
<td>37</td>
<td>28</td>
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<tr>
<td>Age (years)</td>
<td>56 ± 12</td>
<td>70 ± 14</td>
<td>64 ± 16</td>
<td>56 ± 16</td>
<td>76 ± 2</td>
<td>51 ± 3</td>
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<tr>
<td>Sex (% male)</td>
<td>52</td>
<td>83</td>
<td>76</td>
<td>55</td>
<td>43</td>
<td>39</td>
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<td>Hypertension</td>
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<td>18</td>
<td>9</td>
<td>49</td>
<td>7</td>
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</tr>
<tr>
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<td>5</td>
<td>9</td>
<td>11</td>
<td>7</td>
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<td>34</td>
<td>45</td>
<td>11</td>
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<td>13</td>
<td>9</td>
<td>5</td>
<td>7</td>
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<tr>
<td>Obstruction</td>
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<td>0</td>
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<td>Miscellaneous</td>
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<tr>
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<td>12</td>
<td>0</td>
<td>3</td>
<td>11</td>
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<tr>
<td>Imunosuppression</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>3</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>On rRT/after Ntx (months, range)</td>
<td>n.a.</td>
<td>121 (8–389)</td>
<td>33 (5–124)</td>
<td>n.a.</td>
<td>6.1 (2.9–12.4)</td>
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</table>

Means ± SEM. ARF, acute renal failure; HD, haemodialysis; PD, peritoneal dialysis; Ntx, renal transplantation.

### Table 2. Characterization of the patient groups

<table>
<thead>
<tr>
<th></th>
<th>ARF</th>
<th>HD</th>
<th>PD</th>
<th>CKD</th>
<th>Ntx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (10&lt;sup&gt;6&lt;/sup&gt;/μL, m: 4.5–5.9, f: 4.0–5.2)</td>
<td>3.9 ± 0.4</td>
<td>3.7 ± 0.5</td>
<td>3.7 ± 0.4</td>
<td>4.1 ± 0.1</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Thrombocytes (10&lt;sup&gt;3&lt;/sup&gt;/μL, 150–450)</td>
<td>270 ± 91</td>
<td>231 ± 69</td>
<td>277 ± 46</td>
<td>227 ± 12</td>
<td>251 ± 12</td>
</tr>
<tr>
<td>Leucocytes (G/L, 4.4–11.3)</td>
<td>9.5 ± 5.2</td>
<td>6.7 ± 2.3</td>
<td>8.4 ± 2.6</td>
<td>6.6 ± 0.3</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td>% lymphocytes (25–40)</td>
<td>16 ± 9</td>
<td>23 ± 8</td>
<td>20 ± 10</td>
<td>21 ± 1</td>
<td>n.a.</td>
</tr>
<tr>
<td>iNKT cells (μL)</td>
<td>0.06 ± 0</td>
<td>0.2 ± 0.4</td>
<td>0.14 ± 0.05</td>
<td>0.33 ± 0.1</td>
<td>n.a.</td>
</tr>
<tr>
<td>iNKT cells (% of ly.)</td>
<td>0.6 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>2.6 ± 0.6</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>Creatinine (μmol/L, m: 59–104, f: 45–84)</td>
<td>448 ± 178</td>
<td>746 ± 270</td>
<td>660 ± 311</td>
<td>153 ± 20</td>
<td>155 ± 7</td>
</tr>
<tr>
<td>Urea (mmol/L, 3.3–6.7)</td>
<td>32 ± 19</td>
<td>21 ± 6.3</td>
<td>23 ± 7</td>
<td>17 ± 1</td>
<td>10.2 ± 0.7</td>
</tr>
<tr>
<td>eGFR (MDRD)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>29 ± 2</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>CRP (mg/L, &lt;8)</td>
<td>44 ± 6</td>
<td>14.3 ± 24</td>
<td>31 ± 58</td>
<td>6 ± 3</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>33%</td>
<td>74%</td>
<td>73%</td>
<td>16%</td>
<td>13%</td>
</tr>
<tr>
<td>Steroids</td>
<td>0%</td>
<td>18%</td>
<td>9%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>0%</td>
<td>13%</td>
<td>0%</td>
<td>13%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Means ± SEM. ARF, acute renal failure; HD, haemodialysis; PD, peritoneal dialysis; Ntx, renal transplantation; n.a., not assessed; normal ranges are given, separate for men (m) and woman (f), if applicable.
but one mycophenolate (1.4 ± 0.4 g/d) but some received cyclosporine A and some tacrolimus as calcineurin inhibitor. The choice of calcineurin inhibitor did not significantly influence the proportion of iNKT cells among CD3⁺ or CD4⁺ cells and proportions of CD56⁺ and CD161⁺ iNKT cells were very similar (data not shown). These data suggest a reversal of iNKT cell depression after renal transplantation.

**DISCUSSION**

We provide a first detailed description of iNKT lymphocytes in renal impairment. iNKT cell numbers decreased with renal function and were depressed in patients on renal replacement therapy, both HD and PD.

Depletion of iNKT cells, that are important in response to infection, was pronounced compared with other T cells. This argues for increased susceptibility of iNKT cells to uraemia effects. Indeed, also among classical T cells, effector memory cells are predominantly affected in renal impairment [14–18] and iNKT cells are similar to them in many respects [23]. Leucocyte activation by foreign surfaces in dialysis is considered a major pro-inflammatory factor in patients with end-stage renal disease. We found that an HD session significantly further decreased the proportion of iNKT cells. However, the decrease in iNKT cells was also observed in PD patients who are not exposed to foreign surfaces and even more in incipient dialysis patients before the first HD session arguing for a role of renal failure in inducing the iNKT cell depression.

In addition to increased susceptibility to infection, end-stage kidney disease patients suffer from imbalanced pro-inflammatory responses that contribute to their increased rate of atherosclerosis and cardiovascular events. CD4⁺iNKT cells increase atherosclerotic lesion formation in mice [47, 48]. However, it has also been described that a hyperlipidaemic,
pro-atherogenic environment can exhaust iNKT cells [49]. While specific soluble and/or cellular factors mediating decrease in renal disease remain to be identified, this mechanism could possibly have contributed to iNKT cell depletion. We observed no association of iNKT cell counts with multiple other clinical characteristics and laboratory values in HD patients, indicating it is a separate entity. This makes iNKT cells interesting as a possible new prognostic marker with possible additional value to established parameters. This hypothesis can be tested prospectively in our cohorts.

iNKT cells infiltrate the kidney in animal models of glomerulonephritis executing both pro- and anti-inflammatory functions [25, 26]. In the human blood, we did not observe significant differences in iNKT cell counts between patients with terminal renal failure with primary immune or other renal disease; however, there was a trend toward less recovery after transplantation of the former. This trend, and also the presence and functionality of iNKT cells in the human kidney, needs to be further investigated, preferentially in disease-specific subgroups of patients.

The NK cell markers CD56 and CD161 on iNKT cells were significantly decreased in uraemia and reverted after renal transplantation. While CD56 appears to be stable after thymic emigration, CD161 is up-regulated during peripheral T cell maturation [27, 28]. CD161 expression was inhibited by interleukin 6, a cytokine that is increased in dialysis patients [50] but also in CKD (unpublished data). Possibly, IL-6 levels play a role in altered CD161 on iNKT cells and their function in renal failure.

To address functional differences between human iNKT cell subsets, we started an investigation of cytokine expression. The NK cell marker-positive subgroups that were most depleted in renal impairment preferentially produced IL-4 and IFN-γ, cytokines required for host response and for activation of other immune cells by iNKT cells [22]. The pattern of cytokine production of iNKT cell subsets was very similar in HD patients with CKD.

**FIGURE 3:** iNKT lymphocyte CD56 and CD161 surface molecule expression is reduced in end-stage renal failure. (A–C) iNKT lymphocyte CD4, CD56 and CD161 surface marker expression was assessed in patients with chronic kidney disease (CKD), receiving maintenance HD and healthy controls (A, examples, B and C Dunnet’s after one-way ANOVA of 9 n = 35 ctrl, n = 28 HD, n = 12 CKD).
patients and healthy controls. The lack of the subpopulations that produced most cytokine could contribute to increased susceptibility to infection in renal failure.

The depression in iNKT cell numbers, CD56 and CD161 expression was reverted after renal transplantation. Human iNKT cell recovery has also been studied after allogenic stem cell transplantation. After complete bone marrow ablation for haematologic malignancies, normalization of iNKT cell function and up-regulation of CD161 required months [51] to years [31]. CD161 surface expression 6 months after renal transplantation had reached the level of healthy controls. The focus of the present work was iNKT cell recovery with restored renal function, which, however, may be affected by immunosuppressive therapy. The iNKT cell proportion among

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**FIGURE 4**: Differential cytokine expression in human iNKT cells according to CD56 and CD161 expression. iNKT cell cytokine expression was assessed after re-stimulation by intracellular staining. (A–D) Both CD56+ (A,B) and CD161+ (C,D) iNKT cells from healthy donors produced relatively more IFN-γ than CD56− or CD161− cells from the same donor and the proportion of IL-4+ cells was increased among CD161+ cells (paired t-tests, n = 20 PBMC donors). (E) In iNKT cells from HD patients, subset-specific IFN-γ and IL-4 cytokine production was similar to what was observed in healthy controls (paired t-tests, n = 10 patients).
all T cells reverted to normal arguing against preferential susceptibility of this cell type. With the relatively uniform immunosuppressive regimens in our patients, only the impact of calcineurin inhibitors cyclosporine A and tacrolimus could be tested. No differences were observed. iNKT cell counts correlated with maintenance of complete remission in human allogenic stem cell transplantation [31]. Whether iNKT cell numbers or phenotype are related to graft survival in renal transplantation has to be studied prospectively.

In summary, we provide a first assessment of lipid-reactive iNKT cells in patients with renal failure. A significant depression of this cell type was reversible after renal transplantation. iNKT lymphocyte depression may contribute to immune dysfunction in patients with renal failure.

**REFERENCES**


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**CONFLICT OF INTEREST STATEMENT**

The authors have no financial conflict of interest regarding this manuscript.

**SUPPORTING INFORMATION**

Supplementary data are available online at http://ndt.oxfordjournals.org.
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