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Adaptive NKG2C⁺ NK Cell Response and the Risk of Cytomegalovirus Infection in Kidney Transplant Recipients

Dolores Redondo-Pachón,*†,‡ Marta Crespo,*†,‡ Jose Yélamos,*†,‡ Aura Muntasell,† María José Pérez-Sáez,* Silvia Pérez-Fernández,† Joan Vila,† Carlos Vilches,§ Julio Pascual,*†,‡ and Miguel López-Botet†,‡,§

CMV infection in kidney transplant recipients (KTRs) has been associated with an increased risk for graft loss and reduced host survival. CMV promotes persistent expansions of NK cells expressing the CD94/NKG2C receptor. The NKG2C (KLRC2) gene is frequently deleted, and copy number influences the adaptive response of NKG2C⁺ NK cells. The distribution of NKG2C⁺ NK cells and NKG2C genotypes (NKG2C⁺⁺, NKG2C⁺⁺del, NKG2C⁺deldel) were studied in cross-sectional (n = 253) and prospective (n = 122) KTR cohorts. Assessment of CMV viremia was restricted to symptomatic cases in the retrospective study, but was regularly monitored in the prospective cohort. Overall, the proportions of NKG2C⁺ NK cells were significantly higher in KTRs who had suffered posttransplant symptomatic CMV infection in the cross-sectional study. Yet, along the prospective follow-up (3, 6, 12, and 24 mo), posttransplant NKG2C⁺ NK cell expansions were not observed in every patient with detectable viremia who received preemptive antiviral therapy, suggesting that the adaptive NK cell response may be inversely related with the degree of CMV control. Remarkably, the incidence of posttransplant viremia was reduced among cases with high pretransplant levels of NKG2C⁺ NK cells. The NKG2C genotype distribution was comparable in KTR and healthy controls, and greater proportions of NKG2C⁺ cells were detected in NKG2C⁺⁺ than in NKG2C⁺⁺del patients. Yet, a trend toward increased NKG2C⁺⁺del and reduced NKG2C⁺⁺⁺ frequencies associated with symptomatic infection was appreciated in both cohorts. Altogether, our results indirectly support that adaptive NKG2C⁺ NK cells are involved in the control of CMV in KTRs. The Journal of Immunology, 2017, 198: 94–101.

Human CMV infection in immunocompetent individuals is generally asymptomatic and the virus establishes a lifelong latent infection, undergoing reactivations that allow its successful transmission, reaching a high prevalence in all human populations (1, 2). Immunosuppression of kidney transplant recipients (KTRs) favors CMV infection or reactivation, which increases the rate of graft loss and reduced host survival, requiring antiviral therapy (3–5). Prophylactic antiviral drugs are administered to CMV⁺ KTRs transplanted from a CMV⁺ donor or KTRs receiving intensive immunosuppression. In most CMV⁺ KTRs, preemptive therapy upon detection of CMV viremia, regardless of clinical expression, is currently used with positive results; yet, patients who might spontaneously control the infection are exposed to antiviral drug toxicity and the overall costs are increased (4). The identification of biomarkers predicting the risk for posttransplant CMV infection is warranted to improve its clinical management. Assessing the pretransplant frequency of T cells specific for viral Ags (IE1 and pp65) has been proposed to fulfill this goal (6). CMV infection promotes the expansion of a mature NK cell subset that displays high surface expression levels of the CD94/NKG2C activating lectin-like receptor (NKG2C[high]). Expansions of NKG2C⁺ cells have been reported in CMV⁺ healthy adults and children (7–9), in CMV coinfected individuals suffering from other viral infections (10–12), as well as in CMV-infected immunocompromised individuals, including KTRs (13) and hematopoietic stem cell transplant recipients (14, 15). Remarkably, the magnitude of the NK cell subset redistribution appears rather variable in healthy blood donors and persists under steady-state conditions (8). This adaptive NK cell response is reminiscent of that mediated in mice by “memory” NK cells expressing the Ly49H⁺ NK cell receptor (NKR) specific for the m157 murine CMV glycoprotein (16).

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downregulation of some transcription factors and signaling molecules, for example, FcRIR-γ chain (FcRγy), has been reported (17, 18). The variability of some of these phenotypic features (e.g., CD57 expression or FcRγy loss) in expanded NKG2C+ cells from different individuals suggests that they are gradually acquired along late differentiation (19).

NKG2C^{bright} NK cells are functionally mature, capable of efficiently mediating cytotoxicity and cytokine production (e.g., TNF-α and IFN-γ) triggered via CD94/NKG2C (8). Moreover, Ab-dependent stimulation via CD16 (FcγRIIIa) activates NKG2C^{+} NK cells against CMV-infected cells (20–22). Yet, the molecular and cellular mechanisms underlying the generation of adaptive NKG2C^{bright} NK cells in response to CMV and their role in the control of the infection remain unclear (8, 16, 23, 24). A deletion of the NKG2C gene (officially termed KLRC2) determines the existence of three genotypes (i.e., NKG2C^{+/+}, NKG2C^{+/−}, and NKG2C^{−/−}) in populations of different ethnic origin (25, 26). NKG2C copy number has been positively correlated with expression levels and function of the CD94/NKG2C receptor, as well as with steady-state numbers of circulating adaptive NKG2C^{+} NK cells in healthy individuals (27, 28).

Expansions of NKG2C^{bright} NK cells were reported in KTRs with CMV viremia (13), but there is no information on their implication in antiviral defense. To address this issue, we analyzed the distribution of adaptive NK cells and the NKG2C genotype in relation with CMV infection in cross-sectional and prospective KTR cohorts. Our results support an involvement of adaptive NKG2C^{+} NK cells in the control of CMV infection in renal transplantation.

Materials and Methods

Patient population and clinical data

In a retrospective study, 253 patients transplanted between August 2006 and February 2013, under immunosuppression with tacrolimus, mycophenolate, and steroids, with a graft functioning >3 mo were enrolled. NKG2C genotyping was carried out in 238 cases and peripheral blood immunophenotyping in 191 patients at a median posttransplant time of 21 mo (interquartile range [IQR], 19–25).

In a second prospective study, 122 patients transplanted from March 2013 and receiving the same immunosuppressive treatment with tacrolimus, mycophenolate, and steroids were enrolled. All of them were genotyped for NKG2C, and NK cell phenotypic analysis was performed before and at different time points (3, 6, 12, and 24 mo) after transplantation. Both studies were approved by the Hospital Research Ethical Committee (CEIC-Parc de Salut Mar number 2010/3904/I and 137/C/2012), and patients signed an informed consent.

All patients were transplanted across a negative complement-dependent cytotoxicity cross-match with donor lymphocytes. HLA typing and for 2 mo after prophylaxis and for 2 mo after prophylaxis ended in the remaining cases. All patients with compatible symptoms and any degree of replication received specific treatment. For asymptomatic cases, antiviral therapy was administered in seronegative KTRs with >500 copies per milliliter and in seropositive patients with >1000 copies per milliliter.

Immunophenotypic analysis and NKG2C genotyping

Phenotypic analysis was performed by flow cytometry in fresh peripheral blood samples, obtained by venous puncture in EDTA tubes as described previously (29). Samples were pretreated with saturating concentrations of human aggregated IgG to block FcγR and then labeled with different Ab combinations to define total T and NK cell subsets. For direct immunofluorescence staining, we used the following Abs: allophycocyanin-CD3 (clone SK7), PerCP-Cy5.5 (clone 2D1), and FITC-Cy5.5 (clone NCAM16.2) from BD Biosciences (San Diego, CA). NK cells were defined as CD3−CD56− lymphocytes, and NK cell subsets were identified by indirect immunofluorescence staining with Abs specific for NKG2C (clone MA13B1; R&D Systems, Minneapolis, MN), NKG2A (clone Z199; provided by Dr. A. Moretta), CD161 (clone HP-3G10; provided in our laboratory), and KIR; in that case, staining was performed using a mixture of Abs specific for KIR3DL1/1.2 and 2DS4 (clone 5.133; provided by Dr. M. Colonna), KIR2DL2/SL2/L3 (clone CH-L; provided by Dr. S. Ferrini), KIR3DL1 (clone DX9; provided by Dr. L.L. Lanier), and KIR2DL1/S/1/S4 (clone HP-3E4; generated in our laboratory). PE-conjugated F(ab’)2 rabbit antimouse Ig (Dako, Glostrup, Denmark) was used as secondary Ab. After washing and erythrocyte lysis, samples were acquired with a FACSCanto cytometer, and data were analyzed with the DIVA software (BD Biosciences). Absolute numbers of cells were calculated from blood counts obtained in parallel. DNA was isolated from total blood using the Puregene, BloodCore kit B (Qiagen). NKG2C zygosity was assessed as previously described (26).

Statistical analysis

Categorical variables are expressed as percentages. Continuous variables are expressed as mean and SD or median (IQR), according to normal or nonnormal distributions. Normal distribution was assessed by normal Q–Q plots. Univariate analysis was performed by Student’s t, Mann–Whitney U, Friedman, χ², or Fisher’s tests, as appropriate.

The Cox proportional hazards model was used to test the association between the %NKG2C^{+} NK cells and CMV infection, adjusted for confounders. Linearity assumption of the model was tested: none of the non-linear parameters achieved statistical significance when continuous variables were included in the model using a smoothing spline. The proportional hazard assumption for all variables included in the model was also tested.

To find the best cutoff for the %NKG2C^{+} NK cells that discriminated KTRs at higher and lower risk for development of CMV infection, a bootstrapping procedure (31) was used performing 1000 replicates. In each replicate, a sample with replacement of size equal to the study sample was

| Table I. Distribution of NK cell subsets in KTRs from the retrospective study stratified according to symptomatic posttransplant CMV infection |
|-----------------|-----------------|-----------------|-----------------|
|                  | CMV Symptomatic Infection (n = 30) | No CMV Symptomatic Infection (n = 161) | p |
| % NK 12.8 ± 10.9 | 10.5 ± 7.5 | 0.55 |
| % NKG2C^{bright} | 34.8 ± 24.9 | 25.1 ± 20.1 | 0.038 |
| % NKG2A^{bright} | 32.8 ± 18.7 | 41.4 ± 19.4 | 0.013 |
| Posttransplant time (mo), median (IQR) | 21.5 (18–27) | 21 (19–25) | 0.96 |

Data are expressed as mean ± SD.

*Symptomatic CMV infection with confirmed viremia (see Materials and Methods).

Referred to total NK cells.
Table II. Characteristics of KTRs in the prospective study

<table>
<thead>
<tr>
<th></th>
<th>All Patients (n = 122)</th>
<th>CMV Viremia* (n = 39)</th>
<th>No CMV Viremia (n = 83)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age (y, mean ± SD)</td>
<td>54.5 ± 14.4</td>
<td>61.9 ± 12.4</td>
<td>50.5 ± 14.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female recipient (n, %)</td>
<td>43 (35.2)</td>
<td>14 (35.9)</td>
<td>29 (34.9)</td>
<td>0.53</td>
</tr>
<tr>
<td>Donor age (y, mean ± SD)</td>
<td>57.0 (14.7)</td>
<td>64.9 (12.7)</td>
<td>53.1 (14.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Deceased donor (n, %)</td>
<td>103 (84.4)</td>
<td>36 (92.3)</td>
<td>67 (80.7)</td>
<td>0.19</td>
</tr>
<tr>
<td>Female donor (n, %)</td>
<td>62 (51.2)</td>
<td>21 (53.8)</td>
<td>41 (49.4)</td>
<td>0.42</td>
</tr>
<tr>
<td>Retransplantation (n, %)</td>
<td>13 (10.6)</td>
<td>1 (2.5)</td>
<td>12 (14.4)</td>
<td>0.041</td>
</tr>
<tr>
<td>Pre-KT CMV serostatus (n, %)</td>
<td></td>
<td></td>
<td></td>
<td>0.29*</td>
</tr>
<tr>
<td>High risk (D+/R+)</td>
<td>9 (7.4)</td>
<td>4 (10.2)</td>
<td>5 (6.0)</td>
<td></td>
</tr>
<tr>
<td>Intermediate risk (D+/R+, D-/R+)</td>
<td>109 (89.3)</td>
<td>35 (89.8)</td>
<td>74 (89.2)</td>
<td></td>
</tr>
<tr>
<td>Low risk (D-/R-)</td>
<td>4 (3.3)</td>
<td>0</td>
<td>4 (4.8)</td>
<td></td>
</tr>
<tr>
<td>Thymoglobulin induction (n, %)</td>
<td>15 (12.3)</td>
<td>2 (5.1)</td>
<td>13 (15.6)</td>
<td>0.44</td>
</tr>
<tr>
<td>Valganciclovir prophylaxis (n, %)</td>
<td>21 (17.2)</td>
<td>5 (12.8)</td>
<td>16 (19.2)</td>
<td>0.37</td>
</tr>
<tr>
<td>Peak PRA &gt;5% (n, %)</td>
<td>20 (16.4)</td>
<td>6 (15.4)</td>
<td>14 (16.8)</td>
<td>0.83</td>
</tr>
<tr>
<td>Pretransplant PRA &gt;5% (n, %)</td>
<td>9 (7.4)</td>
<td>2 (5.1)</td>
<td>7 (8.4)</td>
<td>0.15</td>
</tr>
<tr>
<td>Pretransplant DSA (n, %)</td>
<td>8 (6.5)</td>
<td>1 (2.5)</td>
<td>7 (8.4)</td>
<td>0.43</td>
</tr>
<tr>
<td>Delayed graft function (n, %)</td>
<td>29 (26.1)</td>
<td>9 (23)</td>
<td>20 (24.1)</td>
<td>0.94</td>
</tr>
<tr>
<td>Biopsy-proven rejection (n, %)</td>
<td></td>
<td></td>
<td></td>
<td>0.47</td>
</tr>
<tr>
<td>Acute cellular</td>
<td>15 (12.3)</td>
<td>3 (7.7)</td>
<td>12 (14.4)</td>
<td></td>
</tr>
<tr>
<td>Acute humoral</td>
<td>3 (2.4)</td>
<td>1 (2.5)</td>
<td>2 (2.4)</td>
<td></td>
</tr>
<tr>
<td>CMV PCR at diagnosis (copies/ml, median (IQR))</td>
<td>NA (759 (338–11,829))</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV infection time after KT (d, median (IQR))</td>
<td>NA (58.5 (41.5–112.5))</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follow-up (mo), median (IQR)</td>
<td>15 (8–22.5)</td>
<td>15.4 (8.5–20.5)</td>
<td>15.9 (8.5–24)</td>
<td>0.76</td>
</tr>
</tbody>
</table>

*Posttransplant detection of CMV viremia by qPCR (see Materials and Methods) including symptomatic and asymptomatic cases.

High versus intermediate-risk comparison.

In D+/R- cases, viremia may correspond to CMV reactivation and/or reinfection.

KTR, kidney transplantation; NA, not applicable; PRA, panel-reactive Abs; R, recipient.

Results

Cross-sectional analysis of NKG2C+ NK cells in KTRs

As a first approach to assess the adaptive NK cell response to CMV in KTR, a retrospective study was carried out in a cohort of 253 cases immunosuppressed at transplantation who did not receive CMV prophylaxis (Supplemental Table I). Viremia was confirmed in 47 KTRs (18.6%) with clinically recorded symptomatic infection, who therefore received antiviral therapy. KTR groups stratified according to CMV infection were similar in donor and recipient CMV serology, delayed graft function, or acute rejection; unadjusted death-censored graft survival was lower in patients with CMV infection than in those without infection (p = 0.026).

Information on posttransplant distribution of the NKG2C+ NK cell subset was obtained in 191 cases, complemented by the analysis of additional NKRIs (i.e., NKG2A, CD161, and KIR). Significantly higher proportions of NKG2C+ and concomitantly lower proportions of NKG2A+ NK cells were detected in KTRs who had developed posttransplant symptomatic CMV infection (n = 30), as compared with the remaining cases (n = 161) in whom the putative incidence of subclinical infection was undefined (Table I). No significant differences were observed in the distribution of CD161+ and KIR+ NK subsets (data not shown). In a Cox regression analysis, thymoglobulin induction (hazard ratio [HR], 2.65; 95% CI, 1.13–6.21; p = 0.025) and the percentage of NKG2C+ NK cells (HR, 1.021; 95% CI, 1.005–1.037; p = 0.010) were found to be independently and directly associated with symptomatic CMV infection; the association with age detected in the univariate analysis was not significant (data not shown). This cross-sectional study further supported the relationship between CMV and the adaptive expansion of NKG2C+ NK cells in immunosuppressed KTR, which was detectable several years after transplant (median follow-up at analysis: 54 mo; Supplemental Table I).

Prospective analysis of NKG2C+ NK cell distribution in KTRs monitored for posttransplant CMV viremia

The retrospective study did not provide information on the incidence of subclinical CMV replication nor on pretransplant levels of

Table III. Pretransplant distribution of NK cell subsets in KTRs from the prospective study stratified according to baseline CMV serology

<table>
<thead>
<tr>
<th>Age, mean ± SD (y)</th>
<th>CMV-Seronegative (n = 13)</th>
<th>CMV-Seropositive (n = 109)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>% NK, mean ± SD</td>
<td>49.9 ± 15.1</td>
<td>55.1 ± 14.3</td>
<td>0.25</td>
</tr>
<tr>
<td>% NKG2C**, mean ± SD</td>
<td>15.3 ± 8.9</td>
<td>13.2 ± 8.1</td>
<td>0.62</td>
</tr>
<tr>
<td>% NKG2A*, mean ± SD</td>
<td>8.3 ± 4.1</td>
<td>21.2 ± 17.7</td>
<td>0.014</td>
</tr>
<tr>
<td>% KIR*, mean ± SD</td>
<td>58.2 ± 16.8</td>
<td>46.7 ± 18.1</td>
<td>0.029</td>
</tr>
<tr>
<td>% CD161*, mean ± SD</td>
<td>54.5 ± 15.5</td>
<td>56.7 ± 16.6</td>
<td>0.72</td>
</tr>
</tbody>
</table>

*Referred to the proportions of total NK cells.
NKG2C⁺ NK cells. To overcome these limitations, we conducted a prospective study assessing the course of the adaptive NK cell response in a second KTR cohort (N = 122) treated similarly with tacrolimus, mycophenolate, and steroids (Table II). A total of 109 cases (89%) were CMV⁺ at transplantation, and 21 received prophylactic valganciclovir. Posttransplant CMV viremia was regularly monitored by PCR, being detected at a median of 58 d (IQR, 41–112) in 39 cases (32%), who subsequently received antiviral therapy.

The NK cell immunophenotype was studied pretransplant and posttransplant at different time points. In agreement with previous data not shown). These observations pointed out a remarkable association of high baseline levels of NKG2C⁺ NK cells with a reduced risk for posttransplant CMV infection.

Overall, NKG2C⁺ NK cells remained rather stable along the follow-up in KTRs without detectable CMV viremia, including cases with low or high baseline proportions of NKG2C⁺ cells. By contrast, a progressive increase in the proportions of NKG2C⁺ cells was perceived in KTRs with CMV viremia, reaching statistical significance at 24 mo (Table VI). An analysis of individual evolution patterns revealed that only some KTRs with a positive PCR for CMV, who subsequently received preemptive antiviral therapy, developed posttransplant expansions of NKG2C⁺ NK cells during the follow-up; representative examples are displayed in Fig. 2.

Relation of the NKG2C genotype with posttransplant CMV infection in KTRs

NKG2C gene copy number variation influences expression levels and function of the receptor, as well as steady-state numbers of NKG2C⁺ NK cells, which tend to be higher in NKG2C⁺/⁺ than in NKG2C⁺/del healthy subjects. As shown in Table VII, the NKG2C genotype distributions appeared in both KTR cohorts comparable...
with those found in healthy donors. Moreover, the relation of NKG2C copy number with the magnitude of steady-state NKG2C+ NK cell expansions reported in CMV+ healthy donors (27, 28) was confirmed pretransplant and posttransplant in the prospective and retrospective KTR cohorts, respectively. In both groups, significantly higher proportions of NKG2C+ NK cells were detected in NKG2C+/+ than in NKG2C+del cases (Fig. 3).

Considering the relation of the NKG2C genotype with the expansion of NKG2C+ adaptive NK cells, and their apparent negative association with the risk for posttransplant CMV infection, we addressed whether this event might be also under the influence of the genotype with the clinical impact of posttransplant infection. Paradoxically, the homozygous NKG2C deletion appeared underrepresented among cases with symptomatic infection, particularly in the retrospective cohort; yet, the low frequency of this genotype rendered the observation difficult to interpret. Inclusion of the NKG2C genotype to the multivariate Cox model for CMV risk (Table V) did not alter the results (NKG2C+/+ genotype versus others: HR, 0.88; 95% CI, 0.44–1.75; p = 0.719), and the significance for basal NKG2C+ NK cells (HR, 0.96, 95% CI 0.94–0.98; p = 0.006) was maintained.

### Discussion

In this report we explored the relation of adaptive NKG2C+ NK cells with the response of KTRs to CMV, based on the complementary study of two cohorts. In a first cross-sectional analysis, patients who had experienced symptomatic posttransplant CMV infection displayed after several years higher proportions of NKG2C+ NK cells, in line with a previous report relating the expansion of adaptive NK cells with CMV infection in KTRs (8, 13). The interpretation of these results was ambiguous, because cases with pretransplant and posttransplant expansions of NKG2C+ cells were not discriminated and asymptomatic infections were not identified. In a second prospective study, KTRs were regularly monitored for CMV viremia, assessing their NK cell immunophenotype pretransplant and posttransplant. Along the follow-up, postinfection adaptive NK cell expansions were detected only in some cases with viremia and, exceptionally, in KTRs with negative CMV-specific PCR during the follow-up, supporting their development in response to untreated subclinical CMV replication. These observations indicated that detection of CMV viremia in KTRs followed by preemptive antiviral therapy was not indestructibly associated with an expansion of NKG2C+ cells, and suggested that a prompt CMV control may hamper or delay the adaptive NK cell response in KTRs. No significant correlation was found between the peak of viral load and posttransplant levels of NKG2C+ NK cells (data not shown). It is plausible that persistent/recurrent low levels of viral replication, after withdrawal of antiviral therapy, may account for the delayed progressive NKG2C+ NK cell expansions observed in some patients, revealing an inefficient control of the pathogen.

Remarkably, the incidence of posttransplant CMV viremia in the prospective study was lower in CMV+ KTRs displaying high pretransplant levels of circulating NKG2C+ NK cells, suggesting that they might play a protective role. This effect was confirmed to be independent of thymoglobulin induction and/or antiviral prophylaxis. Age is a known variable related with CMV infection in kidney transplantation, as observed in both cohorts (Table II, Supplemental Table I). Yet, Cox analysis indicated that the relation

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**Table VI. Longitudinal analysis of NKG2C+ NK cells in KTRs stratified according to posttransplant CMV infection**

<table>
<thead>
<tr>
<th>Follow-up</th>
<th>% NKG2C+ NK Cells, Mean ± SD</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients reaching the follow-up time point at submission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No CMV viremiaa</td>
<td>n = 83</td>
<td>23.7 ± 19.5</td>
</tr>
<tr>
<td></td>
<td>n = 80</td>
<td>22.6 ± 13.3</td>
</tr>
<tr>
<td></td>
<td>n = 70</td>
<td>22.5 ± 18.6</td>
</tr>
<tr>
<td></td>
<td>n = 28</td>
<td>18.2 ± 13.3</td>
</tr>
<tr>
<td>CMV viremiaa</td>
<td>n = 39</td>
<td>15.1 ± 12.3</td>
</tr>
<tr>
<td></td>
<td>n = 37</td>
<td>17.4 ± 12.7</td>
</tr>
<tr>
<td></td>
<td>n = 34</td>
<td>20.1 ± 13.1</td>
</tr>
<tr>
<td></td>
<td>n = 13</td>
<td>24.2 ± 15.9</td>
</tr>
</tbody>
</table>

*aDetection of CMV by qPCR (see Materials and Methods) including symptomatic and asymptomatic cases.

*bStatistical comparison between baseline and 24 mo.

*cStatistical comparison between both groups.
between the levels of % NKG2C+ cells and the risk for posttransplant CMV viremia was independent of age. Actually, there is no evidence supporting a relation between aging and the development of adaptive NKG2C+ cells in CMV+ individuals (32). Despite the limited in vitro direct response of adaptive NK cells against CMV-infected cells, presumably under the influence of viral immune evasion mechanisms, they have been shown to efficiently mediate virus-specific Ab-dependent cytotoxicity and cytokine production triggered through the FcγRIIIa (CD16) receptor (20–22). It is plausible that pretransplant expansions of NKG2C+ cells, in combination with specific IgG, may contribute to posttransplant CMV control in seropositive KTRs, partially compensating for T cell immunosuppression. In this regard, CD16 downregulation and expression of activation markers in NK cells have been reported in KTRs (33). In some cases, this phenotypic profile might reflect an IgG-dependent NK cell activation triggered by CMV or donor-specific alloantibodies (29). On the other hand, CMV-induced adaptive NKG2C+ NK cells may be eventually boosted through CD16 along the Ab-dependent response to other pathogens (18). As indicated by sequential monitoring, regular immunosuppressive therapy (i.e., tacrolimus and mycophenolate) did not affect posttransplant levels of adaptive NK cells in PCR-negative KTRs. Yet, CMV viremia was observed in a case with high basal levels of NKG2C+ NK cells receiving intensive antirejection therapy, which included long-lasting treatment for lupus before transplantation and thymoglobulin induction. Further studies are warranted to assess the impact of different immunosuppressive regimens on the basal levels, development, and effector functions of adaptive NK cells.

Our study was focused on the analysis of NKG2C+ cell expansion as a conventional hallmark of the adaptive NK cell response to CMV. Detection of other phenotypic features in adaptive NKG2C+ NK cells (e.g., CD57 expression and FcγRIIIa loss), reflecting their late differentiation, is variable (9, 18, 19, 27). Assessing the relation of these markers with the putative antiviral effect of adaptive NK cells deserves attention in larger KTR cohorts. In contrast, the association of high pretransplant levels of adaptive NKG2C+ NK cells with a lower risk for CMV infection might indirectly reflect the involvement of other immune effector cells (34). In particular, the levels of pretransplant CMV-specific TcRαβ T cells have been reported to predict the risk for posttransplant infection (6, 35), and increased numbers of TcRγδ T cells were associated with CMV control (36). Recent reports support that adaptive NK cells and Ag-specific T cells may develop independently in response to CMV (37, 38), and further studies are warranted to evaluate their relative contribution to the control of the viral infection in KTRs.

Previous studies revealed an influence of NKG2C gene copy number on surface expression levels and function of the activating CD94/NKG2C receptor, as well as on steady-state numbers of circulating NKG2C+ NK cells, which were reduced in NKG2C+/del as compared with NKG2C+/+ healthy individuals (27, 28); moreover, the lack of NKG2C was suggested to be associated with altered control of CMV in childhood (27, 28). The NKG2C genotype relation with the magnitude of the adaptive NK cell response was confirmed in both KTR cohorts, which displayed an overall distribution of the NKG2C deletion comparable with that of the control population. By contrast, an increased frequency of the NKG2C+/del genotype and a reciprocal reduction of NKG2C+/+ cases were detected among KTRs suffering symptomatic CMV infection. Although these differences did not reach statistical significance, the coincident trend in both cohorts suggested that NKG2C copy number might subtly influence the outcome of CMV infection in KTRs, modulating the magnitude and function of the adaptive NKG2C+ NK cell response (8).

Should NKG2C+ cells play a relevant role in the posttransplant response to CMV, an increased frequency of the NKG2C+/del genotype associated with infection would be predictable. Yet, an opposite reduction of the homozygous deletion was unexpectedly observed among this KTR group, particularly in the retrospective

Table VII. NKG2C genotype distribution in KTRs according to CMV infection

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Healthy Donors (n = 313)</th>
<th>Total (n = 238)</th>
<th>No Symp. (n = 194)</th>
<th>Symp. (n = 44)</th>
<th>Total (n = 122)</th>
<th>PCR+ (n = 83)</th>
<th>PCR (n = 39)</th>
<th>PCR + Symp. (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKG2C+/+</td>
<td>200 (64)</td>
<td>145 (61)</td>
<td>122 (63)</td>
<td>23 (52)</td>
<td>69 (57)</td>
<td>48 (58)</td>
<td>21 (54)</td>
<td>11 (48)</td>
</tr>
<tr>
<td>NKG2C+/del</td>
<td>94 (30)</td>
<td>80 (34)</td>
<td>60 (31)</td>
<td>20 (46)</td>
<td>44 (36)</td>
<td>29 (35)</td>
<td>15 (38)</td>
<td>11 (48)</td>
</tr>
<tr>
<td>NKG2C+/del</td>
<td>19 (6)</td>
<td>13 (5)</td>
<td>12 (6)</td>
<td>1 (2)</td>
<td>9 (7)</td>
<td>6 (7)</td>
<td>3 (8)</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>

aViremia assessed to confirm CMV symptomatic infection.
bViremia systematically monitored by qPCR in all patients (see Materials and Methods).
cNo symptomatic infection.
dSymptomatic infection. The imbalance between the NKG2C+/+ and NKG2C+/del genotype frequencies (bold) observed in this group from both cohorts did not reach statistical significance.

eUndetected viremia by qPCR.
fDetected viremia by qPCR.
study. The relatively low frequency of the NKG2C<sup>del/del</sup> genotype in the general population (25, 26, 28) together with the limited number of cases with CMV symptomatic infection limits the conclusiveness of this analysis, which should be validated in a larger study with a longer follow-up. Yet, this intriguing observation might reflect a reduced graft survival in NKG2C<sup>del/del</sup> KTRs with symptomatic CMV replication, consistent with a potential role of adaptive NKG2C<sup>C</sup> NK cells in antiviral defense. Alternatively, the homozygous deletion might be associated with a lower incidence of CMV infection. In this regard, expansions of NKG2C<sup>C</sup> NK cells expressing activating KIRs were identified in NKG2C<sup>C</sup> del/del hematopoietic stem cell transplant recipients suffering CMV infection (39), supporting the existence of alternative adaptive NK cell subsets in individuals with KIR-B haplotypes (i.e., which encode more activating KIR), a genetic feature previously associated with a lower incidence of CMV infection in KTR (40, 41). Nevertheless, no relation between expansions of NKG2C<sup>C</sup> cells and KIR-A and KIR-B haplotypes was previously observed (7); moreover, NKG2C<sup>C</sup> NK cell populations displaying adaptive phenotypic features independently of activating KIR expression have been recently reported (19, 42).

In summary, to our knowledge, this study provides the first evidence indirectly supporting a role of adaptive NKG2C<sup>C</sup> NK cells in defense against CMV in KTRs. The association of high pretransplant levels of NKG2C<sup>C</sup> NK cells with a reduced incidence of posttransplant CMV viremia suggests that they may confer some protection against viral reactivation or reinfection. From a practical standpoint, high baseline levels of NKG2C<sup>C</sup> cells might predict a lower risk for posttransplant CMV replication and disease in KTRs receiving regular immunosuppression, particularly in NKG2C<sup>C</sup>C<sup>+</sup> CMV<sup>+</sup> patients. This information might contribute to stratifying KTRs for cost-effective use of CMV prophylaxis, which is not free of adverse events (43, 44). In contrast, posttransplant expansions of adaptive NKG2C<sup>C</sup> NK cells are reactive to the incidence of CMV infection and, once established, may contribute to controlling viral replication. In that case, the individual evolution pattern of the adaptive NK cell response might become helpful to monitor CMV control. Yet, the observational design and relatively reduced number of cases are limitations of this report. Larger prospective studies comparatively analyzing pretransplant adaptive NK cells and CMV-specific T lymphocytes are warranted, because both might become complementary useful biomarkers in the clinical management of the viral infection in KTRs.

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References


