Cytomegalovirus-Responsive γδ T Cells: Novel Effector Cells in Antibody-Mediated Kidney Allograft Microcirculation Lesions

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ABSTRACT

Human cytomegalovirus infection in transplant recipients has been associated with adverse renal allograft outcome and with a large γδ T-cell response, but whether both mechanisms are connected is unknown. We previously showed that most expanded circulating cytomegalovirus-responsive γδ T cells express the Fcγ receptor CD16, suggesting that γδ T cells may participate in allograft lesions mediated by donor-specific antibodies through antibody-dependent cellular cytotoxicity. Here, we show that cytomegalovirus-specific CD16pos γδ T cells can perform antibody-dependent cellular cytotoxicity against stromal cells coated with donor-specific antibodies in vitro. In vivo, graft-infiltrating γδ T cells localized in close contact with endothelial cells only in patients who experienced cytomegalovirus infection and were more frequent within peritubular capillaries and glomeruli from antibody-mediated acute rejections than within those from T cell–mediated acute rejections. Finally, a persistently increased percentage of circulating cytomegalovirus-induced γδ T cells correlated inversely with the 1-year eGFR only in kidney recipients with donor-specific antibodies. Collectively, these data support the conclusion that cytomegalovirus-induced γδ T cells are involved in, and may serve as a clinical biomarker of, antibody-mediated lesions of kidney transplants. Moreover, these findings offer a new physiopathologic link between cytomegalovirus infection and allograft dysfunction in recipients with donor-specific antibodies.


In kidney transplant recipients (KTRs), the importance of the recipient’s humoral response against the allograft has been recognized to play a key role in immunologic injuries contributing to graft deterioration.1–6 From an immunologic point of view, donor-specific antibody (DSA)–mediated lesions are considered to rely on complement-fixing DSA-mediated lysis, direct DSA-mediated apoptosis, or antibody-dependent cell-mediated cytotoxicity (ADCC) by natural killer (NK) cells. Until recently, complement was the most recognized way of leading to graft endothelial cell injury. Indeed, deposition of C4d, a breakdown product of complement component C4, in peritubular capillaries still represents the only specific tool providing the “immunopathologic evidence” of DSA interaction with graft tissue.7–11 However, it does not encompass all DSA-mediated lesions.12

Several groups reappraised the multiplicity of mechanisms leading to antibody-mediated rejections (AMR).13 Glomerulitis and peritubular capillaritis are defined by an accumulation of polymorphonuclear cells, macrophages, and lymphocytes around capillaries. These infiltrates are associated with DSA and indicate a poor prognosis.14–16 Among these infiltrates, NK cells have recently been shown to be involved in
DSA-mediated lesions of kidney microcirculation, suggesting that ADCC could play a role in DSA-mediated lesions through DSA interaction with the low-affinity Fc receptor for IgG (FcyRIIIA-CD16) expressed on NK cells. Signaling through CD16 is instrumental for ADCC as it leads to cytolytic granule content release and subsequent death of target cells.

Besides NK cells, and unlike conventional αβ T cells, γδ T cells can also express CD16 at high levels, enabling them to efficiently mediate ADCC. In human transplantation, γδ T lymphocytes have been strongly linked to cytomegalovirus (CMV) infection, itself associated with rejection. A specific and persistent expansion of a γδ T-cell subset normally located in the epithelia (called Vδ2+γδ T cells and mainly composed of Vδ1 and Vδ3 T cells) is observed in peripheral blood during CMV infection in all solid-organ transplantations. From our experience, CMV is the only clinical cause of Vδ2+γδ T-cell expansion in KTR. This tight association between CMV infection and γδ T-cell expansion has been confirmed in many other pathophysiologic contexts. In vitro, clones of Vδ2+γδ T cells display T-cell receptor (TCR)–dependent cytotoxicity against both CMV-infected cells and carcinoma cells. Accordingly, their expansion in kidney transplant recipients correlates with both reduced cancer risk and resolution of CMV infection, suggestive of their antiviral function.

Interestingly, we recently observed that most (around 80%) Vδ2+γδ T cells from CMV-infected individuals expressed CD16, whereas CMV-specific CD8+αβ T cells or Vδ2+γδ T cells did not. CMV infection deeply reshapes the CD16+ lymphocyte compartment composition in CMV+ transplant recipients who exhibit an equal amount of CD16+ NK cells and CD16+ γδ T cells at the periphery. The latter are able to produce high levels of IFN-γ when recognizing IgG-opsonized CMV particles. This cooperation between γδ T cells and the humoral response could represent an interesting control mechanism of CMV reactivation in chronically infected tissues and of CMV spread in blood.

Collectively these results raise the possibility that, in the context of transplantation and in the presence of DSA, reorganization of the CD16+ lymphocyte compartment following CMV infection could have a deleterious effect on the graft. The aim of the present study was to evaluate whether CMV-induced CD16+ γδ T cells were able to mediate ADCC against graft endothelial cells in the presence of DSA, a process that could participate in the association between CMV and DSA-mediated rejection.

RESULTS

Model of KTR DSA Binding to Endothelial and Fibroblastic Cells
To assess the potential allo cytotoxic effect of CMV-induced γδ T cells in the presence of DSA, we used allogeneic stromal cell lines recognized by DSA. To this purpose, we assessed the ability of sera from eight KTRs with DSA (sensitized KTRs, S3–S10) and from two nonsensitized KTRs (S1 and S2) to bind three allogeneic HLA-typed “stromal” cell lines: an endothelial cell line (IVEC), primary foreskin fibroblasts (FSF), and MRC5. Cell line–specific HLA antibodies (CLSA) levels in the sera were first evaluated using the HLA class I single antigen bead (SAB) assay (Tables 1, 2 and 3).

As expected, control sera (S1 and S2) did not contain CLSA. Sera S3, S4, S7, and S8 contained high levels of CLSA. Although containing DSA, sera S5, S6, S9, and S10 contained low levels of CLSA.
The capacity of CLSA to bind to the allogeneic cells was next confirmed by flow cytometry (Figure 1A). The most important stainings were obtained when the three cell lines were incubated with sera S3, S4, S7, and S8, which contained the highest levels of CLSA (Figure 1B). Accordingly, a strong correlation was observed between the CLSA mean fluorescence intensity (MFI) analyzed by SAB and the MFI of cell line staining analyzed by flow cytometry \( (r^2=0.74; P<0.001) \) (Figure 1C). These results suggested that the stromal cell–binding activity of patient’s sera is mostly mediated by CLSA and that this assay is a reliable in vitro model to investigate DSA-dependent ADCC.

**γδ T Cell–Mediated ADCC against Endothelial and Fibroblastic Cells**

We then tested the capacity of CMV-induced CD16pos γδ T-cells to mediate ADCC against endothelial and fibroblastic cell lines coated with CLSA. To this end, a CD16pos γδ T cell line was generated from a CMV–infected donor. FSF, IVEC, and MRC5 cell lines, preincubated with sera containing (S3, S4, S7, and S8) or not containing (S1 and S2) CLSA, were cocultured with CD16pos γδ T cells in a 4-hour chromium release assay. CD16pos γδ T cell line was able to kill the three allogeneic cell lines in the presence of CLSA-positive serum (Figure 2). Killing in the absence of serum or in the presence of CLSA-negative serum was equal or close to background, ruling out a direct allogeneic cytotoxicity of γδ T cells. Likewise, there was no direct cytotoxicity of CLSA on target cells in the absence of γδ T cells (data not shown). Finally, the percentage of chromium release at a 30:1 effector:target ratio by γδ T cells closely correlated with the IgG-binding MFI analyzed by flow cytometry \( (r^2=0.44 \text{ [cumulated correlation for the three cell lines]; } P=0.01) \) (Figure 2B). Collectively, these data demonstrated the capacity of CD16pos γδ T cells to mediate DSA-dependent ADCC against endothelial and fibroblastic cells.

**CLSA-Dependent ADCC by γδ T Cells Relies on CD16 and Perforin**

We next investigated the mechanisms by which γδ T-cells mediate CLSA-dependent ADCC. First, we demonstrated that CD16 was required as γδ T cell–mediated ADCC was prevented with use of a blocking anti-CD16 mAb or a CD16-negative γδ T cell line (Figure 3A for FSF preincubated with serum S8). Similar results were obtained using MRC5 and IVEC and using other CLSA-positive sera (data not shown). Second, we tested whether productive ADCC required the release of cytolytic granules. CD16pos γδ T-cells expressed high levels of granzyme B and perforin (Figure 3B). Concanamycin A, an inhibitor of cytotoxic granule content release, abrogated ADCC by CD16pos γδ T cell line in a dose-dependent manner (Figure 3C). In conclusion, the DSA-dependent γδ T cell–

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**Figure 1.** Anti-class I HLA antibodies from sensitized kidney transplant recipients sera bind on fibroblastic and endothelial cells. (A) Representative binding on FSF of IgG from different sera using a goat anti-human IgG antibody coupled to FITC and analyzed by flow cytometry (numbers indicate the MFI). Pool S+, pool of sensitized sera; S1, nonsensitized serum; S3 and S4, sera containing CLSA; S9, sensitized serum without CLSA. (B) MFI obtained with each serum on all three cell lines. Data are representative of three different experiments. (C) Linear regression analysis of CLSA MFI analyzed by SAB assay versus the MFI of the IgG binding analyzed by flow cytometry on the three cell lines.
ADCC relies on CD16, the triggering of which elicited cytotoxic granule content release, similar to NK cell–mediated ADCC.

**γδ T Cells Are Present in AMR Microcirculation Lesions of CMV-Infected Patients**

Peritubular capillaritis and glomerulitis are early signs of the microcirculation lesions characterizing AMR. To know whether γδ T cells could be involved in this process, we examined their potential colocalization with these organic lesions. Sections of kidney graft diagnosed with acute AMR (n=10) were stained with an anti-CD31 mAb (to localize the endothelial cells) and an anti-γδ TCR mAb. Interestingly, γδ T cells were found in close contact with endothelial cells in peritubular capillaritis and glomerulitis lesions associated with acute AMR (Figure 4A, Suplementary Figure 1). This association was specific, as it was not found for peritubular capillaries or glomeruli of allograft presenting with acute T cell–mediated rejection (acute TCMR, n=13). Furthermore, when we distinguished CMV-experienced patients (R− patients with postgraft CMV primary infection and all R+ patients) from CMV-naive patients (R− patients without postgraft CMV infection), γδ T cells were more frequent in peritubular capillaries and glomeruli of patients with AMR (mean, 34.8 cells/mm³ and 2.6 cells/mm³, respectively) than in those of patients with TCMR (3.8 cells/mm³ and 0.1 cells/mm³, respectively) only among CMV-experienced patients (Figure 4B, Supplemental Figure 1). Of note, two of the seven biopsy specimens from CMV+ patients were negative for C4d staining and showed the highest levels of γδ T cells/mm², suggesting that γδ T-cell presence was independent of complement activation. Moreover, these seven biopsy specimens were negative for CMV antigens (not shown), suggesting that γδ T-cell presence in lesions was related more to the presence of DSA than to that of CMV. In CMV-naive patients, the rate of γδ T cells in peritubular capillaries and glomeruli was low in both TCMR and AMR (Figure 4B). γδ T cells could also be found in the interstitium but were more frequent during TCMR than during AMR, whatever the CMV status of the patient. Together, these results suggest that CMV-induced γδ T cells localize into the AMR microcirculation lesions, where they may mediate graft rejection through ADCC.

**Correlation of γδ T-Cell Counts with eGFR at 12 Months after Transplant in Patients with DSA**

If the assumption that CMV-induced γδ T cells are involved in AMR is true, their expansion in patients with DSA should be associated with renal dysfunction. In a recent study, we observed that among sensitized KTRs, those who had the highest risk for graft rejection and the lowest eGFR 12 months after transplantation were the patients who had anti-HLA DSA the day of the graft. We tested a potential link between circulating γδ T cells at 12 months after transplant and DSA-mediated rejection in this cohort of 21 KTRs with DSA. For a control group, we used 162 KTRs without DSA, whose characteristics are summarized in Table 4. Among the 21 consecutive sensitized KTRs, baseline donors’ and recipients’ characteristics, as well as DSA MFI levels, did not significantly differ between those with rejection and...
those without rejection (Table 5). Notably, 19 of these 21 patients had experienced CMV infection before or after the transplantation. No statistical differences of the percentage of total γδ or Vδ2neg γδ T cells were observed between patients with TCMR, AMR, or without rejection (Figure 5A). However, the circulating γδ T cells and Vδ2neg γδ T cells were strongly and inversely correlated with the eGFR 12 months after transplant ($r^2=0.44$ [$P=0.003$] and $r^2=0.43$ [$P<0.01$], respectively) (Figure 5B).

Figure 3. CLSA-dependent ADCC by γδ T cells depends on CD16 and perforin. (A) CD16pos (left panel) or CD16neg (right panel) γδ T cell–mediated lysis of the FSF primary cell line preincubated with the serum S8 (+++) or S1 (−) in the presence or absence of a blocking anti-CD16 mAb or control mAb. (B) Expression of intracellular granzyme B and perforin in CD16pos γδ T cells. Numbers indicate percentages of positive cells. (C) As in part A with the addition or no addition of indicated concentrations of concanamycin A (CMA). Viability of γδ T cells was >80% even after incubation of 7 hours with the highest concanamycin A concentration (data not shown). Data are representative of three different experiments. Ctrl, control.

Figure 4. γδ T cells are present in antibody-mediated peritubular capillaritis and glomerulitis of CMV-infected patients. Triple-immunofluorescence staining for CD31 (green), γδ T cells (red), and nuclei (blue) on graft biopsy specimens from patients with acute AMR ($n=10$) and acute TCMR ($n=13$). (A) Representative staining of γδ T cells in microcirculation lesions associated with acute AMR (upper panel) and in glomerulitis (lower panel). Arrows indicate γδ T cells and arrowheads indicate endothelial cells of the micro-circulation. Original magnification ×600. (B) Quantification of γδ T cells in peritubular capillaries and glomeruli of acute AMR or TCMR graft biopsy specimens in CMV-experienced (CMV+′′, upper panels) and CMV-naive (CMV−′′, lower panels) patients.
Conversely, in the control group of 162 nonsensitized KTRs, no correlation between γδ or Vδ2neg γδ T cells and post-transplant eGFR ($r^2<0.001$ [P=0.2] and $r^2<0.001$ [P=0.7]), respectively) (Figure 5C) was observed, even when we focused on the 98 CMV-experienced patients ($r^2=0.02; P=0.2$ [data not shown]). In summary, high percentages of CMV-induced Vδ2neg γδ T cells were associated with a poor eGFR only in KTRs with DSA, suggesting cooperation between CMV, γδ T cells, and DSA to induce graft dysfunction.

**DISCUSSION**

Allogeneic reaction is considered the result of direct or indirect recognition of foreign MHC molecules by alloreactive T lymphocytes. Therefore, γδ T cells are usually viewed as non-alloreactive because they do not recognize peptides bound to MHC molecules. However, γδ T cells express a broad range of activatory molecules that allow them to respond to stressed cells and that could potentially participate in a different type of reactivity against allografts. These molecules include the γδ TCR itself, which can recognize self-stress antigens, or NKG2D, which binds stress-induced MHC-like ligands (MHC class I–related chain and UL16-binding protein). Here, we describe the implication of CD16 in the alloreactive potential of γδ T cells responding to CMV infection.

We first present a model of anti-HLA ADCC, using sera from patients sensitized to HLA-A/B antigens expressed by “model” stromal cell lines. Using this model, we demonstrated that CMV-induced γδ T cells can make DSA dose-dependent ADCC. The lytic capacity of γδ T cells was strongly dependent on patients’ sera presence in the assay, excluding direct recognition of putative stromal cell line stress antigens by γδ T-cell activatory receptors (e.g., TCR, NKG2D, etc.). Of note, slight IVEC lysis can be observed with sera from nonsensitized KTRs (15% at a 30:1 effector:target ratio), suggesting the presence of antibodies to other antigens specifically expressed on IVEC. Antibodies to endothelial cell antigens other than HLA antigens have been previously detected in KTRs, particularly in relation to CMV infection.

Figure 5. γδ T-cell levels correlate with eGFR at 12 months after transplant in patients with DSA. (A) Median, 25th–75th percentiles and 10th–90th percentiles of γδ T cell (right panel) or Vδ2neg γδ T cell (left panel) percentages in patients with acute TCMR, with acute AMR, or without rejection. (B) Linear regression analysis between circulating γδ T-cell percentages (right panel) or Vδ2neg γδ T-cell percentages (left panel) and the 12 month post-transplant eGFR in KTRs with DSA the day of the graft. Plain circles are CMV-experienced patients and open circles are CMV-naive patients. (C) Same analysis as in part B in anti-HLA nonsensitized KTRs.
Table 4. Characteristics of the 162 KTRs without DSA according to occurrence of TCMR (no AMR was observed in this cohort)

<table>
<thead>
<tr>
<th>Variable</th>
<th>TCMR (n=23)</th>
<th>No Rejection (n=139)</th>
<th>P Value</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age at time of graft (yr)</td>
<td>47 (16–70)</td>
<td>45 (20–58)</td>
<td>0.7</td>
<td>47 (16–70)</td>
</tr>
<tr>
<td>Men/women (n/n)</td>
<td>17/6</td>
<td>98/41</td>
<td>0.6</td>
<td>115/47</td>
</tr>
<tr>
<td>Transplantation condition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor age (yr)</td>
<td>38 (7–68)</td>
<td>44 (21–64)</td>
<td>0.08</td>
<td>39 (7–68)</td>
</tr>
<tr>
<td>Expanded-criteria donor (%)</td>
<td>5 (22)</td>
<td>21 (15)</td>
<td>0.06</td>
<td>26 (16)</td>
</tr>
<tr>
<td>Ischemia time (hr)</td>
<td>21 (5–35)</td>
<td>21 (8–37)</td>
<td>0.9</td>
<td>21 (5–37)</td>
</tr>
<tr>
<td>Delayed graft function, n (%)</td>
<td>7 (30)</td>
<td>24 (17)</td>
<td>0.1</td>
<td>31 (19)</td>
</tr>
<tr>
<td>Immunologic variables at grafting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA mismatch: A-B-DR</td>
<td>3 (1–5)</td>
<td>3 (0–6)</td>
<td>0.7</td>
<td>3 (0–6)</td>
</tr>
<tr>
<td>Induction immunosuppressive treatment (n/n)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tacrolimus/cyclosporine</td>
<td>8/15</td>
<td>69/70</td>
<td>0.2</td>
<td>77/85</td>
</tr>
<tr>
<td>Mycophenolate mofetil/azathioprine</td>
<td>20/3</td>
<td>121/18</td>
<td>0.9</td>
<td>141/21</td>
</tr>
<tr>
<td>ATG/anti–IL-2R antibodies/no induction</td>
<td>2/14/7</td>
<td>11/67/61</td>
<td>0.6</td>
<td>13/81/68</td>
</tr>
<tr>
<td>CMV status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregraft CMV infection</td>
<td>11 (48)</td>
<td>71 (51)</td>
<td>0.6</td>
<td>82 (51)</td>
</tr>
<tr>
<td>Postgraft CMV infection</td>
<td>4 (17)</td>
<td>35 (26)</td>
<td>0.5</td>
<td>39 (24)</td>
</tr>
<tr>
<td>Median γ6 TL, % (minimum–maximum)</td>
<td>3 (0–16.9)</td>
<td>3.8 (0.6–25.7)</td>
<td>0.3</td>
<td>3.75 (0–25.7)</td>
</tr>
<tr>
<td>Median Vd2neg γ6 TL, % (minimum–maximum)</td>
<td>1.79 (0–10.3)</td>
<td>2.2 (0.1–24.6)</td>
<td>0.3</td>
<td>2.15 (0–24.6)</td>
</tr>
</tbody>
</table>

Unless otherwise noted, values are expressed as the median (range) or number (percentage). ATG, antithymocyte polyclonal antibodies; TL, T lymphocytes.

Table 5. Characteristics of the 21 KTRs with DSA according to occurrence of rejection

<table>
<thead>
<tr>
<th>Variable</th>
<th>Acute TCMR (n=7)</th>
<th>Acute AMR (n=6)</th>
<th>No Rejection (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age at time of graft (yr)</td>
<td>55 (22–70)</td>
<td>46 (33–61)</td>
<td>52 (25–64)</td>
</tr>
<tr>
<td>Men/women (n/n)</td>
<td>2/5</td>
<td>4/2</td>
<td>4/4</td>
</tr>
<tr>
<td>Risk factors of HLA sensitization (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of blood transfusion</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>History of pregnancy</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>&gt;1 kidney transplant</td>
<td>4</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Transplantation conditions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor age (yr)</td>
<td>56 (18–70)</td>
<td>46 (22–59)</td>
<td>48 (18–63)</td>
</tr>
<tr>
<td>Expanded-criteria donor (%)</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Ischemia time (hr)</td>
<td>23 (13–33)</td>
<td>25 (23–34)</td>
<td>20 (14–34)</td>
</tr>
<tr>
<td>Delayed graft function (n)</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Immunologic variables at grafting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA mismatch: A-B-DR-DQ</td>
<td>4 (1–7)</td>
<td>4.5 (1–5)</td>
<td>4.5 (2–7)</td>
</tr>
<tr>
<td>MFI ratio of T cell–positive FCXM</td>
<td>2.20 (0.28–2.46)</td>
<td>1.98 (0.97–7.69)</td>
<td>1.47 (1.18–2.22)</td>
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<tr>
<td>MFI ratio of B cell–positive FCXM</td>
<td>2.83 (1.38–33.02)</td>
<td>3.84 (0.74–25.06)</td>
<td>3.84 (1.38–8.32)</td>
</tr>
<tr>
<td>Day 0 anti-class I DSA</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Day 0 anti-class II DSA</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>MFI of day 0 anti-class I DSA</td>
<td>600 (0–7500)</td>
<td>1250 (0–7900)</td>
<td>300 (0–7900)</td>
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<tr>
<td>MFI of day 0 anti-class II DSA</td>
<td>0 (0–15,800)</td>
<td>550 (0–3000)</td>
<td>500 (0–8600)</td>
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<tr>
<td>MFI of day 0 anti-class I+II DSA</td>
<td>2400 (600–15,800)</td>
<td>2750 (1100–7900)</td>
<td>2200 (600–9700)</td>
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<td>Induction immunosuppressive treatment (n)</td>
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<tr>
<td>Tacrolimus/mycophenolate mofetil</td>
<td>7</td>
<td>5</td>
<td>8</td>
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<td>ATG/ anti–IL-2R antibodies</td>
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<td>Induction with IVIG</td>
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<td>CMV status</td>
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<tr>
<td>Pregraft CMV infection (n)</td>
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<td>Postgraft CMV infection (n)</td>
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<td>2</td>
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<td>Pre- or postgraft CMV infection (n)</td>
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<td>7</td>
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<td>Median γ6 TL, % (minimum–maximum)</td>
<td>5.4 (2.8–9.5)</td>
<td>6.5 (2.9–14.3)</td>
<td>4.2 (1.2–12.9)</td>
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<tr>
<td>Median Vd2neg γ6 TL, % (minimum–maximum)</td>
<td>3.19 (1.6–9.3)</td>
<td>5.9 (0.6–13.6)</td>
<td>3.0 (0.5–12.1)</td>
</tr>
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</table>

Unless otherwise noted, values are expressed as the median (range) or number of patients. FCXM, flow cytometric cross-match.
combination with endothelial cell staining through CD31, we could precisely define γδ T-cell localization within the graft. T cells were observed in close contact with peritubular capillaries and glomerulitis associated with acute AMR in CMV-experienced patients. This localization is consistent with γδ T-cell involvement in antibody-mediated microcirculation injuries, such as that reported for NK cells17 and macrophages.47–49 The fact that higher numbers of γδ T cells can be found in these areas in grafts from CMV-experienced patients compared with grafts from CMV-naive patients suggests that only CMV-induced γδ T cells contribute to DSA-mediated microcirculation injuries, in agreement with their expansion and CD16 overexpression in response to CMV. Because of the limited choice in antibodies against CD16 and γδ TCR suitable for tissue immunofluorescence double-staining, CD16 expression by γδ T cells in the graft remains to be established. Staining of CD16 alone was positive but might also be attributed to NK cells or polymorphonuclear cells (data not shown). However, because higher γδ T-cell numbers were found in peritubular capillaries and glomeruli in acute AMR compared with acute TCMR, we can postulate that DSA and their likely binding to CD16 were required for this specific localization. In addition, there was no increase (and even a significant decrease, P=0.01 using a Mann–Whitney comparison) in γδ T-cell number within the interstitium lymphocyte infiltrate in CMV-experienced patients compared with CMV-naive patients during TCMR, suggesting that CMV-mediated γδ T-cell blood expansion is not the only reason for their migration into the graft.

This suspected γδ T-cell role in acute AMR is supported by the association of decreased graft function in DSA-positive patients with a high percentage of circulating CMV-induced γδ T cells. This is in marked contrast with CMV-specific CD8 αβ T cells, which have been shown to associate with less alloreactivity and improved graft function,39 consistent with their absence of CD16 expression.35 γδ T-cell ADCC could represent a new physiopathologic contribution to the well known but poorly understood association between CMV infection and the increased occurrence of rejection,20,51 poor long-term graft function,21,52–54 and low graft survival.55,56 Hence, both CMV and DSA are required to see a correlation between circulating γδ T cells and graft function deterioration, consistent with a model where CMV infection evokes activation and expansion of CD16pos γδ T cells in undetermined anatomic sites, and DSA triggers their ADCC function within graft microcirculation. This implication of γδ T cells in graft rejection seemingly contrasts with the previously reported Vδ1 T-cell expansion in the peripheral blood and grafts of operationally tolerant liver transplant recipients.57–59 However, Sánchez-Fueyo et al. recently revised their interpretation of Vδ1 expansions, confirming that they relate to CMV infection and are not restricted to tolerant liver recipients.24

Even though the Banff diagnostic criteria of antibody-mediated rejection requires positive C4d peritubular capillary staining, it is now well accepted that C4d does not explain all antibody-mediated lesions.12,60 Complement-independent pathways have been proposed, and their characterization could help define novel markers of DSA-mediated microcirculation damage. NK cells are suspected to play a role because NK-cell transcripts or transcripts induced by IFN-γ suspected to be produced by NK cells are increased in AMR biopsy specimens.18,61 Moreover, peripheral blood NK cells from HLA-sensitized patients produce IFN-γ in a CD16-dependent manner when exposed in vitro to allogeneic cells plus alloantibodies.62 CMV-responsive γδ T cells share many features with NK cells, such as common expression of activating (e.g., Nkp80, NKG2D, CD16), and inhibiting (e.g., CD158, CD85j) receptors;26,31 expression of specific cytotoxic molecules, such as granulysin (J. Déchanet-Merville, unpublished data); and production of IFN-γ.32,35 Transcripts for some of these molecules have been found in kidney biopsy specimens from patients with DSA and have been attributed to NK cells.7 We know, however, from previous studies that engagement of CD16 can also induce IFN-γ production by γδ T cells,35 and that this production depends on a Th1 cytokinic environment. Transplant glomerulopathies were associated with a predominantly Th1 and cytotoxic profile, which could provide this context.63 It is then conceivable that both NK cells and γδ T cells could contribute to AMR through ADCC.

In conclusion, CMV elicits the emergence of an unconventional T-cell subpopulation that does not have intrinsic alloreactivity but has the potential to be activated by alloantibodies through CD16. These γδ T cells are able to control CMV and to limit tumor occurrence but also appear as new players in complement-independent AMR. Because they are found selectively in peritubular capillaries in acute AMR, their detection could be used as a clinical marker to aid in diagnosis, particularly in C4d-negative humoral rejection. More easily, CD16pos Vδ2neg γδ T-cell expansion in peripheral blood can also be monitored and considered as a poor prognostic factor in DSA-positive patients.

CONCISE METHODS

This study was approved by the Institutional Review Board of the Bordeaux Hospital.

Cell Lines and HLA Typing

FSFs were prepared in the laboratory with the help of Catherine Pain (National Institute of Health and Medical Research U1035, Bordeaux) by a classic tissue-dissociation procedure. Fetal lung fibroblasts (MRC5) were purchased from Eurobio. IVECs were obtained from human umbilical vein endothelial cells immortalized with SV40 virus and kindly provided by the Pasteur Institute.64 DNA HLA class I typing of cell lines was performed using the routine LabType SSO HR assay (One Lambda, Canoga Park, CA) following the manufacturer’s instructions. Results of HLA typing were as follows: FSF, A*02,*29; B*35,*51; for MRC5, A*02,*29; B*07,*44; and for IVEC, A*02,*30; B*07,*44.

Characterization of Anti-HLA Antibodies in Patient Sera

Sera from sensitized KTRs were tested for HLA class I antibodies using the SAB assay (LabScreen single antigen LS1A04 and LS2A01; One
from target cells in medium alone was always measured by a chromium release assay. Target cells (FSF, MRC5, IVEC) were labeled with $^{51}$Cr and analyzed on a FACScanto cytometer (BD Biosciences). A pool of immunized sera (pool S+) covering all HLA specificities was used as a positive control.

**Generation of Polyclonal γδ T Cell Lines**

A long-term CD16$^+$ Vδ2$^{\text{neg}}$ γδ T cell line was selected on the basis of persistent expression of CD16 in culture as follows: Vδ2$^{\text{neg}}$ γδ T cells from PBMCs of a CMV-seropositive blood donor were sorted on a FACSAria (BD Biosciences) using a combination of anti-CD3, anti-Vδ2, and anti-TCRγδ. The sorted Vδ2$^{\text{neg}}$ γδ T cells were expanded in culture in RPMI medium supplemented with 10% human serum, 1000 U/ml IL2 (Chiron), 1 μg/ml leuco-agglutinin (Sigma-Aldrich), and irradiated allogeneic PBMCs (35 Gy). After 1 month in culture, the Vδ2$^{\text{neg}}$ γδ T cell line was phenotyped for CD16 expression. CD16$^{\text{neg}}$ and CD16$^{\text{pos}}$ cells were sorted by flow cytometry, expanded as previously described, and then used in the experiments.

**Phenotyping of γδ T Cells by Flow Cytometry**

mAbs directed against the following molecules were purchased from Beckman-Coulter: TCR-Cα, TCR-Vβ, and CD16. mAbs directed against CD3, perforin, and granzyme B were from BD Biosciences. Staining was performed on γδ T cell lines as previously described and analyzed on a FACScanto cytometer (BD Biosciences) with the use of the FACSDiva software, version 6.1.2 (BD Biosciences). At least 10,000 T lymphocytes were analyzed for six-color staining. Permeabilization and fixation of cells for intracellular staining were performed as described elsewhere.

**Cell Cytotoxicity Assays**

Cytolytic activity of γδ T cells was measured by a standard chromium release assay. Target cells (FSF, MRC5, IVEC) were labeled with $^{51}$Cr (1.85 MBq/10$^6$ cells) for 1 hour at 37°C, washed, and incubated with selected sera for 20 minutes. After washes, 3000 cells/well were incubated in triplicate with γδ T cells at the indicated effector:target ratio. After 4 hours at 37°C, $^{51}$Cr released in the supernatant was measured. The percentage of specific lysis was calculated as ((experimental release–spontaneous release)/(maximum release–spontaneous release)) × 100. The spontaneous $^{51}$Cr release from target cells in medium alone was always <15% of the control maximal $^{51}$Cr release obtained with 1% Triton X-100. In some experiments, γδ T-effector cells were preincubated in plates at 37°C for 2.5 hours in 50 μl of culture medium with different concentrations of concanamycin A (Calbiochem; Sigma-Aldrich), which is known to prevent cytotoxic granule content release through a pH increase of the vacuoles.

**In Situ Immunofluorescence Studies**

Renal tissues were from selected graft biopsy fragments exhibiting different type of acute rejections (acute AMR) and acute TCMR. Kidney biopsy specimens were distinguished according to the status of the kidney transplant recipients related to CMV: those coming from patients who had never experienced CMV infection (seronegative on the day of the graft and no infection after transplantation) and those from patients who had been in contact with CMV before (R+) or after (R−) CMV primary infection) transplantation. All kidney biopsy specimens were graded according to the last Banff criteria meeting report. Seven biopsy specimens with AMR lesions and from CMV-experienced patients were also assessed for the presence of CMV antigens using the mAb 810–500 from Abcys. Immunofluorescence was performed on frozen graft-biopsy fragments using rabbit anti-CDS1 mAb (clone ab28364, 1/75; Abcam, Inc.) to stain endothelial cells, and mouse pan-γδ mAb (clone IMMUS10, 1/50, Beckman-Coulter). Differential staining revelation was performed using Alexa-488–coupled anti-rabbit antibody (green staining) and Alexa-533–coupled anti-mouse (red staining), both from Invitrogen. Nuclei were stained with 0.5 μg/ml DAPI (blue staining). Staining analysis was done on an inverted Leica DMI 6000 microscope (Leica Microsystems) equipped with a Quantum camera (Roper Sciences). A control group of patients without DSA was selected in our cohort. From 1998 to 2002, we retrospectively identified 162 consecutive nonsensitized KTRs who underwent transplantation with a negative cross-match, who had not developed DSA at 1 year after transplantation. For the purpose of the study, d0 and year 1 sera were retested and analyzed by MetaMorph software. Cellular infiltrate (expressed in number of stained cells per mm$^2$) was assessed in high-power fields on the entire cortical region of the biopsy specimens by observers blinded to the origin of the slides. Cells were considered to be part of the capillaritis using the Banff criteria.

**Patients**

Twenty-one sensitized KTRs transplanted with anti-HLA DSA the day of the graft (determined by both the SAB assay and the flow cytometric cross-match) were analyzed retrospectively for this study to appraise the potential association between γδ T cells and the incidence of biopsy–proven acute rejection and 1-year eGFR (calculated using the Modification of diet in Renal Disease formula). A control group of patients without DSA was selected in our cohort. From 1998 to 2002, we retrospectively identified 162 consecutive nonsensitized KTRs who underwent transplantation with a negative flow cytometric cross-match, who had not developed DSA at 1 year after transplant. For the purpose of the study, d0 and year 1 sera were retested for class I and class II antibodies using SAB assays (LabScreen single antigen LS2A01 for class II, One Lambda). γδ T-cell subsets are measured routinely in our center in all transplant recipients.

**Statistical Analyses**

Graphs of linear regression were performed using Statview software (Abacus Concepts, Berkeley, CA). Cellular infiltrates are expressed as means and ranges. The statistical differences between groups of AAMR and ACMR were tested with the unpaired Mann–Whitney test. A P value<0.05 represents statistical differences.
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DISCLOSURES

None.

REFERENCES


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