American Journal of Transplantation 2016; 16: 2384–2394
Wiley Periodicals Inc.



doi: 10.1111/ajt.13781

# Easier Control of Late-Onset Cytomegalovirus Disease Following Universal Prophylaxis Through an Early Antiviral Immune Response in Donor-Positive, Recipient-Negative Kidney Transplants

**H. Kaminski[1], L. Couzi[1,2], I. Garrigue[3,4], J.-F. Moreau[2,5], J. Déchanet-Merville[2] and P. Merville[1,2,*]**

[1]*Department of Nephrology, Transplantation and Dialysis, Bordeaux, France*
[2]*National Center of Scientific Research (CNRS), Research Unit 5234, Bordeaux, France*
[3]*Virology Laboratory, Bordeaux, France*
[4]*National Center of Scientific Research, Mix Unit of Research 5164, Bordeaux, France*
[5]*Immunology Laboratory, Pellegrin Hospital, Bordeaux, France*
**Corresponding author: Pierre Merville, pierre.merville@chu-bordeaux.fr*

**Universal prophylaxis for cytomegalovirus (CMV) prevention is viable but, compared with a preemptive strategy, leads to higher incidence of late-onset disease (LOD) associated with poor patient and graft survival. The purpose of this study was to compare LOD with early onset disease (EOD), with a focus on the highest risk kidney transplant recipients (KTRs): CMV seronegative recipients transplanted from seropositive donors (D+R−). Since CMV control depends on both antiviral treatment and specific immune response, we also compared Vδ2-negative ($V\delta2^{neg}$) γδ T cell expansion involved in CMV infection resolution. EOD was defined as occurring <3 mo and LOD as occurring >3 mo after transplantation. Depending on the period, universal prophylaxis or preemptive treatment was used. Overall, 168 D+R− KTRs were included between 2003 and 2011. LOD was associated with a lower peak DNAemia ($p = 0.04$), fewer recurrences (odds ratio 0.16; 95% confidence interval 0.05–0.55; $p = 0.01$) and shorter anti-CMV curative treatment (40 vs. 60 days, $p < 0.0001$). As a corollary, we found that $V\delta2^{neg}$ γδ T cell expansion was faster in LOD than in EOD (31 vs. 168 days after the beginning of CMV disease, $p < 0.0001$). In D+R− KTRs, universal prophylaxis is associated with more LOD, which had better infection management and a faster immune response. These results support the use of universal prophylaxis over a preemptive strategy and reappraise outcomes of LOD.**

**Abbreviations: +, seropositive; ATG, anti–thymocyte globulin; AUC, area under the curve; CI, confidence interval; CMV, cytomegalovirus; D+R−, seronegative recipients transplanted from seropositive donors; eGFR, estimated GFR; EOD, early onset disease; IV, intravenous; KTR, kidney transplant recipient; LOD, late-onset disease; OR, odds ratio; QNAT, quantitative nucleic acid test; SD, standard deviation; −, seronegative; $V\delta2^{neg}$, Vδ2-negative**

**Received 03 January 2016, revised 07 February 2016 and accepted for publication 25 February 2016**

## Introduction

In the present era of universal prophylaxis, late-onset cytomegalovirus (CMV) represents a challenge for the transplant physician, especially in the highest risk group, namely, seronegative recipients transplanted from seropositive donors (D+R−). CMV incidence remains elevated in these patients, at between 16% and 37%, depending on the duration of preventive therapy (1,2). In one study directly comparing prophylactic and preemptive approaches, it was clearly demonstrated that late DNAemia occurs much more frequently in the prophylactic versus the preemptive setting (3,4). In addition, late DNAemia has been associated with poor prognosis, with a reduction in graft survival and higher patient mortality (5–10). Moreover, it has been claimed that the preemptive strategy, usually associated with CMV DNAemia or early onset disease (EOD), may have the advantage of boosting antiviral immunity by exposure to asymptomatic DNAemia compared with universal prophylaxis (11).

Both late-onset disease (LOD), with its poor outcomes and impaired immune response against the virus, and immunosuppressive burden are considered the main drawbacks of universal prophylaxis in comparison to the preemptive strategy associated with EOD. Nevertheless, studies depicting the poor prognosis of LOD have rarely focused on the high-risk D+R− population (5) and have defined LOD as all CMV events >3 mo after transplantation including recurrences (5), which are already known to have poorer prognosis (12,13).

No study has ever directly compared LOD and EOD in terms of control of CMV infection, general outcomes or anti-CMV cell-mediated immunity kinetics. In this context, we and others have shown that Vδ2-negative ($V\delta2^{neg}$) γδ T cells are key effector components in the control of CMV infection (14–16). This subset, which is typically located within the epithelia, is characterized by the use of Vδ1, Vδ3 or Vδ5 segments (collectively called $V\delta2^{neg}$ γδ T cells), whereas the most common subset in the peripheral blood uses the association of Vδ2 and Vγ9 variable regions (Vγ9/Vδ2 T cells). After CMV infection, but not after other viral infections (e.g. herpes simplex virus, Varicella zoster virus, Epstein–Barr virus and influenza), $V\delta2^{neg}$ γδ T cells undergo massive expansion in the blood of kidney transplant recipients (KTRs) (14,17). Recently, we demonstrated that $V\delta2^{neg}$ γδ T cell expansion in the peripheral blood of KTRs was associated with the resolution of infection, and we observed a higher risk of recurrence at the end of a curative treatment in the absence of $V\delta2^{neg}$ γδ T cell expansion (18).

To retrospectively reappraise the outcomes of LOD, defined as the first episode of CMV disease >100 days after transplantation in a population of D+R− KTRs, we compared three populations of KTRs—patients with LOD, with EOD and without CMV disease (defined as asymptomatic patients with or without CMV DNAemia)—for variables regarding severity of infection, evolution of the immune response through $V\delta2^{neg}$ γδ T cells, and graft and patient outcomes.

## Materials and Methods

### *Study design and patients*

As shown in Figure 1, 168 D+R− patients who received a deceased or living donor kidney allograft at Bordeaux University Hospital from January 1, 2003, to December 31, 2011, were included in this retrospective study, and the virological (whole-blood real-time CMV quantitative acid nucleic test [QNAT]) and immunological (peripheral blood immunophenotyping) determinations were collected for ≥2 years after transplantation.

From January 1, 2003, to November 30, 2006, patients received universal prophylaxis for 3 mo using valganciclovir 900 mg once daily. From December 1, 2006, to June 30, 2010, patients were preemptively followed and treated when the CMV QNAT was positive (i.e. 250 IU/mL). Finally, from July 1, 2010, to December 31, 2011, patients received 6 mo of universal prophylaxis using valganciclovir 900 mg once daily.

CMV disease was defined as CMV syndrome or CMV tissue-invasive disease, based on standardized criteria (19). LOD was defined as the first episode of CMV disease occurring >3 mo (100 days) after transplantation. EOD was defined as the first episode of CMV disease occurring <3 mo (100 days) after transplantation. Absence of CMV replication was defined as two consecutive CMV-negative QNATs. Intravenous (IV) ganciclovir (5 mg/kg twice daily) or oral valganciclovir (900 mg twice daily) was given for curative treatment and was always followed by oral valganciclovir (900 mg/day), as described previously (20). The anti-CMV treatment was discontinued once absence of CMV replication was obtained. The dose was carefully adjusted at each outpatient visit, according to the manufacturer's recommendations, using the Cockcroft–Gault formula. Recurrent DNAemia and recurrent disease were defined as a second or later episode of positive CMV DNAemia with or without symptoms either at scheduled visits or with evidence of clinical recurrence in patients with proven absence of CMV replication.

The immunosuppressive regimen was based on calcineurin inhibitors with a tacrolimus target trough concentration of 10–12 ng/mL for the first 3 mo and then 5–10 ng/mL. The cyclosporine A target trough concentration was 150–200 ng/mL for the first 3 mo and then 75–125 ng/mL. Mycophenolic acid was used mainly as an antiproliferative drug. Steroids were decreased quickly in the first month to 5 mg/day or stopped. Anti–thymocyte globulin (ATG) was used in immunized patients. Expanded criteria donor and delayed graft function were defined as described previously (21,22). Pretransplant HLA sensitization was defined as the presence of anti-HLA antibodies in the recipient, using a single-antigen flow bead assay (One Lambda Inc, Canoga Park, CA). All acute rejections, which included both antibody-mediated and T cell–mediated acute rejections, were biopsy proven. Graft failure was defined as return to dialysis. This study was approved by the institutional review board of the Bordeaux University Hospital.

### *CMV monitoring*

CMV IgG serology was performed (Enzygnost anti-CMV/IgM and IgG [Dade Behring, Marburg, Germany] and Access CMV IgG and IgM

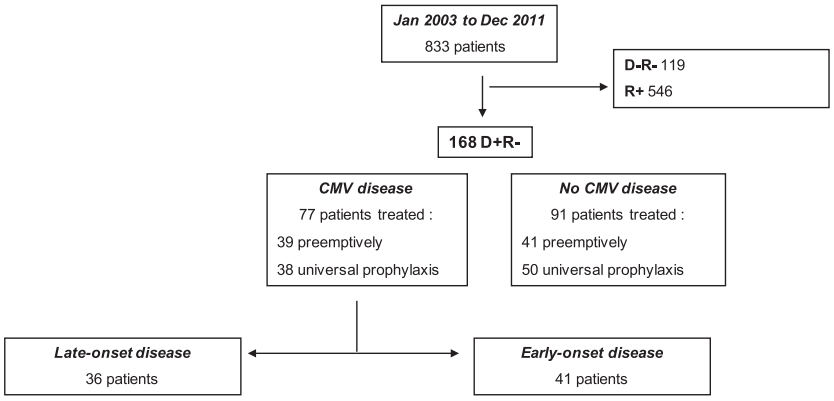


**Figure 1: Flow chart of the study design.** +, seropositive; −, seronegative; D, donor; R, recipient.

[Beckman Coulter, Brea, CA]), following the manufacturer's recommendations. Whole-blood CMV QNAT was performed using real-time polymerase chain reaction, as described previously (23), and the results were converted to international units per milliliter using calibration to the World Health Organization International Standard (24). The sensitivity of the whole-blood CMV QNAT was 250 IU/mL. CMV DNAemia was considered positive when detectable (i.e. >250 IU/mL). CMV negation was defined as CMV DNAemia <250 IU/mL.

Baseline viral load was defined as the viral load of the first positive CMV DNAemia during the first episode of CMV disease, and the peak viral load was defined as the maximum viral load during the first episode of CMV disease. CMV QNAT was performed once a week for the first 3 mo, once a month between months 3 and 6, and then every 2 months up to 1 year and if CMV disease was suspected clinically. During the virological monitoring of CMV disease, the assay was performed once a week until two consecutive negative CMV DNAemia QNATs occurred. Antiviral drug resistance was suspected when persistent viral replication was observed after >2 weeks of appropriate antiviral therapy and was confirmed by full-length sequencing of the *UL97* and *UL54* genes (25), performed at the French National Cytomegalovirus Reference Center (Limoges, France). Sequences were compared with the AD169 reference sequence using the Gene Librarian 3.2 software (Visible Genetics Inc., Siemens, France) (26,27).

#### *Flow cytometry analysis and monitoring of Vδ2$^{neg}$ γδ T cells*

Blood samples were analyzed at the Bordeaux University Hospital immunology laboratory by flow cytometry using a FC500 flow cytometer from Beckman Coulter. All blood samples were withdrawn on EDTA anticoagulant in Vacutainer 5-mL tubes (BD Biosciences, Mountain View, CA) and kept at room temperature until processed. Following the manufacturer's recommendations, labeling was carried out on whole blood and red blood cells lysed at room temperature with a Versalyse (Beckman Coulter France, Villepinte, France) lysing solution added to Iotest fixative solution (Beckman Coulter, Macon, France). Events were acquired with the dedicated CXP-1 software.

Vδ2$^{neg}$ γδ T cells were detected with anti-Vδ2 and anti–PAN-δ, purchased from Beckman Coulter France. Vδ2$^{neg}$ γδ T cell count was obtained using the flow count bead kit from Beckman Coulter following a lyse and no-wash procedure, according to the manufacturer's recommendations.

At our center, the surveillance of Vδ2$^{neg}$ γδ T cells was based on measurement at day 0 of the graft; at months 3, 6 and 12; and then annually. In case of positive CMV DNAemia, additional Vδ2$^{neg}$ γδ T cell determinations were performed. Vδ2$^{neg}$ γδ T cell expansion was defined as demonstrated previously (18). The measure of Vδ2$^{neg}$ γδ T cell expansion and the time of Vδ2$^{neg}$ γδ T cell expansion from CMV disease were determined as described previously (18).

#### *Statistical analysis*

Analyses were performed with conventional statistical methods using R statistical software (version 3.10.1; R Foundation for Statistical Computing, Vienna, Austria) and, specifically, the lme4 and ROCR packages (28). The Mann–Whitney, Kruskal–Wallis and chi-square tests were used, if appropriate. A $p < 0.05$ was considered statistically significant. The cumulative incidence functions depending on the type of disease were computed using the Nelson–Aalen estimator (29) (no disease, EOD or LOD), and the test of the type of disease effect on patient death, graft failure and acute rejection was performed with the Fine and Gray model (30). To identify risk factors, bivariable analyses (i.e. chi-square analysis for categorical variables and Wilcoxon rank sum test for continuous variables) were performed to compare variables in the groups with and without CMV disease. Variables with $p < 0.20$ from bivariable analyses were assessed further and were eligible for inclusion in the multivariable model.

## Results

#### *Baseline characteristics of patients*

Overall, 168 D+R− KTRs were included in this monocentric retrospective study between January 2003 and December 2011. Among them, 91 (54.2%) did not develop CMV disease; 36 (21.4%) developed LOD, and 41 (24.4%) developed EOD. The characteristics of these three groups are depicted in Table 1.

Following univariate analysis comparing CMV disease (EOD and LOD) and absence of disease, the following risk factors were associated with CMV disease: HLA sensitization, expanded criteria donors, total ischemia time, ATG and delayed graft function. Following multivariate analysis, ATG was the only independent factor associated with CMV disease (odds ratio [OR] 3.2; 95% confidence interval [CI] 2.6–5.5; $p = 0.001$) (Table 2).

No significant differences were observed between EOD and LOD for baseline characteristics of transplantation: donor and recipient ages, transplantation range, HLA sensitization, expanded criteria donors, total ischemia time and delayed graft function. Neither baseline immunosuppressive regimen nor type of induction treatment (anti–IL-2R antibody or ATG) was significantly different. Consequently, although this study extended over three time periods in terms of CMV management, we did not isolate day 0 of transplantation as a specific risk factor associated with LOD compared with EOD in this cohort of D+R− patients.

#### *Characteristics of CMV diseases*

LOD occurred preferentially following universal prophylaxis (86% of LOD after universal prophylaxis, 14% after a preemptive strategy), whereas EOD was observed preferentially following a preemptive strategy (83% of EOD after a preemptive strategy, 17% after universal prophylaxis; chi-square, $p < 0.0001$) (Table 3). Peak viral load was significantly lower in the course of LOD than EOD ($p = 0.04$), whereas the baseline viral load was significantly higher in LOD ($p = 0.001$). Despite this, treatment duration to obtain absence of CMV replication was shorter during LOD compared with EOD ($p < 0.0001$). We previously reported that peak viral load and treatment failure were risk factors for antiviral drug resistance during preemptive therapy (25). In this study, we observed that antiviral drug resistance occurred less frequently during LOD than EOD (11% vs. 58.5%, $p = 0.008$). The initial treatment with IV ganciclovir or valganciclovir did not differ between the two groups ($p = 0.12$). The clinical presentation was not different between EOD and LOD. Notably, the prevalence and

**Table 1:** Baseline characteristics of patients

| Criteria | D+R− LOD n = 36 | D+R− EOD n = 41 | D+R− No disease n = 91 | p-value |
|---|---|---|---|---|
| Recipients | | | | |
| Age, years (mean ± SD) | 48.2 ± 13.8 | 49.1 ± 12.2 | 47.4 ± 13.9 | 0.9 |
| Sex (male/female) | 27/9 | 28/13 | 65/26 | 0.7 |
| HLA sensitized (yes/no) | 10/26 | 11/30 | 21/70 | 0.01[1] |
| Nephropathy, n (%) | | | | 0.17 |
| Glomerular | 12 (33) | 19 (46) | 33 (36) | |
| Tubulointerstitial | 7 (19) | 3 (7) | 10 (11) | |
| Vascular | 4 (11) | 3 (7) | 11 (12) | |
| Diabetes | 0 | 3 (7) | 3 (3) | |
| Hereditary | 7 (19) | 7 (17) | 19 (21) | |
| Malformation | 2 (5.5) | 2 (5) | 4 (4) | |
| Unknown | 4 (11) | 4 (10) | 9 (10) | |
| Hemodialysis/peritoneal dialysis, n | 36/0 | 35/4 | 81/3 | 0.5 |
| Transplantation, n (%) | | | | 0.3 |
| 1 | 32 (89) | 33 (80) | 73 (80) | |
| 2 | 1 (3) | 4 (10) | 15 (16) | |
| ≥3 | 3 (8) | 3 (7) | 3 (4) | |
| Donors | | | | |
| Age, years (mean ± SD) | 48.3 ± 14.1 | 47.4 ± 15.1 | 45.5 ± 15.7 | 0.6 |
| Expanded criteria donors (yes/no) | 12/24 | 14/27 | 19/71 | 0.01[2] |
| Living donors | 1 | 0 | 4 | 0.3 |
| HLA A/B/DQ/DR mismatches, n | 3.6 | 3.7 | 3.36 | 0.8 |
| Total ischemia time, h (mean ± SD) | 18.2 ± 8.4 | 17.4 ± 5.7 | 17.5 ± 6.4 | 0.09 |
| Delayed graft function (yes/no) | 12/24 | 16/25 | 26/65 | 0.6 |
| Immunosuppressive treatment, n | | | | |
| Cyclosporine A/tacrolimus/mTOR inhibitor | 12/22/2 | 8/31/2 | 24/66/1 | 0.7 |
| Mycophenolic acid | 36 | 40 | 90 | 0.9 |
| Corticosteroids | 36 | 41 | 91 | 1 |
| Anti–IL-2R antibody/ATG | 23/13 | 27/14 | 64/27 | 0.02[3] |
| Corticosteroid duration, days (mean ± SD) | 511 ± 943 | 497 ± 923 | 500 ± 925 | 0.8 |

ATG, anti-thymocyte globulins; D+R−, seronegative recipients transplanted from seropositive donors; EOD, early onset disease; LOD, late-onset disease: mTOR, mammalian target of rapamycin; SD, standard deviation.
[1]Global chi-square was significant (p = 0.01). The p-values for comparisons between groups are as follows: LOD versus EOD, p = 0.4 (no significant); LOD versus no disease, p = 0.001; EOD versus no disease, p = 0.001.
[2]Global chi-square was significant (p = 0.02). The p-values for comparisons between groups are as follows: LOD versus EOD, p = 0.4 (not significant); LOD versus no disease, p = 0.001; EOD versus no disease, p = 0.001.
[3]Global chi-square was significant. The p-values for comparisons between groups are as follows: LOD versus EOD, p = 0.4; LOD versus no disease, p = 0.001; EOD versus no disease, p = 0.001.

**Table 2:** Multivariate analysis for cytomegalovirus disease

| Criteria | OR (95% CI) | p-value |
|---|---|---|
| HLA sensitized | 1.25 (0.6–2.5) | 0.53 |
| Total ischemia time | 0.95 (0.83–1.98) | 0.12 |
| Expanded criteria donor | 1.9 (0.95–3.8) | 0.07 |
| ATG | 3.2 (2.6–5.5) | 0.001 |

ATG, anti–thymocyte globulin; CI, confidence interval; OR, odds ratio.

the clinical pattern of organ-invasive CMV disease incidence were similar between LOD and EOD (p = 0.25), (Table 3).

### *The risk of recurrent DNAemia and disease was significantly reduced following LOD*

We next analyzed the rate of recurrent CMV DNAemias or diseases in patients with LOD or EOD. As shown in Figure 2, the risk of recurrent DNAemias was significantly reduced after LOD compared with EOD; they occurred in 13 of 36 (36%) patients with LOD and 33 of 41 (80%) patients with EOD (OR 0.09, 95% CI 0.03–0.025, p < 0.001). The risk of recurrent CMV disease was also reduced after LOD compared with EOD. Only three of 36 (3%) D+R− patients undergoing LOD had a recurrent CMV disease compared with 12 of 41 (30%) patients with EOD (OR 0.16, 95% CI 0.05–0.55, p < 0.01).

**Table 3:** Characteristics of cytomegalovirus diseases

| Infection parameters | LOD, n = 36 | EOD, n = 41 | p-value |
|---|---|---|---|
| Preemptive versus universal prophylaxis, n | 5/29 | 34/7 | <0.0001 |
| Time of onset infection from transplantation, days, median (quartiles 1–3) | 230 (127–271) | 30 (25–39) | <0.0001 |
| Peak viral load, IU/mL, median (quartiles 1–3) | 45 086 (10 600–272 550) | 94 925 (41 950–585 000) | 0.04 |
| Baseline viral load, IU/mL, median (quartiles 1–3) | 25 494 (4469–189 150) | 1597 (999–32 350) | 0.001 |
| Treatment duration to obtain eradication, days, median (quartiles 1–3) | 40 (25–50) | 60 (40–90) | <0.0001 |
| Valganciclovir versus IV ganciclovir, n | 4/25 | 10/29 | 0.12 |
| Antiviral drug resistance, yes/no, n | 4/32 | 15/26 | 0.008 |
| Syndrome versus organ-invasive disease, n | 18/14 | 24/15 | 0.25 |
| Organ-invasive diseases | | | |
| Gastrointestinal | 9 | 11 | 0.3 |
| Hematological | 3 | 2 | |
| Pulmonary | 3 | 4 | |
| Retinitis | 0 | 1 | |
| Hepatitis | 1 | 1 | |
| Multiple | 3 | 4 | |

IV, intravenous; LOD, late-onset disease; EOD, early-onset disease.

### *Vδ2$^{neg}$ γδ T cell expansion occurred faster in LOD than in EOD*

The association of LOD with lower peak DNAemia, shorter time to obtain absence of replication and less recurrence would suggest that an efficient immune response takes place more quickly in these patients. As demonstrated previously, Vδ2$^{neg}$ γδ T cells are critical players in CMV control in humans (14–16), and early Vδ2$^{neg}$ γδ T cell expansion was recently identified in patients with late-onset CMV infection (18).

We compared the longitudinal evolution and the time to Vδ2$^{neg}$ γδ T cell expansion in EOD and LOD patients (Figure 3). With the longitudinal approach (Figure 3A), Vδ2$^{neg}$ γδ T cells increased faster in peripheral blood of patients who had LOD than in those with EOD. In LOD patients, Vδ2$^{neg}$ γδ T cell percentage became significantly higher than the baseline level (analyzed before CMV disease) at day 20 after CMV disease ($p < 0.05$). In EOD patients, Vδ2$^{neg}$ γδ T cell percentage became significantly higher than the baseline level at day 140 after CMV disease ($p < 0.05$). As defined previously (18), we next calculated the time from CMV disease diagnosis to Vδ2$^{neg}$ γδ T cell expansion in LOD and EOD patients. We found that the median of time to Vδ2$^{neg}$ γδ T cell expansion was 31 days after the beginning of CMV disease in LOD and

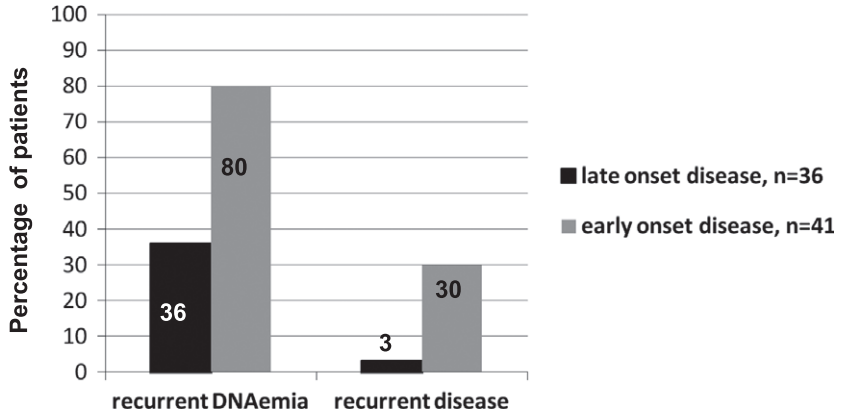


**Figure 2: Reduced risk of recurrent CMV DNAemia and CMV disease following LOD.** After CMV disease eradication, defined by two negative successive CMV quantitative nucleic acid tests, antiviral treatment was stopped, and data about recurrent CMV DNAemia and recurrent disease were collected. Overall, 33 of 41 patients with EOD versus 13 of 36 patients with LOD had recurrent CMV DNAemia (OR 0.09, 95% CI 0.03–0.25, $p < 0.001$). Moreover, 12 of 41 patients with EOD versus three of 36 patients with LOD had recurrent CMV disease (OR 0.16, 95% CI 0.05–0.55, $p < 0.01$). CI, confidence interval; CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease; OR, odds ratio.

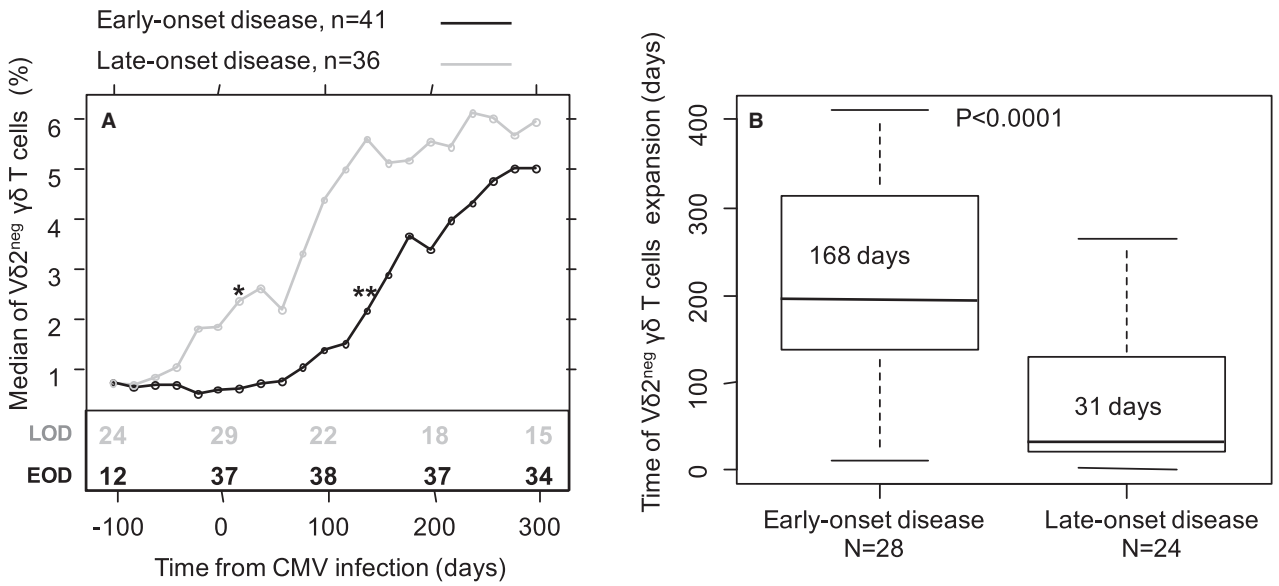


**Figure 3: Vδ2$^{neg}$ γδ T cell response in EOD and LOD patients from the beginning of CMV disease.** (A) Kinetics of Vδ2$^{neg}$ γδ T cells in peripheral blood of patients with LOD and EOD. In LOD patients (gray line) and EOD patients (black line), Vδ2$^{neg}$ γδ T cell percentage interpolated data every 20 days are represented from day 0 of CMV disease (first positive CMV quantitative nucleic acid test). *$p < 0.05$ in LOD patients, comparison of Vδ2$^{neg}$ γδ T cell percentages before and after Vδ2$^{neg}$ γδ T cell expansion. **$p < 0.05$ in EOD patients, comparison of Vδ2$^{neg}$ γδ T cell percentages before and after Vδ2$^{neg}$ γδ T cell expansion. The p-values were obtained using the Mann–Whitney test. (B) Box plots showing time from CMV disease diagnosis to Vδ2$^{neg}$ γδ T cell expansion (days) in patients with LOD and EOD. Patients with >100 days between two determinations of Vδ2$^{neg}$ γδ T cells were excluded from this analysis, as described previously (18). The p-value was obtained with the Mann–Whitney test. CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease; Vδ2$^{neg}$, Vδ2-negative.

168 days after the beginning of CMV disease in EOD (Figure 3B) ($p < 0.0001$). These observations demonstrate a faster anti-CMV immune response after LOD. This relationship was also observed in the subgroup of preemptively treated patients who had LOD (14% of preemptively treated patients) (Figure S1). As a corollary, the 17% of patients treated with universal prophylaxis who had EOD (all because of intolerance or nonadherence) had a delay in Vδ2$^{neg}$ γδ T cell expansion (Figure S1).

Considering only asymptomatic DNAemia, we also found that patients with late-onset DNAemia had more rapid expansion of their Vδ2$^{neg}$ γδ T cells compared with patients with early onset DNAemia (Figure S2).The type of induction, ATG versus anti–IL-2R antibody, did not influence the kinetics of Vδ2$^{neg}$ γδ T cells (percentage or absolute count) among patients who had CMV disease (Figure S3). Results of Vδ2$^{neg}$ γδ T cells are expressed in percentages of total lymphocytes, but similar results were obtained with absolute counts (Figure S4).

### *EOD occurred during profound lymphocytic depletion*

The impact of the profound immunosuppression early after transplantation in EOD patients is illustrated in Figure 4. We described the immune status of other T cell subpopulations in EOD compared with LOD patients. We observed that EOD occurred during a profound lymphocytic depletion (total lymphocyte count in Figure 4A) concerning both CD4 (Figure 4B) and CD8 T cells (Figure 4C), reflecting the highest burden of immunosuppressive treatment, in the first weeks after transplantation. Nevertheless, it is interesting to note that there was no significant difference in T cell absolute values at day 0 of infection between patients with LOD and EOD (917 vs. 809 for total T cell count; 423 vs. 341 for CD4 T cells; 230 vs. 169 for CD8 T cells). In fact, EOD occurred during the decrease of both CD4 and CD8 T cells, whereas LOD occurred while the T cells count was stable.

### *Comparison of clinical outcomes at 3 years after transplantation for patients with LOD, EOD, and no CMV disease*

We analyzed the usual clinical outcomes (acute rejection, renal function, graft and patient survival) at 3 years after transplantation.

As shown in Figure 5, acute rejection (LOD, 33.3%; EOD, 26.8%; no disease, 24.2%; $p = 0.6$), graft failure (LOD, 8.3%; EOD, 14.6%; uninfected, 7.7%; $p = 0.3$) and patient death (LOD, 2.8%; EOD, 2.4%; no disease, 0%; $p = 0.29$) were not significantly different among

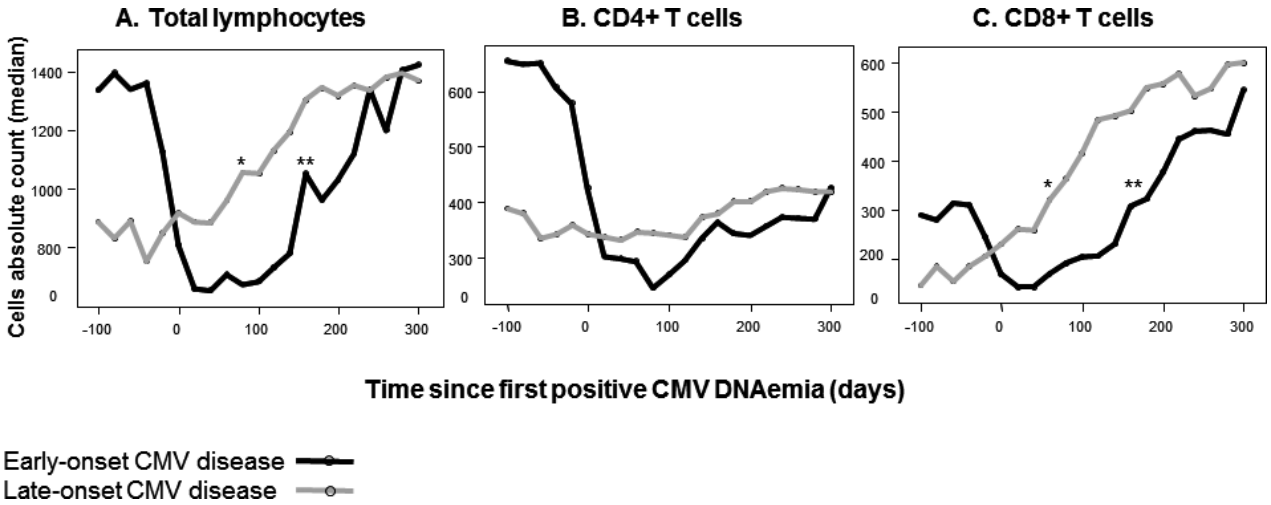


**Figure 4: Total lymphocytes and CD4 and CD8 T cells in EOD and LOD patients from the beginning of CMV disease.** Kinetics of total lymphocyte cells (A) and CD4 (B) and CD8 (C) T cells in peripheral blood of patients with LOD and EOD. In LOD patients (gray line) and EOD patients (black line), absolute counts of total lymphocytes (A) and CD4 (B) and CD8 (C) T cells (interpolated data) every 20 days are represented from day 0 of CMV disease (first positive CMV QNAT). *$p < 0.05$ in LOD patients, comparison of $V\delta2^{neg}$ $\gamma\delta$ T cell percentages before and after $V\delta2^{neg}$ $\gamma\delta$ T cell expansion. **$p < 0.05$ in EOD patients, comparison from baseline value. The p-values were obtained using the Mann–Whitney test. CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease; $V\delta2^{neg}$, Vδ2-negative.

patients who had LOD, who had EOD and who did not develop CMV disease. Moreover, using the MDRD equation, estimated GFR (eGFR) was not different among EOD (mean 47.5 mL/min; standard deviation [SD] 20.6), LOD (51.5 mL/min; SD 15.4) and absence of disease (55.2 mL/min; SD 20.4) at 1 year after transplantation ($p = 0.45$). At 3 years after transplantation, eGFR was not different between EOD and LOD (41.8 mL/min; SD 18 for EOD; 42 mL/min; SD 12 for LOD; $p = 0.7$), but eGFR was better in patients without disease (52.6 mL/min; SD 18.7) compared with those with EOD ($p = 0.02$) and LOD ($p = 0.02$). Interestingly, we also observed an opposite correlation between 3-year eGFR and the area under the curve (AUC) of CMV DNAemia using a linear regression with a p-value of 0.026. That means that CMV disease and the intensity and duration of the viremia (reflected by the AUC) negatively affect 3-year eGFR.

Considering asymptomatic CMV DNAemia and not disease, we found the same results: Acute rejection ($p = 0.27$), graft failure ($p = 0.3$) and patient death ($p = 0.17$) were not significantly different among patients with late, early and no infection.

## Discussion

In this study, we compared LOD versus EOD in D+R− KTRs and found that LOD had better outcomes because of a lower peak viral load, a shorter time to stop viral replication following treatment, less antiviral drug resistance, fewer virological and clinical recurrences and a faster immune response. Moreover, LOD and EOD had the same detrimental effects on graft and patient survival at 3 years after transplantation. Today, LOD appears mainly after the discontinuation of universal prophylaxis in D+R− patients. LOD has often been recognized for its negative impact on graft (10) and patient survival (6,10,31) compared with patients free of CMV disease. This issue is considered the main drawback for universal prophylaxis, but the sole alternative to this strategy is preemptive treatment, which leads to EOD. We found that eGFR was lower in both EOD and LOD patients than in patients without disease. This finding confirms the poorer prognosis associated with LOD but shows that EOD is also associated with more chronic allograft dysfunction than is seen in patients free of CMV disease, as has already been described by Kliem et al (32). Indeed, patients using a preemptive strategy (i.e. a marker of early disease) had lower graft survival than patients who received universal prophylaxis (32), but direct comparison between EOD and LOD was not done.

LOD occurs mainly in D+R− patients for whom CMV disease is more life-threatening (33,34) and more difficult to eradicate and who are at higher risk of recurrences and antiviral drug resistance (35,36). In the randomized VICTOR trial comparing IV ganciclovir versus valganciclovir in the treatment of CMV disease, only 58% of patients had viral eradication at day 21, and 85% had viral eradication

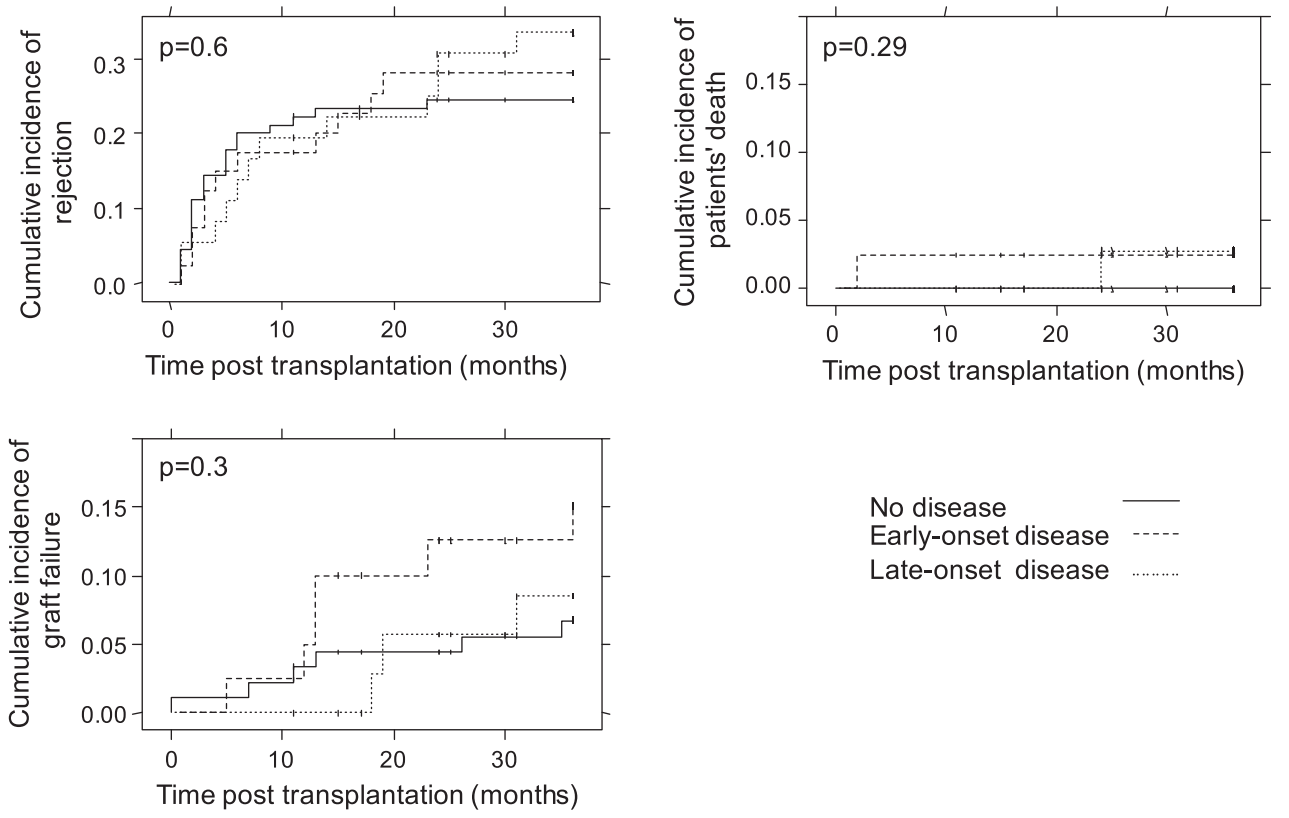


**Figure 5: Comparison of clinical outcomes at 3 years after transplantation among patients with LOD, EOD, or no CMV disease.** Incidence of acute rejection was 33.3% in LOD patients, 26.8% in EOD patients and 24.2% in patients free of CMV disease (p = 0.6). Incidence of graft failure was 8.3% in LOD patients, 14.6% in EOD patients and 7.7% in patients without CMV disease (p = 0.3). Incidence of patient death was 2.8% with LOD, 2.4% with EOD and 0% in patients free of CMV disease (p = 0.29). The Fine and Gray model was used to perform the three analyses. CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease.

at day 49 (37). In this study, we showed that median treatment duration to stop viral replication in D+R− patients is 40 days in LOD versus 60 days in EOD. Consequently, LOD is associated with a savings of 20 days of treatment, which is important in terms of both safety and costs. This finding contrasts with the fact that the baseline viral load is higher in LOD, probably because the virological monitoring is less stringent at the end of universal prophylaxis. This suggests that in LOD, the control of infection is more effective and easier to implement compared with EOD. Recurrences of CMV infection are another worrying problem in the management of KTRs, with a previously described frequency of ≈30% (37,38), but analysis following EOD versus LOD had not yet been achieved. In this study, we found that EOD was associated with a very high rate (80%) of CMV DNAemia recurrence and with 30% of symptomatic recurrences, complicating the management of these patients. In contrast, LOD was associated with 36% of CMV DNAemia recurrence but only 3% of symptomatic recurrences. Because EOD occurs mainly after a preemptive strategy, we believe that this strategy is not appropriate in D+R− patients and argue strongly for universal prophylaxis in these high-risk patients. Moreover, in naïve D+/R− patients, universal prophylaxis is thought to hamper the development of a specific immune response, causing more late-onset CMV infections than the preemptive approach (3,39), with increased morbidity (10). This observation could argue for the choice of a preemptive strategy. Conversely, we found that the time to $V\delta2^{neg}$ $\gamma\delta$ T cell expansion was shorter in patients with LOD, most of whom were treated with universal prophylaxis. Other studies found that universal prophylaxis did not suppress CMV-specific antibodies and T cell response (40,41). We hypothesized that the delay from transplantation obtained with prophylaxis could allow (i) local priming of $V\delta2^{neg}$ $\gamma\delta$ T cells in tissues in the absence of systemic dissemination of the virus and (ii) a decrease of the immunosuppressive burden. When a late-onset infection occurs following the end of the universal prophylaxis, $V\delta2^{neg}$ $\gamma\delta$ T cells are more prone to undergo an efficient expansion, and LOD is managed more easily. Indeed, it has been described previously that a context of high frequencies of memory cells (as could be the case during prophylaxis) could favor rapid memory differentiation and preservation of proliferative potential on viral boosting

(CMV disease) (42), thereby mimicking a kind of vaccine approach.

Actually, no data in humans shows such a local priming of Vδ2neg γδ T cells in tissues after local CMV infection or reactivation; however, this hypothesis stems from a mouse model developed by our group that demonstrated that tissular γδ T cells (spleen, lungs, intestine, and liver) participated in early protection against mouse CMV infection and underwent differentiation into effector memory cells on CMV challenge (43). Concerning the burden of immunosuppression, EOD occurs at the highest level, which could explain the delayed $V\delta2^{neg}$ γδ T cell expansion, whereas a faster expansion of $V\delta2^{neg}$ γδ T cells after LOD could be explained by the decrease in this burden of immunosuppression after 3 mo.

We did not measure CMV-specific CD8 T cells in this work, but we previously described a concomitant expansion of γδ T cells and CMV-specific CD8+ T cells after CMV infection (44).

Due to its retrospective design, our study has some limitations. In particular, we were not able to isolate specific risk factors associated with LOD, whereas LOD has been associated previously with donor age, poor eGFR and ATG (6,45,46). Following multivariate analysis, we found that ATG was the only factor associated with CMV disease, whether for LOD or EOD. ATG is a well-known risk factor of CMV disease (6,47), particularly in D+R− patients, and, not surprisingly, we also found it was the only risk factor. Interestingly, we found that ATG induction did not hamper $V\delta2^{neg}$ γδ T cell expansion following CMV infection, confirming a close observation of the effect of ATG on the development of CMV-specific αβ cell T cell response (48).

In conclusion, the present work emphasized that LOD has a more favorable outcome in high-risk naïve patients because of a faster anti-CMV immune response. Given the close link between LOD and universal prophylaxis, our study strongly argues for recommending this strategy in the management of D+R− patients.

## Acknowledgments

We thank Catherine Rio as a nurse coordinator. We also acknowledge the technicians from the Laboratories of Virology and Immunology in Bordeaux University Hospital and the Centre National de Référence des Cytomégalovirus (Limoges) for their significant contribution to this study.

## Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

## References

1. Humar A, Lebranchu Y, Vincenti F, et al. The efficacy and safety of 200 days valganciclovir cytomegalovirus prophylaxis in high-risk kidney transplant recipients. Am J Transplant 2010; 10: 1228–1237.
2. Helantera I, Kyllonen L, Lautenschlager I, Salmela K, Koskinen P. Primary CMV infections are common in kidney transplant recipients after 6 months valganciclovir prophylaxis. Am J Transplant 2010; 10: 2026–2032.
3. Khoury JA, Storch GA, Bohl DL, et al. Prophylactic versus pre-emptive oral valganciclovir for the management of cytomegalovirus infection in adult renal transplant recipients. Am J Transplant 2006; 6: 2134–2143.
4. Florescu DF, Qiu F, Schmidt CM, Kalil AC. A direct and indirect comparison meta-analysis on the efficacy of cytomegalovirus preventive strategies in solid organ transplant. Clin Infect Dis 2014; 58: 785–803.
5. Reischig T, Hribova P, Jindra P, et al. Long-term outcomes of pre-emptive valganciclovir compared with valacyclovir prophylaxis for prevention of cytomegalovirus in renal transplantation. J Am Soc Nephrol 2012; 23: 1588–1597.
6. Luan FL, Kommareddi M, Ojo AO. Impact of cytomegalovirus disease in D+/R− kidney transplant patients receiving 6 months low-dose valganciclovir prophylaxis. Am J Transplant 2011; 11: 1936–1942.
7. Reischig T. Advances in cytomegalovirus-preventive strategies in solid organ transplantation: Defending pre-emptive therapy. Expert Rev Anti Infect Ther 2012; 10: 51–61.
8. Limaye AP, Bakthavatsalam R, Kim HW, et al. Late-onset cytomegalovirus disease in liver transplant recipients despite antiviral prophylaxis. Transplantation 2004; 78: 1390–1396.
9. Eid AJ, Razonable RR. New developments in the management of cytomegalovirus infection after solid organ transplantation. Drugs 2010; 70: 965–981.
10. Arthurs SK, Eid AJ, Pedersen RA, et al. Delayed-onset primary cytomegalovirus disease and the risk of allograft failure and mortality after kidney transplantation. Clin Infect Dis 2008; 46: 840–846.
11. Abate D, Saldan A, Fiscon M, et al. Evaluation of cytomegalovirus (CMV)-specific T cell immune reconstitution revealed that baseline antiviral immunity, prophylaxis, or preemptive therapy but not antithymocyte globulin treatment contribute to CMV-specific T cell reconstitution in kidney transplant recipients. J Infect Dis 2010; 202: 585–594.
12. Viot B, Garrigue I, Taton B, et al. Two-year post-transplantation cytomegalovirus DNAemia in asymptomatic kidney transplant recipients: Incidence, risk factors, and outcome. Transpl Infect Dis 2015; 17: 497–509.
13. Legendre C, Pascual M. Improving outcomes for solid-organ transplant recipients at risk from cytomegalovirus infection: Late-onset disease and indirect consequences. Clin Infect Dis 2008; 46: 732–740.
14. Dechanet J, Merville P, Berge F, et al. Major expansion of gammadelta T lymphocytes following cytomegalovirus infection in kidney allograft recipients. J Infect Dis 1999; 179: 1–8.
15. Knight A, Madrigal AJ, Grace S, et al. The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. Blood 2010; 116: 2164–2172.
16. Vermijlen D, Brouwer M, Donner C, et al. Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. J Exp Med 2010; 207: 807–821.

17. Pitard V, Roumanes D, Lafarge X, et al. Long-term expansion of effector/memory Vdelta2-gammadelta T cells is a specific blood signature of CMV infection. Blood 2008; 112: 1317–1324.
18. Kaminski H, Garrigue I, Couzi L, et al. Surveillance of gammadelta T cells predicts cytomegalovirus infection resolution in kidney transplants. J Am Soc Nephrol 2016; 27: 637–645.
19. Kotton CN, Kumar D, Caliendo AM, et al. Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. Transplantation 2013; 96: 333–360.
20. Asberg A, Humar A, Rollag H, et al. Oral valganciclovir is noninferior to intravenous ganciclovir for the treatment of cytomegalovirus disease in solid organ transplant recipients. Am J Transplant 2007; 7: 2106–2113.
21. Metzger RA, Delmonico FL, Feng S, Port FK, Wynn JJ, Merion RM. Expanded criteria donors for kidney transplantation. Am J Transplant 2003; 3(Suppl 4): 114–125.
22. Koning OH, Ploeg RJ, van Bockel JH, et al. Risk factors for delayed graft function in cadaveric kidney transplantation: A prospective study of renal function and graft survival after preservation with University of Wisconsin solution in multi-organ donors. European Multicenter Study Group. Transplantation 1997; 63: 1620–1628.
23. Garrigue I, Doussau A, Asselineau J, et al. Prediction of cytomegalovirus (CMV) plasma load from evaluation of CMV whole-blood load in samples from renal transplant recipients. J Clin Microbiol 2008; 46: 493–498.
24. Freyer JF, Heath A, Anderson R, Minor PD and the collaborative study group. Collaborative study to evaluate the proposed 1st WHO international standard for human cytomegalovirus for nucleic acid amplification-based assays. WHO ECBS Report 2010; 1–40.
25. Couzi L, Helou S, Bachelet T, et al. High incidence of anticytomegalovirus drug resistance among D+R- kidney transplant recipients receiving preemptive therapy. Am J Transplant 2012; 12: 202–209.
26. Chou S, Marousek G, Boivin G, et al. Recombinant phenotyping of cytomegalovirus sequence variants detected after 200 or 100 days of valganciclovir prophylaxis. Transplantation 2010; 90: 1409–1413.
27. Hantz S, Garnier-Geoffroy F, Mazeron MC, et al. Drug-resistant cytomegalovirus in transplant recipients: A French cohort study. J Antimicrob Chemother 2010; 65: 2628–2640.
28. Team RC. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. 2012. Available from: http://www.R-project.org.
29. Aalen O. Non parametric estimation of partial transition probabilities in multiple decrement models. Ann Stat 1978; 6: 534–545.
30. Gray R. A class of K-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat 1988; 16: 1141–1154.
31. Santos CA, Brennan DC, Yusen RD, Olsen MA. Incidence, risk factors and outcomes of delayed-onset cytomegalovirus disease in a large retrospective cohort of lung transplant recipients. Transplantation 2015; 99: 1658–1666.
32. Kliem V, Fricke L, Wollbrink T, Burg M, Radermacher J, Rohde F. Improvement in long-term renal graft survival due to CMV prophylaxis with oral ganciclovir: Results of a randomized clinical trial. Am J Transplant 2008; 8: 975–983.
33. Brennan DC. Cytomegalovirus in renal transplantation. J Am Soc Nephrol 2001; 12: 848–855.
34. Reischig T, Jindra P, Svecova M, Kormunda S, Opatrny K Jr, Treska V. The impact of cytomegalovirus disease and asymptomatic infection on acute renal allograft rejection. J Clin Virol 2006; 36: 146–151.
35. Boivin G, Goyette N, Rollag H, et al. Cytomegalovirus resistance in solid organ transplant recipients treated with intravenous ganciclovir or oral valganciclovir. Antivir Ther 2009; 14: 697–704.
36. Myhre HA, Haug Dorenberg D, Kristiansen KI, et al. Incidence and outcomes of ganciclovir-resistant cytomegalovirus infections in 1244 kidney transplant recipients. Transplantation 2011; 92: 217–223.
37. Asberg A, Humar A, Jardine AG, et al. Long-term outcomes of CMV disease treatment with valganciclovir versus IV ganciclovir in solid organ transplant recipients. Am J Transplant 2009; 9: 1205–1213.
38. Helantera I, Schachtner T, Hinrichs C, et al. Current characteristics and outcome of cytomegalovirus infections after kidney transplantation. Transpl Infect Dis 2014; 16: 568–577.
39. Reischig T, Jindra P, Hes O, Svecova M, Klaboch J, Treska V. Valacyclovir prophylaxis versus preemptive valganciclovir therapy to prevent cytomegalovirus disease after renal transplantation. Am J Transplant 2008; 8: 69–77.
40. La Rosa C, Limaye AP, Krishnan A, Blumstein G, Longmate J, Diamond DJ. Primary response against cytomegalovirus during antiviral prophylaxis with valganciclovir, in solid organ transplant recipients. Transpl Int 2011; 24: 920–931.
41. San-Juan R, Navarro D, Garcia-Reyne A, et al. Effect of delaying prophylaxis against CMV in D+/R- solid organ transplant recipients in the development of CMV-specific cellular immunity and occurrence of late CMV disease. J Infect 2015; 71: 561–570.
42. Fraser KA, Schenkel JM, Jameson SC, Vezys V, Masopust D. Preexisting high frequencies of memory CD8+ T cells favor rapid memory differentiation and preservation of proliferative potential upon boosting. Immunity 2013; 39: 171–183.
43. Khairallah C, Netzer S, Villacreces A, et al. gammadelta T cells confer protection against murine cytomegalovirus (MCMV). PLoS Pathog 2015; 11: e1004702.
44. Couzi L, Pitard V, Netzer S, et al. Common features of gammadelta T cells and CD8(+) alphabeta T cells responding to human cytomegalovirus infection in kidney transplant recipients. J Infect Dis 2009; 200: 1415–1424.
45. Jamal AJ, Husain S, Li Y, Famure O, Kim SJ. Risk factors for late-onset cytomegalovirus infection or disease in kidney transplant recipients. Transplantation 2014; 97: 569–575.
46. Manuel O, Kralidis G, Mueller NJ, et al. Impact of antiviral preventive strategies on the incidence and outcomes of cytomegalovirus disease in solid organ transplant recipients. Am J Transplant 2013; 13: 2402–2410.
47. Kanter J, Pallardo L, Gavela E, et al. Cytomegalovirus infection renal transplant recipients: Risk factors and outcome. Transpl Proc 2009; 41: 2156–2158.
48. San-Juan R, Navarro D, Garcia-Reyne A, et al. Effect of long-term prophylaxis in the development of cytomegalovirus-specific T-cell immunity in D+/R- solid organ transplant recipients. Transpl Infect Dis 2015; 17: 637–646.

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Figure S1: $V\delta2^{neg}$ $\gamma\delta$ T cell response in LOD after a preemptive strategy compared with EOD after**

**universal prophylaxis.** Kinetics of $V\delta2^{neg}$ γδ T cells in peripheral blood of the subgroup of patients with LOD after a preemptive strategy compared with the subgroup of patients with EOD after universal prophylaxis. In LOD patients (gray line) and EOD patients (black line), $V\delta2^{neg}$ γδ T cell percentages (interpolated data) every 20 days are represented from day 0 of CMV disease (first positive CMV quantitative nucleic acid test). CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease; $V\delta2^{neg}$, Vδ2-negative.

**Figure S2: $V\delta2^{neg}$ γδ T cell response in patients with early onset DNAemia and late-onset DNAemia from the beginning of CMV disease.** Kinetics of $V\delta2^{neg}$ γδ T cells in peripheral blood of patients with early onset DNAemia and late-onset DNAemia. In the late-onset DNAemia group (black line) and the early-onset DNAemia group (gray line), $V\delta2^{neg}$ γδ T cell percentages (interpolated data) every 20 days are represented from day 0 of CMV infection (first positive CMV quantitative nucleic acid test). CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease; $V\delta2^{neg}$, Vδ2-negative.

**Figure S3: $V\delta2^{neg}$ γδ T cell response in patients with CMV disease after anti–IL-2R antibody and ATG.** Kinetics of $V\delta2^{neg}$ γδ T cells in peripheral blood of patients with CMV disease after anti–IL-2R antibody and ATG. In after anti–IL-2R antibody (black line) and ATG (gray line), $V\delta2^{neg}$ γδ T cell percentages or absolute counts ($mm^3$; interpolated data) every 20 days are represented from day 0 of CMV infection (first positive CMV quantitative nucleic acid test). ATG, anti–thymocyte globulin; CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease; $V\delta2^{neg}$, Vδ2-negative.

**Figure S4: $V\delta2^{neg}$ γδ T cell response in patients with EOD and LOD in absolute count from the beginning of CMV disease.** Kinetics of $V\delta2^{neg}$ γδ T cells in peripheral blood of patients with EOD and LOD in absolute count ($mm^3$). In the LOD group (gray line) and the EOD group (black line), $V\delta2^{neg}$ γδ T cell absolute count ($mm^3$; interpolated data) every 20 days are represented from day 0 of CMV infection (first positive CMV quantitative nucleic acid test). CMV, cytomegalovirus; EOD, early onset disease; LOD, late-onset disease; $V\delta2^{neg}$, Vδ2-negative.