

# An Early Immediate Early Protein IE-1–Specific T-Cell Polyfunctionality Is Associated With a Better Control of Cytomegalovirus Reactivation in Kidney Transplantation



**To the Editor:** Immune response quality is considered as a major factor in chronic viral infections.<sup>1</sup> Cytomegalovirus (CMV) remains one of most frequent and deleterious viral infections in kidney transplantation. For the prevention of CMV disease, prophylactic or preemptive treatments are commonly proposed. Because of side effects, new methods for tailoring of anti-CMV treatments are needed to adapt to an individual's recipient risk.<sup>2–4</sup> Assessment of the host immune system capacity to control CMV replication might be useful to predict viral reactivation control, but is not yet widely recommended.<sup>5–9</sup> In this study, we characterized the specific memory T-cell responses against immediate early protein 1 (IE-1) and phosphoprotein 65 (pp65), 2 immunodominant proteins from the CMV virion, to better understand the immune mechanisms of viral control in CMV-seropositive patients receiving kidney transplantation.

## PATIENTS, MATERIAL, AND METHODS

According to France's bioethics specifications, we realized a prospective monocentric observational study. Consecutive CMV-seropositive patients receiving *de novo* kidney transplant (KTR) and CMV-seropositive healthy donors (French blood bank donors) were included from January to November 2013. In the KTR group, CMV-DNAemia was monitored weekly in whole blood during the 6 months' follow-up. CMV infection or disease was defined according to International Guidelines and American Society of Transplantation recommendations for use in clinical trials.<sup>3,4,10</sup> Antiviral therapy was initiated in case of CMV infection (defined by an increase of CMV-DNAemia kinetic more than 10 times between 2 consecutive samples, without symptoms, treated by preemptive valganciclovir) or CMV disease (curative ganciclovir treatment, followed by preemptive therapy). On renal transplant day (D0), 6 weeks, 3 months, and 6 months after transplantation,

peripheral blood mononuclear cells were stimulated overnight with CMV peptide pools (IE-1 or pp65). After stimulation, cells were stained for memory T-cell subpopulation identification<sup>11–13</sup> (stem-cell memory [T<sub>SCM</sub>], central-memory [T<sub>CM</sub>], effector-memory [T<sub>EM</sub>], and terminally differentiated effector T cells [T<sub>D</sub>]), and then for intracellular cytokine secretions (Figure 1, Supplementary Material and Methods online). Lymphocyte polyfunctionality was evaluated by the capacity of CMV-specific T cells to perform single, double, and triple cytokine secretions. To ensure the robust analysis of CMV-specific T-cell subpopulations, only samples with CMV-specific T-cell responses more than twice the background (no antigen) and a minimum of 40 cytokine-secreting cells were considered positives and included (Supplementary Figure S1).

## RESULTS

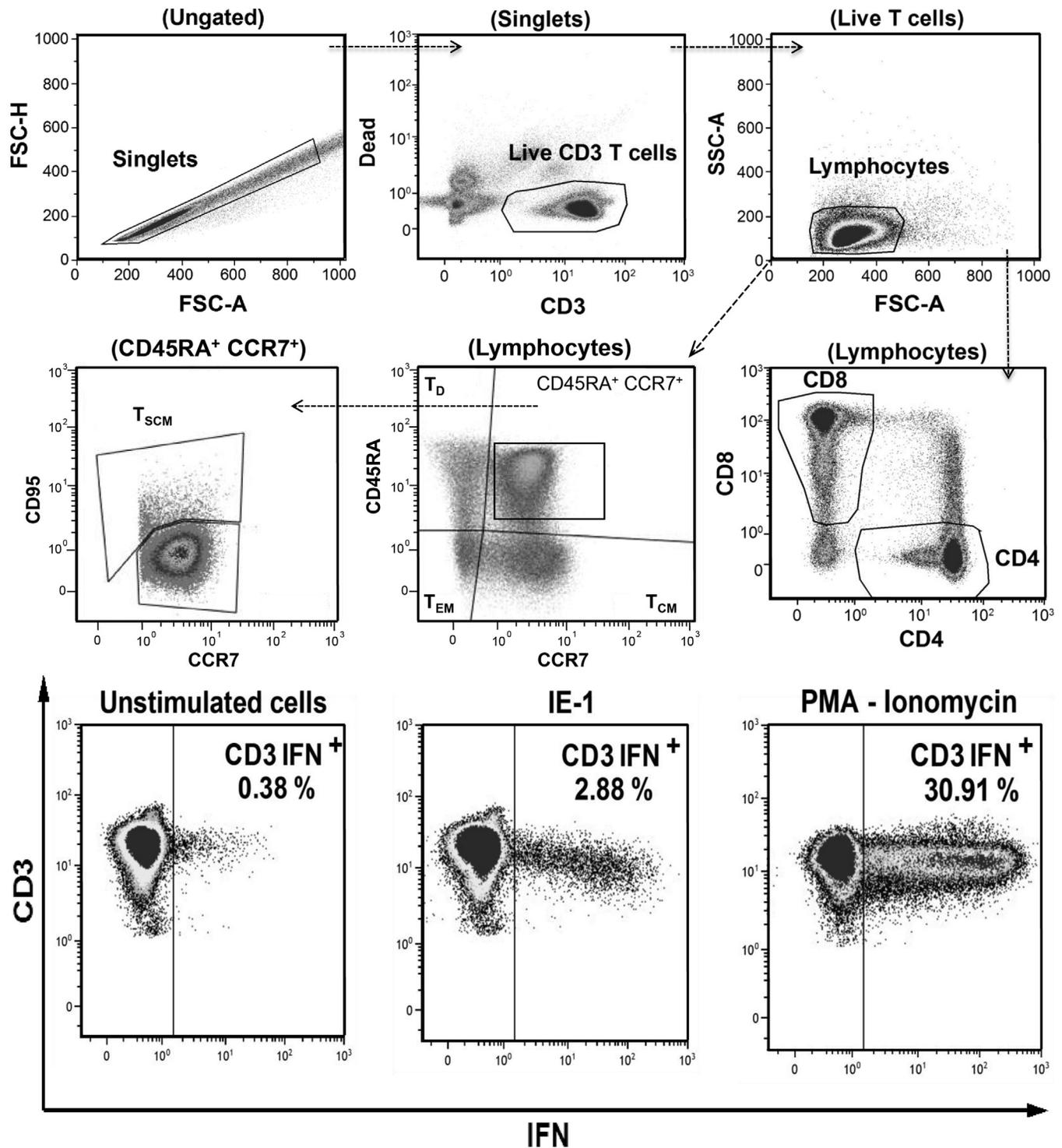
### Clinical Data

Fifteen CMV-seropositive KTR were followed for 6 months after transplantation. As the control group, 17 CMV-seropositive healthy donors were included. Controls were comparable to KTR for age and gender ( $P > 0.05$ ). Two groups of KTR were identified per the occurrence of CMV infection/disease: 7 KTR presented CMV infection/disease requiring antiviral treatment (CMV<sup>+</sup> KTR), whereas 8 KTR did not (CMV<sup>-</sup> KTR). Median CMV-viral load peak was 55,800 copies/ml (1070–801,000) with a median time of 6 weeks after transplantation. During the follow-up period, the CMV<sup>+</sup> KTR group presented more viral infectious complications, without a statistical difference concerning the immunosuppressive regiment, nor in graft function (Table 1).

### At D0, CMV-Specific Memory T Cells Were Less Frequent, With Lower CD8 T<sub>SCM</sub> and T<sub>CM</sub> Counts in Patients Awaiting Renal Transplantation

In pretransplantation, CD4 and CD8 T-cell total counts were similar in KTR groups compared with healthy donors and, on the sixth week after transplantation, CMV<sup>+</sup> KTR presented CD8 lymphopenia compared with CMV<sup>-</sup> KTR ( $P = 0.04$ ; Figure 2a). On D0, CMV-specific CD8 T cells were lower in KTR groups compared with healthy donors (IE-1:  $P = 0.004$ , pp65:  $P = 0.04$ ; Figure 2b) and, on the third month after transplantation, a lower IE-1–specific CD4 T-cell count was found in the CMV<sup>+</sup> KTR compared with the CMV<sup>-</sup> KTR group ( $P = 0.04$ ; Figure 2b).

In pretransplantation, compared with healthy donors, CMV-specific T<sub>SCM</sub> and T<sub>CM</sub> were lower in CMV<sup>+</sup> KTR (IE-1-CD4 and CD8 T<sub>CM</sub>, IE-1-CD8 T<sub>SCM</sub>,



**Figure 1.** Gating strategy for the identification of IE-1 and pp65-specific memory T cells. One million fresh peripheral blood mononuclear cells were incubated overnight in AIM-V medium alone (unstimulated cells) or with CMV peptides (IE-1 or pp65 peptide pools) or with positive control (PMA-ionomycin). After activation, cells were stained by surface markers (antibodies anti-CD3, -CD4, -CD8, -CCR7, -CD45RA, -CD95) and then fixed and permeabilized for intracellular cytokine staining (antibodies anti-IL2<sup>+</sup>, TNF $\alpha$ <sup>+</sup>, or IFN $\gamma$ <sup>+</sup>) on CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T lymphocytes. In IE-1, pp65, and PMA/ionomycin stimulation, the same gating strategy was used to define the cytokine-secreting cells. The percentage of cytokine-producing CD4 or CD8 T cells was determined after subtracting the background in unstimulated controls. Shown are examples on a representative healthy donor of IFN $\gamma$ -secreting CD3<sup>+</sup> T cells (percentage of CD3<sup>+</sup> T cells expressing IFN $\gamma$ ) in unstimulated cells, after IE-1 and PMA/ionomycin (Continued)

**Table 1.** Characteristics and clinical evolution of CMV-seropositive patients with *de novo* kidney transplantation, according to the occurrence of CMV infection/disease, during the 6 months of follow-up

Time		CMV <sup>+</sup> KTR (n = 7)	CMV <sup>-</sup> KTR (n = 8)	P value
D0	Male (n)	4	7	0.282
	Age (yr)	70 (49–77)	50 (31–74)	0.088
	Diabetic and/or vascular nephropathy (n)	4	4	1.000
	Malformative uropathy (n)	1	1	1.000
	Glomerulopathy (n)	2	3	1.000
	Dialysis (mo)	45 (23–104)	23 (0–159)	0.219
	Hemodialysis (n)	6	6	1.000
	First kidney transplant (n)	6	6	1.000
	I and II class HLA mismatches (n)	4 (2–8)	5.5 (2–8)	0.788
	Thymoglobulin (n)	3	3	1.000
	Tacrolimus (n)	3	6	0.315
	Mycophenolate mofetil (n)	6	8	0.467
	Corticosteroids (n)	6	8	0.467
	CMV-specific IgG antibody (UA/ml)	222 (101–250)	250 (125–250)	0.689
	W6	CMV viral load peak (copies/ml)	55,800 (1070–801,000)	0 (0–2867 <sup>a</sup> )
Delay between CMV infection/disease and transplant day (wk)		6.3 (2.3–11)	0	*0.016
Valganciclovir (n)		5	–	–
Ganciclovir or valganciclovir (n)		2	–	–
Creatininemia (μmol/l)		204 (98–412)	149 (93–210)	0.456
M6	Creatininemia (μmol/l)	183 (96–542)	140 (78–210)	0.149
	Graft rejection (n)	2	1	0.569
	Graft lost (n)	2	0	0.200
	Viral infectious complications other than CMV infection/disease (n)	6	2	*0.041

Median (Minimum–Maximum), Mann-Whitney. \* $P < 0.05$ , \*\* $P < 0.005$ .

Seven KTR presented CMV infection/disease requiring treatment (CMV<sup>+</sup> KTR), whereas 8 KTR did not (CMV<sup>-</sup> KTR). There was no statistical difference concerning the immunosuppressive regimen, nor in graft function from the transplant day to the sixth month. During the follow-up period, patients from the CMV<sup>+</sup> KTR group had more viral infectious complications such as EBV or HSV reactivation, compared with the CMV<sup>-</sup> KTR group.

CMV, cytomegalovirus; CMV<sup>+</sup> KTR, CMV-seropositive kidney transplant recipients with CMV-infection/disease; CMV<sup>-</sup> KTR, CMV-seropositive kidney transplant recipients without CMV-infection/disease; D0, transplantation day; M6, 6th month after kidney transplantation; n, number; W6, 6th week after kidney transplantation.

<sup>a</sup>Two patients from the CMV<sup>-</sup> KTR group presented a latent CMV, without antiviral treatment requirement (asymptomatic lower CMV viremia with spontaneous normalization between 2 consecutive samples).

pp65-CD8 T<sub>SCM</sub> and T<sub>CM</sub>, all  $P < 0.05$ ; Figure 3a and b). In healthy donors, compared with CMV<sup>+</sup> KTR, IE-1-specific CD8 T<sub>EM</sub> and T<sub>D</sub> were also higher ( $P = 0.04$  and  $P = 0.009$ , respectively; Figure 3a). Although the CMV-specific T-cell differentiation states were similar between the 2 KTR groups on the transplant day, on the third and sixth months after transplantation, IE-1-specific CD8 T<sub>CM</sub> counts were lower in CMV<sup>+</sup> KTR ( $P = 0.01$  and  $P = 0.003$ , respectively; Figure 3c).

### IE-1-Specific Lymphocyte Polyfunctionality Increased Early in KTR Without CMV Infection/Disease

Concerning the IE-1-specific lymphocyte polyfunctionality, in pretransplantation, IE-1-specific one-cytokine-secreting CD8 T cells were more frequent in healthy donors compared with KTR ( $P = 0.0001$ ,  $P = 0.005$ , respectively; Figure 4a), without differences between the 2 KTR groups (Figure 4a). However, we observed, on the sixth week, higher double- and triple-cytokine-secretion counts of IE-1-CD4 T cells ( $P = 0.01$ ,  $P = 0.05$ , respectively; Figure 4b) in CMV<sup>-</sup> KTR

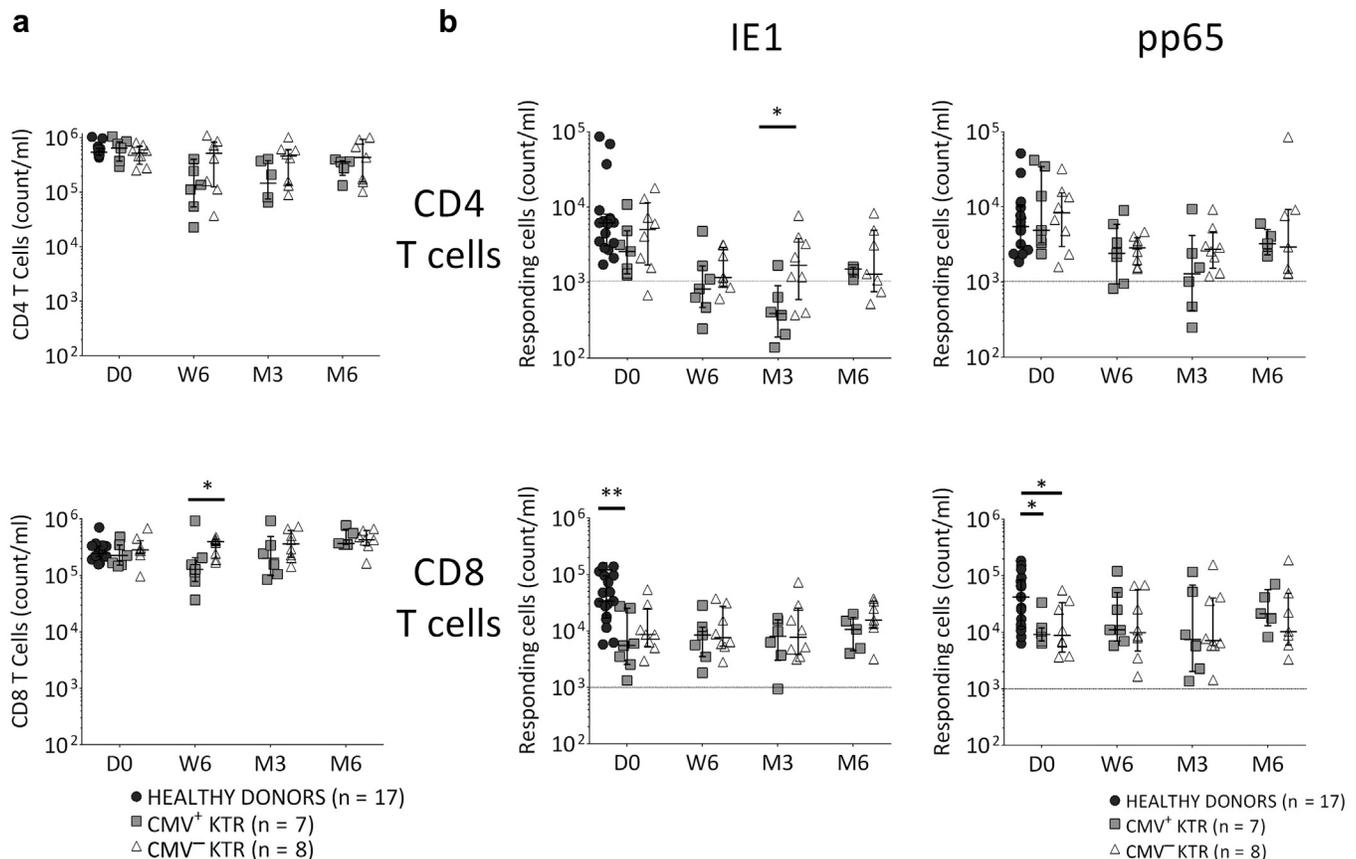
compared with CMV<sup>+</sup> KTR. On the third and sixth months after transplant, triple-cytokine-secreting CD8 T-cell counts were higher in CMV<sup>-</sup> KTR ( $P = 0.04$ ,  $P = 0.03$ , respectively; Figure 4b).

Concerning the pp65-specific lymphocyte polyfunctionality, there was no difference between KTR with or without CMV infection/disease during the follow-up period (Supplementary Figure S2b).

## DISCUSSION

Our study aimed to better understand the immune mechanisms of viral control in CMV-seropositive patients receiving kidney transplantation. Immunological memory is the key feature of the adaptive immune system to provide life-long protection against pathogens.<sup>11,14</sup> Here, CMV-specific memory T cells were numerically lower in patients awaiting kidney transplantation. In KTR with CMV infection/disease, we found a decrease of CMV-specific T-cell subsets with lower T<sub>SCM</sub> and T<sub>CM</sub> counts in pretransplantation, compared with healthy donors. A premature immunological aging in patients with end-stage renal failure

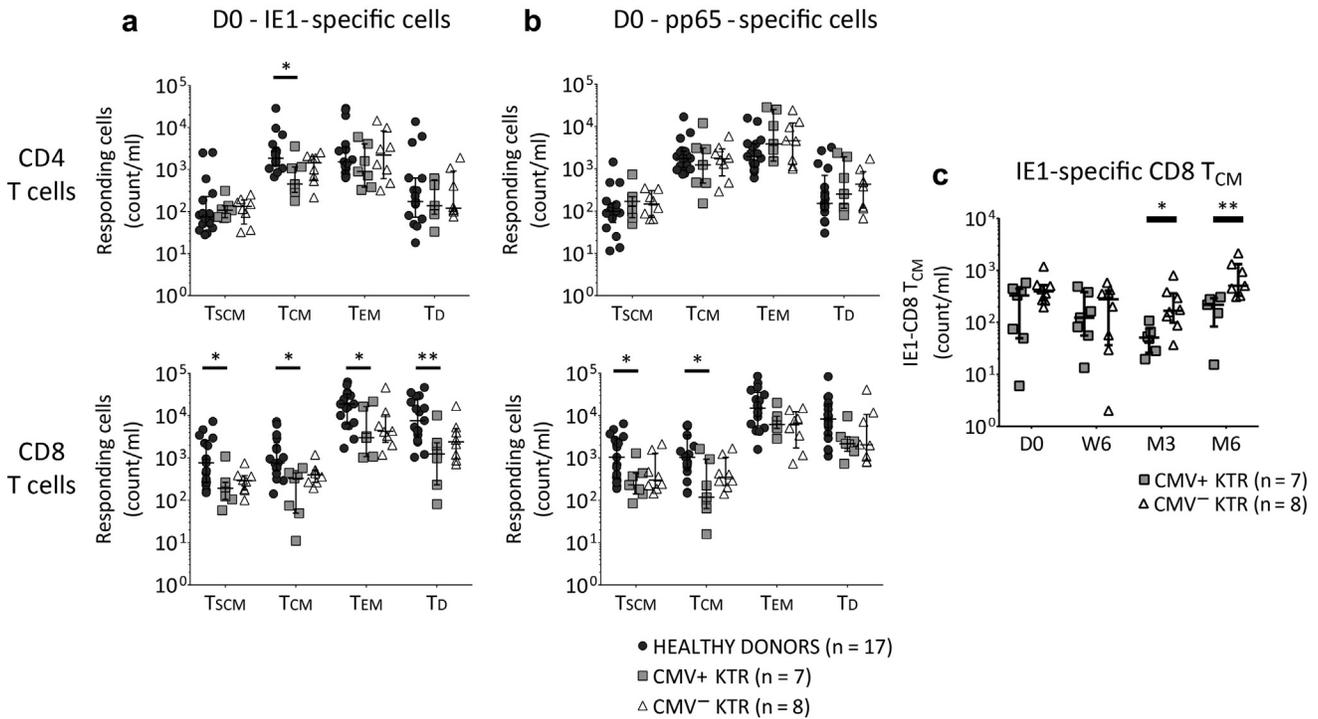
**Figure 1.** (Continued) stimulation. Similar results were found for IL2 and TNF $\alpha$  secretions, and after pp65 stimulation (data not shown). Lymphocyte polyfunctionality was evaluated by the capacity of the CMV-specific T cells to perform single, double, and triple cytokine secretions. Mono-, bi-, and trifunctional T cells were identified by Boolean gates. Total frequency of polyfunctional cells was determined by summing the total number of cytokine-secreting cells. CD3 IFN $\gamma$ <sup>+</sup>, interferon $\gamma$ -producing CD3 T cells; IE-1, immediate early protein 1; IFN $\gamma$ , interferon $\gamma$ ; pp65, phosphoprotein 65; T<sub>CM</sub>, central-memory T cells; T<sub>D</sub>, terminally differentiated effector T cells; T<sub>EM</sub>, effector-memory T cells; T<sub>SCM</sub>, stem-cell memory T cells.



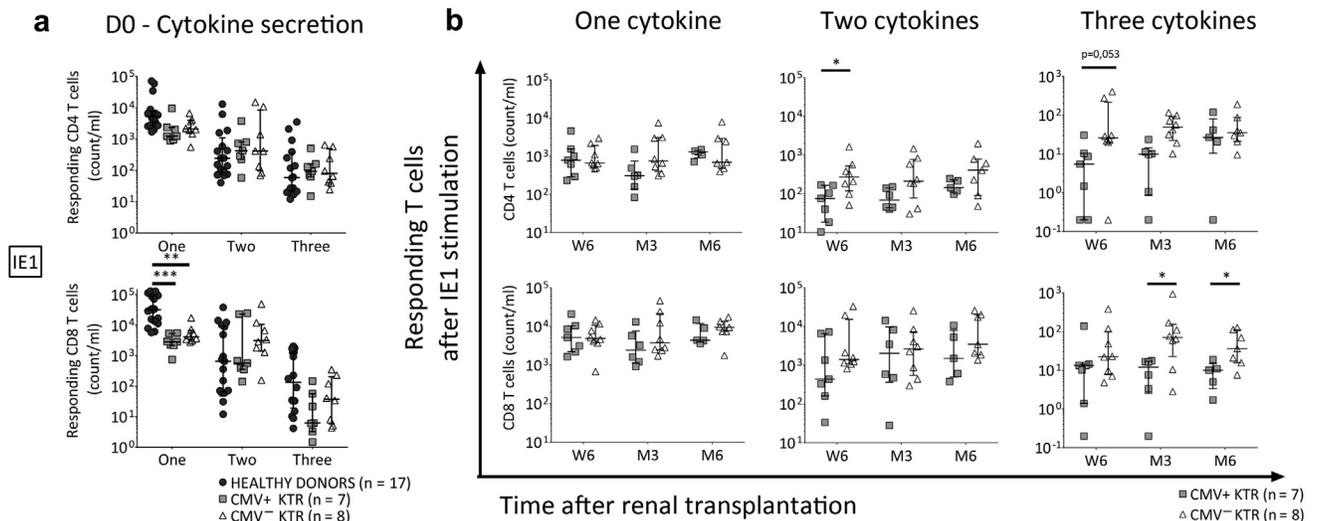
**Figure 2.** Evolution of CD4 and CD8 T-cell counts and CMV-specific CD4 and CD8 T-cell counts in healthy donors and CMV-seropositive KTR, from transplant day to 6 months after transplantation. (a) On the transplant day, there were no differences in absolute cell numbers of CD4 and CD8 T cells between healthy donors and KTR groups. From the sixth week after transplantation, CMV<sup>+</sup> KTR presented CD8 T-cell lymphopenia, compared with the CMV<sup>-</sup> KTR group. (b) On the transplant day, absolute cell numbers of CMV-specific CD8 T cells were lower in CMV-seropositive KTR, compared with healthy donors. From the third month after transplantation, CMV<sup>+</sup> KTR presented lower absolute cell numbers of IE-1-specific CD4 T cells, compared with the CMV<sup>-</sup> KTR group. Median and 25th and 75th percentiles, Mann-Whitney or Kruskal-Wallis tests for comparison of 2 or 3 groups. \* $P < 0.05$ , \*\* $P < 0.005$ . The dotted line represents a strong CMV-specific response. CMV<sup>+</sup> KTR, CMV-seropositive kidney transplant recipients with CMV infection/disease; CMV<sup>-</sup> KTR, CMV-seropositive kidney transplant recipients without CMV infection/disease; D0, transplantation day; IE-1, immediate early protein 1; M3, third month after kidney transplantation; M6, sixth month after kidney transplantation; n, number; pp65, phosphoprotein 65; W6, sixth week after kidney transplantation.

was observed in previous studies, showing a selective loss of naive and T<sub>CM</sub> T cells.<sup>15–18</sup> In healthy aged donors, CMV-specific response may represent over 10% of the CD8 T-cell pool.<sup>19</sup> Here, CMV-specific CD8 T cells mainly produced one cytokine in healthy donors. This relative monofunctionality in healthy donors could be explained by the expansion of IE-specific T<sub>EM</sub> and T<sub>D</sub> CD8 T cells. Interestingly, the higher T<sub>SCM</sub> and T<sub>CM</sub> counts in healthy donors can suggest increased self-renewal and multipotency capacities, providing a potential reservoir for T-cell memory.<sup>13</sup> Lymphocyte phenotype and functionality are intrinsically linked and effector T cells may exercise effector functions in situations of viral reactivation.<sup>20</sup> CMV-specific T-cell differentiation state was not an early predictive factor allowing identifying recipients at CMV infection/disease risk. Although immunosuppressive regimens were similar in our

study, quantitative and qualitative changes have been described to occur in CMV-specific CD8 T cells in response to immunosuppressive treatment, with predominantly effector phenotype and cytotoxic capacities.<sup>21</sup> In liver or lung transplantation, polyfunctional CMV-specific T cells have been found to be associated with a lower risk of CMV reactivation.<sup>22–24</sup> Also, precedent studies found that higher frequencies of IE-1 but not pp65-specific CD8 T cells correlated with protection against CMV disease.<sup>25–27</sup> Here, we found a higher IE-1-specific lymphocyte polyfunctionality in KTR without CMV infection/disease compared with those with CMV infection/disease, with a superior double-functionality of CD4 T cells at an early period, that is, 6 weeks after transplantation and a superior trifunctionality of CD8 T cells at 3 and 6 months after transplantation. Importantly, polyfunctionality of CMV-specific T cells in pretransplantation was not



**Figure 3.** CMV-specific memory CD8 T<sub>SCM</sub> and T<sub>CM</sub> are lower in kidney transplant recipients with CMV infection/disease. (a) On the transplant day, absolute numbers of IE-1-specific CD4 T<sub>CM</sub> were lower in CMV<sup>+</sup> KTR, compared with healthy donors. The distribution of IE-1-specific CD8 T-cell differentiation was more important in healthy donors, with an expansion of T<sub>SCM</sub>, T<sub>CM</sub>, T<sub>EM</sub>, and T<sub>D</sub>. (b) On the transplant day, without a difference in pp65-specific CD4 T-cell differentiation, absolute cell numbers of pp65-specific CD8 T<sub>SCM</sub> and T<sub>CM</sub> were lower in CMV<sup>+</sup> KTR, compared with healthy donors. (c) On the third and sixth months after transplantation, CMV<sup>+</sup> KTR presented a lower absolute cell numbers of IE-1-specific CD8 T<sub>CM</sub> compared with the CMV<sup>-</sup> KTR group. Median and 25th and 75th percentiles, Mann-Whitney or Kruskal-Wallis tests for comparison of 2 or 3 groups. \**P* < 0.05, \*\**P* < 0.005. CMV<sup>+</sup> KTR, CMV-seropositive kidney transplant recipients with CMV infection/disease; CMV<sup>-</sup> KTR, CMV-seropositive kidney transplant recipients without CMV infection/disease; IE-1, immediate early protein 1; n, number; pp65, phosphoprotein 65; T<sub>CM</sub>, central-memory T cells; T<sub>D</sub>, terminally differentiated effector T cells; T<sub>EM</sub>, effector-memory T cells; T<sub>SCM</sub>, stem-cell memory T cells.



**Figure 4.** IE-1-specific lymphocyte polyfunctionality is precociously more important in KTR without CMV infection/disease. An early increase of IE-1-specific lymphocyte polyfunctionality in kidney transplant recipients without CMV infection/disease was observed. (a) On the transplant day, the absolute number of IE-1-specific CD8 T cells producing 1 cytokine was higher in healthy donors, compared with KTR groups. No differences were found in T cells secreting double and triple cytokines on D0. (b) From the sixth week after transplantation, double-cytokine-producing CD4 T cells were higher in KTR without CMV infection/disease. On the third and sixth months after transplant, concomitant secretion of 3 cytokines by IE-1-specific CD8 T cells was higher in CMV<sup>-</sup> KTR. Median and 25th and 75th percentiles, Mann-Whitney or Kruskal-Wallis tests for comparison of 2 or 3 groups. \**P* < 0.05, \*\**P* < 0.005, \*\*\**P* < 0.0005. CMV<sup>+</sup> KTR, CMV-seropositive kidney transplant recipients with CMV infection/disease; CMV<sup>-</sup> KTR, CMV-seropositive kidney transplant recipients without CMV infection/disease; D0, transplantation day; IE-1, immediate early protein 1; M3, third month after kidney transplantation; M6, sixth month after kidney transplantation; n, number; W6, sixth week after kidney transplantation.

different between KTR with or without CMV infection/disease. Despite the small number of patients and a relatively limited lymphocyte polyfunctionality evaluation, our pilot study was prospective and assessed the CMV-specific cellular immunity in the absence of systematic prophylactic treatment. In conclusion, our study suggests that an early posttransplantation emergence of polyfunctional IE-1-specific T cells is associated with a decreased risk of CMV infection/disease, and therefore, the identification of such T cells might possibly be used as a predictive biomarker of viral replication control in the renal transplant setting. Further multicenter studies with higher numbers of patients are needed to confirm this hypothesis.

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## DISCLOSURE

All the authors declared no competing interests.

## SUPPLEMENTARY MATERIAL

### Supplemental Material and Methods.

**Figure S1.** Absolute number of T cells (CD4, CD8, CMV-specific CD4 and CD8 T cells) included in CMV-specific memory T-cell analyses during the study follow-up. CMV<sup>+</sup> KTR, CMV-seropositive kidney transplant recipients with CMV-infection/disease; CMV<sup>-</sup> KTR, CMV-seropositive kidney transplant recipients without CMV-infection/disease; D0, day of transplantation; IE-1, Immediate Early protein 1; M3, third month after kidney transplantation; M6, sixth month after kidney transplantation; n, number; pp65: Phosphoprotein 65; W6, sixth week after kidney transplantation. Median and 25<sup>th</sup> and 75<sup>th</sup> percentiles. The dotted line represents a strong CMV-specific response.

**Figure S2.** Evaluation of pp65-specific T-cell polyfunctionality in KTR with or without CMV infection/disease, from the transplant day to 6 months. On the transplant day, the absolute number of pp65-specific T cells producing one cytokine was higher in healthy donors, while double-cytokine secreting pp65-specific T-cells were higher in KTR group. There were no differences between KTR with or without CMV-infection/disease during the follow-up period. CMV<sup>+</sup> KTR, CMV-seropositive kidney transplant recipients with CMV-infection/disease; CMV<sup>-</sup> KTR, CMV-seropositive kidney transplant recipients without

CMV-infection/disease; D0, transplantation day; M3, third month after kidney transplantation; M6, sixth month after kidney transplantation; n, number; pp65, Phosphoprotein 65; W6, sixth week after kidney transplantation. Median and 25<sup>th</sup> and 75<sup>th</sup> percentiles, Mann-Whitney or Kruskal-Wallis tests for comparison of 2 or 3 groups. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ .

Supplementary material is linked to the online version of the paper at [www.kireports.org](http://www.kireports.org).

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