MAJOR ARTICLE

Common Features of γδ T Cells and CD8+ αβ T Cells Responding to Human Cytomegalovirus Infection in Kidney Transplant Recipients

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Background. Kidney transplant recipients infected with cytomegalovirus (CMV) undergo a persistent γδ T cell expansion in their peripheral blood. The anti-CMV function of these cells was previously demonstrated by their ability to kill CMV-infected cells in vitro.

Methods. To gain insight into the role of γδ T cells within the antiviral immune network, we compared the expansion kinetics of these T cells with that of CMV pp65–specific CD8+ αβ T cells in the peripheral blood of twenty-one kidney transplant recipients.

Results. Both the percentage and the absolute number of pp65-specific CD8+ T cells and γδ T cells showed a concomitant increase and persistence in most of the kidney transplant recipients with CMV infection. Both cell subsets exhibited an effector/memory phenotype (CD28−, CD27−, and CD45RA+) that predominated for the entire follow-up period.

Conclusions. In conclusion, CMV-specific CD8+ αβ T cells and γδ T cells share common expansion kinetics and a common effector phenotype, suggesting that these cell types act similarly in response to CMV infection.

In kidney transplant recipients, symptomatic cytomegalovirus (CMV) infection is associated with morbidity, acute rejections [1], and graft losses [2]. Efficient antiviral treatments exist; however, effective use of these treatments requires accurate viral and immunological monitoring. The well-known players of cellular adaptive immunity implicated in the resolution of CMV infection are CD4+ T cells and CD8+ T cells. In CMV-seropositive individuals, ~10% of the CD4+ and CD8+ memory T cells are specific for CMV [3]. Both CD4+ and CD8+ T cells can be cytotoxic against CMV peptide–loaded cells [4, 5]; however, CMV-specific CD8+ αβ T cells are the principal actors of the anti-CMV cellular immune response, as these cells can elaborate at least 2 effector functions (interferon-γ production and cytotoxicity) to kill CMV-infected fibroblasts [6, 7]. Moreover, adoptive transfer of CMV-specific CD8+ αβ T cells efficiently restricts CMV infection in recipients of allogeneic bone marrow [7]. Interestingly, these CMV-specific CD8+ T cells display a singular effector memory T_{EMRA} phenotype (CD45RA+CD27−) [8], which differs from the central memory phenotype (CD45RA−CD27+) expressed by CD8+ T cells specific for other latent viruses [9].

Several years ago, we reported the implications of a subset of γδ T cells in the anti-CMV response in kidney transplant recipients. These unconventional T cells are involved in diverse microbial infections in humans and are a minor subset (~4%) of peripheral blood T cells. The predominant population of circulating γδ T cells (~3% of T cells) express a T cell receptor encoded by the Vγ9 and Vδ2 gene segments, whereas other populations of γδ T cells use the Vδ1, Vδ3, or Vδ5 segments

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to form their T cell receptor (collectively designated as $V\delta2^{neg}$ $\gamma\delta$ T cells and comprising ~1% of circulating T cells) and normally reside in epithelia. CMV infection induces a major, persistent, and specific expansion of the Vδ2 negative ($V\delta2^{neg}$) $\gamma\delta$ T cells in the peripheral blood of kidney transplant recipients, and this cell population often represents >10% of the total lymphocyte count [10]. $V\delta2^{neg}$ $\gamma\delta$ T cell expansion kinetics differ widely from patient to patient. The duration and intensity of CMV infection, however, are greater in patients with late $\gamma\delta$ T cell expansion than in those with an early expansion. In fact, CMV infection regresses after the $V\delta2^{neg}$ $\gamma\delta$ T cell expansion, suggesting that these cells are involved in the resolution of this infection [11]. Extending these results, our recent studies in healthy blood donors demonstrated a specific correlation between CMV seropositivity and an increase of circulating $V\delta2^{neg}$ $\gamma\delta$ T cell percentage and repertoire restriction [12]. Furthermore, in vitro analyses showed that $V\delta2^{neg}$ $\gamma\delta$ T cell clones or cell lines from kidney transplant recipients with CMV infection are strongly reactive against CMV-infected fibroblasts. These cells are able to kill CMV-infected cells and yield large amounts of interferon-γ that limit CMV replication in vitro. This reactivity required T cell receptor engagement, but did not involve major histocompatibility complex class I molecules [13].

In general, $\gamma\delta$ T cells are considered to be intermediates between innate and adaptive immunity because of their rapid and massive responses to very diverse immune challenges. These features have been well demonstrated in mice, but a clear understanding of the timing of the $\gamma\delta$ T cell response to an infectious agent in humans is lacking. As a first approach to understand the timing of $V\delta2^{neg}$ $\gamma\delta$ T cell action during CMV infection in kidney transplant recipients, we compared the expansion kinetics and phenotype of peripheral blood $V\delta2^{neg}$ $\gamma\delta$ T cells with those of the CMV-specific $CD8^+$ $\alpha\beta$ T cells, as a reference cell type involved in the anti-CMV cellular immune response [14].

PATIENTS AND METHODS

Patients. Twenty-one consecutive human leukocyte antigen (HLA)–A2 kidney transplant recipients were included in this pilot prospective study that was conducted from November 2004 through April 2006. This research protocol was approved by the relevant local institutional review board (CPPRB Bordeaux A), and all patients gave their written consent. Subjects were longitudinally monitored for ~1 year to analyze the cellular immune response during and after CMV infection. Immunosuppressive treatment for all patients involved a combination daclizumab, cyclosporine or tacrolimus, mycophenolate mofetil, and prednisolone. Delayed graft function was defined as the need for hemodialysis within the first week after transplant. Acute rejection was diagnosed by kidney allograft biopsy. Only CMV-seronegative recipients who received CMV-seropositive allografts (donor positive [D^+], recipient negative [R^-]) were given oral valganciclovir for 3 months. CMV infection was defined by 2 consecutive positive whole blood CMV polymerase chain reaction (PCR) results. CMV infection was treated with intravenous ganciclovir for 3 weeks.

Quantitative CMV PCR in whole blood. Quantitative CMV PCR was performed in whole blood specimens as described elsewhere [15]. Analysis was performed weekly for the first 3 months after transplantation, then twice each month during the next 3 months, and then monthly.

Flow cytometric analysis. Blood was drawn in ethylene diamine tetraacetic acid, and flow cytometric analysis was performed within 24 h after obtaining the sample. The detection of pp65-specific CD8 $\alpha\beta$ T cells was performed through labeling with an HLA-A2 tetramer loaded with a pp65 peptide (NLVPMVATV; Proimmune). Five hundred μL of blood were incubated for 30 min at room temperature with the antibodies or HLA tetramers, then the blood was lysed and fixed with 2.5 mL of BD FACS lysing solution (BD Biosciences) for 10 min at room temperature. Cells were washed once with 3 mL of cell wash (BD Biosciences) and were resuspended in 200 μL of cell wash. All cells were analyzed on a BD FACSCanto cytometer with use of the FACSDiva software, version 5.0 (BD Biosciences). The following antibodies were purchased from BD Biosciences: anti-CD3 allophycocyanin (APC)-CY7; anti-CD8 PE-CY7; anti-CD27 fluorescein isothiocyanate (FITC); anti-CD45RA PE-CY5; anti-CD94 FITC; anti-CD28 PE; anti-CD158a,h FITC; anti-CD158b,j FITC; anti-CD45RA APC; anti-CD94 APC; anti-CD56 FITC; anti-CD16 FITC; anti-CD4 PE-CY7; anti-Perforin PE; and anti-Granzyme B PE. The following antibodies were purchased from Beckman-Coulter: anti-NKG2A PE, anti-NKG2D PE, anti-CD158e PE, anti-CD158i PE, anti-pan delta PE-CY5, anti-Vdelta2 PE-CY7, anti-CD45 PE-CY7, anti-CD45RO PE and anti-CD11a FITC. Permeabilization and fixation of cells for intracellular stainings were performed as described elsewhere [12]. All data are expressed as absolute counts (per mm^3) obtained by multiplying the percentages obtained with the flow cytometric analysis by the total lymphocyte count determined using an automatic hemocytometer (cell-dyn350; Abbott). The $\gamma\delta$ T cells and CMV-specific $CD8^+$ T cells were also expressed as percentages of total lymphocytes and $CD8^+$ T cells, respectively.

Statistical analysis. Comparisons were performed using conventional statistics. The Mac Nemar χ^2 test for qualitative variables, the Student's t test, or the Mann-Whitney test were used when appropriate. Analyses were performed with Statview Software (Abacus Concepts). Significance was defined as a P value <.05.

RESULTS

Baseline patient characteristics. Among the 21 patients included in this study, 8 developed CMV infection. The remain-

Table 1. Comparison of Baseline Clinical and Immunological Data between Cytomegalovirus (CMV)–Infected and Noninfected Patients

Characteristic	CMV-infected patients (n = 8)	Control patients (n = 13)	P
Demographic and clinical data			
Age, years	49.5 ± 10	49.3 ± 9.5	.9
Sex, M/F	5/3	10/3	.6
Duration of follow-up, mean months ± SD	408 ± 83	324 ± 126	.1
CMV status			
D^+ R^-	3	8	
R^+	5	5	.4
No. of HLA mismatches	3.1 ± 1.1	3.4 ± 1	.8
Cold ischemia, h	18 ± 7	18 ± 6	.9
Delayed graft function	5	2	.08
Receipt of cyclosporine/tacrolimus	4/4	7/6	.9
Acute rejection	1	4	.7
M6 serum creatinine value, μmol/L	147 ± 46	134 ± 39	.4
M12 serum creatinine value, μmol/L	149 ± 45	138 ± 47	.5
Baseline immunological data			
Total lymphocyte count, cells/mm^3	1357 ± 687	1549 ± 771	.6
NK cell count, cells/mm^3	146 ± 101	194 ± 119	.4
Vδ2 γδ T lymphocytes, %	1.86 ± 1.5	2.17 ± 1.7	.7
Vδ2 γδ T lymphocytes, cells/mm^3	28.1 ± 22	42.5 ± 52.3	.8
Vδ2-negative γδ T lymphocytes, %	1.53 ± 1.85	1.24 ± 1.26	.9
Vδ2 negative γδ T lymphocytes, cells/mm^3	17.7 ± 22.3	20.9 ± 21.9	.7
$CD4^+$ T lymphocyte count, cells/mm^3	594 ± 362	760 ± 367	.1
$CD8^+$ T lymphocyte count, cells/mm^3	260 ± 130	361 ± 302	.5
Among R^+ recipients			
pp65-HLA A2–specific $CD8^+$ T cells, %	0.59 ± 0.69[a]	0.28 ± 0.42[a]	.5
pp65-HLA A2–specific $CD8^+$ T cells, cells/mm^3	1.68 ± 2.50[a]	0.95 ± 1.07[a]	.9

NOTE. Data are no. of patients or mean values ± standard deviation. D^+, CMV-positive donor; HLA, human leukocyte antigen; NK, natural killer; R^-, CMV-negative recipient; R^+, CMV-positive recipient.

[a] n = 5.

ing 13 patients comprised the control group. Baseline data of the 2 groups of patients are summarized in Table 1. No statistical differences between the 2 groups regarding age, sex ratio, follow-up duration, donor/recipient CMV status (D^+/R^-, R^+, D^-/R^-), HLA mismatches, cold ischemia duration, delayed graft function occurrence, use of anti-calcineurin, acute rejection, and serum creatinine values were noted. Although not statistically significant, more D^+R^- patients were present in the control group (8 of 13) than in the CMV group (3 of 8), probably because only these patients received anti-CMV prophylactic treatment.

When the basal levels of several immunological parameters were compared on the day of the graft (day 0) in each group of patients, no statistical differences were observed between the 2 groups with regard to total lymphocyte count, $CD3^-CD16^+CD56^+$ natural killer (NK) cell count, Vγ9/Vδ2 T cell count and percentage, $Vδ2^{neg}$ γδ T cell count and percentage, $CD4^+$ T cell count, or $CD8^+$ T cell count were found (Table 1). The pp65-specific $CD8^+$ αβ T cells were detected via labeling with an HLA-A2 tetramer loaded with a pp65 peptide. As expected at day 0, R^- patients in either group did not have any pp65-specific $CD8^+$ T cells. Among R^+ patients, no statistical differences were observed between the infected and control groups regarding pp65-specific $CD8^+$ T cell count and percentage.

Concomitant and parallel expansion and persistence of $Vδ2^{neg}$ γδ T cells and CMV-specific $CD8^+$ T cells during CMV infection. The 21 patients were then longitudinally monitored for the immunological parameters cited above. No significant changes were observed for the total lymphocyte count, NK cell count, $CD4^+$ T cell count, $CD8^+$ T cell count, or Vγ9/Vδ2 T cell count or percentage during the follow-up period for any of the 21 studied patients (data not shown). We observed alterations in the percentages of pp65-specific $CD8^+$ T cells and/or $Vδ2^{neg}$ γδ T cells in the peripheral blood of CMV-infected kidney transplant recipients (Figure 1). When patients were analyzed individually, only 5 of 8 CMV-infected patients demonstrated pp65-specific $CD8^+$ T cell expansion (Figure 1, P1–

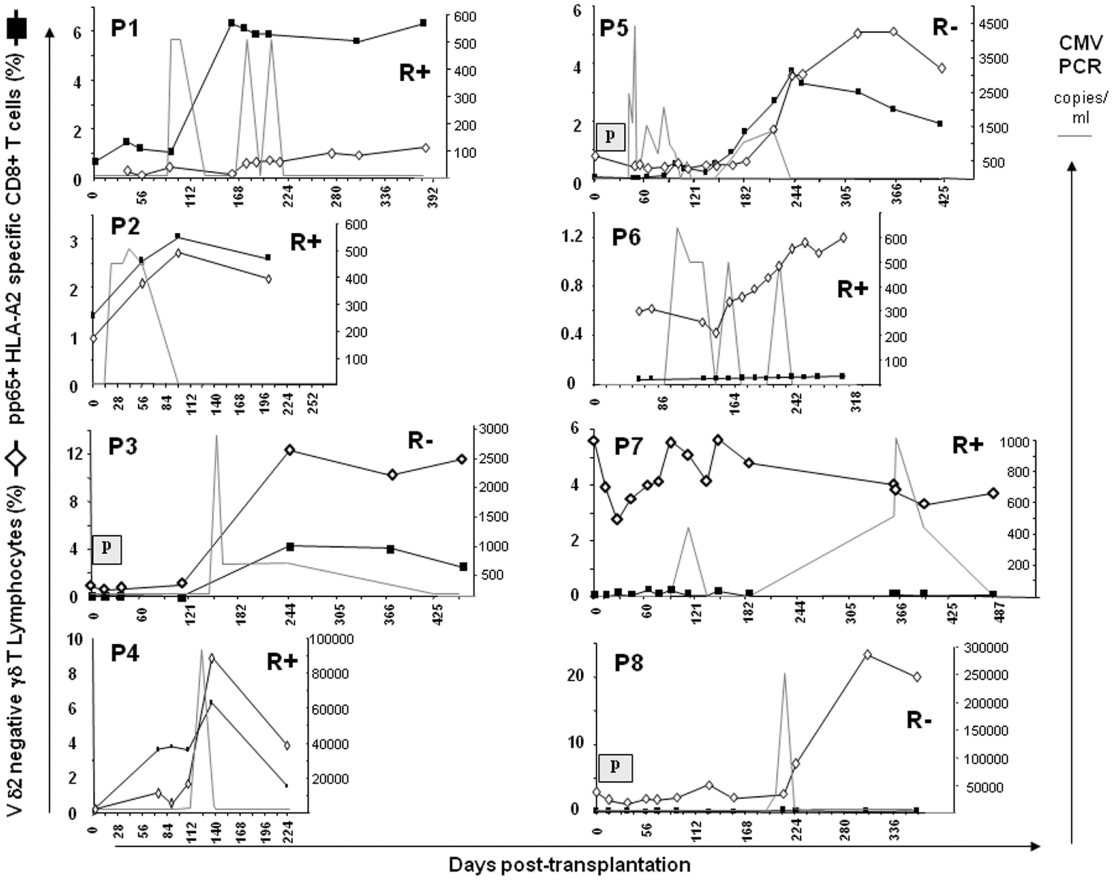

Figure 1. Concomitant expansion and parallel persistence of pp65 human leukocyte antigen (HLA)-A2–specific $CD8^+$ T cells and Vδ2-negative γδ T cells in peripheral blood of CMV-infected kidney transplant recipients. $CD8^+$ and γδ T cells subsets were analysed in the peripheral blood of eight patients (P1 to P8) with cytomegalovirus (CMV) infection. For CMV-specific $CD8^+$ T cell staining, blood was incubated with the anti-CD3 allophycocyanin (APC)-CY7 and anti-CD8 PE-CY7 antibodies as well as the pp65-HLA-A2 tetramers APC. For Vδ2-negative γδ T cell staining, blood was incubated with the anti-CD3 APC-CY7, anti-pan delta PE-CY5, and anti-Vdelta2 PE-CY7 antibodies. The γδ T cells and CMV-specific $CD8^+$ T cells are expressed as percentages of total lymphocytes and $CD8^+$ T cells, respectively. CMV polymerase chain reaction (PCR) results are represented as a thin line. The duration of the anti-CMV prophylaxis given to the 3 R^- patients is indicated by a grey rectangle containing a P (prophylaxis).

P5). The other 3 infected patients, who lacked this expansion (P6–P8), may have responded to other CMV antigenic peptides that we could not assess in this study. With regard to the $V\delta2^{neg}$ γδ T cells, all but 1 of the 8 CMV-infected patients displayed expansion of this cell subset after CMV infection. Of note, the only patient who did not demonstrate this cell expansion was an R^+ patient who already had a high peripheral blood $V\delta2^{neg}$ γδ T cell percentage on the day of the graft (Figure 1, P7), in accordance with that observed for other CMV-seropositive healthy individuals [12].

To analyze the effect of CMV on T cell subsets in the entire patient cohort, we compared the basal (at day 0) and the highest (peak) values of pp65-specific $CD8^+$ T cell and $V\delta2^{neg}$ γδ T cell percentages reached during the follow-up (Figure 2*A*). Among CMV-infected patients ($n = 8$), the basal mean value (± standard deviation [SD]) of pp65-specific $CD8^+$ T cell percentage was 0.39% ± 0.59% and reached 3.00% ± 2.64% after the infection ($P = .03$). Similarly, the basal mean value (±SD) of the $V\delta2^{neg}$ γδ T cell percentage was 1.53% ± 1.86% and reached 7.31% ± 7.55% after CMV infection ($P = .01$). In contrast, among patients not infected with CMV ($n = 13$), we did not observe any significant fluctuation between the basal level (±SD) and the highest mean values (±SD) of either pp65-specific $CD8^+$ T cells (0.11% ± 0.28% vs 0.23% ± 0.42%; $P = .2$) or $V\delta2^{neg}$ γδ T cells (1.25% ± 1.27% vs 1.69% ± 1.56%; $P = .6$). Similar results were observed when considering the absolute counts (Figure 2*B*). Of note, the mean absolute number (±SD) of $V\delta2^{neg}$ γδ T cells measured after CMV infection was greater than the mean absolute number (±SD) of pp65-specific $CD8^+$ T cells (108.9 ± 116.3 cells/mm^3 vs 14.7 ± 16.6 cells/mm^3; $P = .02$). Taken together, these results reveal the existence of concomitant expansion kinetics

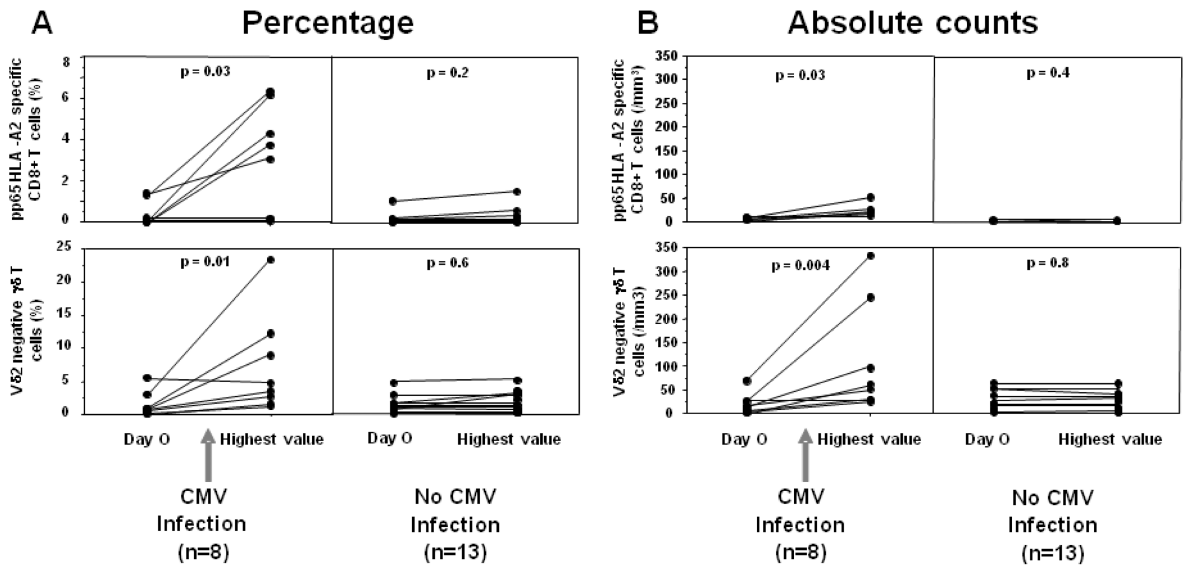

Figure 2. Comparison of the basal and maximum values of pp65-specific CD8⁺ T cell and V$\delta2^{neg}$ $\gamma\delta$ T cell percentages of kidney transplant recipients. Twenty-one patients were longitudinally monitored. The basal (day 0) and the highest values of pp65-specific CD8⁺ T cell and V$\delta2^{neg}$ $\gamma\delta$ T cell percentages and the absolute counts reached during the whole follow-up period are shown. *A,* $\gamma\delta$ T cells and cytomegalovirus (CMV)–specific CD8⁺ T cells are expressed as percentages of total lymphocytes and CD8⁺ T cells, respectively. *B,* Absolute counts (cells/mm^3) were obtained by multiplying the percentages by the total lymphocyte and CD8⁺ T cell counts, respectively.

of CMV-specific $\alpha\beta$ CD8⁺ and $\gamma\delta$ T cells in the peripheral blood of kidney transplant recipients, suggesting a similar type of response to CMV for both subsets.

V$\delta2^{neg}$ $\gamma\delta$ T cells and CMV-specific CD8⁺ T cells are terminally differentiated effectors. CMV has a peculiar propensity to drive the majority of responding CD8⁺ T cells to express a phenotype of terminally differentiated T cells (CD27⁻CD28⁻CCR7⁻CD45RA⁺) [8, 9]. Given the similar response of $\gamma\delta$ T cells and CMV-specific CD8⁺ T cells that we observed, we next determined whether these cell types also exhibited a similar effector phenotype during CMV infection in kidney transplant recipients. Thus, we determined the status of their CD27 and CD45RA membrane expression, which distinguishes naive (CD45RA⁺CD27⁺), effector memory T_{EMRA} (CD45RA⁺CD27⁻), effector memory T_{EMh} (CD45RA⁻CD27⁻), and central memory T cells (CD45RA⁻CD27⁺) in both $\alpha\beta$ and $\gamma\delta$ T cell populations [8, 16]. When analyzed at the peak of their expansion (the highest value) in CMV-infected kidney transplant recipients, the majority of pp65-specific CD8⁺ T cells were terminally differentiated effector memory T cells (CD45RA⁺CD27⁻) also called T_{EMRA} (mean, 56%; median, 43.5%) (Figure 3*A*), as reported elsewhere [8]. The whole CD8⁺ T cell population, however, presented a different picture with a balanced mixture of the 3 different subsets: effector memory T_{EMRA}, 33.5% (median, 27.7%); naive T cells, 28.8% (median, 32.3%); effector memory T_{EMh}, 11.2% (median, 9.6%); and central memory T cells, 26.5% (median, 26.7%).

Strikingly, V$\delta2^{neg}$ $\gamma\delta$ T cells displayed a very similar phenotype to the primarily terminally differentiated effector memory T_{EMRA} phenotype (mean, 63.7%; median, 61.5%). We observed significantly more effector memory T_{EMRA} ($P = .006$) and significantly less naive ($P = .03$) V$\delta2^{neg}$ $\gamma\delta$ T cells in CMV-infected patients, compared with patients without CMV infection (Figure 3*B*), suggesting that the T_{EMRA} phenotype on V$\delta2^{neg}$ $\gamma\delta$ T cells was driven by CMV infection. This distribution was in stark contrast to that of Vγ9/Vδ2 T cells, which do not participate in the anti-CMV response [17] and principally express a central memory cell phenotype in both CMV-infected and CMV-uninfected patients [18].

It is noteworthy that among both CMV-specific $\alpha\beta$ CD8⁺ T cells and V$\delta2^{neg}$ $\gamma\delta$ T cells, the second predominant subset was the naive CD45RA⁺CD27⁺ subset (Figure 3). Such an substantial amount of CMV-specific naive cells was quite surprising. Among CD45RA⁺CD27⁺ T cells, true naive cells can be distinguished from recently primed T cells through their bright expression of CD27 and dull expression of CD11a [19]. As depicted in Figure 4*A*, the CD45RA⁺CD27⁺ V$\delta2^{neg}$ $\gamma\delta$ T cells and pp65-specific CD8⁺ T cells turned out to be mainly CD27-dull CD11a-high (Figure 4*A*), a phenotype considered to identify non-naive cells and most probably recently primed T cells [19].

During longitudinal monitoring, we noted that R⁺ patients already demonstrated, before the infection, a predominant effector T_{EMRA} phenotype among pp65-specific CD8⁺ T cells and V$\delta2^{neg}$ $\gamma\delta$ T cells, whereas in R⁻ patients, this phenotype became

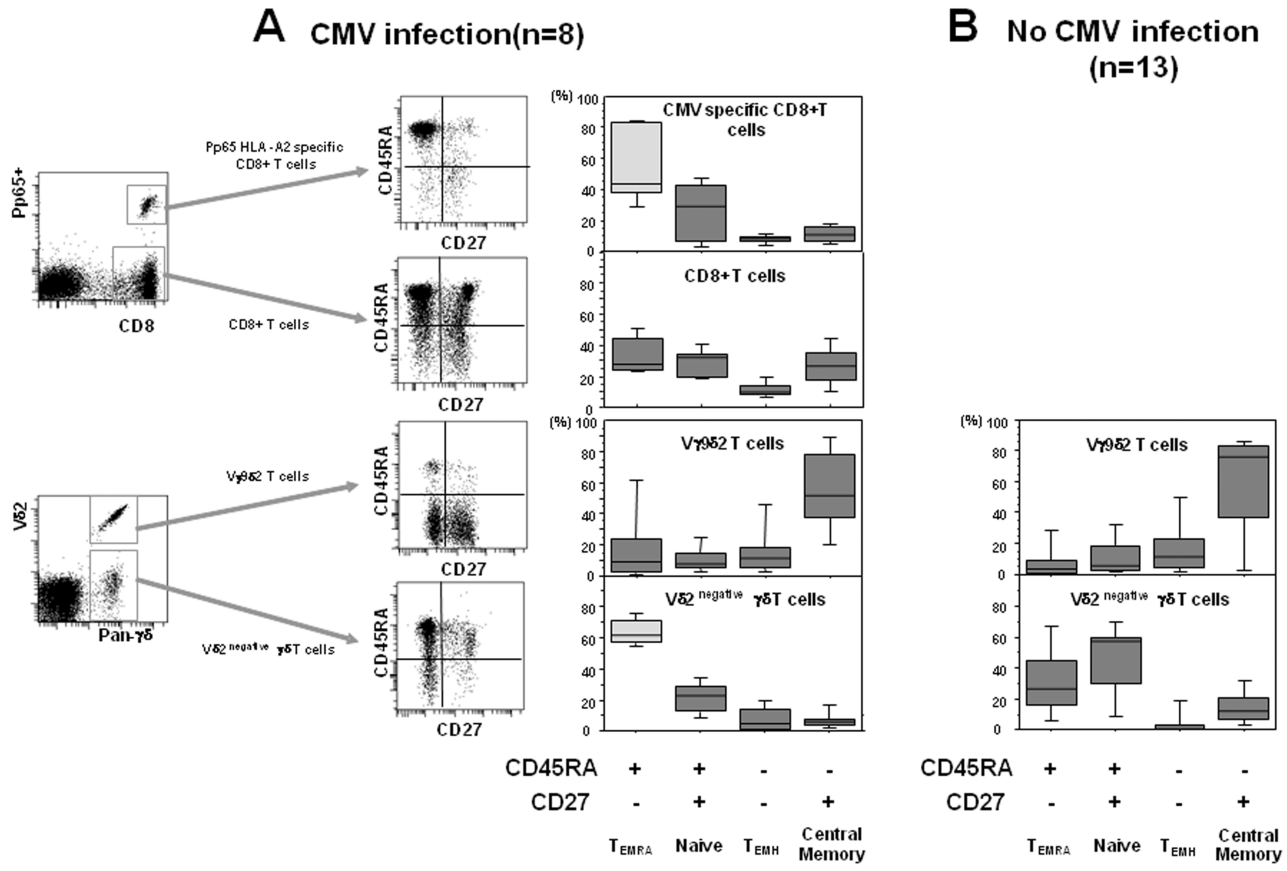

Figure 3. Predominance of terminally differentiated effector memory cells CD45RA$^+$ CD27$^-$ (T_{EMRA}) among pp65-specific CD8$^+$ T cells and V$\delta2^{neg}$ $\gamma\delta$ T cells after cytomegalovirus (CMV) infection in kidney transplant recipients. Cell staining was performed using anti-CD27 fluorescein isothiocyanate and anti-CD45RA PE-CY5 or allophycocyanin (APC) antibodies. The phenotypes of the pp65-specific CD8$^+$ T cells and V$\delta2^{neg}$ $\gamma\delta$ T cells analyzed at the peak in kidney transplant recipients are shown: naïve (CD45RA$^+$CD27$^+$), effector memory T_{EMRA} (CD45RA$^+$CD27$^-$), effector memory T_{EMh} (CD45RA$^-$CD27$^-$), or central memory T cells (CD45RA$^-$ CD27$^+$). The V$\delta2^{neg}$ $\gamma\delta$ T cell phenotypes of the 8 CMV-infected kidney transplant recipients have been studied, whereas only the 5 patients with pp65-specific CD8$^+$ T cell expansion were analyzed at the peak. Results are expressed as medians.

prevalent after infection (Figure 5). This common effector cell phenotype shared by CMV-specific $\alpha\beta$ CD8$^+$ and $\gamma\delta$ T cells suggests a similar behavior and function of both T cell subsets in the antiviral host defense.

Perforin, granzyme, CD28, and NK receptor expression by V$\delta2^{neg}$ $\gamma\delta$ T cells and CMV-specific CD8$^+$ T cells during CMV infection. To extend the comparison between V$\delta2^{neg}$ $\gamma\delta$ T cells and pp65-specific CD8$^+$ T cells after CMV infection, we analyzed several markers specific of effector/memory T cells. We first observed that the T_{EMRA} V$\delta2^{neg}$ $\gamma\delta$ T cells and pp65-specific CD8$^+$ T cells of the CMV-infected patients displayed high expression of perforin and granzyme B (Figure 4*B*).

We also analyzed their cell surface expression of CD28, a marker known to be down-modulated on fully differentiated effector $\alpha\beta$ T cells [8, 9]. In the 8 patients who experienced CMV infection, the pp65-specific CD8$^+$ T cells and V$\delta2^{neg}$ $\gamma\delta$ T cells displayed a much lower percentage of CD28$^+$ cells than the other CD8$^+$ T cells and Vγ9/Vδ2 $\gamma\delta$ T cells (Table 2). Moreover, CMV-infected patients displayed a lower CD28 expression on V$\delta2^{neg}$ $\gamma\delta$ T cells than did CMV-uninfected patients.

Next, the expression of the NK cell receptors for major histocompatibility complex class I molecules or related molecules was investigated, because these molecules are important for regulating $\gamma\delta$ T cell reactivity [20]. Also, these receptors reflect the maturation of T cells following antigen exposure or CMV infection [21, 22]. We looked at NKG2A-CD94$^+$, which is an inhibitory receptor for HLA-E that is induced by CMV on infected cells [23]. Both V$\delta2^{neg}$ $\gamma\delta$ T cells and pp65-specific CD8$^+$ T cells of CMV-infected patients comprised low percentages ($\pm$SD) of NKG2A-CD94$^+$ cells (4.1% $\pm$ 5.4% and 14.4% $\pm$ 7.9% of cells, respectively). Interestingly, expression of CD158 a/h and CD158 b/j molecules was higher on V$\delta2^{neg}$ $\gamma\delta$ T cells than on pp65-specific CD8$^+$ T cells and Vγ9/Vδ2 T cells. Finally, CMV-infected patients had a lower NKG2A-CD94$^+$ expression but a higher CD158 b/j expression on V$\delta2^{neg}$ $\gamma\delta$ T cells than did CMV-uninfected patients. This high CD158b/j

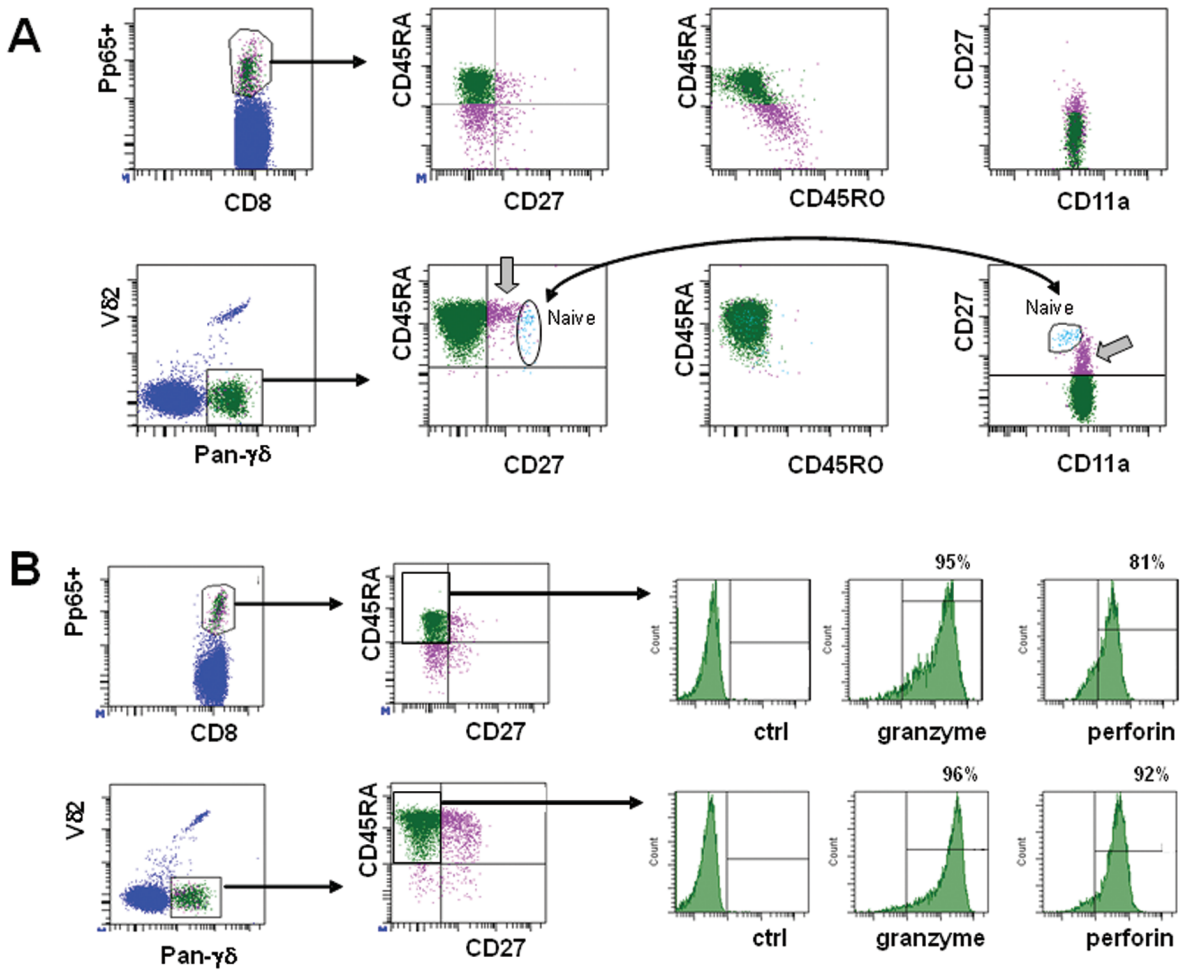

Figure 4. *A,* High expression of CD11a by CD45RA$^+$CD27$^+$ pp65-specific CD8$^+$ T cells and V$\delta2^{neg}$ $\gamma\delta$ T cells from cytomegalovirus (CMV)–infected patients. The markers CD45RO and CD11a were added to our previous multicolor staining (CD3, pan-$\gamma\delta$, Vδ2, CD45RA, CD27). For pp65-specific CD8$^+$ T cells of the 4 patients analyzed, only few CD45RA$^+$CD27$^+$ cells were observed and no CD45RA$^+$ CD27$^+$ cells were CD11a dull (ie, real naive cells). Among the V$\delta2^{neg}$ $\gamma\delta$ T cells of the representative patient presented in panel *A,* only 12.2% of the CD45RA$^+$ CD27$^+$ cells were CD11a dull (real naive cells). The majority of the CD45RA$^+$CD27$^+$ V$\delta2^{neg}$ $\gamma\delta$ T cells were CD11a high and thus non-naive (*gray arrow*). *B,* T_{EMRA} pp65-specific CD8$^+$ T cells and V$\delta2^{neg}$ $\gamma\delta$ T cells from CMV-infected patients express high levels of perforine and granzyme B. Intra cellular staining of permeabilized cells was performed with specific anti-perforin and anti-granzyme B antibodies. This patient is representative of the 8 CMV-infected patients included in the study. ctrl, control.

expression could also be driven by CMV infection and may be involved in the regulation of V$\delta2^{neg}$ $\gamma\delta$ T cell functions that are distinct from that of pp65-specific CD8$^+$ T cells.

DISCUSSION

In this study, we compared V$\delta2^{neg}$ $\gamma\delta$ T cells to CMV-specific CD8$^+$ $\alpha\beta$ T cells during the course of CMV infection in kidney transplant recipients. Despite variable expansion between patients, similar expansion kinetics were shared by the 2 subsets in most of the patients. Therefore, these data highlight the redundancy of the immune system and indicate that mechanisms involved in the response of both V$\delta2^{neg}$ $\gamma\delta$ T cells and pp65 HLA-A2–specific CD8$^+$ T cells may be similar. Because we can only analyze the cells in the peripheral blood, the early response of each cell subset to the CMV in the infected tissues is out of reach. V$\delta2^{neg}$ $\gamma\delta$ T cells are normally located in the epithelia where they are considered to be sentinels readily responding in the early phase of an immune challenge. The circulating subsets analyzed here may reflect the phase of the antiviral response when the multiplying effector T cells migrate out of the infected sites to the periphery. Nonetheless, the similar timing of the CD8$^+$ $\alpha\beta$ and $\gamma\delta$ T cell subsets detected in the blood suggests that these cell types are involved and proliferate concomitantly during the antiviral response. This is not the case for CMV-specific CD4$^+$ T cells, which display an independent and heterogeneous expansion [5, 14].

The V$\delta2^{neg}$ $\gamma\delta$ T cells and the pp65-specific CD8$^+$ T cells shared the same terminally differentiated effector memory

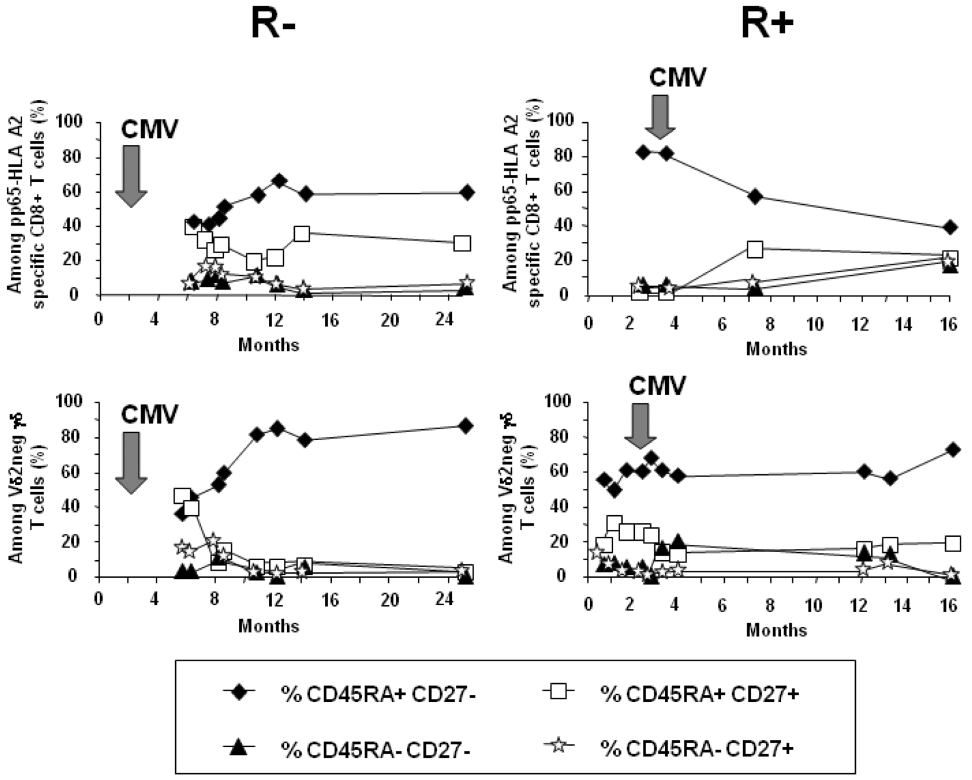

Figure 5. The effector phenotypes of T_{EMRA} of $V\delta2^{neg}$ $\gamma\delta$ T cells and pp65 HLA-2-specific $CD8^+$ T cells are stable over time. Analyses of peripheral blood from 2 representative cytomegalovirus (CMV)–infected kidney transplant recipients are depicted. Data are lacking for the first graft weeks of R^- kidney transplant recipients because of the absence of peripheral blood expansion of these 2 lymphocyte subsets at this time. The data shown in this figure are representative of the 8 CMV-infected patients included in the study.

T_{EMRA} phenotype. It has been shown that T_{EMRA} CMV-specific $CD8^+$ T cells can proliferate in vitro on activation with CD137-expressing APC or interleukin-15 [24, 25] and T_{EMRA} $V\gamma9V\delta2$ T cells can proliferate ex vivo with interleukin-15 [26]. Such accumulation of CMV-specific cells over time has also been described in the murine model of CMV, a phenomenon called memory inflation. It was recently shown that the inflationary murine CMV–specific T cells are terminally differentiated T cells able to divide in response to viral antigen in vivo and are a dynamic population with a short half-life in the blood that

Table 2. Cell Surface Expression of CD28 and Natural Killer (NK) Receptors for Major Histocompatibility Complex Class I Molecules or Related Molecules

	CMV-infected patients (n = 8)						CMV-uninfected patients (n = 13)		
Marker[a]	$CD8^+$ T cells, %	CMV-specific $CD8^+$ T cells, %	P	$V\gamma9/V\delta2$ T cells, %	$V\delta2$-negative $\gamma\delta$ T cells, %	P	$V\gamma9/V\delta2$ T cells, %	$V\delta2$-negative $\gamma\delta$ T cells, %	P
CD28	30.6 ± 13.9	6.5 ± 3	.04	61.7 ± 27.5	10.8 ± 10 [d]	.01	55.3 ± 30.6	33.8 ± 20.9 [d]	.02
NKG2A-CD94	9.5 ± 13.7	4.1 ± 5.4	.3	40.7 ± 18.7 [c]	14.4 ± 7.9 [d]	.01	64.7 ± 20.7 [c]	36.8 ± 26.9 [d]	.02
CD158 a/h	1.4 ± 1.2	3.6 ± 7.4 [b]	.9	2.7 ± 3.5	21.2 ± 30.2 [b]	.01	0.9 ± 1.1	7.8 ± 17.8	.002
CD158 b/j	3.8 ± 4.6	3.7 ± 5.8 [b]	.7	10.3 ± 9.1	50.1 ± 24.2 [bd]	.01	6.8 ± 8.4	26.4 ± 25.6 [d]	.004

NOTE. Data are mean values ± standard deviation, unless otherwise indicated. CMV, cytomegalovirus.

[a] The human leukocyte antigen A2–specific $CD8^+$ T cells and $V\delta2^{neg}$ $\gamma\delta$ T cells analyzed at the peak in kidney transplant recipients were stained with the anti-CD28 PE, anti-NKG2D PE, anti-CD94 fluorescein isothiocyanate (FITC) or allophycocyanin, anti-NKG2A PE, anti-CD158a,h FITC, anti-CD158b,j FITC, anti-CD158e PE, and anti-CD158i PE antibodies.

[b] $V\delta2^{neg}$ $\gamma\delta$ T cells versus CMV-specific $CD8^+$ T cells, $P = .04$.

[c] $V\delta2$ $\gamma\delta$ T cells of CMV-infected patients versus $V\delta2$ $\gamma\delta$ T cells of CMV-uninfected patients, $P = .03$.

[d] $V\delta2^{neg}$ $\gamma\delta$ T cells of CMV-infected patients versus $V\delta2^{neg}$ $\gamma\delta$ T cells of CMV-uninfected patients, $P < .01$.

are constantly replaced by an influx of differentiated cells [27]. In humans, this influx could be represented by the recently primed $CD45RA^+CD27^{+(dull)}CD11a^{+(bright)}$ T cells [19].

In R^- transplant recipients, we can presume that naive cells differentiate in T_{EMRA} cells, either directly or more probably via an intermediate T_{EMh} phenotype, as proposed elsewhere for Vγ9Vδ2 T cells [18]. In R^+ transplant recipients, the slight increase of $CD45RA^+CD27^+$ CMV-specific αβ T cells might correspond to the new recruitment of recently primed cells [27].

Similar to Vγ9Vδ2 T cells in healthy donors and CMV-specific $CD8^+$ T cells in kidney transplant recipients, cells expressing the T_{EMRA} phenotype display a high cytotoxic potential [5, 16, 18]. The low cell surface expression of CD28 by both $V\delta2^{neg}$ γδ T cells and pp65 HLA-A2–specific $CD8^+$ T cells is also consistent with this effector phenotype [9]. To summarize, the similarities between the $V\delta2^{neg}$ γδ T cell and pp65 HLA-A2–specific $CD8^+$ T cell phenotypes suggest a similar cytolytic function against CMV-infected cells in vivo. Thus, protection against CMV disease was associated with a rapid recovery of CMV-specific $CD8^+$ T cells in bone marrow transplanted patients [28] and with a rapid expansion of $V\delta2^{neg}$ γδ T cells in kidney transplant recipients [11].

On binding their ligand, NK receptors transmit activating or inhibitory signals to the cells. CMV infection induces the expression of activating NK receptors, such as NKG2D and the heterodimer NKG2C-CD94, at the cell surface of CMV-specific $CD8^+$ T cells [21, 22, 29]. Similar to CMV-specific $CD8^+$ T cells, all $V\delta2^{neg}$ γδ T cells expressed NKG2D (data not shown), a major activating NK receptor, whereas only a few of these cells expressed CD94-NKG2A, which is an inhibitory NK receptor. This result is consistent with the effector phenotype described above. CD158 was expressed on a small proportion of pp65-specific $CD8^+$ T cells, consistent with results reported elsewhere [8], but was also present on a large fraction of $V\delta2^{neg}$ γδ T cells. Because the quality of the signal delivered by CD158 molecules depends on the composition of the intracellular part of the individual receptor or associated molecules, it is difficult to speculate on their precise action; however, these NK receptors may play an important role in the control of T cell activation when the virus persists and coexists with its host for many years [21]. Previously, we showed that CD158j expression confers an enhanced T cell receptor–induced cytotoxic activity to $V\delta2^{neg}$ γδ T cells isolated from a CMV-infected kidney transplant recipients, compared with their CD158-negative counterparts [30].

Finally, this pilot study demonstrates that CMV-specific $CD8^+$ T cells and $V\delta2^{neg}$ γδ T cells are committed in a similar fashion to the anti-CMV host response. These results confirm and extend our previous report demonstrating that expansion of $V\delta2^{neg}$ γδ T cells is specific to CMV infection [10, 12, 13]. In addition, we noted that this expansion was larger in terms of absolute count than that of pp65-specific $CD8^+$ T cells. The use of anti-CMV prophylaxis does not seem to interfere with this marker, which can be monitored in both R^- and R^+ recipients. Thus, $V\delta2^{neg}$ γδ T cell detection could be a realistic candidate for CMV infection monitoring in kidney transplant recipients. Furthermore, phenotyping of these cells by flow cytometry is easy and fast. In the future, larger studies are necessary to evaluate the potential role of these newly described effector cells in the prevention of CMV infection or disease in kidney transplant recipients.

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References

1. Sageda S, Nordal KP, Hartmann A, et al. The impact of cytomegalovirus infection and disease on rejection episodes in renal allograft recipients. Am J Transplant **2002**; 2:850–6.
2. Nett PC, Heisey DM, Fernandez LA, Sollinger HW, Pirsch JD. Association of cytomegalovirus disease and acute rejection with graft loss in kidney transplantation. Transplantation **2004**; 78:1036–41.
3. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific $CD4^+$ and $CD8^+$ T cells dominate the memory compartments of exposed subjects. J Exp Med **2005**; 202:673–85.
4. van Leeuwen EM, Remmerswaal EB, Heemskerk MH, ten Berge IJ, van Lier RA. Strong selection of virus-specific cytotoxic $CD4^+$ T-cell clones during primary human cytomegalovirus infection. Blood **2006**; 108:3121–7.
5. Gamadia LE, Remmerswaal EB, Weel JF, Bemelman F, van Lier RA, Ten Berge IJ. Primary immune responses to human CMV: a critical role for IFN-gamma-producing $CD4^+$ T cells in protection against CMV disease. Blood **2003**; 101:2686–92.
6. Betts MR, Price DA, Brenchley JM, et al. The functional profile of primary human antiviral CD8+ T cell effector activity is dictated by cognate peptide concentration. J Immunol **2004**; 172:6407–17.
7. Cobbold M, Khan N, Pourgheysari B, et al. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. J Exp Med **2005**; 202:379–86.
8. Gamadia LE, Rentenaar RJ, Baars PA, et al. Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers. Blood **2001**; 98:754–61.
9. Appay V, Dunbar PR, Callan M, et al. Memory $CD8^+$ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med **2002**; 8:379–85.
10. Dechanet J, Merville P, Lim A, et al. Implication of gammadelta T cells in the human immune response to cytomegalovirus. J Clin Invest 1999; 103:1437–49.
11. Lafarge X, Merville P, Cazin MC, et al. Cytomegalovirus infection in transplant recipients resolves when circulating gammadelta T lymphocytes expand, suggesting a protective antiviral role. J Infect Dis **2001**; 184:533–41.
12. Pitard V, Roumanes D, Lafarge X, et al. Long term expansion of effector/memory $V\delta2^-$ γδ T cells is a specific blood signature of CMV infection. Blood **2008**; 112:1317–24.

13. Halary F, Pitard V, Dlubek D, et al. Shared reactivity of Vδ2$^-$ γδ T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. J Exp Med **2005**; 201:1567–78.
14. Sester M, Sester U, Gartner BC, Girndt M, Meyerhans A, Kohler H. Dominance of virus-specific CD8 T cells in human primary cytomegalovirus infection. J Am Soc Nephrol **2002**; 13:2577–84.
15. 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: 493–8.
16. Angelini DF, Borsellino G, Poupot M, et al. FcgammaRIII discriminates between 2 subsets of Vγ9Vδ2 effector cells with different responses and activation pathways. Blood **2004**; 104:1801–7.
17. 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.
18. Dieli F, Poccia F, Lipp M, et al. Differentiation of effector/memory Vδ2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med **2003**; 198:391–7.
19. De Rosa SC, Andrus JP, Perfetto SP, et al. Ontogeny of gamma delta T cells in humans. J Immunol **2004**; 172:1637–45.
20. De Libero G. Control of γδ T cells by NK receptors. Microbes Infect **1999**; 1:263–7.
21. van Stijn A, Rowshani AT, Yong SL, et al. Human cytomegalovirus infection induces a rapid and sustained change in the expression of NK cell receptors on CD8$^+$ T cells. J Immunol **2008**; 180:4550–60.
22. Lopez-Botet M, Angulo A, Guma M. Natural killer cell receptors for major histocompatibility complex class I and related molecules in cytomegalovirus infection. Tissue Antigens **2004**; 63:195–203.
23. Tomasec P, Braud VM, Rickards C, et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science **2000**; 287:1031.
24. Waller EC, McKinney N, Hicks R, Carmichael AJ, Sissons JG, Wills MR. Differential costimulation through CD137 (4–1BB) restores proliferation of human virus-specific "effector memory" (CD28(-) CD45RA(HI)) CD8(+) T cells. Blood **2007**; 110:4360–6.
25. van Leeuwen EM, Gamadia LE, Baars PA, Remmerswaal EB, ten Berge IJ, van Lier RA. Proliferation requirements of cytomegalovirus-specific, effector-type human CD8$^+$ T cells. J Immunol **2002**; 169:5838–43.
26. Caccamo N, Meraviglia S, Ferlazzo V, et al. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vγ9Vδ2 naive, memory and effector T cell subsets. Eur J Immunol **2005**; 35:1764–72.
27. Snyder CM, Cho KS, Bonnett EL, van Dommelen S, Shellam GR, Hill AB. Memory inflation during chronic viral infection is maintained by continuous production of short-lived, functional T cells. Immunity **2008**; 29:650–9.
28. Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood **1991**; 78:1373–80.
29. Huard B, Karlsson L. KIR expression on self-reactive CD8$^+$ T cells is controlled by T-cell receptor engagement. Nature **2000**; 403:325–8.
30. Lafarge X, Pitard V, Ravet S, et al. Expression of MHC class I receptors confers functional intraclonal heterogeneity to a reactive expansion of gammadelta T cells. Eur J Immunol **2005**; 35:1896–905.