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Age-Dependent Association between Low Frequency of CD27/CD28 Expression on pp65 $CD8^+$ T Cells and Cytomegalovirus Replication after Transplantation[▽]

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In this cross-sectional study of 42 solid organ transplant recipients, the association of human cytomegalovirus (HCMV) replication and age with the phenotype of the HCMV-specific $CD8^+$ T cells was analyzed by using the CMV pp65 HLA-A*0201 pentamer. A correlation between the proportion of $CD28^-$ HCMV-specific $CD8^+$ T cells and age was observed in patients without HCMV replication ($r = 0.50$; $P = 0.02$) but not in patients with HCMV replication ($r = -0.05$; $P = 0.83$), a finding which differs from that observed for total $CD8^+$ T cells. Within the group of patients younger than 50 years of age, patients with HCVM replication after transplantation had higher percentages of $CD28^-$ HCMV-specific $CD8^+$ T cells (85.6 compared with 58.7% for patients without HCMV replication; $P = 0.004$) and $CD27^-$ HCMV-specific $CD8^+$ T cells (90.7 compared with 68.8% for patients without HCMV replication; $P = 0.03$). However, in patients older than age 50 years, a high frequency of these two subpopulations was observed in patients both with and without previous HCMV replication (for $CD28^-$ HCMV-specific $CD8^+$ T cells, 84.4 and 80.9%, respectively [$P = 0.39$]; for $CD27^-$ HCMV-specific $CD8^+$ T cells 86.6 and 81.5%, respectively [$P = 0.16$]). In conclusion, the present study shows that in the group of recipients younger than age 50 years, HCMV replication after transplantation is associated with a high percentage of $CD27^-$ and $CD28^-$ HCMV-specific $CD8^+$ T cells. These results suggest that the increased percentage of $CD27^-$ or $CD28^-$ HCMV-specific subsets can be considered a biomarker of HCMV replication in solid organ transplant recipients younger than age 50 years but not in older patients. Further studies are necessary to define the significance of these changes in HCMV-associated clinical complications posttransplantation.

Although a robust innate immune response is quickly induced just after the entry of human cytomegalovirus (HCMV) (21), the adaptive response of $CD8^+$ T cells plays a major role in the control of HCMV infection (9, 19, 36). The development of HLA multimer technology and the analysis of surface markers (CD45RA, CCR7, CD27, CD28) have allowed the study of the CMV-specific $CD8^+$ T-cell immune response and its phenotypic characteristics. Naïve cells ($CD45RA^+$ $CCR7^+$), which lose the ability to express CD45RA after interaction with the antigen, become memory cells and comprise two subpopulations: central memory cells ($CD45RA^-$ $CCR7^+$) and effector memory cells ($CD45RA^-$ $CCR7^-$). Subsequently, a fraction of the HCMV-specific effector memory cells may revert to RA expression ($CD45RA^+$ $CCR7^-$) after the acute phase of infection (32, 34). CD28 and CD27 costimulatory molecules, however, are also useful markers in determining the phenotype of $CD8^+$ T cells and allow their classification into early memory cells ($CD27^+$ $CD28^+$), intermediate memory cells ($CD27^-$ $CD28^+$), and late memory cells ($CD27^-$ $CD28^-$), which progressively increase their levels of perforin production (3, 32).

Latent HCMV infection is associated with the massive clonal expansion of virus-specific memory $CD8^+$ T cells (47), which is especially pronounced in HCMV-seropositive elderly patients. In these patients, the population of HCMV-specific $CD8^+$ T cells shows a highly differentiated phenotype with a high percentage of $CD28^-$ lymphocytes and a series of functional changes associated with a process of immune senescence. It has been suggested that HCMV may contribute significantly to this senescence process (29, 33, 45, 50). In fact, the combination of HCMV seropositivity with a high percentage of $CD28^-$ cells and inversion of the $CD4^+$/$CD8^+$ ratio define the so-called immune risk phenotype, which some longitudinal studies have associated with decreased survival in individuals older than 80 years of age (1, 23, 24, 29, 53).

Because of immunosuppression, there is a higher frequency of CMV reactivation in CMV-seropositive transplant recipients and of primary infection in CMV-seronegative recipients receiving a CMV-seropositive organ (donor positive and recipient negative [D^+/R^-] patients) (13–15). HCMV replication may induce the accumulation of HCMV-specific $CD8^+$ T cells observed in some studies, which may account for as much as 10 to 50% of the total $CD8^+$ T-cell pool (26, 27). Furthermore, other studies show that these cell clones may have a highly

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differentiated phenotype (5, 6, 18) and may show functional changes similar to those observed in HCMV-seropositive elderly individuals (10, 17, 26). These observations led us to propose the hypothesis that transplant patients with HCMV replication after transplantation may have a population of HCMV-specific $CD8^+$ T cells with a more differentiated phenotype and a higher percentage of $CD28^-$ and/or $CD27^-$ cells than transplant patients without replication. In order to test this hypothesis, a cross-sectional study was carried out with patients who had received a solid organ transplant at least 1 year earlier to determine the frequency and phenotype of the HCVM-specific $CD8^+$ T-cell population and its relationship to age and HCMV replication posttransplantation.

MATERIALS AND METHODS

Study population and design. The study was carried out at a single center (Reina Sofia University Hospital, Córdoba, Spain). From October 2007 to April 2008, 42 patients who had undergone a transplant ($n = 12$ lung transplants, $n = 30$ kidney transplants) at least 1 year earlier were included in the study. All patients had the HLA-A*02 allele and correct posttransplantation monitoring for HCMV replication. Monitoring for HCMV replication was considered to be correct when the HCMV load was determined at least weekly during the hospitalization, at least every 2 weeks until the third month, and monthly until the first year. The patients studied received the transplants between March 2005 and November 2006. To prevent HCMV replication, preemptive therapy was used when evidence of asymptomatic HCMV infection was detected by PCR, and universal prophylaxis was administered only to individuals at high risk, such as D^+/R^- patients, recipients receiving antilymphocyte therapy, and lung recipients. The study was approved by the ethics committee of the Reina Sofia University Hospital.

Determination of anti-HCMV immunoglobulin G antibodies and HCMV load. The presence of anti-HCMV immunoglobulin G antibodies pretransplantation was determined for the donors and the recipients by chemoluminescence assay (Diasorin SA).

The HCMV load was determined by quantitative PCR (Cobas Amplicor, Roche). Viral DNA was isolated from citrate-anticoagulated whole-blood samples, according to the manufacturer's instructions. The detection limit of this test is 400 genomes/ml, so values above this limit were considered positive viral loads.

Immunosuppression and prevention of HCMV infection. Initial immunosuppression consisted of cyclosporine-tacrolimus combined with mofetil mycophenolate-azathioprine and steroids. Episodes of acute rejection were treated with steroid boluses or recycling of steroid tapers. Lung transplant patients received prophylaxis with intravenous ganciclovir (5 mg/kg of body weight/day)-valganciclovir (900 mg/day) for 6 months. Kidney transplant patients received preemptive therapy (900 mg/12 h) or universal prophylaxis (900 mg/day) with valganciclovir for 3 months. Episodes of viral replication were treated for at least 2 weeks or until the PCR result was negative.

Isolation of mononuclear cells from peripheral blood. Peripheral blood mononuclear cells (PBMCs) were obtained from 20 ml of peripheral whole blood in tubes with acid-citrate-dextrose by Ficoll-Histopaque density gradient centrifugation. Purified PBMCs were cryopreserved at −80°C in fetal calf serum containing 10% dimethyl sulfoxide. PBMCs were obtained during routine follow-up visits posttransplantation. No evidence of acute HCMV infection at the time of PBMC extraction was found.

Study of HCMV-specific $CD8^+$ T-cell population. The PBMC samples were thawed in a 37°C water bath; and the lymphocytes were centrifuged at 1,800 rpm for 5 min or until a pellet was obtained, resuspended in phosphate-buffered saline, counted, and aliquoted at approximately 500,000 cells per tube.

For the study of the HCMV-specific $CD8^+$ T-cell population, an HLA-A*0201 pentamer bound to an immunodominant epitope of HCMV (pp65, NLVPMV ATV) labeled with allophycocyanin (Proimmune) was used. Although the analysis of virus-specific T cells with multimers is limited by the allele and the peptide used, no differences in the phenotype or function of multimer-positive cells depending on the HLA allele considered have been reported. Thus, the findings obtained with HLA-A2 pentamers can be generalized to other virus-specific CD8 populations (3, 26). The cells were incubated with 10 μl of the pentamer for 10 min at room temperature in the dark. The cells were then washed in MACS buffer (bovine serum albumin supplemented with EDTA) and incubated with anti-CD8 monoclonal antibodies labeled with peridinin chlorophyll protein (Becton Dickinson) and monoclonal antibodies against other surface molecules (CD45RA, CCR7, CD28, CD27) labeled with phycoerythrin and fluorescein isothiocyanate (FITC; Becton Dickinson) for 30 min on ice in the dark. Intracellular perforin and granzyme B staining was performed by incubating the PBMCs with fluorescence-labeled conjugated monoclonal antibodies to CD8 (Becton Dickinson) and HCMV-pentamer complexes. The PBMCs were washed once, fixed, and subsequently permeabilized by using an IntraStain kit (Dako Cytomation), according to the manufacturer's instructions. The cells were then incubated with antiperforin (FITC) and anti-granzyme B (FITC), as indicated above. Analysis of the cells was performed with a four-color FACSCalibur flow cytometer and Cellquest software (Becton Dickinson).

Statistical analysis. Statistical analysis was performed by using the SPSS (version 15.0) program. The total population of $CD8^+$ T cells was counted and expressed as a proportion of the total lymphocytes, and the population of HCMV-specific $CD8^+$ T cells was counted and expressed as a proportion of the total population of $CD8^+$ T cells. Determination of whether there was a correlation between age and the percentage of lymphocyte subpopulations was carried out by using the nonparametric Spearman rank correlation test. Comparison of the percentages of different subpopulations among the different groups was carried out by using the Mann-Whitney U test. In this case, patients were stratified according to age into two groups: those older than 50 years of age and those younger than 50 years of age. Univariate and multivariate linear regression analyses were used to identify the variables associated with the $CD27^-/CD28^-$ HCMV-specific $CD8^+$ T-cell percentages. The following variables were included in the analyses: donor-recipient HCMV serostatus (D^+/R^- or R^+), HCMV replication (negative or positive) posttransplantation, age (younger and older than 50 years), and the organ transplanted (kidney or lung). Values were considered statistically significant when the P value was <0.05.

TABLE 1. Demographic characteristics of the study population

Characteristic	Value for patients with ages of:	
	<50 yr	>50 yr
Total no. of patients	26	16
No. (%) patients of the following sex:		
Male	19	11
Female	7	5
Median (range) age (yr)	34.5 (15–49)	60 (52–75)
Median (range) time of follow-up (days)	935 (418–1,105)	666 (436–1,018)
No. (%) of patients with the following organ transplant:		
Renal	16 (61.5)	14 (87.5)
Lung	10 (38.5)	2 (12.5)
No. of patients receiving immunosuppression		
Tacrolimus-cyclosporine	17/9	13/3
Mycophenolate-azathioprine	23/3	16/0
Steroids	26	16
Everolimus		1
Thymoglobulin	3	1
No. of patients receiving antiviral treatment		
Preemptive therapy	12	12
Universal prophylaxis	14	4
No. (%) of patients with the following HCMV serostatus:		
R^+	19 (73.1)	14 (87.5)
D^+/R^-	7 (26.9)	2 (12.5)

RESULTS

Demographic data. Forty-two patients (30 men, 12 women) who had the HLA-A*02 allele were studied. The median age of the group was 46.5 years (age range, 15 to 75 years). Twenty-six patients were younger than 50 years of age, and 16 patients were over that age. The demographic characteristics of these groups are indicated in Table 1.

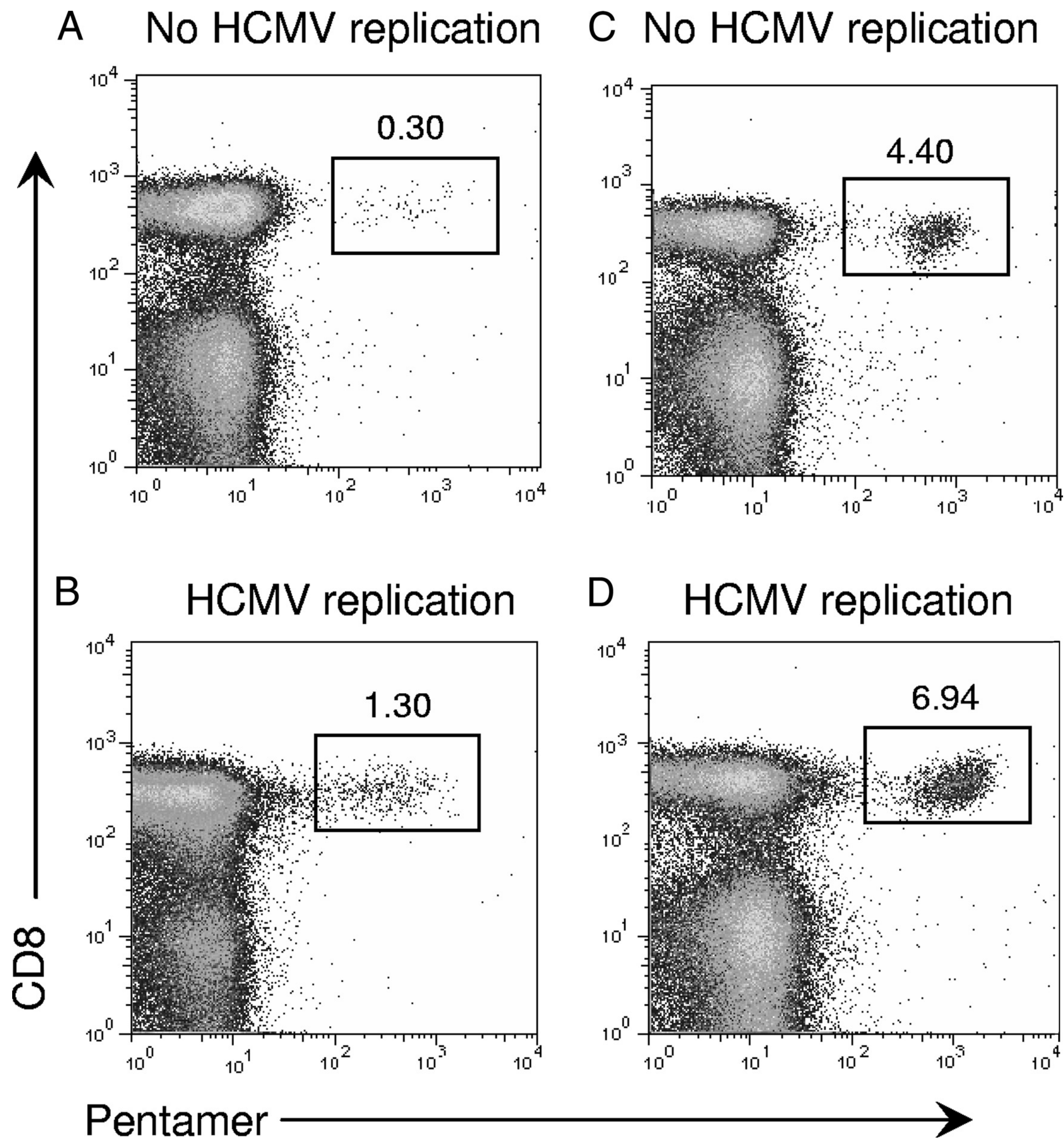

FIG. 1. Plots of pentamer-labeled HCMV-specific $CD8^+$ T cells from four representative transplant recipients. (A) HCMV D^+/R^- patient and no HCMV replication (n = 3; median, 0.41%; range, 0.30 to 0.60%); (B) HCMV D^+/R^- patient and HCMV replication (n = 6, median%, 1.24; range, 0.12 to 4.15%); (C) HCMV R^+ patient and no HCMV replication (n = 19, median%, 2.45; range, 0.14 to 22.00%); (D) HCMV R^+ patient and HCMV replication (n = 14, median, 3.79%; range, 0.14 to 13.20%). Values indicate the percentage of pentamer-positive cells with reference to the total number of CD8 T cells. n, number of patients for each situation; total number of patients, 42.

Frequency of HCMV-specific $CD8^+$ T cells determined by use of HLA pentamer. Figure 1 shows the representative results of a plot analysis of HCMV-specific $CD8^+$ T cells performed with the HLA-A*02 pentamer from four transplant recipients, according to the donor and the recipient HCMV serostatus and HCMV replication. No significant differences in the frequency of HCMV-specific $CD8^+$ T cells were observed when the patients were compared by age (older or younger than 50 years) or the presence of HCMV replication episodes after transplantation (data not shown).

Quantification of $CD28^-$ and $CD27^-$ lymphocyte subpopulations in total $CD8^+$ T cells. The median percentage of total $CD8^+$ T cells was 20.18% (range, 5.02 to 43.39%), and no significant differences were observed between patients with and without replication in the group younger than age 50 years (22.21% [range, 10.25 to 41.57%] and 20.05% [range, 5.02 to 40.58%], respectively; P = 0.49) or the group older than age 50 years (19.53% [range, 9.23 to 43.39%] and 19.33% [range, 11.55 to 27.87%], respectively; P = 0.74).

The proportion of $CD28^-$ total $CD8^+$ T cells (Fig. 2A) increased with age, so that there was a linear correlation between these two parameters both in patients with HCMV replication (r = 0.49; P = 0.02) and in those with no replication (r = 0.44; P = 0.05). This explains why the proportion of this

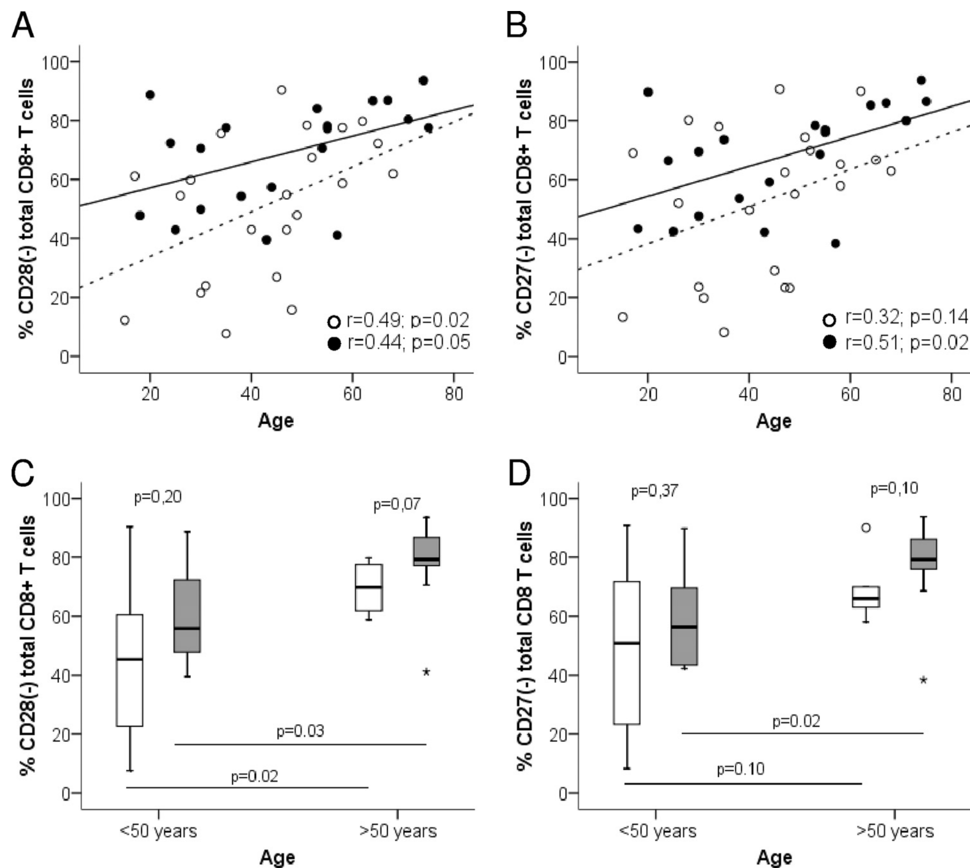

FIG. 2. (A) Correlation between age and CD28$^-$ total CD8$^+$ T-cell percentage in patients with HCMV replication (●; linear regression, solid line) and without HCMV replication (○; linear regression, dashed line). (B) The same analysis described for panel A for CD27$^-$ total CD8$^+$ T cells. (C and D) Box plots for CD28$^-$ and CD27$^-$ total CD8$^+$ T-cell frequency, respectively, according to age (<50 and >50 years) (open boxes, patients without HCMV replication; shaded boxes, patients with HCMV replication). The median value of each data set is shown as a horizontal line within the box, which encompasses the 25th and 75th percentiles. The whiskers extending from either end of the box represent the range of the data.

lymphocyte subpopulation was significantly higher in patients older than age 50 years than in those younger than age 50 years both in patients with HCMV replication (79.3% [range, 41.1 to 93.6%] and 55.8% [range, 39.5 to 88.7%], respectively; P = 0.03) and in those with no replication (69.8% [range, 58.7 to 79.7%] versus 45.7% [range, 7.8 to 90.4%], respectively; P = 0.02) (Fig. 2C). However, no significant differences in the median percentage of CD28$^-$ total CD8$^+$ T cells were observed between patients with and without HCMV replication in the group of patients younger than age 50 years (55.8% and 45.7%, respectively; P = 0.20) or older than age 50 years (79.3% and 69.8%, respectively; P = 0.07) (Fig. 2C).

With regard to the CD27 marker, in patients without HCMV replication, there was a trend toward a correlation between the percentage of CD27$^-$ total CD8$^+$ T cells and age; however, the difference was not statistically significant (r = 0.32; P = 0.15) (Fig. 2B). The same result was observed when the values were compared by age group, as the proportion of CD27$^-$ total CD8$^+$ T cells in patients older than age 50 years was not significantly higher than that in patients younger than age 50 years without HCMV replication (66% [range, 57.94 to 90.03%] and 50.9% [range, 8.2 to 90.8], respectively; P = 0.10) (Fig. 2D). In patients with HCMV replication, however, there was a statistically significant linear correlation between the percentage of CD27$^-$ total CD8$^+$ T cells and age (r = 0.51; P = 0.02) (Fig. 2B). This result was confirmed when the values were compared by age group, as the percentage of CD27$^-$ total CD8$^+$ T cells was significantly higher in patients older than age 50 years than in patients younger than age 50 years (79.2% [range, 38.4 to 93.8] and 56.4% [range, 42.2 to 89.7], respectively; P = 0.02). No significant differences in the median percentage of CD27$^-$ total CD8$^+$ T cells were observed between patients with and without HCMV replication in the group of patients younger than age 50 years (56.4% and 50.9%, respectively; P = 0.37) and the group older than age 50 years (79.4% and 66.0%, respectively; P = 0.10) (Fig. 2D).

Quantification of CD28$^-$ and CD27$^-$ lymphocyte subpopulations in HCMV-specific CD8$^+$ T cells. In patients without HCMV replication, the percentage of CD28$^-$ HCMV-specific CD8$^+$ T cells showed a linear correlation with age, so that the older that the patient was, the higher the frequency of this population became (r = 0.50; P = 0.02) (Fig. 3A). When the same patients were compared by age group, patients older than age 50 years had a significantly higher percentage of CD28$^-$ HCMV-specific CD8$^+$ T cells than patients younger than age 50 years (80.9% [range, 54.2 to 96.2%] and 58.7% [range, 8.7

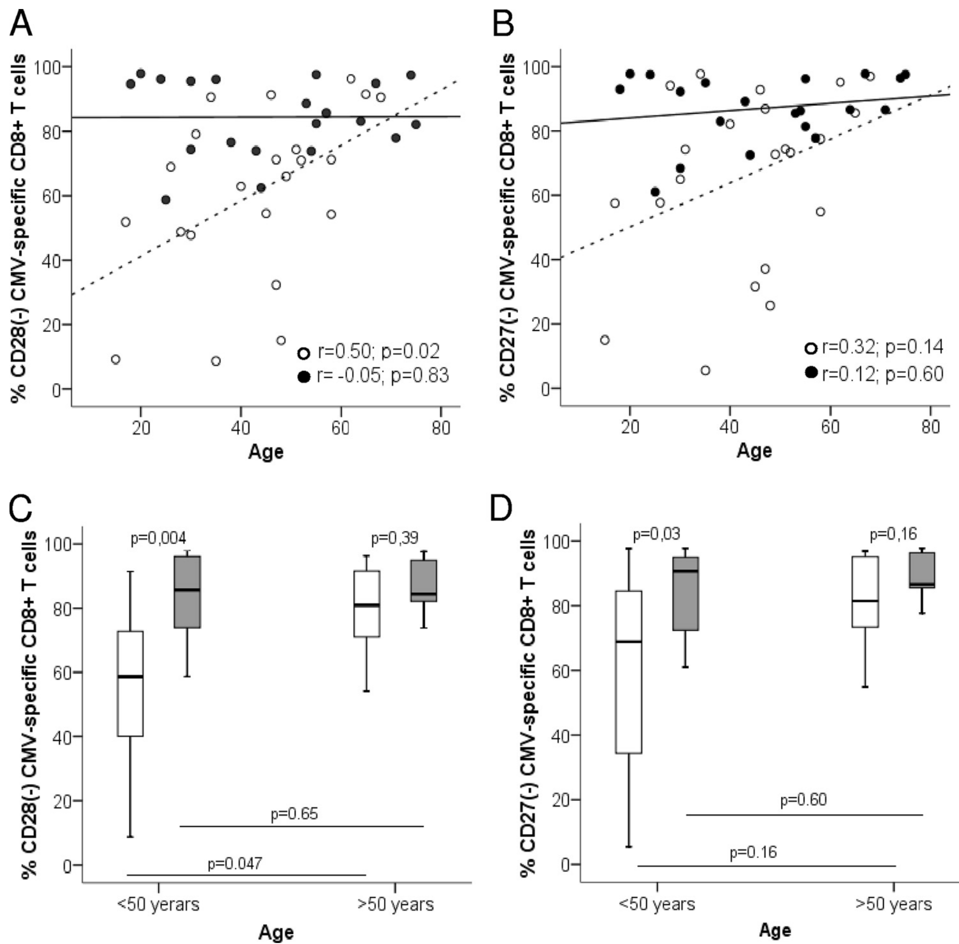

FIG. 3. (A) Correlation between age and $CD28^-$ HCMV-specific $CD8^+$ T-cell percentage in patients with HCMV replication (●; linear regression, solid line) and without HCMV replication (○; linear regression, dashed line). (B) The same analysis described for panel A for $CD27^-$ HCMV-specific $CD8^+$ T cells. (C and D) Box plots for $CD28^-$ and $CD27^-$ HCMV-specific $CD8^+$ T-cell frequency, respectively, according to age (<50 and >50 years) (open boxes, patients without HCMV replication; shaded boxes, patients with HCMV replication). HCMV-specific $CD8^+$ T-cell data are expressed as the proportion of total $CD8^+$ T cells. The median value of each data set is shown as a horizontal line within the box, which encompasses the 25th and 75th percentiles. The whiskers extending from either end of the box represent the range of the data.

to 91.3%], respectively; $P = 0.047$) (Fig. 3C). In patients with HCVM replication, the correlation between age and the percentage of $CD28^-$ HCMV-specific $CD8^+$ T cells observed in patients without replication was not observed ($r = -0.051$; $P = 0.83$). In this group, all patients had more than 58% $CD28^-$ HCMV-specific $CD8^+$ T cells, regardless of their age, including those transplant recipients between 18 and 30 years of age, in whom 94 to 97% of HCMV-specific $CD8^+$ T cells were $CD28^-$ (Fig. 3A). When patients showing HCMV replication were compared by age, no significant differences in the percentage of $CD28^-$ HCMV-specific $CD8^+$ T cells were observed between patients younger than or older than 50 years of age (85.6% [range, 58.7 to 97.2%] and 84.4% [range, 73.8 to 97.6%], respectively; $P = 0.65$) (Fig. 3C).

With regard to the CD27 marker, although there was a trend toward a linear correlation between the percentage of $CD27^-$ HCMV-specific $CD8^+$ T cells and age in patients without viral replication, it was not statistically significant ($r = 0.32$; $P = 0.14$) (Fig. 3B). Similar results were observed when the same patients were distributed into two age groups (those younger than and those older than 50 years of age). Even though patients in the older subgroup without HCMV replication showed an increase in the proportion of $CD27^-$ HCMV-specific $CD8^+$ T cells (81.5% [range, 54.9 to 97.0%] versus 68.8% [range, 5.5 to 97.7%] for those in the younger subgroup; $P = 0.16$), the differences were not statistically significant (Fig. 3D). The trend toward a correlation between the percentage of $CD27^-$ HCMV-specific $CD8^+$ T cells and age observed in patients without HCMV replication disappeared in patients with virus replication, and all patients had a high percentage of $CD27^-$ lymphocytes (over 60%), regardless of their age. As with CD28, patients between 18 and 30 years of age had 92 to 97% $CD27^-$ HCMV-specific $CD8^+$ T cells (Fig. 3B). These results were confirmed when the same values were analyzed by age group, as no differences in the percentage of $CD27^-$ HCMV-specific $CD8^+$ T cells were observed between patients younger than and older than 50 years of age (90.7% [range, 61.1 to 97.8%] and 86.6% [range, 77.8 to 97.8%], respectively; $P = 0.60$) (Fig. 3D).

When the percentages of $CD27^-$ and $CD28^-$ HCMV-spe-

TABLE 2. Subpopulations of HCMV-specific $CD8^+$ T cells obtained on the basis of CD27 and CD28 receptor expression in patients with and without HCMV replication according to age[a]

Subpopulation	<50 yr (n = 26)			>50 yr (n = 16)		
	HCMV replication[a]		P	HCMV replication		P
	No (n = 16)	Yes (n = 10)		No (n = 6)	Yes (n = 10)	
$CD28^-$	58.68 (8.67–91.30)	85.62 (58.73–97.92)	0.004	80.88 (54.24–96.25)	84.41 (73.83–97.60)	0.386
$CD27^-$	68.85 (5.48–97.74)	90.73 (61.06–97.79)	0.031	81.55 (54.87–97.00)	86.64 (77.78–97.80)	0.159
$CD27^+$ $CD28^+$	34.92 (5.40–91.67)	10.62 (2.65–32.76)	0.003	20.69 (2.25–32.56)	12.08 (0.81–24.17)	0.329
$CD27^+$ $CD28^-$	7.62 (1.39–38.39)	22.76 (2.46–52.40)	0.011	6.77 (2.33–54.92)	9.81 (5.98–21.67)	0.664
$CD27^-$ $CD28^-$	38.34 (2.25–76.97)	60.19 (28.60–76.75)	0.102	62.63 (11.69–94.00)	72.76 (53.33–90.65)	0.278

[a] The data are expressed as the median percentage (range).

cific $CD8^+$ T cells were analyzed separately in patients younger than and older than age 50 years, no significant differences in the percentage of $CD28^-$ HCMV-specific $CD8^+$ T cells were found in the group of patients older than age 50 years with and without HCMV replication (84.4% [range, 73.8 to 97.6%] and 80.9% [range, 54.2 to 96.2%], respectively; $P = 0.39$), and no significant differences in the percentage of $CD27^-$ HCMV-specific $CD8^+$ T cells were found in the group of patients older than age 50 years with and without HCMV replication (86.6% [range, 77.8 to 97.8%] and 81.5% [range, 54.9 to 97.03%], respectively; $P = 0.16$). However, for the group of recipients younger than age 50 years, when patients with HCMV replication were compared with patients without replication, patients with HCMV replication showed a significant increase in the percentage of $CD28^-$ HCMV-specific $CD8^+$ T cells (85.6% [range, 58.7 to 97.9%] and 58.7% [range, 8.7 to 91.3%], respectively; $P = 0.004$) and $CD27^-$ HCMV-specific $CD8^+$ T cells (90.7% [range, 61.1 to 97.8%] and 68.8% [range, 5.8 to 97.7%], respectively; $P = 0.03$) (Fig. 3C and 3D).

However, as shown in Table 2, despite the increase in the percentages of $CD27^-$ and $CD28^-$ cells observed in patients younger than age 50 years when the percentages were analyzed separately, when both markers were analyzed together, the $CD27^-$ $CD28^-$ lymphocyte population, defined as late-phenotype cells, showed no significant increase with replication in the same group of patients (38.3% [range, 2.2 to 77.0%] and 60.2% [range, 28.6 to 76.7%], respectively; $P = 0.10$), possibly since not all $CD28^-$ lymphocytes are $CD27^-$ but a certain proportion of $CD28^-$ lymphocytes are $CD27^+$. It was precisely the $CD27^+$ $CD28^-$ population, or intermediate-phenotype cells, that increased significantly (7.6% [range, 1.4 to 38.4%] and 22.8% [range, 2.5 to 52.4], respectively; $P = 0.01$) in patients younger than age 50 years with HCMV replication, while a parallel decrease was also observed in the most immature population, or early-phenotype cells ($CD27^+$ $CD28^+$) (34.9% [range, 5.4 to 91.7%] and 10.6% [range, 2.6 to 32.8%], respectively; $P = 0.003$).

Factors associated with the $CD27^-$/$CD28^-$ HCMV-specific $CD8^+$ T cell percentages. A series of univariate and multivariate linear regression models were used to determine the variables associated with the frequency of $CD27^-$ and $CD28^-$ cells within the pool of HCMV-specific $CD8^+$ T cells (Table 3). The univariate models showed that patients with HCMV replication had 23.27% more HCMV-specific $CD8^+$ $CD28^-$ cells than patients without HCMV replication and that patients older than age 50 years had 18.31% more HCMV-specific $CD8^+$ $CD28^-$ cells than patients younger than age 50 years. The analysis of the percentage of $CD27^-$ cells showed that HCMV replication posttransplantation, age, and the type of organ transplanted were associated with the percentage of HCMV-specific $CD8^+$ $CD27^-$ cells. Thus, patients with HCMV replication had 21.04% more HCMV-specific $CD8^+$ $CD27^-$ cells than patients without virus replication, patients older than age 50 years had 16.00% more HCMV-specific

TABLE 3. Linear regression analyses relating patient characteristics with the percentage of $CD28^-$ or $CD27^-$ HCMV-specific CD8 T cells

HCMV-specific $CD8^+$ T-cell type and patient characteristic[a]	Univariate analysis		Multivariate analysis[b]	
	Mean difference in % of cells (95% CI[c])	P	Mean difference in % of cells (95% CI)	P
$CD28^-$				
HCMV serostatus	9.22 (−8.77 to 27.21)	0.307		
HCMV replication	23.27 (10.27 to 36.27)	0.001	20.22 (7.34 to 33.09)	0.003
Age	18.31 (4.06 to 32.56)	0.013	13.45 (0.21 to 26.69)	0.047
Organ transplanted	−7.48 (−23.86 to 8.90)	0.362		
$CD27^-$				
HCMV serostatus	15.87 (−1.52 to 33.27)	0.072	21.84 (6.70 to 36.99)	0.006
HCMV replication	21.04 (7.76 to 34.32)	0.003	24.61 (12.17 to 37.05)	<0.001
Age	16.00 (1.57 to 30.43)	0.031		
Organ transplanted	−18.15 (−33.55 to −2.76)	0.022		

[a] The patient characteristics are donor-recipient HCMV serostatus (D^+/R^- or R^+), HCMV replication (negative or positive) posttransplantation, age (younger and older than 50 years), and type of organ transplanted (kidney or lung).
[b] In the multivariate analysis, only results with a P value of <0.05 after adjustment of the other variables are indicated.
[c] CI, confidence interval.

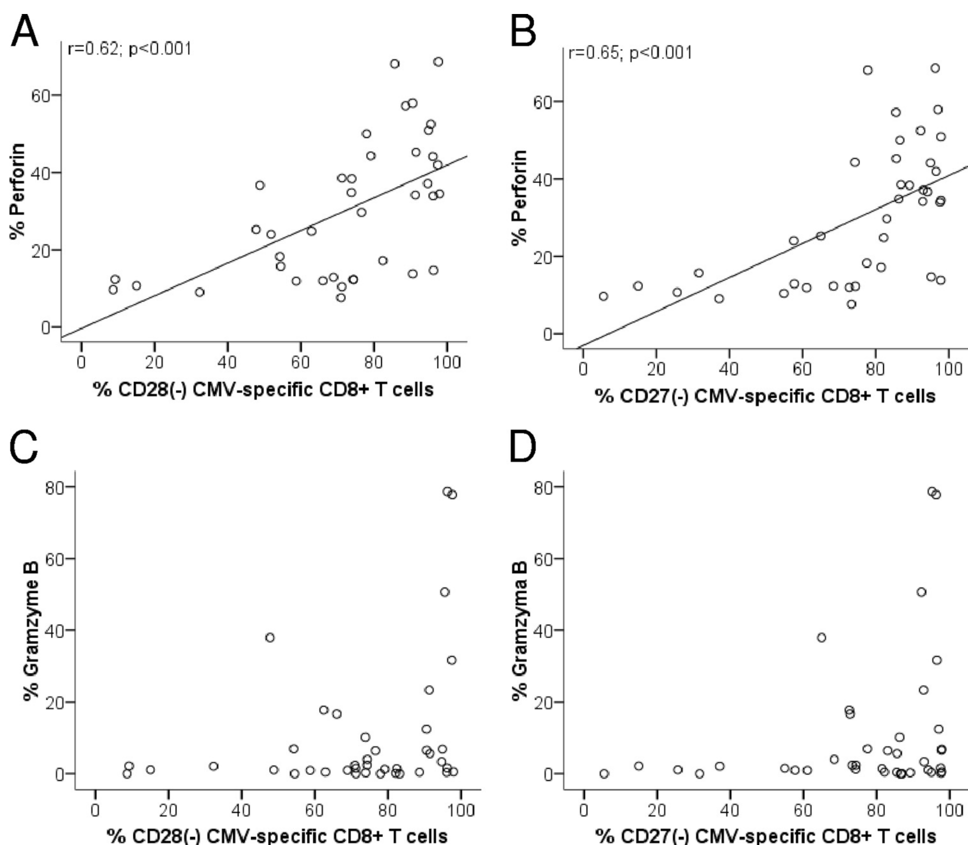

FIG. 4. Scatter plots showing correlations between percentages of $CD28^-$ and $CD27^-$ HCMV-specific $CD8^+$ T cells and the amounts of perforin (A and B) and granzyme B (C and D) produced by these subpopulations. Spearman's rank correlation test was used for statistical analysis; a P value of <0.05 was considered significant.

$CD8^+$ $CD27^-$ cells than patients younger than age 50 years, and patients who received a kidney transplant had 18.15% more HCMV-specific $CD8^+$ $CD27^-$ cells than patients who received a lung transplant.

The multivariate linear regression analysis demonstrated that patients with HCMV replication had 20.22% more HCMV-specific $CD8^+$ $CD28^-$ cells than patients without HCMV replication and that patients older than age 50 years had 13.45% more HCMV-specific $CD8^+$ $CD28^-$ cells than patients younger than age 50 years, after adjustment for the other variables. The analysis also showed that donor-recipient HCMV serostatus and posttransplantation HCMV replication were associated with the percentage of HCMV-specific $CD8^+$ $CD27^-$ cells. Thus, R^+ patients had 21.84% more HCMV-specific $CD8^+$ $CD27^-$ cells than D^+/R^- patients and patients with HCMV replication had 24.61% more HCMV-specific $CD8^+$ $CD27^-$ cells than patients without virus replication. Age and the organ transplanted were not associated with the percentage of $CD27^-$ HCMV-specific $CD8^+$ T cells in the multivariate analysis.

Production of perforin and granzyme B by $CD27^-$ and $CD28^-$ HCMV-specific $CD8^+$ T-cell subpopulations. With regard to perforin production, it can be seen in Fig. 4 that there was a linear correlation between the percentage of $CD28^-$ HCMV-specific $CD8^+$ T cells and the percentage of this cytotoxic molecule ($r = 0.62$; $P < 0.001$) (Fig. 4A). The same correlation was observed with the percentage of $CD27^-$ HCMV-specific $CD8^+$ T cells ($r = 0.65$; $P < 0.001$) (Fig. 4B), indicating that the greater that the proportion of cells with this differentiated phenotype ($CD28^-$ or $CD27^-$) among HCMV-specific $CD8^+$ T cells is, the greater that the level of production of perforin becomes, which would imply a greater cytotoxic capacity of these subpopulations. On the contrary, no correlation was observed between granzyme B (median, 2.13% [range, 0.01 to 78.68%]) and the percentages of $CD28^-$ HCMV-specific $CD8^+$ T cells ($r = 0.27$; $P = 0.90$) (Fig. 4C) or $CD27^-$ HCMV-specific $CD8^+$ T cells ($r = 0.19$; $P = 0.24$) (Fig. 4D).

DISCUSSION

This study focused on the phenotype of HCMV-specific $CD8^+$ T cells in kidney and lung transplant recipients according to age and HCMV replication. The results showed a statistically significant association between HCMV replication and the downregulation of CD27 and CD28 in recipients younger than 50 years of age but not in older recipients.

The association between HCMV replication and a high percentage of $CD27^-$ T cells in younger individuals was observed only for HCMV-specific $CD8^+$ T cells and not for total $CD8^+$ T cells, which rules out the idea that it is a general and nonspecific finding. The multivariate linear regression analysis in-

dicates that the main factors associated with the percentage of HCMV-specific $CD8^+$ $CD27^-$ cells are donor-recipient HCMV serostatus and posttransplantation HCMV replication rather than age or the type of organ transplanted. These results agree with those published previously that reported that HCMV infection is associated with the loss of CD27 expression (33) and that emphasized the correlation that exists between the decreased frequency of CD27 expression and the increased synthesis of cytotoxic molecules (perforin and granzymes) (3), which were also observed in our patients. However, there is certain controversy about the meaning of the loss of CD27 expression by $CD8^+$ T cells. Whereas some authors suggest that they would correspond to effector cells with a high cytolytic capacity and, therefore, with a certain protective action (41, 46), other authors have associated the loss of CD27 expression with decreased proliferative capacity and immunosenescence (5, 28). We think that the loss of CD27 expression observed in younger patients (those younger than age 50 years) with prior HCMV replication may be a consequence of their immune system's effort to control the infection, while that observed in patients older than age 50 years would mainly be due to age, and hence, the impact of HCMV replication would be less.

We also observed that in the absence of HCMV replication, there was a linear correlation between increased age and the loss of CD28 expression by both HCMV-specific and total $CD8^+$ T cells, as previously stated by other authors (27, 29, 50). However, this correlation was not observed in patients with viral replication, and all patients from the youngest to the oldest (older than age 70 years of age) had a high percentage of $CD28^-$ cells in the pool of HCMV-specific $CD8^+$ T cells but not in total $CD8^+$ T cells. Multivariate linear regression analysis also supports the association of HCMV replication and age with the percentage of $CD28^-$ HCMV pentamer-positive CD8 T cells, whereas no association with donor-recipient HCMV serostatus or the type of organ transplanted was found. This was confirmed when patients were analyzed by age, as HCMV replication in younger patients (those younger than age 50 years) was associated with an increased percentage of $CD28^-$ HCMV-specific $CD8^+$ T cells, which was similar to that observed in patients older than age 50 years without viral replication, whose percentage of $CD28^-$ cells is likely to be increased with age. Similar results have been published by other authors, who showed that active HCMV infection in kidney (16) and lung (52) transplant recipients increases the frequency of $CD28^-$ expression in $CD8^+$ T cells. Therefore, the decreased frequency of CD28 expression observed in younger patients with HCMV replication could be the result of the activation of T cells by interaction with the virus (3, 16, 17, 48). Furthermore, the fact that this association was observed only in the HCMV-specific T-cell population (and not in the total T-cell population) would support the idea of its relationship to viral replication. This replicative pressure would lead naïve $CD28^+$ T cells in patients younger than age 50 years to become memory cells and eventually $CD28^-$ effector cells which might show characteristics of senescent cells (17, 44) and certain similarities to $CD28^-$ cells in older patients. This might partly explain the fact that HCMV replication was associated with a decrease in the percentage of naïve-phenotype cells ($CD45RA^+$ $CCR7^+$) and early-phenotype cells ($CD27^+$ $CD28^+$) and an increase in the percentage of intermediate-phenotype cells ($CD27^+$ $CD28^-$) (3).

This downregulation of $CD28^-$ cells has been described under conditions that cause chronic immune stimulation, such as inflammatory diseases (20, 35), cancer (6, 7, 11, 30, 40), and other latent viral infections, such as infection with human immunodeficiency virus (HIV) or Epstein-Barr virus (3, 48). Therefore, even though all patients with viral replication included in our study had a high percentage of $CD27^-$ or $CD28^-$ cells in the HCMV-specific T-cell population, this subpopulation might have distinct functions, depending on the length of contact with the virus. In this regard, it has been reported that $CD28^-$ cells from younger persons are mostly $CD45RA^+$, while those from elderly persons are usually $CD45RA^-$, which seem to be truly senescent cells (2). The latter cells are characterized by their high degree of differentiation, which is higher than that of $CD8^+$ T-cell populations specific to other persistent viruses such as HIV and Epstein-Barr virus (3, 8, 39, 48). They also show a series of alterations that lead to a state of clonal exhaustion or replicative senescence, characterized by phenotypic and functional changes (12, 18, 22, 43, 49), which in turn lead to decreased levels of secretion of gamma interferon (2, 37, 38, 42), perforin, and granzymes, making them more inefficient for cytotoxicity than $CD45RA^+$ cells (4, 54). In addition, these exhausted clones resemble in some aspects the senescent clones that naturally accumulate throughout aging (5, 29, 47, 50), and age and latent HCMV infection seem to induce similar changes in the T-cell pool (51). Further studies are therefore necessary to clarify this aspect in transplant patients with prior HCMV replication. The importance of clarifying the functionality of the $CD27^-/CD28^-$ HCMV-specific T-cell population lies in the possible consequences that it may have on the incidence of certain posttransplantation diseases, such as rejection (25, 52), the development of other infections (including the new replication of HCMV or other herpesviruses), tumor development, posttransplantation vasculopathy, diabetes, and even mortality (15, 31). An illustrative case would be HIV infection, in which the accumulation of virus-specific clones with features of replicative senescence has been correlated with disease progression (28).

Although we cannot rule out the possibility that our results for patients younger than age 50 years were merely the consequence of stimulation of the immune system by HCMV in immunosuppressed patients, other hypotheses can also be proposed. The first is that the increase in the frequency of expression of $CD27^-/CD28^-$ HCMV-specific $CD8^+$ T cells in younger transplant recipients corresponds to a form of accelerated immune senescence at early ages associated, among other possible factors, with HCMV replication (5, 17, 45). Another alternative hypothesis would be that phenotypic changes are generated before or during transplantation (during which the patient receives dialysis, immunosuppressants, etc.), which may facilitate viral replication. Clarification of the factors involved would require a prospective cohort study to be conducted with patients with and without HCMV replication with the aim of studying the phenotypic and functional changes from before to different points after transplantation.

In conclusion, our study shows that in transplant recipients, HCMV replication is associated with an increased frequency of $CD27^-/CD28^-$ HCMV-specific $CD8^+$ T cells in patients

younger than age 50 years, so the proportion of these subpopulations observed in patients younger than age 50 years is similar to that observed in patients older than age 50 years without HCMV replication. HCMV replication may cause phenotypic changes in younger transplant recipients similar to those induced by age. Further studies are necessary to clarify the functional changes associated with these phenotypic changes and their potential relationship to clinical complications posttransplantation.

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