Human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in adult allogeneic hematopoietic stem cell transplant recipients and immune control of viral infection

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ABSTRACT

Background
Human cytomegalovirus infection is the most frequent viral complication in patients undergoing hematopoietic stem cell transplantation. We investigated the development of human cytomegalovirus-specific T cells in adult recipients of hematopoietic stem cell transplants.

Design and Methods
From May 2003 through October 2006 a total of 45 patients were monitored for human cytomegalovirus-specific T-cell reconstitution. Human cytomegalovirus-infected autologous dendritic cells were used as a stimulus to detect interferon-γ-producing human cytomegalovirus-specific CD8+ and CD4+ T cells during the first year after transplantation. Interleukin-2 production by specific T cells was also determined.

Results
Human cytomegalovirus infection was detected in the blood of 39/45 patients at a median of 29 days after transplantation. Human cytomegalovirus-specific T-cell reconstitution followed reactivation of latent human cytomegalovirus infection at a median time of about 2 months after transplantation. Only donor human cytomegalovirus-seronegativity and bone marrow as a stem cell source were found to delay specific T-cell reconstitution significantly. Levels of three CD8+ and one CD4+ human cytomegalovirus-specific T-cells/μL blood had a positive predictive value of around 80% for identifying patients able to control human cytomegalovirus infection spontaneously. Five patients who received high doses of steroids for treatment of graft-versus-host disease developed human cytomegalovirus infection requiring pre-emptive treatment despite high levels of interferon-γ-producing T cells in response to human cytomegalovirus. Specific interleukin-2 production was not detected in patients with human cytomegalovirus infection requiring treatment, while 90% of patients who spontaneously controlled human cytomegalovirus infection had T cells that produced interleukin-2 and interferon-γ.

Conclusions
Pre-transplant human cytomegalovirus infection of the recipient is a major factor driving human cytomegalovirus-specific immune reconstitution. Control of human cytomegalovirus infection likely requires the presence of both interferon-γ and interleukin-2 producing T cells. Corticosteroid treatment may favor active viral replication even in patients with specific T cells.

Key words: human cytomegalovirus, specific T cells, stem cell transplantation.

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Introduction

Human cytomegalovirus (HCMV) infection is the most frequent viral complication in patients undergoing hematopoietic stem cell transplantation (HSCT).[1] The use of prophylactic and pre-emptive treatment strategies has significantly reduced the morbidity and mortality of HCMV infection.[2-4] However, both HCMV-specific CD8+ and CD4+ arms of the T-cell immune response must be reconstituted in order to acquire long-term protection against HCMV relapse and disease in humans. Early studies used conventional functional assays, i.e. cytotoxicity and lymphoproliferation assays to determine HCMV-specific CD8+ and CD4+ T-cell-mediated immune responses, respectively.[5-7]

In recent years, rapid techniques, such as tetramer staining, and intra- or extra-cellular detection of cytokine production have been developed to assess virus-specific T-cell memory responses. However, the majority of studies evaluating the kinetics of HCMV infection and HCMV-specific T-cell reconstitution after HSCT, while assessing a CD4+ T-cell response against whole viral antigens (infected cell lysate), have focused only on pp65-and IE-1 as targets for a CD8+ T-cell response.[8-11] Although these two proteins have been recognized as immunodominant for the CD8+ T-cell response to HCMV,[12,13] a number of other viral proteins are recognized by T cells[14,15] and quantification of T cells specific for pp65 and IE-1 cannot account for and does not correlate with the actual HCMV-specific T-cell pool. In this respect, it was observed in solid organ transplant recipients that the assessment of only pp65- and IE-1-specific T cells underestimates the actual T-cell immune response against HCMV.[16]

A different methodological approach providing a more comprehensive evaluation of the HCMV-specific T-cell immune response,[17] was recently adopted to study the kinetics of HCMV-specific T-cell reconstitution in children undergoing HSCT.[18] This method, based on the use of HCMV-infected immature dendritic cells as the stimulus, provides a broader and more physiological activation of T cells, along with simultaneous quantification and functional evaluation of both HCMV-specific CD8+ and CD4+ T cells.

In this study, we took advantage of the same technique to investigate the development of HCMV-specific T-cell immunity in adult HSCT recipients, in order to better define the kinetics of T-cell reconstitution in this high risk population and its correlation with immune control of HCMV infection.

Design and Methods

Patients

From May 2003 through October 2006, a total of 45 patients undergoing allogeneic HSCT were monitored for HCMV-specific T-cell reconstitution. The patients' characteristics are reported in detail in Table 1. Donor-recipient histocompatibility was determined by serology for HLA-A and HLA-B antigens, and by a high molecular resolution allelic technique for DRB1. The study was approved by the Fondazione IRCCS Policlinico San Matteo Ethics Committee, and patients gave their written informed consent prior to entering the study.

Table 1. Characteristics of the 45 patients analyzed.

Characteristics	*Number of patients (%)*
Gender (male/female)	27/18
Median age at transplantation (range)	42 (20-67) years
Stem cell source	
bone marrow	12 (27)
peripheral blood	33 (73)
Donor type	
sibling	27 (60)
unrelated	18 (40)
Donor/recipient HCMV serostatus	
D+/R+	28 (62)
D-/R+	9 (20)
D+/R-	8 (18)
Conditioning regimen	
TBI-based	20 (44)
Chemotherapy-based	25 (56)
GvHD grade	
0-I	34 (76)
II-IV	11 (24)

HCMV, human cytomegalovirus; TBI, total body irradiation; GvHD, graft-versus-host disease

Virological follow-up

HCMV infection was considered to be present when HCMV was detected in blood by any assay, in the absence of clinical manifestations or organ function abnormalities, while HCMV disease was defined as either systemic or local HCMV infection, associated with clinical symptoms and/or organ function abnormalities.[22]

Patients were randomized to monitoring of HCMV reactivation by either the antigenemia or the DNAemia assay, according to methods previously described.[20,21] In the antigenemia arm, patients were treated upon first detection of either two or more pp65-positive leukocytes or upon first confirmed positivity, when a single positive cell was detected.[22] In the DNAemia arm, patients were treated when reaching a cut-off of 10,000 DNA copies/mL whole blood. For all patients the duration of therapy was determined by clearance of virus from blood according to the guiding assay, and treatment was stopped after two consecutive negative results. Relapses were treated similarly. In addition, viremia, and donor/recipient HCMV serostatus were evaluated according to previously reported methods.[23,24] Pre-emptive therapy of HCMV infection was intravenous ganciclovir (5 mg/kg twice a day). Ganciclovir was replaced by foscarnet (90 mg/kg twice a day) in the case of ganciclovir-induced neutropenia or increasing levels of viremia.

Patients' treatment

All patients were given a full-intensity myeloablative preparative regimen. Graft-versus-host disease (GvHD) prophylaxis consisted of cyclosporine A associated with a short course of methotrexate and steroids (methylprednisolone 0.5 mg/kg/day until day +30), for patients receiving an allograft from an HLA-identical sibling, whereas 15/18 patients transplanted from an unrelated donor also received anti-thymocyte globulin before HSCT. Acute GvHD was treated with steroids as first-line therapy (methylprednisolone 2-5 mg/kg/day), while patients with steroid-resistant disease were treated with mofetil mycophenolate and extracorporeal photochemotherapy.

Immunological follow-up

Immunological reconstitution was investigated 30, 60, 90, 180 and 360 days after transplantation. HCMV-specific $CD4^+$ and $CD8^+$ T cells were simultaneously quantified by a novel method based on the use of autologous, monocyte-derived, HCMV-infected dendritic cells as previously described.[17] Briefly, following *in vitro* generation from peripheral blood mononuclear cells,[25] dendritic cells were infected for 24 hours with an endotheliotropic and leukotropic strain of HCMV (VR1814).[26] HCMV-infected dendritic cells were then co-cultured overnight with autologous thawed peripheral blood mononuclear cells at a ratio of 1:20 in the presence of 10 μg/mL brefeldin A (Sigma, St. Louis, MO, USA) to prevent cytokine release. Finally, the peripheral blood mononuclear cells were tested for the frequency of HCMV-specific $CD4^+$ and $CD8^+$ interferon (IFN)-γ-producing T cells by cytokine flow cytometry. In some patients, interleukin (IL)-2 production was also determined.

Absolute $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell counts were determined on heparinized peripheral blood samples by direct immunofluorescence flow cytometry (Beckman Coulter Inc, Fullertone, CA, USA). The total number of HCMV-specific $CD4^+$ and $CD8^+$ T cells was calculated by multiplying the percentages of HCMV-specific T-cells positive for IFN-γ by the relevant absolute $CD4^+$ and $CD8^+$ T-cell counts. Based on results obtained by testing 46 HCMV-seropositive and eight HCMV-seronegative healthy blood donors, "responders" (i.e. patients with virus-specific immunity) were defined as those subjects with HCMV-specific $CD4^+$ or $CD8^+$ T cells counts greater than 0.4/μL blood.[17]

Flow cytometry analysis

Following incubation with HCMV-infected dendritic cells, peripheral blood mononuclear cells were tested for intracellular IFN-γ production. The mononuclear cells were washed and incubated for 30 min on ice for surface staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibody (Beckman Coulter Immunotech, Marseilles, France) and phycoerythrin cyanin 5 (PC5)-conjugated anti-CD4 monoclonal antibody (Beckman Coulter Immunotech) in phosphate-buffered saline + 5% fetal bovine serum, containing 5% human immunoglobulin and 0.01% sodium azide. Cells were then washed with phosphate-buffered saline + 5% fetal bovine serum, fixed and permeabilized using the FIX and PERM® kit (Caltag, Burlingham, CA, USA), and stained for 45 min with phycoerythrin (PE)-conjugated anti-IFN-γ monoclonal antibody (Beckman Coulter Immunotech). To determine IFN-γ/IL-2 production, surface staining was performed with PC5-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD8 monoclonal antibodies (Beckman Coulter Immunotech), while PE-conjugated anti-IFN-γ monoclonal antibody was coupled with FITC-conjugated anti-IL-2 monoclonal antibody (Caltag) for intracytoplasmic staining.

Cells were resuspended in 1% paraformaldehyde and analyzed in a FACSCalibur flow cytometer (Becton-Dickinson, San José, CA, USA) equipped with a 488 nm argon laser and a 635 nm red-diode laser, operating with the CellQuest software (Becton-Dickinson). As a routine, $1\text{-}2\times10^5$ viable lymphocytes were collected and at least 2.5×10^4 $CD4^+$ and $CD8^{bright}$ T-cells were analyzed as above. The frequencies of $CD4^+$ and $CD8^{bright}$ T cells producing IFNγ in response to HCMV stimuli were calculated by subtracting the value of the sample incubated with mock-infected dendritic cells (consistently <0.05%) from the test value.

Statistical analysis

Differences between medians were compared by using the Mann-Whitney U test for unpaired data or Wilcoxon's test for paired data.

The curves of the percentages of patients showing HCMV infection or HCMV-specific immune response in the first year after transplantation in the different groups of HSCT recipients were calculated and expressed as cumulative incidences, with death and infection relapse being competing risks,[27,28] and compared by the log-rank test; p values lower than 0.05 were considered statistically significant, while p values from 0.05 to 0.1 were considered statistically non-significant, but are reported in detail. All variables with a p value less than 0.5 in univariate analysis were included in a multivariate analysis performed using the Cox-proportional hazard regression model. Receiver-operator characteristics (ROC) analysis was performed to identify levels of HCMV-specific $CD4^+$ and $CD8^+$ T cells protective against HCMV infection.

Results

HCMV infection and T-cell immune reconstitution

HCMV infection was detected in the blood of 39/45 patients (1 year cumulative incidence: 87%) at a median of 29 days (range, 16-68) after transplantation. In detail, HCMV infection developed in all donor HCMV-negative (D^-), recipient HCMV-positive (R^+) patients and 27/28 D^+R^+ patients (96%), while it occurred in only 3/8 D^+R^- patients

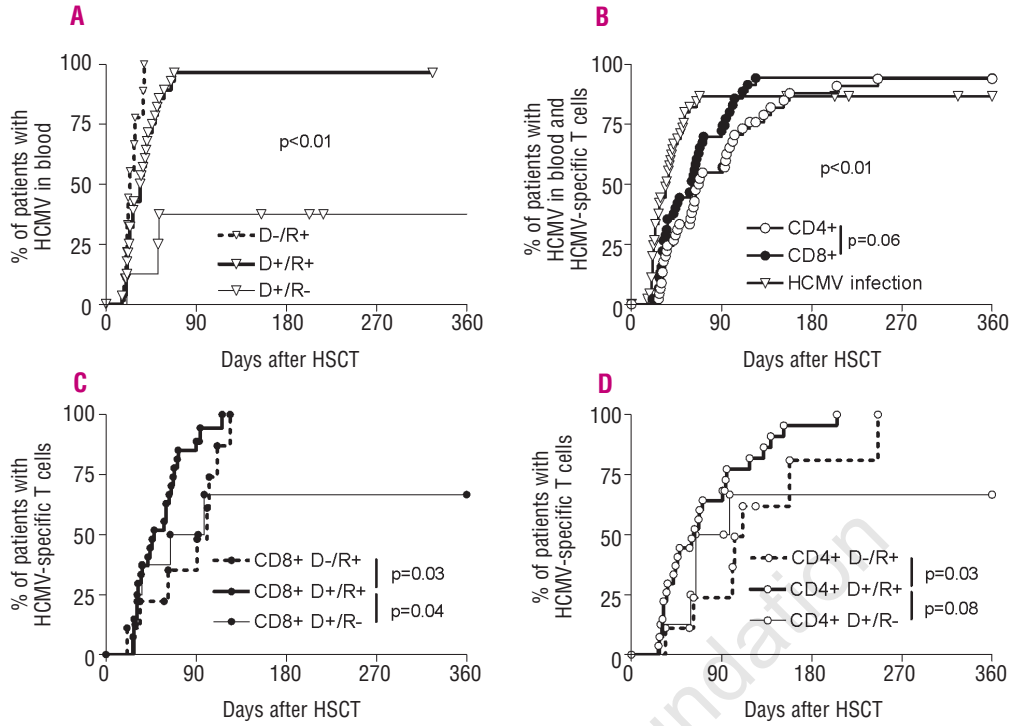

Figure 1. Probability of HCMV infection development and HCMV-specific CD4⁺ and CD8⁺ T-cell immunity reconstitution. A: cumulative incidence curves of HCMV infection according to donor (D) and recipient (R) HCMV-serostatus. B: cumulative incidence curves of HCMV infection and HCMV-specific CD8⁺ and CD4⁺ T-cell reconstitution (i.e. corresponding to a specific T-cell number greater than 0.4 cells/μL blood). C: cumulative incidence curves of HCMV-specific CD8⁺ T-cell reconstitution according to D/R HCMV-serostatus. D: cumulative incidence curves of HCMV-specific CD4⁺ T-cell reconstitution according to D/R HCMV-serostatus.

(38%, $p<0.01$). As shown in Figure 1A, HCMV infection developed earlier in D^-R^+ patients, later in D^+R^+ patients and lastly in D^+R^- patients ($p<0.01$, log-rank test for trend). No patient developed HCMV disease, due to the effective pre-emptive treatment approach. HCMV-specific T-cell development followed HCMV infection, with the appearance of CD8⁺ T cells slightly preceding that of CD4⁺ T cells (median time: 60 *vs.* 67 days, $p=0.06$, Figure 1B). Although the number of HCMV-negative recipients examined was small, it was possible to identify, among patients receiving a transplant from a positive donor, a lower rate of HCMV-specific T-cell reconstitution in HCMV-negative recipients with respect to HCMV-positive recipients. On the other hand, D^-R^+ patients showed delayed T-cell reconstitution with respect to D^+R^+ (median time: 101 days for CD8⁺ and 111 days for CD4⁺ T cells, Figures 1C and D). The 1-year cumulative incidence of development of HCMV-specific CD4⁺ T cells was 93% for D^+R^+, 78% for D^-R^+ and 63% for D^+R^-, and that of specific CD8⁺ T cells was 96% for D^+R^+, 89% for D^-R^+ and 63% for D^+R^-.

Table 2. Factors potentially influencing the development of HCMV-specific T cells.

	CD4⁺			*CD8⁺*		
Parameter	*Probability ratio*	*95%CI*	*p*	*Probability ratio*	*95%CI*	*p*
Conditioning regimen (chemotherapy/total body irradiation)	1.47	(0.60-3.60)	0.40	1.41	(0.49-4.04)	0.52
Stem cell source (bone marrow/peripheral blood)	0.35	(0.14-0.88)	0.09	0.71	(0.29-1.76)	0.46
Donor serostatus (negative/positive)	0.33	(0.12-0.94)	0.04	0.30	(0.11-0.83)	0.02
Donor type (unrelated/sibling)	1.23	(0.22-7.24)	0.80	0.25	(0.04-1.46)	0.15
Administration of antithymocyte globulin (yes/no)	0.89	(0.15-5.49)	0.90	1.55	(0.25-9.59)	0.64
Pre-emptive therapy guiding assay (antigenemia/DNAemia)	1.04	(0.46-2.35)	0.92	1.46	(0.68-3.16)	0.33

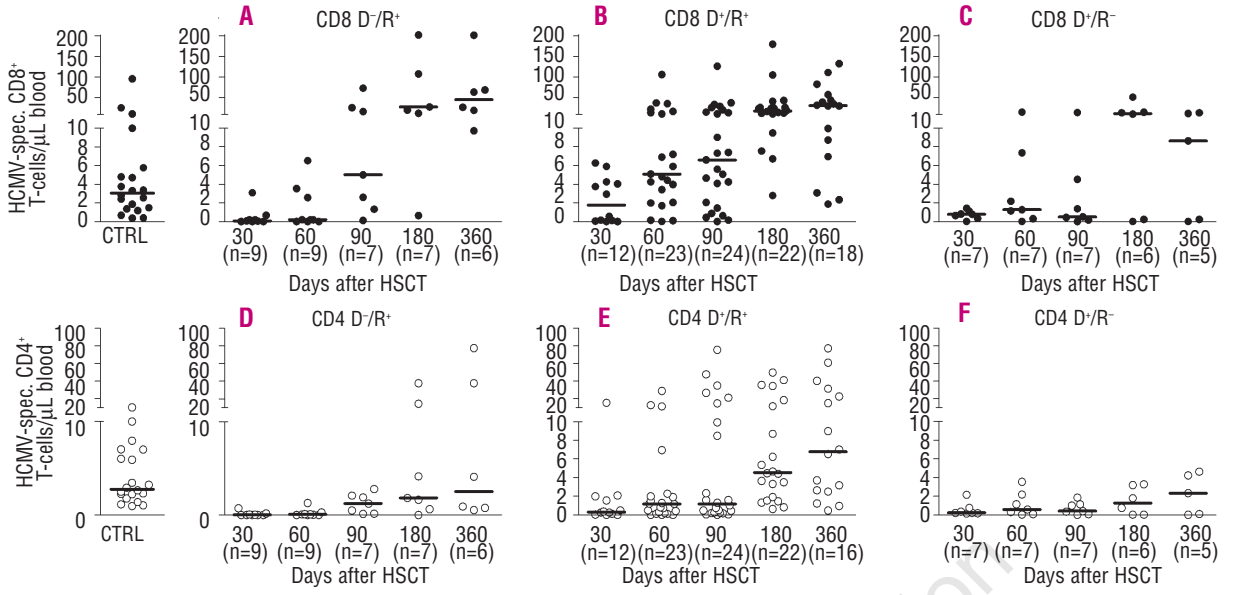

Figure 2. Absolute number of HCMV-specific CD8⁺ and CD4⁺ T-cells in the three groups of HCMV D⁻/R⁺ (A and D), D⁺/R⁺ (B and E) and D⁺/R⁻ (C and F) transplanted patients during the first year after transplantation and in healthy controls (CTRL). Horizontal bars indicate median values.

Among HCMV-positive recipients, two D⁺R⁺ and two D⁻R⁺ died from transplant related complications before specific T-cell reconstitution occurred (two of them developed only CD8⁺ T cells and the other two did not develop either CD8⁺ or CD4⁺). At the last follow-up visit, all four of these patients still showed active HCMV infection in their blood. All the surviving HCMV-positive recipients developed HCMV-specific CD8⁺ and CD4⁺ T cells within 4 to 6 months after HSCT, respectively. On the other hand, after 1 year, 3/8 D⁺/R⁻ patients had not developed either infection or specific immunity, and one of them exhibited an initial (day +30) HCMV-specific T-cell response which disappeared at subsequent control visits.

The total CD8⁺ T cell count was not different in the three groups of D/R patients, reaching that of controls from day +90 (*data not reported*). Levels of HCMV-specific CD8⁺ T cells were lower than those of controls at day +30, similar at days +60 and +90, and higher from day +180 ($p<0.01$). Specific T-cell counts were not significantly different between HCMV-positive and -negative recipients undergoing HSCT from a positive donor (although positive recipients reached higher peak values), whereas they were lower in D⁻R⁺ patients ($p<0.05$) at days +30 and +60 (Figure 2A, B and C).

As observed for CD8⁺ T cells, the total CD4⁺ T-cell count was not different among the three groups of D/R patients (*data not shown*), remaining significantly lower than that of controls throughout the follow-up. The median number of HCMV-specific CD4⁺ T-cells was lower than that of controls at days +30, +60 and +90, reaching that of controls from day +180 onwards. No difference in levels of HCMV-specific CD4⁺ T cells was found between D⁺R⁺ and D⁺R⁻ patients (although, as observed for CD8⁺ T cells, positive recipients reached higher peak values), while patients receiving stem cells from negative donors had lower levels of HCMV-specific CD4⁺ T cells ($p<0.05$) at days +30 and +60 (Figure 2D, E and F). It is notable that HCMV-specific T-cell reconstitution was relatively more pronounced in magnitude than total T-cell reconstitution for both CD8⁺ and CD4⁺ subpopulations. In fact, the ratio of HCMV-specific to total T-cells was higher in HSCT recipients than in controls ($p<0.01$; *data not reported*).

Factors influencing the kinetics of reconstitution of CD4⁺ and CD8⁺ IFN-γ-producing T-cells

Multiple factors were analyzed in a Cox regression model to evaluate their possible influence on the recovery of an HCMV-specific T-cell-mediated immune response: conditioning regimen (total body irradiation- or chemotherapy-based), stem cell source (peripheral blood/bone marrow), donor HCMV serostatus, donor type (sibling/unrelated), anti-thymocyte globulin administration, and randomization arm (antigenemia/DNAemia). Only the use of a seronegative donor (for both T-cell subpopulations) and bone marrow as the source of stem cells (for CD4⁺ T-cells) were associated with a delayed recovery of an HCMV-specific immune response (Table 2).

Relationship of HCMV-specific T-cell reconstitution and control of viral infection

In order to determine the level of HCMV-specific T cells apparently able to control infection, we compared (Figure 3A) the number of specific T cells detected in patients considered as *non-protected* (i.e. those reaching the cut-off for pre-emptive treatment), and in patients considered as *protected* (i.e. those without viremia after antiviral treatment, referred to as having *chemotherapy-resolved infection,* and in those with active HCMV infection which was not treated

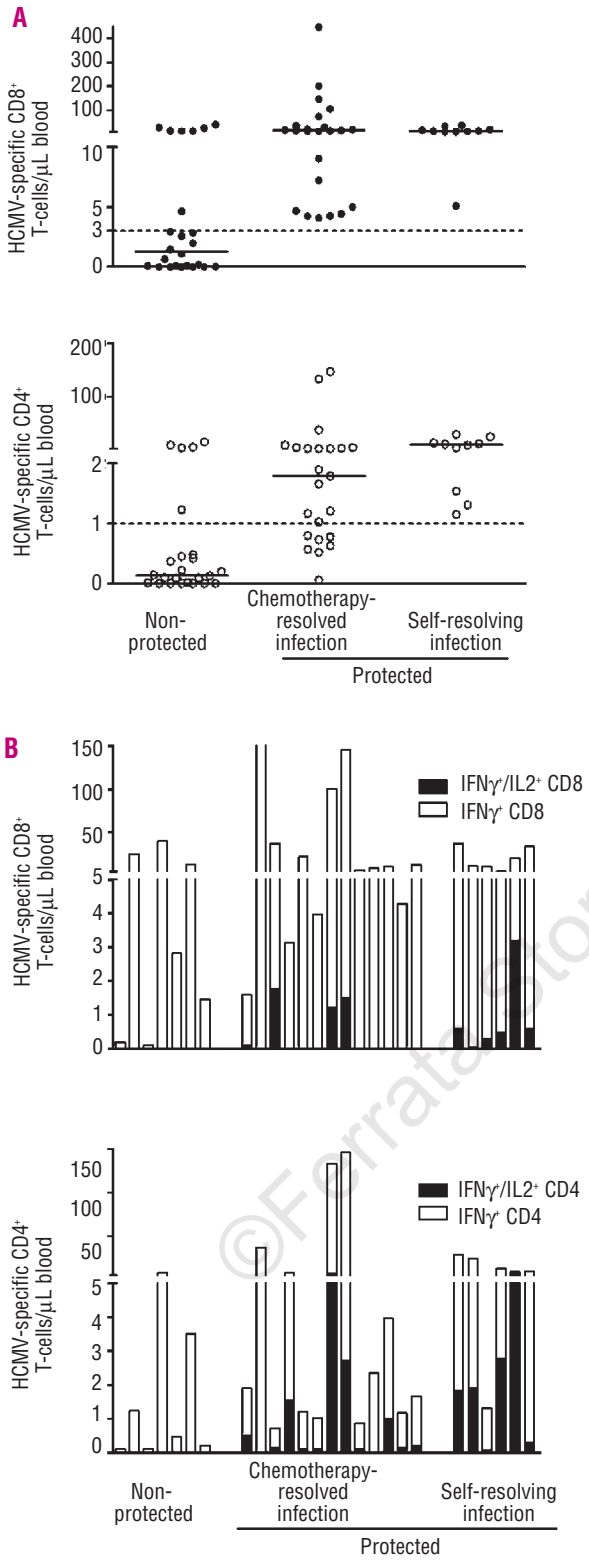

Figure 3. Relationship between absolute number (A) or IL-2 production (B) of HCMV-specific $CD8^+$ and $CD4^+$ T-cells and different types of HCMV infection. *Non-protected* indicates patients reaching the cut-off level for pre-emptive treatment. *Protected* includes patients with sustained disappearance of virus from blood after antiviral treatment (chemotherapy-resolved infection) and patients with spontaneous control of HCMV infection without antiviral treatment (self-resolving infection). In (A), dashed lines indicate cut-off levels for apparent protective immunity. In (B), each vertical bar represents a single patient.

due to spontaneous resolution, referred to as having *self-resolving infection*). ROC analysis showed that a cut-off level of one $CD4^+$ and three $CD8^+$ HCMV-specific T cells/μL blood had 78% sensitivity, 79% specificity, 83% positive predictive value, 73% negative predictive value, and 100% sensitivity, 71% specificity, 82% positive predictive value, 100% negative predictive value, respectively, for identifying patients able to spontaneously control HCMV infection in the absence of treatment. As an exception, five patients, all receiving high doses of steroids for GvHD treatment, developed relapsing HCMV infection requiring pre-emptive treatment in the presence of both $CD8^+$ and $CD4^+$ specific T cells above the cut-off levels.

In a subgroup of samples obtained from 18 patients, the simultaneous production of IFN-γ and IL-2 by HCMV-specific T cells was evaluated (Figure 3B). In patients considered as *non-protected*, no IL-2 production was detected in response to the HCMV-infected dendritic cell stimulus, even in the presence of $IFN\text{-}\gamma^+$ HCMV-specific T cells. On the other hand, the great majority of *protected* patients had HCMV-specific T cells producing both IFN-γ and IL-2, especially in the $CD4^+$ compartment. In detail, 17/19 (89%) patients examined had $IL\text{-}2^+$ HCMV-specific $CD4^+$ T cells (3-86% of $IFN\gamma^+CD4^+$-specific T-cells; 0.06-3.74% of total $CD4^+$ T-cells), while $IL\text{-}2^+$ HCMV-specific $CD8^+$ T cells were detected in 8/19 (42%) patients (1-16% of $IFN\gamma^+$ $CD8^+$-specific T cells; 0.10-1.53% of total $CD8^+$ T cells).

Discussion

The kinetics and magnitude of HCMV infection and HCMV-specific T-cell immune reconstitution were investigated in adult patients undergoing HSCT by means of a novel methodology which provides a more comprehensive assessment of the virus-specific T-cell pool as compared to methods exploiting stimuli given by single peptides or peptide mixtures, or based on MHC-multimer staining.[16,17] Major innovative results of our study are: i) pre-transplant HCMV infection of the recipient is a major factor driving HCMV-specific immune reconstitution; ii) cut-off levels of both HCMV-specific $CD4^+$ and $CD8^+$ T cells presumably conferring protection against HCMV infection were determined; iii) control of HCMV infection appeared to require the presence of both IFN-γ and IL-2-producing T cells.

As for the first point, HCMV-specific immune reconstitution appeared to be relatively more pronounced in magnitude than total T-cell reconstitution. HCMV reactivation (which preceded specific T-cell reconstitution) was detected in the blood of nearly all HCMV-positive recipients, whereas a significantly smaller proportion of negative recipients (less than half) developed detectable HCMV infection in the blood, thus suggesting a minor role of the graft in virus transmission to the recipient. Although the number of the HCMV-negative recipients is too small to

allow robust conclusions to be drawn, it was found that HCMV-specific T-cell reconstitution occurred in all surviving HCMV-positive recipients while about 40% of negative recipients did not develop HCMV-specific T-cell immunity or infection within the 12 months after their transplant. This conclusion is in keeping with previous reports suggesting that recipient HCMV infection may act as a booster for antigen-experienced T cells transferred with the graft.[18,29,30] However, since two HCMV-negative recipients developed HCMV-specific immune reconstitution in the absence of detectable viral infection in blood, an antigen-independent, cytokine-driven expansion of donor memory T cells transferred with the graft could be taken into consideration as an inducer of immune reconstitution in a minority of cases,[31-33] unless episodes of HCMV infection at sites other than blood may have contributed to the virus-specific immune reconstitution.

These findings are in agreement with those of a previous study conducted on children receiving HSCT, in which it was possible to examine a higher proportion of HCMV-negative recipients, and reactivation of latent virus was identified as the main factor leading to immune reconstitution.[18] In pediatric patients, the presence of donor's memory T cells in the graft did not seem to have a major influence on the kinetics and magnitude of immune reconstitution. In fact, children receiving a graft from a seronegative donor or a T-cell-depleted graft did not show a delay in HCMV-specific T-cell reconstitution. Conversely, in the present study, it was observed that adult patients receiving a transplant from an HCMV-negative donor reconstituted HCMV-specific T-cells significantly later than those receiving a graft from a positive donor. This finding, in keeping with reports indicating a better control of HCMV infection[34] and reduced transplant-related mortality[35] in adult patients receiving a graft from a positive donor, supports a major role for donor-derived memory T cells in accelerating immune reconstitution in adults. It can be hypothesized that children, given their still active thymic function, have a better capacity to develop an anti-HCMV primary immune response in the absence of antigen-experienced T cells in the graft. It is known that, in young children, recovery of naïve $CD4^+$ T cells is rapid after chemotherapy,[36] whereas in adults, following thymus atrophy, it is limited and delayed.[37] These data may provide a reasonable explanation for the well-known fact that the capacity to control infections is less in adults than in children.

Among other factors potentially influencing immune reconstitution, only the use of peripheral blood as the source of stem cells was found to be significantly correlated with an earlier recovery of HCMV-specific $CD4^+$ T cells. This may be due to the higher number of memory T cells in peripheral blood than in bone marrow, leading to an earlier HCMV-specific $CD4^+$ T-cell reconstitution.[38] In this respect, $CD8^+$ T cells are less influenced, possibly because they are able to undergo more rapid and massive expansion with respect to $CD4^+$ T cells, in response to HCMV stimulation. The magnitude of the reconstituted HCMV-specific $CD8^+$ T cell pool exceeded that of $CD4^+$ T cells, and a much higher number of virus-specific $CD8^+$ T cells than that observed in healthy controls (reaching even levels >30% of the total $CD8^+$ T cells) accumulated and persisted in peripheral blood. Despite the difference in magnitude, data from this study confirm that the kinetics of HCMV-specific $CD8^+$ and $CD4^+$ T-cell reconstitution are similar[39] (with a median time to immune reconstitution of about 2 months), with $CD8^+$ appearing slightly earlier than $CD4^+$ T cells. This is different from what has been observed in organ transplant recipients in whom $CD8^+$ T cells appeared consistently and significantly earlier than $CD4^+$ T cells (median time: 3 *vs.* 6 months).[40] This may be due to the higher dosage of immunosuppressive drugs which may affect $CD4^+$ more than $CD8^+$ T-cell reconstitution.

As for the second point, due to the very strict virological monitoring and early pre-emptive therapy, no patient suffered from HCMV disease. Thus, HCMV load reaching the cut-off level for pre-emptive therapy was used as a surrogate marker for the risk of developing HCMV disease. On the other hand, a self-resolving infection or the sustained disappearance of HCMV after antiviral treatment was considered an indicator of acquired immune protection against HCMV. Results of the ROC analysis seem to confirm that, as observed for pediatric patients,[18] levels of $CD4^+$ >1 cell/μL and $CD8^+$ >3 cells/μL may be proposed as cut-offs for defining immune protection against a high risk of HCMV disease. However, the positive predictive values of these cut-offs for identifying patients able to control HCMV infection were around 80%, indicating that 20% of patients reaching these levels of HCMV-specific T cells may still develop HCMV infection requiring treatment. In our study, as many as five patients developed HCMV infection requiring pre-emptive treatment in the presence of levels of both $CD4^+$ and $CD8^+$ specific T cells above those defined as *protective*. All these five patients received high dosage steroid treatment for GvHD.

This observation raises concerns about the protective role of a reconstituted HCMV-specific T-cell pool in patients undergoing steroid treatment. Previous studies reported delayed HCMV-specific T-cell reconstitution[41] or the presence of non-functional HCMV-specific T cells[42] in patients receiving high dose steroids. In the present study, it was found that, although developing an active HCMV infection in blood, patients receiving high dose steroids for GvHD treatment did not lose the reconstituted pool of HCMV-specific T cells. Since our patients received pre-emptive therapy, we cannot know whether, in those with apparent T-cell reconstitution but receiving steroid treatment, HCMV infection would have really been uncontrolled, leading to overt disease in the absence of antiviral treatment. On the other hand, we cannot exclude that steroid treatment affects other T-cell functions, preserving IFN-γ production[43] (which is the read-out of the assay

adopted for the identification of HCMV-specific T cells). Thus, in order to better define the protective features of HCMV-specific T-cells, in addition to IFN-γ production, IL-2 production by HCMV-specific T cells was analyzed.

It is noteworthy that, in a recent study, functionally distinct antigen-specific T-cell subpopulations were described based on their ability to produce IFN-γ and/or IL-2 according to duration of antigen exposure and level of antigen load.[44] It was shown that in patients infected by human immunodeficiery virus, control of HCMV infection required the presence of dual IFN-γ and IL-2 producing T cells.[45] In accordance with this report, *non-protected* HSCT recipients experiencing HCMV recurrences (also those with specific T-cell reconstitution but receiving steroid treatment) did not have HCMV-specific T cells able to produce both IL-2 and IFNγ, while the great majority of *protected* patients did (especially in the $CD4^+$ subset). Lack of IL-2 may impair proper acquisition of more differentiated effector functions (such as cytotoxic activity) by HCMV-specific $CD8^+$ T cells. Thus, the absence of IFN-γ/IL-2 producing T-cells might be an indicator of the lack of capacity to control the infection by the recovered HCMV-specific T-cell pool.

In conclusion, our results suggest that, as already observed in young HSCT recipients, pre-transplant HCMV serostatus of the recipient is the main trigger for specific T-cell reconstitution, exceeding in relative magnitude the total T-cell reconstitution. The reconstituted HCMV-specific T-cell pool is generally able to control the infection in the absence of antiviral drugs. However, in some patients, steroid treatment is associated with active viral replication in the presence of specific T cells, thus suggesting an immune impairment that might not be detectable by the sole determination of IFN-γ production by T cells. In the future, routine immunological monitoring should flank virological monitoring in the view of making therapeutic decisions for HSCT recipients. Virological monitoring and interventions with antiviral drugs could be limited in patients with an adequate T-cell immune response. However, determination of IFN-γ production alone may not be sufficient to evaluate the protective capacity of HCMV-specific T cells. Analysis of IL-2 production seems to add additional information to the study of immune recovery. Along the same line, the search for additional cytokines, other effector functions and homing properties should help in the future to better define the protective role of reconstituted HCMV-specific T-cell immunity, particularly in patients receiving high doses of steroids.

Authorship and Disclosures

DL: interpretation of results, data collection, database and statistical analysis; CF,AC: immunological assays; DC: follow-up of patients; EPA: follow-up of patients and interpretation of clinical findings; GG: conception and design of the study, and critical discussion of the draft manuscript. The authors reported no potential conflicts of interest.

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