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Successive emergence of two CD8 subsets in primary CMV infection of allograft recipients

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Abstract Allograft recipients with cytomegalovirus (CMV) infection develop increased proportions of circulating $CD8^+$ lymphocytes. A longitudinal study of 11 kidney and 5 liver allograft recipients with primary CMV infection but no other aetiological factor to explain graft dysfunction revealed selective imbalances in peripheral blood $CD8^+$ T cell subsets. Initially, CMV viraemia was associated with elevated $CD8^{+bright}$ T cell numbers and T cell activation. Activation markers fell to normal when viral cultures became negative (before the end of the 1st month). During the 2nd–6th months, most (12/16) patients continued to have high $CD8^+$ T cell counts (1050–2900 $CD8^+$ cells/mm^3), comprising an uncommon $CD8^+$ T cell subset, as 45–73% of $CD8^{+bright}$ lymphocytes were $CD3^+$ and $TCR\alpha\beta^+$ but were not stained by anti-CD28, CD11b, CD16, CD56 and CD57 antibody. Unexpectedly, $CD8^+CD57^+$ T cells, a hallmark of CMV infection, did not appear until the 2nd–6th months of primary CMV infection, and their numbers increased progressively thereafter. They became the predominant $CD8^+$ T cell subset after about 6 months of infection and their persistence for several (up to 4) years was strongly correlated ($r = 0.87$) with expansion of $CD8^+$ cells. Persistence of CD8 lymphocytosis was, thus, directly related to the rate of expansion of an uncommon $CD8^+CD57^-$ subset and its progressive replacement by $CD8^+CD57^+$ T cells that were chronically elicited by CMV.

Key words T lymphocyte subsets
$CD8^+$ cells · $CD57^+$
Cytomegalovirus · Transplantation

Introduction

Human cytomegalovirus (CMV), a ubiquitous pathogen that is carried lifelong after initial exposure, has profound effects on the CD8 lymphocytic compartment that are increased in immunocompromised hosts, such as solid organ and bone marrow transplant recipients. A remarkable phenotypic feature associated with CMV infection is a consistent increase in the proportion and absolute number of $CD8^+$ T lymphocytes, although CMV-specific T cells are detected in peripheral blood at a frequency of only 1/5000 or lower [1–3]. Whereas infection of T cells by CMV is usually abortive [4, 5], CD8 lymphocytosis persists for several months in immunocompetent subjects, and even longer in allograft recipients [6, 7].

Healthy CMV-seropositive individuals have higher levels of the $CD8^+CD57^+$ T cell subset than CMV-seronegative controls [8, 9], but CMV-infected allograft

recipients maintain a marked expansion of their $CD8^+CD57^+$ lymphocytes [10, 11]. However, the characterisation of the $CD8^+$ cell surface phenotype in CMV-infected individuals remains limited. To investigate in more details how selective imbalances in peripheral blood $CD8^+$ T lymphocyte subsets evolve in CMV-infection, we performed longitudinal analyses of the subset composition within $CD8^+$ T lymphocytes, comparing allograft (kidney or liver) recipients who became primarily infected by CMV.

Our follow-up data showed that persisting $CD8^+$ T cell expansion in CMV infection was accounted for by the successive expansion of two minor (in normal subjects) $CD8^+$ subsets; failure to expand these subsets resulted in transient CD8 lymphocytosis after primary CMV infection.

Patients and methods

Patients

All kidney allograft recipients received sequential quadruple drug immunosuppression with antilymphocyte globulin, azathioprine, prednisolone, and cyclosporin A. The baseline immunosuppressive regimen of liver allograft recipients consisted of triple therapy with prednisolone, azathioprine and cyclosporin A. Among the patients who were seronegative before graft transplantation but who received an organ from a CMV-seropositive donor and developed mild (fever, leucopenia) to severe CMV disease, 16 (11 kidney and 5 liver transplants) were selected because no other aetiological factor of graft dysfunction was identified after the diagnosis of CMV infection. These patients had received prophylactic treatment with anti-CMV-specific immunoglobulin, and only severe CMV disease was treated in three of the liver recipients by a 15-day course of ganciclovir (DHPG). CMV infection was defined by the first positive viraemia.

Analysis of peripheral blood lymphocyte subsets

The whole blood staining technique was used with combinations of FITC and PE-conjugated monoclonal antibodies: anti-CD4 (T4), anti-CD8 (T8) and anti-CD56 (NKH-1) antibodies were from Coulter (Hialeah, F.); anti-CD3 (leu-4), anti-TCR $\alpha\beta$ (TCR-1, α/β), anti-CD28 (leu-28), anti-CD11b (leu-15), anti-CD57 (leu-7), anti-CD16 (leu-11a) and anti-HLA-DR antibodies were from Becton Dickinson (Mountain View, Calif.). Cells were analysed by flow cytometry using an Epics Profile (Coulter). Results are expressed either as an absolute cell count or as a percentage of the total $CD8^+$ lymphocyte population.

Measurement of soluble interleukin-2 receptor and soluble CD8 antigen level

Serum concentrations of soluble interleukin 2 (IL-2) receptor and of soluble CD8 antigen (sCD8) were measured by standard ELISA techniques (Immunotech, and T Cell Science, Cambridge, Mass., respectively).

Results

Primary CMV infection occurred within 3 months after the transplantation of a kidney or a liver from a CMV-seropositive donor to a CMV-seronegative recipient.

CD8 lymphocytosis may be transitory or persistent after primary CMV infection

Whereas CD8 lymphocyte numbers were stable at normal values (15–30% of total lymphocytes) in allograft recipients with no virological changes, primary CMV infection was marked by a sharp increase in the absolute $CD8^+$ lymphocyte counts that peaked in less than 1 week, preceding by a few days or accompanying clinical disease and the first viraemia. Contemporary $CD4^+$ T cells were diminished in percentages, but were normal or slightly decreased in absolute numbers.

After the viraemic phase (after 1 month of infection), two evolving patterns of CD8 lymphocyte numbers were observed. In most patients ($n = 12$, a representative is shown in Fig. 1a, the absolute number of $CD8^+$ cells remained steadily elevated ($1050 - 2900/mm^3$; 42–85% of the total blood lymphocytes) for up to 4 years in our earliest patients. In the second pattern (four patients), the initial peak of $CD8^+$ cell counts was a high, but the percentage and absolute number of $CD8^+$ cells fell significantly (Fig. 1 b). There were, however, no apparent differences in the severity of CMV disease and the viral parameters, the occurrence of rejection before CMV infection, the type of graft (kidney or liver) or the immunosuppressive therapy or prescription of ganciclovir treatment between patients differing by persisting versus falling CD8 counts after 1 month of infection.

Most of the expanded CD8 lymphocyte population (87–97%) expressed brightly the CD8 marker at any time of CMV infection. These cells were also stained by anti-CD3 and anti-$\alpha\beta$ T cell receptor (TCR) antibody, but not by anti-CD16, indicating that the $CD8^{+bright}$ cells were not accounted for by contamination of natural killer cells and, therefore represented $CD8^+$ T lymphocytes.

Initially in all patients, a significant proportion (34–66%) of $CD8^+$ T cells co-expressed brightly the HLA-DR molecule, and the rate of appearance of $CD8^+HLA\text{-}DR^+$ cells was parallel to the increase in the whole CD8 population (Fig. 1c, d). At the viraemic phase, soluble IL-2 receptor and soluble CD8 antigen levels also increased markedly in serum, peaking at around six-fold the basal value for CD8 (Fig. 1e, f).

The number of $CD8^+HLA\text{-}DR^+$ lymphocytes fell to normal values in all patients 2–3 months after the first

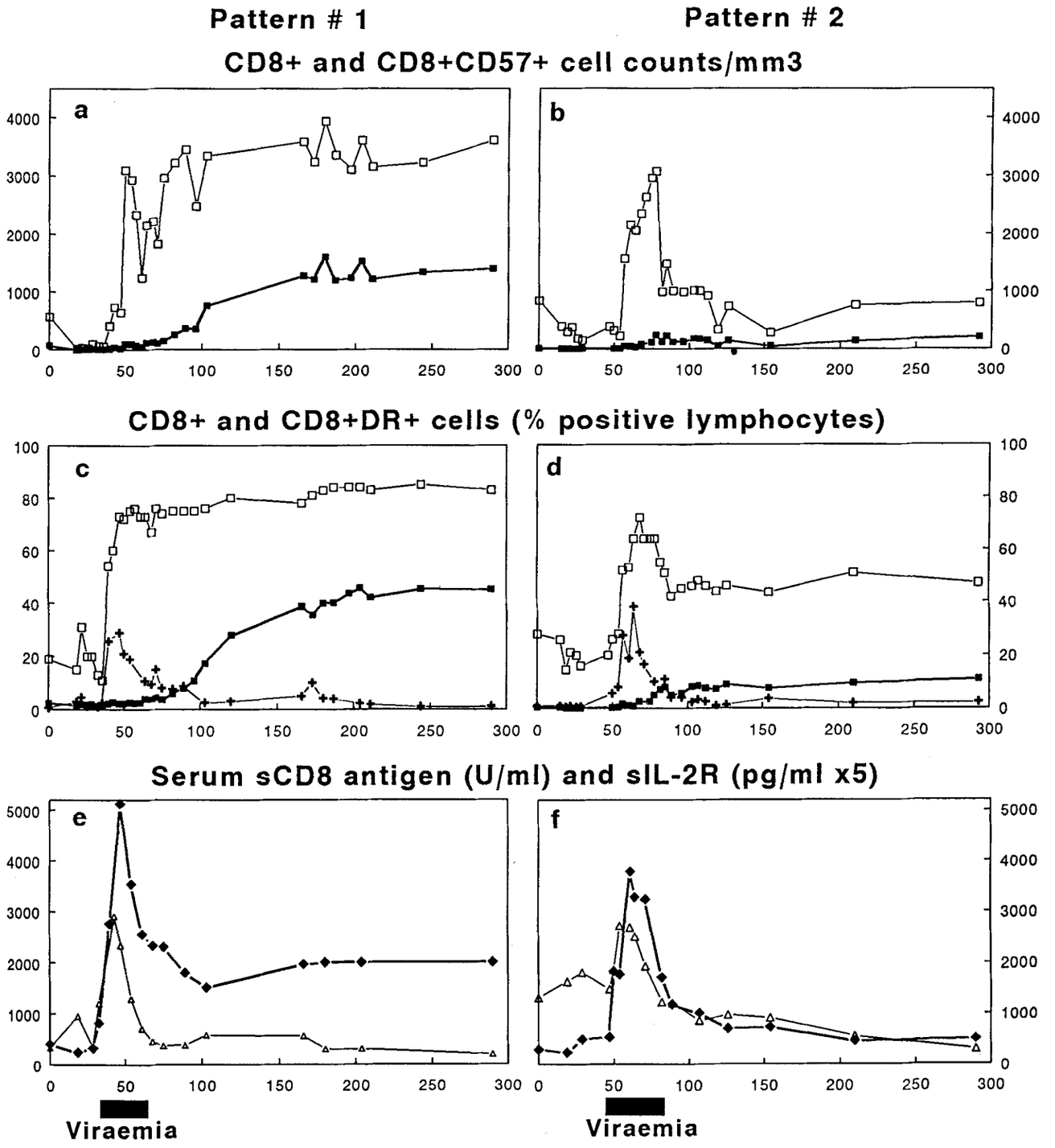

Fig. 1a–f Two evolving patterns of $CD8^+$ T cell expansion in primary CMV infection of allograft recipients: pattern #1 with persistent CD8 expansion (a representative patient, left part of the figure) and pattern #2 with transient elevation of $CD8^+$ cell counts during viraemia (a representative patient, right part of the figure). **a, b** Evolution of total $CD8^{+bright}$ T cell counts (□) and of $CD8^+CD57^+$ T cell counts (■). **c, d** Evolution of the percentages of $CD8^{+bright}$ T cells (□), of $CD8^+CD57^+$ cells (■) and of $CD8^+HLA\text{-}DR^+$ cells (+). **e, f** Evolution of serum levels of soluble CD8 antigen (sCD8) (◆) and of soluble IL-2 receptor (sIL-2R, level × 5 for scale) (△)

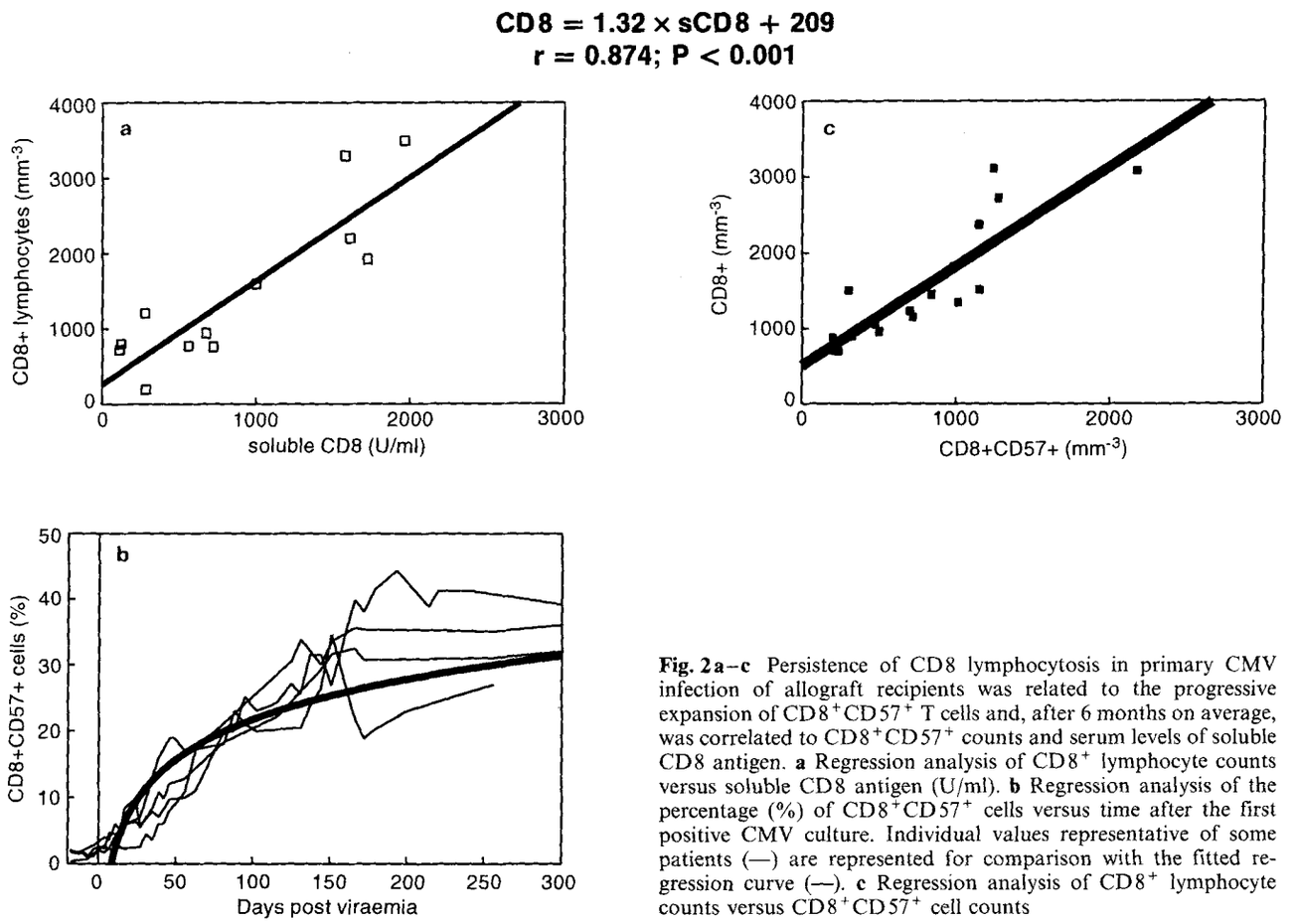

Fig. 2a–c Persistence of CD8 lymphocytosis in primary CMV infection of allograft recipients was related to the progressive expansion of $CD8^+CD57^+$ T cells and, after 6 months on average, was correlated to $CD8^+CD57^+$ counts and serum levels of soluble CD8 antigen. **a** Regression analysis of $CD8^+$ lymphocyte counts versus soluble CD8 antigen (U/ml). **b** Regression analysis of the percentage (%) of $CD8^+CD57^+$ cells versus time after the first positive CMV culture. Individual values representative of some patients (—) are represented for comparison with the fitted regression curve (—). **c** Regression analysis of $CD8^+$ lymphocyte counts versus $CD8^+CD57^+$ cell counts

positive CMV culture (Fig. 1c, d). Soluble IL-2 receptor levels also reverted to normal, and soluble CD8 antigen levels decreased significantly. After the viraemic phase, soluble CD8 antigen level was, tus, directly correlated to the size of CD8 lymphocytosis ($r = 0.88$; $P < 0.001$; Fig. 2a).

Delayed and progressive expansion of $CD8^+CD57^+$ T cells

In all patients during the viraemic period (1st month of infection), only 2–10% of $CD8^+$ T lymphocytes coexpressed the CD57 marker, whether or not elevated CD8 counts were maintained thereafter, and $CD8^+$ $CD57^+$ cell counts were not significantly different from transplant patients with no virological changes (Table 1).

In patients who continued to have elevated $CD8^+$ lymphocyte counts, $CD8^+CD57^+$ cells appeared progressively during the 2nd–6th months. Our longitudinal study of absolute $CD8^+CD57^+$ T cell counts was significant for a delayed and progressive (logarithmic) increase with time, half-maximum values being observed 52 days on average after the first viraemia (Fig. 2b). They became the predominant $CD8^+$ T cell subset (49–74% of $CD8^+$ cells) after the 6th month only (Fig. 1). Then, CD8 lymphocytosis reflected the expansion of $CD8^+CD57^+$ cells ($r = 0.87$; $P < 0.001$; Fig. 2c).

Table 1 $CD8^+$ T cells and their subsets during the successive phases of primary CMV infection in transplant patients with persistent CD8 expansion. Data are given as medians and ranges

	% CD8 in total lymphocytes	% $CD8^+CD28^+$ in $CD8^+$ cells		% $CD8^+CD57^+$ in $CD8^+$ cells		% residual $CD8^+$ lymphocytes[a]	
Viraemia	56 (48–73)	61 (44–71)	33 (27–38)[b]	5 (2–10)	4 (2–7)[b]	34 (19–54)	64 (58–70)[b]
2nd to 6th months	57 (42–74)	24 (8–38)		19 (5–45)		55 (45–73)	
At 6 months	57 (44–85)	26 (12–30)		38 (19–58)		35 (25–48)	
At 2 years	62 (50–70)	19 (12–27)		55 (49–74)		26 (14–29)	
Sero-negatives[c]	21 (15–30)	73 (48–87)		10 (5–15)		3 (0–7)	

[a] Residual $CD8^{+bright}$ T lymphocytes that were not stained by anti-CD28, CD11b, CD56, CD57 antibody

[b] The data represent two subgroups of patients with and without an initial peak in $CD8^+$ $CD28^+$ T lymphocytes. There were no differences between these two subgroups after the 1st month of infection, therefore the data for later periods of infection were pooled

[c] Transplant patients who remained CMV sero-negative had stable CD8 counts and the proportions of their lymphocyte subsets were stable throughout the study period

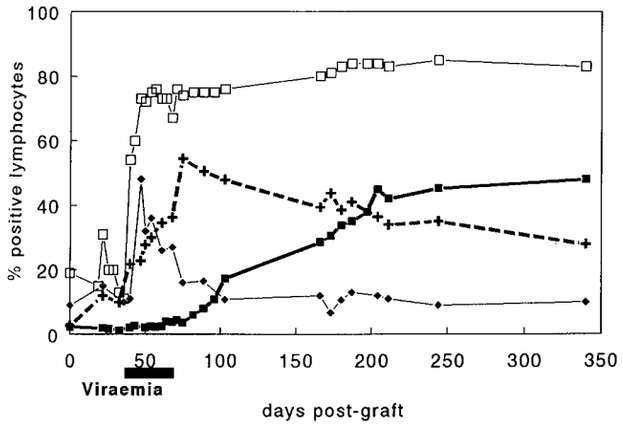

Fig. 3 Evolution of $CD8^+$ T cell subsets in primary CMV infection of allograft recipients with persistent CD8 expansion: a representative patient with an initial elevation of $CD8^+CD28$ T cells (◆) during the viraemic phase. The $CD8^+CD57^+$ T cells (■) rose afterwards, and replaced progressively an uncommon subset of $CD8^{+bright}$ $CD3^+$ $TCR\alpha\beta^+$ but $CD28^-$ $CD11b^-$ $CD56^-$ $CD57^-$ lymphocytes (+) within the whole $CD8^+$ T cell population (□). Data are percentages of total lymphocytes

Initial CD8 expansion reflected the emergence of an unusual $CD8^+CD57^-$ T cell subset that was replaced progressively by $CD8^+CD57^+$ lymphocytes

In the viraemic period, in around half of the patients ($n = 7$), the predominant lymphocytic population was initially T cells with a $CD8^+CD28^+$ phenotype (44–73% of $CD8^+$ T lymphocytes; Fig. 3). While in lower absolute numbers, this was also the predominant subset throughout the evolution of allografts with no virological changes (48–87% of $CD8^+$ cells). In the second half of the patients ($n = 9$), the percentage of $CD8^+CD28^+$ T cells was persistently low even at the time of virological diagnosis (Table 1). There was, however, no relationship between the nature of the predominant CD8 subset at the viraemic phase and the maintenance of elevated $CD8^+$ cell counts thereafter.

For all CMV patients, after the 1st month of primary CMV infection the number of $CD8^+CD28^+$ T lymphocytes was normal in absolute numbers. The percentage was diminished (8–38% of $CD8^+$ T lymphocytes) in patients who continued to have expanded CD8 values, and in these patients, 45–73% of $CD8^+$ T cells did not coexpress any of the following subset-specific markers: CD28, CD11b, CD16, CD56 and CD57 (Fig. 3). This unusual $CD8^+$ T cell subset persisted beyond the 6th months of primary CMV infection but their percentages dropped as the $CD8^+CD57^+$ cells became predominant (Table 1).

Discussion

Our phenotypic analyses revealed that $CD8^+$ T lymphocytes with distinct immunophenotypes expanded as a function of time in primary CMV infection, allowing three phases in the evolution of CD8 lymphocytosis to be distinguished: (1) the 1st month, when viraemia was associated with the emergence of activated $CD8^+$ T cells, as has also been shown in other studies [12, 13]; (2) during the 2nd–6th months, in patients who maintained a CD8 lymphocytosis, $CD8^+CD57^-$ T lymphocytes without activation markers predominated and $CD8^+CD57^+$ T cells began to emerge; (3) $CD8^+CD57^+$ T cells predominated thereafter and persisted for several years (up to 4 years in this series).

Both activated and quiescent $CD8^+$ lymphocytes spontaneously release an immunoreactive soluble CD8 glycoprotein [14, 15]. Elevation in the serum concen-

tration of sCD8 represents a sensitive marker of $CD8^+$ T cell activation (vireamic phase), but also occurs when the pool size of $CD8^+$ lymphocytes increases.

After the 1st month of infection, CD8 lymphocyte counts and their expression of activation markers evolved in the opposite direction in the majority of the patients: CD8 lymphocytosis remained steadily elevated, although viral cultures became negative and activation markers disappeared. However, in contrast with classical reports, in a few of our allograft recipients (4/16), $CD8^+$ T lymphocyte counts fell to normal values in 1–3 months, parallel to the disappearance of activation markers and normalisation of soluble CD8 antigen levels. No clinical features were correlated to the two evolving patterns, even though our definition of primary CMV infection was based on viraemia, which is usually associated with symptomatic CMV disease [16]. The two evolving patterns were not correlated with previous treatment of rejection episodes. We also found no relation between the initial number of activated T cells and the recovery from CMV infection or the development of progressive CMV disease, as reported by van den Berg et al. [17, 18].

The use of lymphocyte markers in the assessment of CMV infection has largely been centred on the well-documented increase in the $CD8^+CD57^+$ T cell population, a hallmark of CMV infection [7–11], but the function of $CD8^+CD57^+$ T cells is still poorly defined [9, 19, 20].

Disproportionate expansion of this subset in active CMV infection accounts for the elevation of the CD8 population, but only after a delay in primary infection. This subset, not yet elevated in the viraemic phase, cannot account for the initial expansion of the CD8 lymphocytic population. In contrast, our data in transplant patients with CMV reactivation or reinfection revealed the simultaneous increase in the CD8 population and in its $CD8^+CD57^+$ subset (data not shown). Forman et al. [10] also report a peak in $CD57^+$ cells concomitant with viraemia in patients who have evidence of reactivated CMV infection after bone marrow transplantation. In addition, CMV carriers have significantly increased numbers of $CD8^+CD57^+$ compared with non-carriers [8, 9]. Taken together, these observations suggest that $CD8^+CD57^+$ T lymphocytes represent "memory" cells in CMV infection that are rapidly mobilised in CMV reactivation but have a late and progressive appearance in primary CMV infection. Initially, the expanded CD8 compartment comprised mostly uncommon T lymphocytes that expressed brightly the CD8 molecule and bore T cell markers (CD3 and TCR$\alpha\beta$), but could be ascribed to any particular subset with the panel of antibodies tested (CD28, CD11b, CD56, CD57, CD16). Lack of detection of subset-specific markers of $CD8^+$ T lymphocytes could not be related to their down-modulation, considering the close time interval of our follow-up analyses. We can only speculate that the $CD3^+CD8^{+bright}CD28^-CD11b^-CD16^-CD56^-CD57^-$ cells belonged to a sub-population rare in healthy subjects, for which no specific marker has been tested or is available, that is directly expanded as the result of a selective process related to primary CMV infection.

Our longitudinal studies indicated altogether that a persisting CD8 lymphocytosis in primary CMV infection in allograft recipients is directly related to the rate of expansion of an uncommon $CD8^+CD57^-$ subset and its progressive replacement by $CD8^+CD57^+$ T cells that are successively elicited by CMV. Two hypotheses can be made: (1) distinct CD8 subsets could follow each other during the 1st 6 months of primary CMV infection, with a slower expansion rate of $CD8^+CD57^+$ lymphocytes; (2) the CD57 marker could be acquired as a result of the maturation of a (initially) $CD8^+CD57^-$ T lymphocyte subset. Whether this results either in a differentiation or activation disorder in $CD8^+$ T cell lineage is currently under investigation. Whatever the mechanism, linkage of the two phenotypes is suggested since, in the few transplant patients who did not maintain a CD8 expansion after the 1st months, the $CD8^+CD57^-$, and later on the $CD8^+CD57^+$ subsets, did not expand even in percentages within $CD8^+$ cells.

As the disproportionate elevation of minor (in normal subjects) $CD8^+$ T cell subsets persisted up to 4 years after primary CMV infection in allograft recipients, this indicated that the combination of immunosuppressive therapy and CMV-derived factors results in the establishment of a novel homeostasis of the peripheral CD8 lymphocytic compartment during primary CMV infection.

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