Management of human cytomegalovirus infection in transplantation: validation of virologic cut-offs for preemptive therapy and immunological cut-offs for protection

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INTRODUCTION

Human cytomegalovirus (HCMV) still causes major viral complications in the post-transplant period of both solid-organ (SO) and hematopoietic stem cell (HSC) transplant (T) recipients (R). Diagnosis of HCMV infection is mostly made by real-time PCR-based methodologies, which allow quantification of viral DNA in both blood and, if required, organ tissues or local secretions. HCMV infection/disease can be prevented by either universal prophylaxis or preemptive therapy. The latter approach has mostly been used in European Transplantation Centers upon reaching predetermined cut-off levels of viral load, predictive of high risk for HCMV disease. In our Department, these cut-offs are higher for SOTR (3x10⁵ DNA copies/ml whole blood) and lower for HSCTR (3x10⁴ DNA copies/ml). Antiviral therapy is continued until viral DNA disappearance from blood or tissues. However, the authentic long-term control of HCMV infection is achieved when HCMV-specific CD⁴⁺ and CD⁸⁺ T-cells are detected in blood or tissues. Proposed immunological cut-off levels conferring protection are: one HCMV-specific CD⁴⁺ and three CD⁸⁺ T-cells/ul blood for HSCTR, and 0.4 HCMV-specific T-cells/ul for both CD⁴⁺ and CD⁸⁺ in SOTR. However, anti-rejection in SOTR and anti-GvHD in HSCTR steroid therapies make patients susceptible to HCMV infection, even in the presence of protective levels of specific T-cells.

KEY WORDS: Human cytomegalovirus, Solid organ transplant, Hematopoietic stem cell transplant, CD⁴⁺ T-cells, CD⁸⁺ T-cells, Viral load cut-off, Pre-emptive therapy, Immunological cut-off

SUMMARY

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INTRODUCTION

Human cytomegalovirus (HCMV) infection (Figure 1A to D) represents a major infectious complication in the post-transplant period for both solid-organ transplant recipients (SOTR) (Fishman and Rubin, 1998) and hematopoietic stem cell transplant recipients (HSCTR) (Boeckh and Ljungman, 1998). In seronegative recipients of transplants from seropositive donors, HCMV can be transmitted through the transplanted organ or HSC (primary HCMV infections), or the administration of non-leukocyte-depleted blood units or blood products. This event occurs in a minor number of cases in HSCTR, whereas it is quite frequent in SOTR. In the great majority of transplant recipients, reactivation of latent HCMV in HCMV-seropositive patients does occur: In addition, in transplant recipients undergoing immunosuppressive regimens, besides reactivation of latent HCMV, a reinfection by a new HCMV strain may occur; thus associating reactivation of latent virus with a new HCMV infection. Clinically, HCMV infection can be either asymptomatic or symptomatic. The presence of clinical manifestations is directly related to the inability of the HCMV-specific T-cell immune response to
control and limit the infection. The balance between virus replication and host defense immune mechanisms is the major pathogenetic factor underlying the clinical presentation of HCMV infection in different transplantation settings. When asymptomatic, HCMV infection consists of detection of virus and virus products in blood during programmed routine examinations, or from local organ sites, in which collection of body secretions or tissue biopsies are performed blindly (Table 1). Monitoring of HCMV infection in these cases, must be associated with monitoring of immunological response in order to adopt the most timely and efficacious preventive approach. Sequential testing for HCMV load in blood is currently performed when using preemptive therapy strategies, which are aimed at detecting HCMV infection prior to appearance of clinical symptoms (see below). The alternative approach to prevention of HCMV infection is HCMV prophylaxis, i.e. administration of antiviral drugs to all patients in the post-transplant period starting from the first day of transplant for a period of 3-6 months (see below). A third approach to treat HCMV infection in the post-transplant period consists of delaying any type of intervention until appearance of HCMV disease (deferred therapy). This may consist of a systemic syndrome or end-organ disease or both (Ljungman et al., 2002).

HCMV systemic syndrome is characterized by fever greater than 38°C for at least two days,

![Figure 1 - HCMV replication in human embryonic lung fibroblast cell cultures.](image)

**TABLE 1 - Spectrum of HCMV infection/disease in humans.**

<table>
<thead>
<tr>
<th>HCMV infection</th>
<th>Clinical symptoms</th>
<th>Diagnosis</th>
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<tbody>
<tr>
<td>Asymptomatic</td>
<td>None</td>
<td>Detection of low level of virus/virus</td>
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<tr>
<td></td>
<td></td>
<td>products in blood or tissue biopsies</td>
</tr>
<tr>
<td>Systemic (HCMV syndrome)</td>
<td>Fever, leukopenia, thrombocytopenia,</td>
<td>Detection of high level of virus/virus</td>
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<tr>
<td></td>
<td>&gt; hepatic transaminases, malaise, headache</td>
<td>products in blood (see below)</td>
</tr>
<tr>
<td>Localized (end-organ disease)</td>
<td>Symptoms/signs of organ involvement (gastrointestinal, pulmonary, hepatic, neural or other)</td>
<td>Detection of virus and virus products in organ biopsies or local secretions in association or not with virus detection in blood</td>
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![Image](image)
malaise, leukopenia, thrombocytopenia, increase in hepatic transaminases greater than twofold the normal upper limit (with exclusion of liver transplant recipients), and presence of HCMV or its products in blood, in the absence of other possible causes. HCMV end-organ disease is characterized by symptoms and signs of organ involvement (gastrointestinal, pulmonary, hepatic, neural, or, less frequently, involving other organ sites) in association with virus detection in biopsy tissues or local body secretions (independently of virus presence in blood), and in the absence of other possible causes of organ disease (Table 1). However, in most patients, systemic and local infections are associated.

Diagnosis of HCMV infection
Apart from transplantation centers using prophylaxis as an HCMV preventive measure, which are not routinely involved in diagnosing HCMV infection (at least until prophylaxis is continued), in all the other centers early diagnosis of HCMV infection is mandatory in view of deciding the most adequate intervention measures aimed at preventing or treating HCMV disease.

A major preliminary consideration in the development of guidelines for diagnosing HCMV infection in transplanted patients concerns the type of specimen to be examined for virus detection. While virus detection in clinical samples such as urine, saliva, cervical secretions or breast milk, may be useful for other purposes, in patients transplant recipients, only two major types of specimens must be taken into consideration: whole blood samples for diagnosis of disseminated infection, and biopsy samples or local secretion samples for diagnosis of end-organ HCMV infection/disease.

Until the mid 1980s, the only available assay was conventional virus isolation in human embryonic lung fibroblasts (HELF). However, this assay was cumbersome and time-consuming, providing results in 2-3 weeks when infectious virus was low in titer. At that time, the availability of monoclonal antibodies (MAbs) led to the development of rapid virus isolation methods based upon use of the shell vial technique (Gleaves et al., 1984), which allowed detection of HCMV-infected cells prior to the appearance of a cytopathic effect (Figure 1A). Virus strain recovery, however, is still of critical importance for studies of cell tropism and susceptibility to antiviral drugs (Erice et al., 1998). The shell-vial assay, which provides results in 24-48 h post-inoculation, gained popularity in the transplantation setting as the “viremia” assay (Gerna et al., 1990). This assay allowed rapid virus detection in blood samples by staining an inoculated HELF monolayer with anti-p72 MAb 24-48h p.i. and visualization of infected cell nuclei by the immunofluorescence (IFA) or the immunoperoxidase antibody (IPA) technique. However, this assay does not allow virus strain recovery.

A major advancement in the diagnosis of disseminated HCMV infection was made at the end of 1980s with the introduction of the antigenemia assay (Figure 2B) developed simultaneously by two groups of researchers in Groningen, the Netherlands, and in Pavia, Italy (Van der Bij et al., 1988; Revello et al., 1989). This assay detected the presence of HCMV pp65 in nuclei of peripheral blood leukocytes (neutrophils and monocytes) by using a pool of pp65-specific MAbs (Gerna et al., 1992) and IFA or IPA. Compared to viremia, antigenemia displayed higher sensitivity and a shorter turnaround time, requiring about two hours to complete. The test performance does not require expensive equipment and is fairly easy to perform. However, it may not be possible with neutrophil counts less than 1,000 cells/ul, and blood samples should be processed within 6-8 hours of collection to prevent decrease in sensitivity.

One of the major achievements of the last decades has been the development of the polymerase chain reaction (PCR) and PCR-based molecular techniques for detection of HCMV genomic DNA and RNA transcripts in clinical samples following amplification of target sequences by a cyclic enzymatic procedure. Besides PCR-based target amplification techniques, methods for detecting HCMV DNA through signal amplification have been developed using either branched DNA probes (Kolberg et al., 1996) or RNA probes (Baldanti et al., 1997; Hebart et al., 1998).

A different amplification technique (nucleic acid sequence-based amplification, NASBA) was also developed for detection of HCMV transcripts in blood of immunocompromised patients (Gerna et al., 1999). More recently, automatic extraction procedures and a real-time readout format have allowed better standardization of PCR conditions.
Viral organ localization, either in the presence or absence of virus in blood, can be diagnosed by examining organ biopsies or local secretions by one of the above reported methods, showing local presence of infectious virus or viral DNA.

**Quantification of HCMV load in blood or tissues**

Quantitative determination of viral load in blood was shown to possess a high positive predictive value for the development of HCMV disease (Grossi et al., 1995).

Among the different methods reported above, rapid virus isolation by the shell vial assay was found to provide quantitative results correlating with HCMV replication (Figure 2A). However, the method was found to lack sensitivity (Gerna et al., 2001). The possibility of using the viremia assay to quantify viral load was based on the observation that a single PBL was able to infect a single HELF (Gerna et al., 1990). In other words, the number of p72-positive HELF nuclei correlated with the number of PBL carrying infectious virus.

Measurement of viral load in patients with disseminated HCMV infection allowed the response to antiviral therapy to be monitored and represented the basis for the adoption of pre-emptive therapy strategies (Grossi et al., 1995; Locatelli et al., 1994). In addition, the shell vial assay allowed

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**FIGURE 2** - (A) Viremia, indicated by the presence, in a shell vial monolayer, of HCMV p72-positive fibroblast nuclei following cocultivation with peripheral blood leukocytes carrying infectious virus, and immunostaining with fluorescein-conjugated p72-specific monoclonal antibody (Gerna et al., 1990) (B) Antigenemia ex-vivo, showing immunofluorescent staining, with a pool of pp65-specific monoclonal antibodies, of pp65-positive peripheral blood polymorphonuclear leukocytes from a patient with AIDS and disseminated HCMV infection (Van der Bij et al., 1988; Revello et al., 1989; Gerna et al., 1992). (C) Antigenemia in vitro, showing pp65-positive polymorphonuclear leukocytes from a healthy blood donor following cocultivation with HCMV-infected human umbilical vein endothelial cells and immunofluorescent staining with the same pool of pp65-specific monoclonal antibodies used in B (Revello et al., 1998). (D) Circulating cytomegalic endothelial cell with a pp65-positive leukocyte (arrow) (From Revello MG and Gerna G, p. 690, Clin Microbiol Rev 2002; 15: 680-715).
quantification of viral load in different biological materials, such as CSF, BAL and tissue biopsies. However, a major advance in the quantification of HCMV load was the introduction of the antigenemia assay (Figure 2B). Threshold values associated with the appearance of HCMV disease were identified following weekly monitoring of antigenemia values in SOTR (Grossi et al., 1995). Thus, pre-emptive therapy of disseminated HCMV infection could be initiated upon reaching cut-off antigenemia values preceding the appearance of clinical symptoms in both SOTR and HSCTR (Locatelli et al., 1994; Grossi et al., 1995; Boeckh, 1999). The antigenemia assay was also of great help in monitoring the response to antiviral treatment, including the emergence of drug-resistant HCMV strains (Baldanti et al., 1998, 2004). In the late 1990s, the in vitro transfer of HCMV pp65 into PBL allowed standardization of the assay (Figure 2C) (Revello et al., 1998; Gerna et al., 1998a), even though some pitfalls of the assay persist:

1) in HSCTR it cannot be used during engraftment (Locatelli et al., 1994; Limaye et al., 1997);
2) a paradoxical rise in antigenemia values may occur during ganciclovir (GCV) treatment (Gerna et al., 1998c, 2003c, 2005a), and thus antigenemia does not correlate directly with viral replication (Gerna et al., 1999b). During the performance of the antigenemia assay, circulating cytomegalic endothelial cells were detected in peripheral blood of immunocompromised patients and fetuses with congenital HCMV infection (Figure 2D).

These limitations were overcome by the introduction of PCR-based molecular assays (Gerna et al., 1991; Humar et al., 1999; Emery et al., 2000; Caliendo et al., 2002; Razonable et al., 2003). Most laboratories performing viral load quantification have been moving to real-time PCR techniques. These techniques are more precise and rapid, possess a broader linear range and a higher output, and have a lower risk of carryover contamination (Cortez et al., 2003; Mengelle et al., 2003; Piiparinen et al., 2004). Viral DNA can be quantified in different blood fractions (leukocytes or plasma) or whole blood (Gerna et al., 1994b). Although no general consensus has been reached and plasma and whole blood both provide prognostic and diagnostic information (Hamprecht et al., 1998; Razonable et al., 2002), whole blood is now accepted as the specimen of choice, since it allows detection of HCMV DNA earlier and in greater amounts compared to plasma.

However, in order to make results obtained by different laboratories comparable, a standardized PCR methodology is warranted, as well as reference to an International Standard or to external quality control panels by all laboratories. Interinstitutional comparison of quantitative DNA values through the exchange of specimens and use of external quality control samples has been initiated among laboratories (Lilleri et al., 2009b; Pang et al., 2009).

Given the general consensus that higher viral load values correlate with an increased risk for development of HCMV disease, it must be specified that viral load changes less than 5-fold (0.7 log10 copies/ml) may not be considered significant (Kotton et al., 2010).

In general, there is a good but not uniform correlation between antigenemia and DNA viral load levels in blood (Caliendo et al., 2002). One recent study comparing viral load in blood by real-time PCR (DNAemia) and antigenemia in SOTR showed that use of DNAemia versus antigenemia for initiation of pre-emptive therapy significantly reduced the number of patients requiring treatment without an increase in HCMV disease (Gerna et al., 2007).

Diagnosis of end-organ disease such as hepatitis or gastrointestinal infection must be performed by immunohistochemistry or in situ hybridization (Lautenschlager et al., 2006). However, quantification of viral DNA in tissue biopsies as well as BAL samples is preferable due to greater sensitivity without loss of specificity. As for BAL samples, in the absence of randomized studies, quantification of viral DNA has been suggested as helpful in predicting pneumonitis (Westall et al., 2004; Chemaly et al., 2005; Gerna et al., 2009). In particular, the HCMV DNA concentration in the epithelial lining fluid diluted in BAL would be critical for differentiating between symptomatic and asymptomatic infections (Zedtwitz-Liebenstein et al., 2004). Diagnosis of other organ syndromes, such as central nervous system disease or retinitis would require detection of viral DNA in CSF or aqueous humour (Gerna et al., 1994a).
As a final consideration, it is important to recall that an International Reference Standard (containing $5 \times 10^6$ DNA copies/mL) has become recently available through WHO (Freyer et al., 2010), whereas a consensus has yet to be reached on the type of blood sample to be used.

**Prevention of HCMV infection/disease**

Two major prevention strategies have been used in the last two decades to avoid HCMV infection/disease in the post-transplant period: universal prophylaxis and pre-emptive therapy. Some transplantation centers have also been using hybrid methods based on the combined use of both strategies.

Use of deferred (symptomatic) therapy, i.e. initiation of antiviral treatment upon appearance of clinical symptoms, is now restricted to a few transplantation centers. Antiviral drugs used for different strategies have been ganciclovir (GCV) and foscarnet (PFA) initially, and more recently valganciclovir (VGCV), and, as a second line therapy, cidofovir (CDV). There were no differences in long-term outcomes of HCMV disease between iv GCV and VGCV (Åsberg et al., 2009), while GCV was found to be equally effective in universal prophylaxis vs pre-emptive therapy (Small et al., 2006).

The major advantage of universal prophylaxis (which is currently based on antiviral drug administration for 3-6 months after transplantation) is its daily administration in the absence of viral testing. However, problems related to this strategy are mostly due to the toxic effects of prolonged antiviral drug administration (myelotoxicity-neutropenia for GCV and VGCV, electrolyte disturbances for PFA, and nefrotoxicity for CDV). In addition, potential drawbacks of universal prophylaxis may include the following:

1) late HCMV disease may affect up to 29% of prophylactically treated patients (Limaye et al., 2004, 2006; Eid et al., 2008; Sun et al., 2008);
2) prophylaxis may interfere with and delay HCMV-specific T-cell reconstitution due to abortive stimulation of viral antigens (Hakki et al., 2003). However, a recent study reports that VGCV prophylaxis does not appear to impair the development of HCMV-specific immunity in lung transplantation (Humar et al., 2009; Snyder et al., 2011);
3) suboptimal drug dosage used according to the prophylaxis schedule may induce the emergence of drug-resistant strains.

On the other hand, pre-emptive therapy refers to administration of antiviral drugs when viral load in blood reaches levels predictive of HCMV disease, but prior to onset of clinical symptoms. The major advantage of preemptive therapy with respect to universal prophylaxis is treatment of a smaller proportion of patients for a shorter period of time. However, the pre-emptive therapy approach requires continuous virological monitoring. Additional advantages of pre-emptive therapy include:

1) savings in terms of drug toxicity;
2) savings in terms of patient management costs (Kusne et al., 1999);
3) according to some authors, an efficacy comparable to that of prophylaxis in preventing the indirect effects of HCMV infection, such as graft failure/rejection and fungal/bacterial infections (Singh et al., 2005, 2006a, 2006b; Khoury et al., 2006). However, although in the absence of statistical support, Snydman et al. (2009) stated a superiority of prophylaxis on the indirect effects of HCMV infection, namely mortality and opportunistic infections.

**Initiation of pre-emptive therapy in SOTR**

Since the beginning of the 1990s, at our Institute (Fondazione IRCCS Policlinico San Matteo) the recommended preventive strategy for SOTR has been pre-emptive therapy with GCV until disappearance of virus from blood. Initially, the antigenemia assay was used for deciding the onset of pre-emptive therapy. It was observed that HCMV disease in $D^+/R^+$ and $D^-/R^+$ SOTR started in the presence of antigenemia levels $\geq 400$ pp65-positive PBL/2x10^5 examined (Grossi et al., 1995). Therefore, an arbitrary cut-off threshold of 100 pp65-positive PBL/2x10^5 examined was established for initiating antiviral treatment (Table 2). In addition, due to the reported higher risk for HCMV disease, a much lower antigenemia cut-off was adopted for $D^+/R^-$ SOTR ($\geq 2$ pp65-positive PBL/2x10^5 examined, or 1 pp65-positive PBL/2x10^5 confirmed twice in a week). This strategy was used for about a decade and was flanked by viremia and DNAemia testing as complementary assays. During this period, it was observed that antigenemia did not correlate closely and consistently with virus replication. The molecular
basis of this discrepancy was later clarified by in vitro studies (Gerna et al., 1998c, 2003c, 2005a). To overcome these problems, efforts were directed towards the development of molecular assays detecting virus products more directly related to virus replication. One of these new approaches was the detection of HCMV late transcripts, i.e. the development of the NASBA assay, allowing specific detection of unspliced mRNAs in a DNA background (Gerna et al., 1999).

A prospective randomized open-label trial investigated whether HCMV pp67 (late) mRNA could represent a valuable alternative to quantitative antigenemia in a group of 82 HTR and LTR divided into two arms. In the NASBA arm, both primary and recurrent infections were treated upon the first confirmed positive NASBA result, while in the antigenemia arm, primary infections were treated upon the first confirmed positive result and recurrent infections upon a cut-off of 100 pp65-positive leukocytes. Results showed that the number of treated/infected patients was significantly higher in the NASBA arm, as was the number of treated/relapsing patients. In addition, in the NASBA arm the overall number of days of therapy was significantly higher. It was concluded that pp67 NASBA could safely replace antigenemia, with some disadvantages in terms of overtreatment of patients and greater duration of overall treatment (Gerna et al., 2003a). In the same study, the retrospective determination of quantitative HCMV DNAemia opened the door for subsequent prospective studies aimed at defining better pre-emptive therapy-based control of HCMV infections in SOTR, as compared to antigenemia. A retrospective analysis of DNAemia and antigenemia in SOTR performed at our Institute showed that a DNAemia cut-off of 300,000 DNA copies/ml whole blood could provide positive and negative predictive values as high as those given by antigenemia, i.e. >90% with reference to patients requiring pre-emptive therapy (Lilleri et al., 2004; Gerna and Lilleri, 2006). On this basis, a trial was designed to compare the DNAemia cut-off of 300,000 DNA copies/ml with the antigenemia cut-off of 100 pp65-positive/2x10⁵ leukocytes for pre-emptive treatment of both primary and reactivated HCMV infections (Table 2) (Gerna et al., 2007). Results showed that, compared to antigenemia, the selected DNAemia cut-off:

1) significantly reduced the number of patients receiving treatment (20% vs 40%);
2) was capable of safely guiding pre-emptive therapy of both primary and reactivated HCMV infections in SOTR;
3) did not significantly modify the overall duration of treatment. Furthermore, no case of HCMV disease occurred in patients treated after reaching the cut-off. However, four patients (three in the antigenemia, and one in the DNAemia arm) suffered from

<table>
<thead>
<tr>
<th>HCMV clinical condition</th>
<th>Viral load in blood</th>
<th>pp65-antigenemia</th>
<th>DNAemia</th>
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<tbody>
<tr>
<td>Disease in SOTR</td>
<td>400 pp65-pos PBL/2x10⁶ PBL examined (Grossi et al., 1995)</td>
<td>1,000,000 DNA copies/ml blood (Gerna et al., 1998b)</td>
<td></td>
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<tr>
<td>Preemptive therapy in SOTR*</td>
<td>100 pp65-pos PBL/2x10⁶ examined (historical) (Gerna et al., 2007)</td>
<td>300,000 DNA copies/ml blood (Gerna et al., 2007)</td>
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</tr>
<tr>
<td>Disease in HSCTR</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Preemptive therapy in HSCTR**</td>
<td>2 pp65-pos PBL/2x10⁵ examined (historical) (Lilleri et al., 2007a; Gerna et al., 2008b)</td>
<td>10,000 DNA copies/ml blood</td>
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*Indicated DNAemia cut-offs were found in randomized trials to be safe for both primary and reactivated infections. Many SOT Centers still start treatment of primary infection (high-risk patients) upon first confirmed virus detection by either antigenemia or DNAemia. **Most HSCT Centers still start treatment upon first confirmed detection of virus in blood by either assay.

HCMV management in transplant recipients
HCMV disease prior to reaching the relevant cut-off. In these patients, organ localization in lungs (two patients) and gastrointestinal tract (two patients) occurred prior to virus dissemination in blood (Einsele et al., 1995; Parente et al., 1998; Sanchez et al., 2001; Humar et al., 2009). Thus, in the presence of local clinical symptoms and absence of virus in blood, local samples (biopsy tissues or secretions) must be taken for examination.

This inconvenience could be avoided by prophylaxis, which however presents the major problem of late-onset HCMV disease after prophylaxis discontinuation. Although it was shown in one study in kidney transplant recipients that prophylaxis reduced HCMV infection, while graft survival was improved at four years post-transplant, thus suggesting a beneficial effect of prophylaxis (Kliem et al., 2008), late-onset disease appears to be associated with higher rates of mortality (Limaye et al., 2006) and graft loss (Arthurs et al., 2008).

A major advantage of the use of a single DNAemia cut-off for both primary and reactivated infections was the lack of a greater risk of HCMV disease or recurrence in primary infections compared with treatment started upon first virus detection in blood (Gerna et al., 2007). This approach allowed a greater and more sustained antigenic stimulus during the development of a primary T-cell-mediated immune response. Still, in several transplantation centers, a differential approach to preventive treatment of primary and reactivated infections is maintained, and recently guidelines promulgated by the TTS of America suggested prolonging prophylaxis from three to six months in D+/R- patients (Kotton et al., 2010). In order to export a pre-determined DNAemia cut-off to different centers, different commercial and in-house-developed methods using an International Standard should be standardized.

As mentioned above, in a recent multi-center study guided by our Institution, we observed an acceptable range of variation (≤0.7 log10 or five-fold) among different centers (Lilleri et al., 2009b). Thus, it was recommended that at participating centers, pre-emptive therapy should be started when reaching levels of ≥5.5 log10 (300,000) copies/ml blood in SOTR.

Besides the cut-off of 300,000 DNA copies/ml whole blood for systemic infections, a study on pre-emptive therapy for systemic and pulmonary HCMV infection in lung transplant recipients tested a tentative cut-off of 5.0 log10 (100,000) DNA copies/ml BAL fluid (Gerna et al., 2009). This cut-off was intermediate between that reported by Westall et al. (2004) and that found by Chemaly et al. (2005). The conclusion of this study was that monitoring of HCMV infection in both blood and BAL as well as pre-emptive therapy for both types (sites) of infection when reaching the pre-determined HCMV cut-offs, should be recommended in LTR to prevent HCMV disease. Although definitive cut-offs for intervention have not reached consensus, other centers have used cut-offs in the range of 5,000 DNA copies/mL plasma (Humar et al., 1999) or 1,000-10,000 copies/mL whole blood (Li et al., 2003; Khoury et al., 2006; Reischig et al., 2008). Since our cut-offs were determined on several hundreds of SOT recipients and we did not encounter problems of HCMV disease, we continue using our cut-offs higher than those adopted by other transplantation centers.

Prophylaxis and pre-emptive therapy have been only partially investigated in the pediatric transplanted patient population. (Seu et al., 1997; Kelly, 2006; Spivey et al., 2007; Lilleri et al., 2007a). A study comparing prophylaxis followed by pre-emptive therapy with pre-emptive therapy alone in pediatric patients undergoing liver transplantation found that, although numbers of both infected and treated patients were comparable in the two arms, the median number of total days of antiviral therapy was significantly higher in the prophylaxis arm. Therefore, the trial was interrupted and prophylaxis replaced with pre-emptive therapy alone (Gerna et al., 2008a).

**Initiation of pre-emptive therapy in HSCTR**

Starting in the 1990s, several European HSCT centers employed confirmed qualitative PCR or antigenemia results to guide pre-emptive therapy (Ljungman et al., 1998a; Hebart et al., 2001; Reusser et al., 2002). Then, in a prospective randomized trial, qualitative determination of HCMV immediate-early transcripts (IE-mRNA) by NASBA was compared to antigenemia and retrospectively to DNAemia as a potential guiding parameter for pre-emptive therapy in HSCTR (Gerna et al., 2003b). Results showed that IE-mRNA is more sensitive than antigenemia and DNAemia in detecting active HCMV infection. However, many
patients had to be submitted to treatment with respect to antigenemia. Thus, efforts were directed towards selection of a safe DNAemia cut-off for pre-emptive therapy of HCMV infections in HSCTR. To this aim, a retrospective analysis of DNAemia levels observed in transplant recipients at the time of initiation of antigenemia-guided pre-emptive therapy was performed (Lilleri et al., 2004). It was found that among HSCTR only 19.6% of patients treated upon first confirmed antigenemia positivity reached DNAemia levels above a cut-off of 10,000 DNA copies/ml whole blood, whereas 94.7% of untreated antigenemia patients had DNAemia levels below this cut-off. Thus, a cut-off of 10,000 DNA copies/ml whole blood showed a positive predictive value of 91.7%, and a negative predictive value of 28.6% with respect to HSCTR requiring antigenemia-based pre-emptive therapy. Therefore in HSCTR, use of a quantitative, rather than a qualitative, approach to pre-emptive therapy appeared suitable to increase the negative predictive value without modifying the positive predictive value with respect to qualitative antigenemia (Lilleri et al., 2004).

This hypothesis was verified in a single-center randomized prospective open-label study aimed at defining the effectiveness of the real-time DNAemia versus antigenemia cut-off as the guiding parameter for pre-emptive treatment in pediatric HSCTR (Table 2) (Lilleri et al., 2007a). Using a cut-off of 10,000 DNA copies/ml whole blood in the DNAemia arm, and first positive antigenemia in the antigenemia arm in a cohort of 178 HSCTR (89 assigned to the DNAemia arm, and 89 in the antigenemia arm), it was found that in the presence of a comparable number of HCMV infections in the two arms a significantly lower number of patients required treatment in the DNAemia arm (18%) with respect to the antigenemia arm (31%) (p=0.026). Thus, a DNAemia cut-off-guided pre-emptive treatment proved safe and effective in controlling HCMV infection in the HSCT setting, thereby avoiding unnecessary antiviral treatment in a significant proportion of patients. Comparable results were obtained in an adult HSCTR population, where the major difference with respect to the pediatric population was that the HCMV infection rate was almost double (80% vs 40%) (Table 2) (Gerna et al., 2008b).

In addition, in agreement with a previous report (Verkruyse et al., 2006) an observational study showed that adoption of a cut-off level of $4 \log_{10}$ (10,000) HCMV DNA copies/ml blood for starting pre-emptive therapy is safe and more cost-effective (significantly reducing the number of patients receiving pre-emptive therapy) than starting treatment upon the first positive result. Furthermore, the utility of the progressive replacement of antigenemia with DNAemia as a guiding method for pre-emptive therapy was supported by the frequent rise in antigenemia levels detected after initiation of pre-emptive therapy in allogeneic HSCTR, in the absence of steroid treatment for GvHD or antiviral drug resistance (Gerna et al., 2005a). In addition, the incidence of HCMV infection and pre-emptive treatment in patients with refractory solid tumors receiving nonmyeloablative allogeneic SCT were shown to be significantly reduced compared to recipients of standard SCT for hematologic malignancies (Zambelli et al., 2005).

**Immunological control**

Immunological control of HCMV infection involves both the innate immune and the adaptive immune response to HCMV.

**a) Innate immunity**

Innate immune response seems to play an important role both in the defense against HCMV infection as well as in priming the adaptive immune response. HCMV stimulates toll-like receptors (TLRs), thus activating signal transduction pathways. These cause secretion of inflammatory cytokines and recruitment of cells of the innate immune system, as well as upregulation of costimulatory molecules important for the activation of adaptive immunity (Boehme and Compton, 2004; Boehme et al., 2006). In the murine model, TLR-mediated signal transduction pathways result in the production of interferon by dendritic cells (DC) and macrophages, and activation of natural killer (NK) cells (Tabeta et al., 2004; Delale et al., 2005). NK cells have been shown to contribute to virus clearance in the experimental model of murine CMV infection (Polič et al., 1998) and to provide protection against murine CMV infection (Bukowski et al., 1985), while some strains of
mice resistant to murine CMV became susceptible upon NK cell depletion (Scalzo et al., 1992). In humans, although the role of NK cells in the defense against HCMV infection has not been investigated in depth, NK activity was shown to increase in the control of both primary and recurrent HCMV infection (Venema et al., 1994; Hadaya et al., 2008; Stern et al., 2008).

b) Adaptive immunity
Adaptive immune responses consist of both humoral and T-cell-mediated immune responses. HCMV is a potent immunogen, eliciting a strong immune response from both arms of the immune system.

**Humoral immune response**
Although the role of the humoral immune response is debated, there are indications that CMV-specific antibodies play a somewhat protective role against both HCMV and murine CMV, namely in limiting dissemination and disease severity (Jonjić et al., 1994; Boppana and Britt, 1995). Thus far, viral glycoproteins gB and gH have been considered the major targets of the HCMV neutralizing antibody response (Britt et al., 1990; Rasmussen et al., 1991). Recently, however, a major target of the HCMV neutralizing antibody response was identified in a glycoprotein complex present in the virus envelope of wild-type HCMV strains cultured in endothelial and epithelial cells. This pentamer contains three gene products of the UL131-128 locus of the HCMV genome, which were identified in 2004 as indispensable for HCMV infection of endothelial cells and transfer of virus and viral products from infected cells to PBL (Hahn et al., 2004). Subsequently, pUL130 and pUL128 were shown to form a complex with gH and gL (in the absence of pUL131), which was required for epithelial and endothelial cell tropism (Wang and Shenk, 2005). Finally, gH/gL/pUL131/pUL130/pUL128 were shown to form a protein pentamer complex mediating entry into epithelial and endothelial cells (Ryckman et al., 2008). As mentioned above, this complex has been preliminarily shown to be the target of a number of potent neutralizing human monoclonal antibodies elicited during both primary and reactivated HCMV infection in both the immunocompetent and the immunocompromised host (Macagno et al., 2010). The protective effect of these neutralizing antibodies remains to be determined.

In the mouse model, memory B-cell transfer provided long-term protection from the lethal course of infection that is invariably seen in immune-deficient animals (Klenovsek et al., 2007). Transfer of memory B-cells was also effective in protecting animals from an already ongoing viral infection. T-cells were not involved in this process. In addition, in the guinea pig model, both passive (Chatterjee et al., 2001) and active immunization (Schleiss et al., 2004) have been shown to reduce both fetal HCMV infection and disease in guinea pigs. In humans, it is well known that transfer of HCMV-specific antibodies from a seropositive mother to a newborn infant is able to protect against HCMV infection transmitted by seropositive blood transfusion units (Yeager et al., 1981). Furthermore, mothers with preconceptional HCMV immunity are known to transmit the infection to the fetus at a much lower rate than mothers experiencing primary HCMV infection (Stagno et al., 1982; Fowler et al., 1992). However, the role of HCMV hyperimmune globulin or HCMV-specific monoclonal antibody in preventing or curing HCMV disease in transplant recipients is controversial (Ljungman et al., 1992, 1998b; Machado et al., 2000; Boeckh et al., 2001). The role of hypogammaglobulinemia as a risk factor for HCMV infection in the post-transplant period remains to be defined.

**Cell-mediated immune response**
However, based on the observation that HCMV disease usually occurs in immunocompromised patients with profound cellular immunodeficiency, it is well accepted that the cell-mediated immune response is the major mechanism of control for HCMV infection/disease. Within the T-cell immune response, HCMV-specific CD8+ T-cells, CD4+ T-cells, and γδ T-cells all seem to play an important role in the control of HCMV reactivation.

As for CD8+ T-cells, using the murine CMV model, it was shown that the selective depletion of lymphocyte subsets revealed that CD8+ T-cells were the most important factor in the immune control of murine CMV infection (Polić et al., 1998). In humans, in the HSCT setting, the development of CD8+ cytotoxic T lymphocytes
(CTL) was shown to correlate with protection and recovery from HCMV disease (Quinnan et al., 1982; Li et al., 1994), whereas lack of a CD8 T-cell response was associated with development of HCMV disease (Reusser et al., 1991). Pioneer studies by Riddell et al. (1992) and Walter et al. (1995) definitively showed that adoptive transfer of CD8 T-cell clones expanded in vitro resulted in immune reconstitution in the post-transplant and finally in protection from HCMV disease in the recipients.

Similarly, in the SOT setting, CD8 T-cell responses, in addition to the CD4 T-cell immune response, were repeatedly reported to protect against HCMV disease in the post-transplant period (Reusser et al., 1999; Sester et al., 2002; Radha et al., 2005; Shlobin et al., 2006). However, in the absence of CD4 T-cell help, CD8 T-cells are not able per se to confer effective long-term protection from HCMV infection (see below). Within the CD8 arm of the T-cell immune response, the proportion of CD8 T-cells committed to develop an anti-HCMV response, is surprisingly large, ranging from 10% in healthy individuals up to 40% in elderly individuals (Gillespie et al., 2000; Khan et al., 2004; Crough et al., 2005; Sylvester et al., 2005). The biological reason for such an extended T-cell immune response is not yet known.

The fine specificity of CD8 T-cell responses and the viral proteins to which they are directed have been extensively investigated. By taking advantage of using overlapping 15-mer peptides from all 213 ORF of the HCMV genome in ex-vivo T-cell assays, it was found that CD8 (and/or CD4) T-cells are directed towards more than 70% ORFs.

The most immunodominant antigens representing targets for CD8 T-cells are pUL123 (IE-1), pUL122 (IE-2) and pUL83 (pp65). The different antigens acting as targets for CD8 T-cells are reported in a recently published review (Crough and Khanna, 2009). It must be borne in mind that the largest response does not necessarily represent the most effective in limiting HCMV replication (Crough and Khanna, 2009).

The two major HCMV-specific T-cell subpopulations (CD4, CD8) can be investigated for both their functional markers, such as the expression of IFN-γ, IL-2, granzyme, and perforin, and their phenotypic markers, including CD45RA, CD45RO,CCR7, CD27, CD28, and so on. High perforin expression has been claimed as an easy-to-measure prognostic marker of severe viral reactivation soon after HSCT (Pietersma et al., 2010). Different sets of T-cell subpopulations can be recognized by analyzing surface expression markers.

In particular, the analysis of the chemokine receptor CCR7 (which enables cells to migrate to the lymph nodes) and the different isoforms of the tyrosine phosphatase CD45 allows the study of T-cell differentiation (Sallusto et al., 1999). Naïve T-cells (CD45RA CCR7) switch from the CD45RA isoform to CD45RO, and memory T-cells, according to the differential CCR7 expression, are divided into central memory T-cells (TCM cells; CD45RA CCR7), which are able to migrate to lymph nodes and display a high proliferation potential, and effector memory T-cells (TEM cells; CD45RA CCR7) which exert effector functions in peripheral tissue.

However, it has been shown that a proportion of TEM cells, in case of persistent infection, can revert to the RA isoform of CD45 (TEMRA cells; CD45RA CCR7) after the acute phase of infection (Callan et al., 1998; Wills et al., 1999). In particular, this event was recently found to occur in an elevated percentage of HCMV-specific CD8 (51%) and CD4 (33%) T-cells during the first year after primary infection, thus reaching levels similar to those of subjects with remote HCMV infection (Lilleri et al., 2008b).

In addition, CD45RA expression correlated with HCMV disappearance from blood, while the level of specific CD45RA T-cells during the first months after primary infection in pregnant women was significantly lower in mothers who transmitted the infection to the fetus than in mothers who did not transmit. These results supported the conclusion that HCMV-specific effector T-cells reverting to the CD45RA phenotype may represent true long-lived memory lymphocytes in the HCMV-specific pool.

Two typical features of the HCMV-specific CD8 T-cell response include the accumulation of an oligoclonal T-cell repertoire (HCMV-specific clonotypes) (Price et al., 2005; Day et al., 2007) and the accumulation of HCMV-specific CD8 T-cells occurring with age (Ouyang et al., 2003; Khan et al., 2004). This event, termed “memory inflation” has been shown to extend to murine
CMV (Karrer et al., 2003) and to the HCMV-specific CD4+ T-cell response (Pourghesari et al., 2007). Expansion of HCMV-specific CD8+ T-cells in the elderly is invariably oligoclonal or even monoclonal and is considered to be a contributing factor in immune senescence (Khan et al., 2002; Pawelec et al., 2005). In addition, it is thought that the HCMV immunodominance might impair immunological responses to other pathogens (Trzonkowski et al., 2003). On the other hand, it seems possible that a “bystander” effect of infection with heterologous stimulation of T-cell populations of other specificities may occur (Crough et al., 2005).

In addition to MHC class I-restricted CD8+ T-cells, evidence has accumulated indicating that also CD4+ T-cells are essential for control of HCMV infection. This conclusion has been reached both in mice (Jonjić et al., 1990; Polić et al., 1998) and humans, with special reference to the transplantation setting (Kumar et al., 2009; BenMarzouk-Hidalgo et al., 2011). In SOT recipients, it was observed that low levels of HCMV-specific CD4+ T-cells correlated with emergence of infectious complications (Sester et al., 2001, 2005), while effector-memory T-cells were required for recovery from infection (Gamadia et al., 2003). Similarly, in HSCT recipients CD4+ T-cells were found to be associated with protection from HCMV disease (Li et al., 1994; Hebart et al., 2002; Tormo et al., 2010). In addition, HCMV-specific CD4+ T-cells were required for expansion of CD8+ CTLs (Einsele et al., 2002).

As with HCMV-specific CD8+ T-cells, a very high frequency of CD4+ T-cells was observed in some blood donors (Sylwester et al., 2005). The most frequent targets of HCMV-specific CD4+ T-cells were products of the TLR14, UL16, UL55, and UL83 genes (Crough and Khanna, 2009). While HCMV-specific CD4+ T-cells have been considered to display only an indirect role (help in maintaining the antibody response and expanding CD8+ T-cells), more recently γδ T-cells have been reported (Elkington et al., 2004; Casazza et al., 2006).

Similar to CD8+ T-cells, an oligoclonal or monoclonal expansion of CD4+ T-cells has been reported in some hematologic clinical conditions (Garrido et al., 2007; Crompton et al., 2008). In addition, regulatory T-cells (Tregs) have been shown to correlate with recovery of HCMV-specific CD8+ T-cells after allogeneic HSCT, preventing GvHD and promoting immune recovery (Pastore et al., 2011). Finally, the γδ T-cell subset, representing less than 6% of T-cells in blood of healthy people, but a much larger fraction in body surfaces exposed to the contact with external material, seems to possess an important role in protection against HCMV disease. Both findings in the mouse model (Ninomiya et al., 2000) and in transplanted patients (Lafarge et al., 2003) indicate that γδ T-cells are involved in the anti-HCMV immune response. Their expansion has been associated with resolution of HCMV infection in HSCT recipients, as confirmed by a recent report (Knight et al., 2010).

Methods for immunological monitoring of HCMV infection

While virologic monitoring is critical for evaluating the severity of HCMV infection, immunologic monitoring, with special reference to the T-cell immune response, is mandatory for drawing prognostic indications. A number of ex-vivo T-cell assays have been developed for measuring the HCMV-specific cellular response. An ideal assay should be able to determine both the specificity of CD4+ and CD8+ T-cells as well as function. Among assays determining HCMV-specific T-cells, the most widely used have been peptide-conjugated MHC class I tetramers. These assays can measure virus-specific CD8+ T-cell frequency, but are epitope-specific and require determination of patients’ HLA type. While some reports have found a correlation between CD8+ T-cells measured by tetramer-based technology and protection (Gratama et al., 2001, 2010), other studies have questioned this conclusion (Crough et al., 2007). Among functional assays, intracellular cytokine staining (ICS) for IFN-γ (or other cytokines), following T-cell stimulation with HCMV peptide mixtures or whole antigen lysate, is not HLA-restricted, and requires, like the tetramer-based method, the availability of a flow cytometer. Both CD4+ and CD8+ can be determined, and several clinical studies have shown a correlation between the presence of HCMV-specific cytokine-producing T-cells and absence of HCMV disease and viremia, or vice versa (Sester et al., 2001, 2005; Shlobin et al., 2006; Kumar et al., 2009; Gerna et al., 2006; Mattes et al., 2008).
The other widely used functional assay is the enzyme-linked immunospot (ELISPOT) assay that determines T-cells producing IFN-γ following stimulation of T-cells with peptide pools or antigen lysates, without differentiating between CD4+ and CD8+ T-cells. IFN-γ released by activated T-cells is captured by a bound antibody and detected by a labelled anti-IFN-γ antibody. ELISPOT is not standardized; however, it seems able to predict HCMV disease (Mattes et al., 2008).

All the above-mentioned assays are also quantitative. Our laboratory developed a novel assay to measure both CD4+ and CD8+ T-cells following stimulation with a number of HCMV antigens (Lozza et al., 2005). This assay was based on differentiation of monocyte-derived autologous dendritic cells (DCs) in 5-7 days in the presence of GM-CSF and IL-4 (Sallusto et al., 1994), followed by DC infection with an endotheliotropic (HU-VEC+) and leukotropic (Leuk+) HCMV strain (Gerna et al., 2005b) and subsequent 18-24 h incubation with PBMC for stimulation (Figure 3).

ICS and flow cytometry analysis allowed the quantification of functional HCMV-specific CD4+ and CD8+ T-cells following stimulation by multiple HCMV antigens. Using this assay, it became evident how clinically relevant it was to quantify T-cells for careful immunologic monitoring of HCMV infection. Previous studies often drew conflicting conclusions, i.e. while one study reported that protection in transplanted patients correlated with IE-1, but not pp65-specific CD8+ T-cells (Bunde et al., 2005), another study concluded that pp65-specific T-cells were also important for protection (Lacey et al., 2006; Crough et al., 2007; Lilleri et al., 2007b).

FIGURE 3 - Flow chart of a novel method for simultaneous quantification of HCMV-specific CD4+ and CD8+ T-cells by using autologous monocyte-derived HCMV-infected immature dendritic cells (Lozza et al., 2005). FACS analysis of CD4+ and CD8+ cytokine-producing HCMV-specific T-cells.
It has become evident that the analysis of a single antigen, as done in most studies, may be insufficient to predict the clinical course of HCMV infection (Sylwester et al., 2005; Lilleri et al., 2007b).

The determination of phenotypic markers in combination with functional assays, such as that developed in our laboratory, has allowed the identification of functionally special T-cell populations at different stages of differentiation. For instance, the reversion of effector memory T-cells to the CD45RA^+ phenotype has been shown to correlate with virus clearance from blood, while a high percentage of these CD45RA^+ CD4^+ T-cells was found to protect against HCMV transmission to the fetus (Lilleri et al., 2008b).

The quantiFERON-CMV assay is a commercially available ELISA-based assay measuring IFN-γ levels released in blood following stimulation with a range of CD8^+ HCMV-specific T-cell epitopes from a number of HCMV viral proteins specific for a wide range of HLA class I alleles (Walker et al., 2007; Westall et al., 2008). This assay is being evaluated in clinical trials (Kumar et al., 2009; Fleming et al., 2010).

**Definition of protective threshold levels of the HCMV-specific T-cell response in clinical trials**

Notwithstanding the different methodological approaches used, it is now agreed that early reconstitution of the T-cell immune response prevents or limits HCMV infection, whereas a delay or lack in the development of the cellular immune response is the pathogenetic basis for severe infection, often leading to a systemic syndrome and/or organ disease in the absence of antiviral treatment. PBMC stimulation with pp65 and/or IE-1 peptide mixtures for rapid monitoring of HCMV-specific T-cell responses in both the immunocompetent and the immunocompromised host was found to underestimate real T-cell responses against HCMV (Lilleri et al., 2007b, 2009c). Due to the partial response to single HCMV proteins or epitopes, the above-mentioned novel assay based on T-cell stimulation by autologous DCs infected with an endotheliotropic HCMV strain was developed in our laboratory to allow a more comprehensive evaluation of the T-cell immune response (Lozza et al., 2005; Gerna et al., 2005b).

Based on results obtained by testing a series of HCMV-seropositive and HCMV-seronegative healthy blood donors, subjects with virus-specific cellular immunity were those with more than 0.4 HCMV-specific CD4^+ and CD8^+ T-cells/µl blood (Lozza et al., 2005).

A prospective clinical study evaluating HCMV-specific CD4^+ and CD8^+ T-cell reconstitution in young allogeneic HSCT recipients found that recovery of both HCMV-specific CD4^+ and CD8^+ T-cell immunity occurred in all 39 HCMV-seropositive (R^+) patients within six months, and in 6/18 (33%) HCMV-seronegative (R) patients within 12 months (Lilleri et al., 2006). In addition, the receiver-operator characteristics (ROC) analysis showed that levels of HCMV-specific CD4^+ less than 1/µl and CD8^+ less than 3 cells/µl (Table 3) are not protective against recurrent infection (Figure 4A). Similar cut-off levels were reported by others (Tormo et al., 2010).

Patients without prompt recovery of HCMV-specific cellular immunity underwent repeated episodes of recurrent infection, as previously reported by others (Krause et al., 1997; Hebart et al., 2002; Boeckh et al., 2003). Another study on HCMV reconstitution in young patients receiving T-cell-depleted allogeneic HSCT showed that, using the same cut-offs as above, immune reconstitution was delayed in these patient groups compared to patients receiving unmanipulated HSCTs (Lilleri et al., 2009). A parallel study on immune control of HCMV infection and HCMV immune reconstitution in adult allogeneic HSCT recipients reported that levels of three CD8^+ and one CD4^+.

**TABLE 3 - Protective threshold levels of HCMV-specific CD4^+ and CD8^+ T-cell responses in transplant recipients.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Protective levels^a of HCMV-specific T-cells/µl</th>
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<tbody>
<tr>
<td></td>
<td>CD4^+</td>
</tr>
<tr>
<td>Immunocompetent</td>
<td>0.4</td>
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<tr>
<td>Immunocompromised</td>
<td>0.4</td>
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<tr>
<td>- SOTR</td>
<td>0.4</td>
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<tr>
<td>- HSCTR</td>
<td>1.0</td>
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^a Upon steroid treatment for rejection episodes in SOTR or GvHD in HSCTR, the reported cut-offs may change.
HCMV-specific T-cells/μl blood (Table 3) had a positive predictive value of about 80% in identifying patients able to control HCMV infection spontaneously (Figure 4B) (Lilleri et al., 2008a). However, corticosteroid treatment may favour HCMV infection even in the presence of the above reported levels of HCMV-specific T-cells.

Figure 5 reports the virological and immunological follow-up of three hematopoietic stem cell transplant recipients as follows: in (A), early development of T-cell response; in (B), a sustained episode of GvHD, despite the presence of both HCMV-specific T-cell subsets; in (C), delayed development of the T-cell response.

A prospective study on SOT recipients monitored HCMV-specific CD4+ and CD8+ T-cells, according to the new methodology using HCMV-infected DCs as a stimulus by dividing patients according to the pattern of T-cell restoration in early (i.e. within 30 days after transplantation) and late responders (Gerna et al., 2006). In late responders, HCMV CD4+ T-cell restoration was significantly delayed with respect to CD8+ T-cell restoration. In addition, higher pre-transplant HCMV-specific T-cell counts predicted earlier immune restoration. Results of the ROC analysis indicated that a cut-off of 0.4 T-cells/μl for both CD4+ and CD8+ T-cells displayed the maximal predictive value. Similar cut-off levels were reported by others (BenMarzouk-Hidalgo et al., 2011).

These conclusions were confirmed by clinical findings showing that the great majority of early responders (85%) underwent self-resolving HCMV infections, whereas the great majority of late responders (76%) had HCMV infections requiring antiviral treatment, even in the presence of HCMV-specific T-cells/μl blood (Table 3).
of specific CD8+ T-cells. In the past, predominant long-term protection conferred by HCMV-specific CD4+ T-cells has been repeatedly reported (Sester et al., 2001, 2002). In addition, it was shown in renal transplant recipients with primary infection that asymptomatic patients had a CD4+ T-cell response preceding the CD8+ T-cell response, whereas in symptomatic patients the specific CD4+ T-cell response was delayed in the presence of a CD8+ T-cell response that was not protective (Gamadia et al., 2003). Thus, HCMV-specific CD4+ T-cells appear to play a major role in long-term protection from HCMV infection/disease.

Figure 6 reports the virological and immunological follow-up of three solid organ transplant recipients showing: in (A) early development, in (C) delayed development of the T-cell response, while in (B) steroid therapy due to a rejection episode caused loss of T-cell immunity.
CONCLUDING REMARKS

From the clinical standpoint, it appears reasonable to conclude that simultaneous immunological and virologic follow-up of individual patients may improve the management of HCMV infection in transplanted patients (Radha et al., 2005; Sester et al., 2005), thereby avoiding treatment of patients with an apparently efficient T-cell immune response. Given the ongoing debate be-

FIGURE 6 - HCMV virological and immunological follow-up of three solid organ transplant recipients. In A, and C, two heart transplant recipients (HTRs) are reported. In B, a lung transplant recipient (LTR) is shown. On the y axis, viral load expressed as copy number/ml whole blood, is reported on the left, while on the right the absolute number of HCMV-specific T cells/µl blood is shown (dotted line indicates predetermined cut-off for both CD4+ and CD8+ T-cells). In A, the maintenance of T-cell immunity during the entire follow-up period prevented HCMV infection. In B, steroid therapy due to a rejection episode caused loss of T-cell immunity and appearance of a peak of HCMV infection in blood, which was treated with ganciclovir (GCV); a high level of HCMV load was also detected in bronchoalveolar lavage (BAL) fluid. However, the subsequent development of HCMV-specific CD4+ (first) and (then) CD8+ T-cell immunity controlled the infection at both sites. In C, lack of T-cell immunity caused the appearance of multiple peaks of HCMV infection in blood, which required two courses of GCV treatment; subsequently, development of HCMV-specific CD8+ (first) and (then) CD4+ T-cell response controlled the infection.
tween prophylaxis and pre-emptive therapy in view of reducing costs of HCMV infection in transplant recipients, the identification of a cohort of patients who are less likely to develop HCMV reactivation may suggest discontinuation of HCMV monitoring. On the other hand, the identification of another cohort of patients who are more likely to develop HCMV reactivation may prompt a prophylactic or therapeutic intervention.

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