Differential kinetics of human cytomegalovirus load and antibody responses in primary infection of the immunocompetent and immunocompromised host

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The comparative long-term kinetics of human cytomegalovirus (HCMV) load and HCMV-specific antibody responses in the immunocompetent and immunocompromised solid-organ transplanted host during primary HCMV infection was investigated. In total, 40 immunocompetent subjects and 17 transplanted patients were examined for viral load as well as for IgG antibody responses to HCMV glycoproteins gH/gL/pUL128L, gH/gL and gB, and neutralizing antibodies in ARPE-19 epithelial cells and human fibroblasts. In parallel, the CD4+ and CD8+ HCMV-specific T-cell responses were determined by cytokine flow cytometry. Transplanted patients reached significantly higher viral DNA peaks, which persisted longer than in immunocompetent subjects. The ELISA-IgG responses to the pentamer, gH/gL and gB were significantly higher in primary infections of the immunocompetent until six months after onset, with the two antibody levels then overlapping from six to 12 months. Antibody levels neutralizing infection of epithelial cells were significantly higher in transplanted patients after six months, persisting for up to a year after transplantation. This trend was not observed for antibodies neutralizing infection of human fibroblasts, which showed higher titres in the immunocompetent host for the entire one-year follow-up. In conclusion, in immunocompromised patients the viral load peak was much higher, while the neutralizing antibody response exceeded that detected in the immunocompetent host starting six months after onset of follow-up, often concomitantly with a lack of specific CD4+ T cells. In this setting, the elevated antibody response occurred in the presence of differentiated follicular helper T cells in the blood, which decreased in number as did antibody titres upon reappearance of HCMV-specific CD4+ T cells.

INTRODUCTION

In the last few years, significant progress has been made in the diagnosis of human cytomegalovirus (HCMV) infections both for viral DNA quantification by real-time PCR (recently standardized with reference to the WHO International Standard; Furione et al., 2012), and antibody response determination to the HCMV envelope glycoprotein complexes gH/gL/pUL128L (the pentameric complex), gH/gL and gB as well as neutralizing antibodies (Genini et al., 2011; Lilleri et al., 2012, 2013).

In 2004, in collaboration with the group of U. Koszinowski (Munchen, Germany), it was documented for the first time that HCMV UL128-131 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes (Hahn et al., 2004). Subsequently, it was shown that the UL128, UL130 and UL131 locus (thus referred to as UL128L) gene products formed a pentameric complex with gH/gL (gH/gL/pUL128/pUL130/pUL131), which was required for infection of both endothelial and epithelial cells (Wang & Shenk, 2005; Ryckman et al., 2008; Revello & Gerna, 2010). Multiple antigenic sites of the pentamer have been shown to elicit very potent neutralizing mAbs with high frequency, unlike gB which induces a predominant number of binding but non-neutralizing mAbs (Macagno...
et al., 2010; Pötzsch et al., 2011; Fouts et al., 2012). In addition, gB-based vaccines have shown only partial protection against vertical HCMV transmission in pregnant women and against HCMV infection in transplanted patients (Pass et al., 2009; Griffiths et al., 2011). Furthermore, neutralizing antibodies have been shown to display far higher titres when measured in epithelial rather than fibroblast cells (Cui et al., 2008; Gerna et al., 2008).

During follow-up of a series of primary HCMV infections in the immunocompetent (pregnant and non-pregnant) host and the solid-organ immunocompromised transplanted patient (HCMV-seronegative patients receiving organs from seropositive donors, thus developing primary HCMV infections), differential features in the kinetics of viral load and antibody responses in the two clinical conditions were observed. These features included: (i) viral DNA appearance in the blood of the immunocompetent subject which elicited an immediate antibody response, whereas in the immunocompromised patient the antibody response was delayed by several months; (ii) the earlier antibody response in immunocompetent subjects reached a higher level with respect to immunocompromised patients within three months or more (according to different assays) after onset of infection; afterwards, the antibody response in immunocompromised patients appeared to overlap with that of immunocompetent subjects; and (iii) very high antibody levels in the immunocompromised host reaching titres of $1:10^5$–$1:10^6$ were often observed in the absence of HCMV-specific CD4$^+$ T cells. The current study was therefore conducted to verify the consistency and reproducibility of these preliminary observations.

The main objective of this study was to comparatively investigate the kinetics of HCMV load and different types of antibody response to HCMV glycoprotein complexes (gB, gH/gL/pUL128L and gH/gL) and neutralizing antibodies [determined in both ARPE-19 epithelial cells and human embryonic lung fibroblast (HELF) cells] in the immunocompetent subject and the immunocompromised solid-organ transplanted patient. Both parameters were evaluated with reference to the development of HCMV-specific T-cell immune responses, which behaved very differently in the two patient populations examined (Lozza et al., 2005; Lilleri et al., 2009).

**RESULTS**

**Clinical characteristics and management of HCMV infection in two patient populations**

All transplanted patients ($n=17$) received one or more courses of pre-emptive therapy with ganciclovir or valganciclovir for an overall median duration of 78 (17–179) days after reaching a level of 300 000 DNA copies ml$^{-1}$ in the blood (or in the presence of an organ localization, shown by virus detection in organ biopsy by PCR). In all transplanted patients, HCMV disappeared from the blood after antiviral therapy. Immunocompetent subjects were either asymptomatic ($n=10$ pregnant women), symptomatic ($n=13$; 3 pregnant women and 10 non-pregnant subjects) or paucisymptomatic ($n=17$ pregnant women). The subjects did not receive antiviral treatment.

**Fig. 1.** Human cytomegalovirus (HCMV) DNA (a) appearance, (b) peak and (c) persistence in blood of 40 immunocompetent subjects and 17 solid-organ transplanted patients. Median values with interquartile ranges are shown. Mann–Whitney U test (a, b) and log-rank test (c) were adopted for statistical analysis.
HCMV load in primary HCMV infections

The onset of HCMV infection was determined on the basis of criteria reported in the Methods for immunocompetent (pregnant and non-pregnant) subjects, and with reference to the day of transplantation for transplanted patients. Within the limits of these criteria, the median first viral DNA detection (Fig. 1a) occurred significantly earlier (\( P < 0.03 \)) in immunocompetent subjects (22 days, range 2–68, days) than in transplanted patients (29, range 19–52, days). In addition, the median peak DNA level [copies (ml whole blood)\(^{-1}\)] was significantly higher (\( P < 0.01 \)) in transplanted patients (189 800, range 5000–774 000 copies ml\(^{-1}\)) than in immunocompetent individuals (56, range 25–6762) (Fig. 1b). Finally, the median persistence of viral DNA (Fig. 1c, Kaplan–Meier curves) in the blood of transplanted patients (235, range 120–670 days) was significantly higher (\( P < 0.01 \), log-rank test) than in immunocompetent subjects (85, range 33–194, days).

Kinetics of HCMV load in relation to antibody and T-cell responses

First viral DNA detection occurred earlier in immunocompetent patients compared with transplanted patients. In addition, the antibody response occurred significantly (\( P < 0.01 \)) earlier in the immunocompetent patients independently of the antibody assay performed (Fig. 2a). Similarly, the HCMV-specific CD4\(^+\) and CD8\(^+\) T-cell responses were delayed in

\[ \text{DNA NT-ARPE} \]
\[ \text{NT-HELF} \]
\[ \text{Pent-lgG} \]
\[ \text{gHgL-lgG} \]
\[ \text{gB-lgG} \]
\[ \text{spec CD4}^+ \]
\[ \text{spec CD8}^+ \]

\[ \text{Time after onset of infection/transplant (days)} \]

**Fig. 2.** (a) Time (days) to first antibody detection after transplantation in immunocompromised transplanted patients and after presumed onset of infection in immunocompetent subjects. (b) Time (days) required to reach peak antibody titre after reaching the DNA peak in the transplanted and the immunocompetent patient. Median values with interquartile ranges are shown. Mann–Whitney U test was adopted for statistical analysis.
transplant recipients with respect to the immunocompetent host. However, the CD8⁺ T-cell response appeared at a time comparable to that of the antibody response, whereas the CD4⁺ T-cell response appeared later (Fig. 2a).

In transplanted patients the median viral DNA peak (Fig. 2b) was reached significantly later (45 days, range 27–132 vs 22 days, range 5–48), and the median peak of the antibody response was also reached significantly later with four of the five types of antibody determined, with the exception of the ELISA IgG antibodies to the pentamer whose levels were reached in the two populations at a comparable time. Similarly, the T-cell response peaks (for both HCMV-specific CD4⁺ and CD8⁺ T cells) were reached later in transplant recipients (Fig. 2b).

**Kinetics of the ELISA IgG antibody response to the three major HCMV glycoprotein complexes in the immunocompetent and the immunocompromised host during primary HCMV infection**

The kinetics of the IgG antibody response to the pentamer gH/gL/pUL128L, the dimer gH/gL and gB glycoprotein complexes in the immunocompetent and the immunocompromised patient were significantly different \( P<0.0001 \), as shown in Figs 3a, 4a and 5a, respectively. When considered individually, the antibody responses to the three glycoprotein complexes were significantly higher in the immunocompetent host until 180 days after onset. Subsequently, at days 181–360, the three types of antibody response observed in both the immunocompetent and the immunocompromised host substantially overlapped until 12 months (Figs 3b, 4b and 5b). However, a number of serum samples taken at both time intervals (91–180 and 181–360 days post-transplant) were 4–16-fold higher in titre than the relevant highest antibody titres of the immunocompetent subjects. This was the case for antibody titres to all three major glycoprotein complexes, although the difference in antibody titre between the two populations studied was less pronounced for antibodies to gB (Figs 3b, 4b and 5b).

**Kinetics of the neutralizing antibody response as determined in ARPE-19 epithelial cells and in human embryonic lung fibroblasts (HELF) in the immunocompetent and the immunocompromised host**

The kinetics of the neutralizing antibody response determined in ARPE-19 epithelial cells was significantly different \( P<0.0001 \) in the two patient populations (Fig. 6a). Similarly, the kinetics of the antibody response in the two patient populations was significantly different when the neutralizing activity was measured in HELF cells (Fig. S1, available in the online Supplementary Material). The neutralizing activity in ARPE-19 cells was higher in the immunocompetent patients until 90 days after onset of follow-up, with activity then increasing in the immunocompromised patient after 180 days (Fig. 6a). The above reported finding of a markedly higher titre to the three major glycoprotein complexes in the immunocompetent as compared with the immunocompromised host until 180 days follow-up was partially confirmed for antibodies neutralizing infection of ARPE-19 epithelial cells, but only up to 90 days after transplantation. Subsequently, at 91–180 days the titres were overlapping, while at 181–360 days after transplantation, neutralizing antibody titres were significantly higher in transplanted patients (Fig. 6b). The great majority of transplanted patients showed neutralizing antibody titres 4–16-fold higher as compared with immunocompetent subjects (Fig. 6a).
Kinetics of precursor CCR7lo PD-1hi CXCR5+ CD4+ follicular helper T cells (Tfh) in immunocompetent and immunosuppressed patients

Antibody responses to primary HCMV infection in immunocompetent individuals started early concomitantly with the appearance of differentiated Tfh cells in blood, an aliquot of which (approximately 30%) expressed ICOS. Once the antibody titre reached the plateau, both precursor Tfh and ICOS+ Tfh cells decreased in number (Fig. 7a).

In Fig. 7b, the case of a heart-transplanted patient with primary HCMV infection is reported. In this patient, in the absence of HCMV-specific CD4+ T cells, IgG and neutralizing antibody responses appeared later than those currently observed in immunocompetent individuals, yet concomitantly with the differentiation of circulating Tfh precursor CCR7lo PD-1hi CXCR5+ ICOS+ T cells. In Table 1, the kinetics of blood Tfh cells and different types of antibody response are reported for a heart-transplanted patient, showing the neutralizing antibody increase concomitantly with the increase in ICOS+ Tfh cells, and the neutralizing antibody titre decrease upon ICOS+ Tfh-cell decrease, following reconstitution of HCMV-specific CD4+ T cells.

DISCUSSION

The main objective of this study was to investigate the differential kinetics of HCMV load, and IgG and
neutralizing antibody responses in the immunocompetent and the immunocompromised host affected by primary HCMV infection. The IgG antibody responses to the glycoprotein complexes representing the pentamer (gH/gL/pUL128L), the dimer gH/gL (Hahn et al., 2004; Ryckman et al., 2008) and gB were investigated. In addition, the neutralizing antibody response was determined in parallel by inoculating virus-serum mixtures onto ARPE-19 epithelial cells and HELF cells. The double neutralization assay was based on the recent discovery that HCMV requires the pentamer to infect epithelial/endothelial/dendritic cells (Gerna et al., 2005; Ryckman et al., 2008), while infection of HELF cells requires the presence of gH/gL/gO on the HCMV envelope (Zhou et al., 2013). It has been demonstrated and repeatedly confirmed that neutralizing titres determined in epithelial cells are far higher than those obtained in HELF cells (Cui et al., 2008; Gerna et al., 2008).

The HCMV viral load measured in whole blood (expression of the enhanced replication rate occurring in transplanted patients still lacking immune control of viral infection) was markedly higher in immunocompromised patients due to the delayed development of the T cell-mediated immune response caused by the immunosuppressive therapy. Conversely, in the immunocompetent individual, the rapid mounting of the T-cell response aided B-cell antibody production and allowed prompt control of viral replication (Lilleri et al., 2009), thus reducing antigen exposure and prolonged stimulation of the antibody response. In contrast, the antibody response was delayed and poorly developed in transplanted patients until 90 days after transplantation. At 181–360 days post-transplantation, the antibody response in transplanted patients increased, first overlapping and occasionally exceeding that of immunocompetent subjects, as was the case for neutralizing antibodies preventing infection of epithelial cells. This process occurred mostly in the absence of HCMV-specific CD4+ T cells in peripheral blood as detected by cytokine flow cytometry. Under these conditions, precursor CD4+ Tfh cells circulating in peripheral blood (PD-1hi CCR7lo CXCR5+ ICOS+) increased before the antibody response in these patients, thus suggesting their CD4+ T-cell helper function for B cells. In several of the transplanted patients examined in this study the antibody response reached titres not previously observed in immunocompetent individuals, i.e. titres ranging from 1:1 × 10^5 to 1:1 × 10^6 for both HCMV-specific ELISA IgG antibodies to viral glycoprotein complexes, and neutralizing antibodies preventing infection of epithelial cells. A likely explanation for the higher antibody titres observed in these patients at later time points may reside in the prolonged viraemia, reflecting an extended period of virus replication and, therefore, antigenic stimulation. High titres persisted for several months until the reappearance of specific CD4+ T cells when, due to virus replication control, titres started dropping concomitantly with a decrease in Tfh cells until they reached levels comparable to those of immunocompetent subjects in most cases. This mechanism appeared to be responsible for the delayed and enhanced antibody response of transplanted patients during primary HCMV infection.

It has recently been demonstrated in humans and mice that circulating precursor CCR7lo PD-1hi CXCR5+ CD4+ T cells are indicative of Tfh-cell activity, which promotes effective antibody responses upon antigen reexposure (He et al., 2013). These precursor cells may rapidly differentiate into mature Tfh cells after encountering HCMV to promote a potent antibody response. In both immunocompetent and immunocompromised patients, the kinetics of CCR7lo PD-1hi CXCR5+ T cells somewhat paralleled the profile of ICOS+ T cells, thus suggesting that this co-stimulator is required to keep precursor Tfh cells at an efficient level to become mature Tfh cells to promote the

Fig. 6. (a) Kinetics of the median neutralizing activity preventing infection of ARPE-19 epithelial cells by the HCMV isolate VR1814 in the two populations during one-year follow-up: non-linear regression curves are shown and compared to the extra sum-of-square F test. (b) Neutralizing titres at different time intervals after onset of infection: median values with interquartile ranges are shown. Mann–Whitney U test was adopted for statistical analysis. ns, non-significant.
antibody response. In this study on primary HCMV infections, the long delay observed in transplanted patients between virus appearance in blood and antibody response in the absence of HCMV-specific CD4$^+$ T cells is most likely required for the differentiation of CCR7$^{hi}$ PD-1$^{lo}$ ICOS$^+$ T cells outside germinal centres. Following exposure to the antigen, these precursor T cells rapidly differentiate into mature Tfh cells to promote an effective antibody response.

In conclusion, in transplant recipients undergoing primary HCMV infection the antibody response is delayed with respect to that in immunocompetent subjects. However, transplanted patients develop a high antibody response (especially antibodies neutralizing HCMV infection of epithelial cells) concomitantly with or slightly earlier than HCMV-specific CD8$^+$ T-cell appearance, but before reconstitution of HCMV-specific CD4$^+$ T cells. The significance of this high antibody response and the interaction of humoral and cell-mediated immunity in protection from HCMV infection require further clarification in future studies. However, the delayed appearance of high antibody titres in transplant recipients, in whom primary HCMV infection is often severe, may suggest that vaccines capable of inducing high antibody titres (Griffiths et al., 2011), or passively transferred monoclonal antibodies, might have a role in protecting these patients.

**METHODS**

**Immunocompetent and immunocompromised patient populations.** The immunocompetent subject population affected by primary HCMV infection comprised 30 pregnant women (aged 20–40 years) and 10 non-pregnant subjects in the same age range, including 7 males and 3 females. The immunocompromised transplanted patient population comprised 17 D$^+/R^2$ solid-organ transplant recipients (8 kidney, 7 heart, 1 single lung and 1 heart-lung) with primary HCMV infection, with a median age of 52 (range 13–75) years.

**Diagnosis of primary HCMV infection.** In the group of immunocompetent subjects with primary HCMV infection, diagnosis was ascertained along two major lines (Revello & Gerna, 2002; Revello et al., 2011). In 10 non-pregnant subjects, diagnosis was based on the presence of HCMV-related clinical symptoms that presumably indicated onset of infection (high fever lasting 2–4 weeks in the absence of other identifiable causes and in association with one of the following symptoms/signs: lymphadenopathy, sore throat,
Table 1. Kinetics of total and HCMV-specific CD4⁺, CD8⁺ and follicular helper T cells in a heart-transplanted patient showing a sharp increase in Nt antibody titres at 189 days and their decrease at 350 days after transplantation upon increase/decrease in precursor and differentiated Tfh cells

<table>
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<tr>
<th>Days after transplant</th>
<th>HCMV DNA (copies ml⁻¹)</th>
<th>CD4⁺/µl⁻¹</th>
<th>CD8⁺/µl⁻¹</th>
<th>CD4⁺/HCMV-spec/µl⁻¹</th>
<th>CD8⁺/HCMV-spec/µl⁻¹</th>
<th>Antibody titre</th>
<th>CXCR5⁺ PD1⁺ / % of CD4⁺</th>
<th>ICOS⁺ (% of CD4⁺/CXCR5⁺/PD-1⁺/CCR7⁻)</th>
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<tbody>
<tr>
<td></td>
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<td>ARPE-19 Nt</td>
<td>HELF-Nt</td>
<td>Pentamer IgG</td>
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<td>1280</td>
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Patient number: SOTR-054; age: 33 years; transplanted organ: heart; HCMV serology: D⁺/R⁻. Nt, neutralization test; SOTR, solid-organ transplant recipient.

*Precursor Tfh cells.
†Differentiated Tfh cells.
arthromyalgias, headache, elevated liver enzymes levels, lymphocytosis). Virological and serological tests then confirmed a suspected diagnosis. In the group of seronegative women entering pregnancy, only 3/30 women displayed overt clinical symptoms, thus raising the suspect of a primary infection, while in 17/30 women mild or non-specific symptoms prompted the performance of viral tests. In the remaining 10 cases, the detection of primary infection was by chance during routine pregnancy follow-up (asymptomatic primary infection). In the latter cases, the date of infection onset was determined in the middle interval between the last negative and the first positive serological result. In the group of 17 immunocompromised patients, the onset of infection was dated with the day of transplantation.

**Viral and serological assays.** Conventional diagnostic assays performed in this study for the diagnosis of primary HCMV infection were ELISAs (DiaSorin) for determination of HCMV-specific IgG and IgM antibody by ETI-CYTOK-G and ETI-CYTOK-M, respectively, according to the manufacturer’s instructions. In addition, the HCMV-specific avidity index (AI) was measured with an in-house-developed ELISA, as reported (Revello et al., 2010; Furione et al., 2013). When clinical symptoms suggested the onset of primary infection, diagnosis was later confirmed by serological and viral assays, whereas in paucisymptomatic/asymptomatic women, primary infection was suspected based on the presence of at least two of the four following criteria: IgG seroconversion, presence of IgM antibody, low IgG AI and the presence of viral DNA in blood (DNAemia) (Revello et al., 2011). Infection onset timing was determined in symptomatic infections based on both symptoms/signs and viral assays, whereas in asymptomatic infections, it was based on IgG and IgM antibody kinetics in association with low AI. The unconventional assays were ELISAs for determination of IgG antibodies to the pentamer (gH/gl/UL128L), gh/gl and g8, as reported previously (Lilleri et al., 2012). The three glycoprotein complexes were captured in 96-well polystyrene microplates (Corning) with an in-house-developed murine anti-gH mAb (mH1P73), or an anti-g8 mAb (HCMV 37, Abcam). Net OD was determined by subtracting OD of serum incubated in the absence of antigen from OD of serum incubated in the presence of antigen.

Neutralizing assays were performed in 96-well microtitre plates by inoculating virus-serum dilution mixtures in duplicate onto monolayers of epithelial (ARPE-19) or fibroblast (HELF) cells, as reported previously (Lilleri et al., 2013). HCMV isolate VR1814 was used for assays in ARPE-19 cells, while laboratory strain AD169 was used for assays in HELF cells. The differential neutralizing activity of human sera when tested in ARPE-19 or HELF cells was previously reported (Gerna et al., 2008).

Quantitative DNAemia results were expressed as HCMV DNA copies (ml blood) (Gerna et al., 2006). When requested, results were also translated into IU ml⁻¹ with reference to the WHO International HCMV DNA Standard (Furione et al., 2012). Viral DNA was quantified weekly in transplant recipients, and monthly in immunocompetent individuals.

**T-cell assays.** HCMV-specific T cells were determined ex vivo (Lozza et al., 2005) following stimulation with autologous monocyte-derived (Sallusto & Lanzavecchia, 1994), HCMV-infected (VR1814) immature dendritic cells. Peripheral blood mononuclear cells (PBMCs) were tested for frequencies of IFN-γ-producing CD4⁺ and CD8⁺ T cells by cytokine flow cytometry (Lilleri et al., 2009). PBMCs were washed, then incubated with Live/Dead Fixable Violet Dye (Invitrogen) and V500 (clone RPA-T8)-conjugated anti-CD8 (BD Biosciences) for cell surface staining. Cells were then washed and permeabilized (FACS Permeabilizing Solution, BD Biosciences) and incubated with an intracellular mix of the following mAbs: PerCP-Cy5.5 (clone UCHT1)-conjugated anti-CD3, APC-Cy7 (clone RPA-T4)-conjugated anti-CD4 and PE-Cy7 (clone B27)-conjugated anti-IFN-γ (BD Biosciences). Finally, cells were washed, resuspended in 1% (v/v) paraformaldehyde and analysed with a FACSCanto II Flow cytometer (BD Biosciences). As a routine, 1–2 × 10⁶ viable lymphocytes were collected, and at least 2.5 × 10⁴ CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells were analysed. The frequency of CD4⁺ and CD8⁺ T cells producing IFN-γ was calculated by subtracting the value of the sample incubated with mock-infected DCs from the test value. Absolute CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells were determined on whole blood samples with a direct immunofluorescence flow cytometry method (TruCOUNT tubes, BD Biosciences). 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.

Cell surface staining of Th cells was performed by incubating thawed PBMCs with a mix of the following mAbs: PECy7 (clone EH12.1)-conjugated anti-CD279 (PD-1), BV-510 (clone R8B2)-conjugated anti-CD28, APC-Cy7 (clone RPA-T4)-conjugated anti-CD4 (BD Biosciences), APC (clone IA-3)-conjugated anti-CD278 (ICOS) (eBiosciences) and BV421 (clone G043H7)-conjugated anti-CD197 (CCR7) (BioLegend). Finally, cells were washed, resuspended in 1% (v/v) paraformaldehyde and analysed.

**Statistical analysis.** Nonlinear regression models were used to express the kinetics of different serological parameters (IgG antibodies to gH/gl/UL128L, gh/gl and g8; neutralizing antibodies titres in ARPE-19 and HELF cells). The curves were compared by the log-rank test. Time to HCMV disappearance was calculated with Kaplan–Meier curves, which were compared by the log-rank test.

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**REFERENCES**


Fouts, A. E., Chan, P., Stephan, J. P., Vandlen, R. & Feierbach, B. (2012). Antibodies against the gH/gl/UL128L, gh/gl and g8 neutralizing antibodies titres in ARPE-19 and HELF cells. The curves were compared by the extra-sum-of-square F test (Prism 5.0 Software; GraphPad). Medians of the glycoprotein complex-specific and neutralizing antibody titres at different time intervals as well as HCMV load of different patient group populations were compared by the Mann–Whitney U-test. Time to HCMV disappearance was calculated with Kaplan–Meier curves, which were compared by the log-rank test.

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