Follicular helper T-cells and virus-specific antibody response in primary and reactivated human cytomegalovirus infections of the immunocompetent and immunocompromised transplant patients

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Analysis of human cytomegalovirus (HCMV) primary infection in immunocompetent (n=40) and immunocompromised transplant patients (n=20) revealed that the median peak antibody titre neutralizing infection of epithelial cells was 16-fold higher in immunocompromised patients. The mechanism of this finding was investigated by measuring: (i) HCMV DNAemia; (ii) HCMV neutralizing antibodies; (iii) ELISA IgG antibody titre to HCMV glycoprotein complexes gHgLpUL128L, gHgLgO and gB; and (iv) HCMV-specific (IFN-γ+) CD4+ and CD8+ T-cells. Circulating CXCR5+ CD4+ (memory T follicular helper – Tfh-cells) were identified as activated Tfh cells (ICOS+PD-1++CCR7lo) and quiescent cells. In the early stages of primary infection, activated Tfh cells increased in number. Concomitantly, both neutralizing and IgG antibodies to HCMV glycoproteins reached a peak, followed by a plateau. A stop in antibody rise occurred upon appearance of HCMV-specific CD4+ T-cells, HCMV clearance and progressive reduction in activated Tfh cells. The main differences between healthy and transplant patients were that the latter had a delayed DNA peak, a much higher DNA load and delayed activated Tfh cells and antibody peaks. Similar events were observed in clinically severe HCMV reactivations of transplant patients. A preliminary analysis of the specificity of the activated Tfh cell response to viral proteins showed a major response to the pentamer gHgLpUL128L and gB. In conclusion, in the absence of T-cell immunity, one of the first lines of defence, during primary infection, is conferred by antibodies produced through the interaction of Tfh cells and B-cells of germinal centres, resulting in differentiation of B-cells into antibody producing plasma cells.

INTRODUCTION

Follicular helper T-cells (Tfh) represent a subset of CD4+ T-cells that provide help to B-cells for the production of high-affinity antibodies and generation of memory B-cells and long-lived plasma cells. Tfh cells are present within and at the border of germinal centres (GC) in the secondary lymphoid organs. Their memory compartment circulates in peripheral blood (Chevalier et al., 2011; Morita et al., 2011). Tfh cells were first described in human blood and tonsils as CD4+ T-cells expressing CXCR5, a chemokine receptor that is required for homing to the B-cell follicles (Forster et al., 1994; Breitfeld et al., 2000; Scharf et al., 2000; Kim et al., 2001). However, unlike GC Tfh cells, blood memory Tfh cells (Schmitt et al., 2014) do not
express Bcl-6 protein, a TFH-associated transcription factor, thus indicating that Bcl-6 is dispensable for maintenance of these cells (Bentebibel et al., 2013; He et al., 2013).

Human blood memory TFH cells include several subsets with unique phenotypic and functional properties (Schmitt et al., 2014). Although a consensus has not yet been reached among laboratories, a recent proposal has been put forward for the identification of different subsets based on three sets of parameters: (i) presence/absence of the chemokine receptors CXCR3 and CCR6; (ii) presence/absence of the immuno-regulatory molecule PD-1 and the chemokine receptor CCR7; and (iii) presence/absence of the co-stimulatory molecule ICOS (Ueno et al., 2015).

The first set of parameters identifies three major subsets: CXCR3+CCR6- identifies blood memory TFH1 cells resembling Tfh1 cells; (ii) CXCR3+CCR6- identifies blood memory Tfh2 cells resembling Tfh2 cells; and (iii) CXCR3+CCR6+ identifies blood memory Tfh17 resembling Tfh17 cells. Blood memory Tfh2 and blood memory Tfh17 cells promote naïve B-cells to produce immunoglobulins and to switch isotype. In contrast, blood memory Tfh1 cells lack the capacity to help naïve B-cells. Thus, only blood memory Tfh2 and Tfh17 are efficient B-cell helper cells (Morita et al., 2011; Locci et al., 2013; Boswell et al., 2014).

The second (PD-1 and CCR7) and third (ICOS) sets of parameters define developmentally and functionally distinct subpopulations within the three major subsets: one activated (ICOS-PD-1+CCR7lo) and two quiescent subsets, (ICOS-PD-1+CCR7hi and ICOS-PD-1-CCR7hi) (He et al., 2013; Locci et al., 2013; Boswell et al., 2014). Finally, blood memory Tfh1 cells may help memory B-cell differentiation into plasma cells via secretion of IL-21 and IL-10 but only when they become ICOS-PD-1+CCR7lo activated cells (Bentebibel et al., 2013).

Recently, we had the opportunity to investigate the serum antibody response to primary human cytomegalovirus (HCMV) infection in the immunocompetent (Ic) and the immunocompromised transplant (Tx) patient. Preliminary observations showed that both neutralizing and ELISA IgG antibody titres to the HCMV glycoprotein pentamer gB were higher in the Tx patient (Gerna et al., 2015). However, it has recently been shown by our group that antibodies to the pentamer are mostly highly neutralizing, whereas antibodies to gB are mostly non-neutralizing (Kabanova et al., 2014).

To investigate the basis for such a differential antibody response to HCMV, we correlated the kinetics of the antibody response to the kinetics of activated Tfh cells in two groups of patients with primary HCMV infection – Ic and immunocompromised solid-organ Tx patients. An additional group of Tx patients with reactivated HCMV infection was tested for comparison with the group of Tx patients with primary HCMV infection. Results showed that a protracted presence of activated Tfh cells in the absence of HCMV-specific CD4+ T-cells and the presence of HCMV DNAemia, might be related to the exaggerated antibody response observed in Tx patients in comparison with Ic patients.

RESULTS

Distribution of circulating Tfh, activated Tfh and activated Tfh cell subsets in HCMV-seropositive and -seronegative healthy adults

The frequency of CD4+ CXCR3+ T-cells (Tfh1), activated Tfh1 cells and Tfh17 cell subsets in peripheral blood was preliminarily determined in a group of 10 HCMV-seropositive and 10 HCMV-seronegative healthy subjects. It was found that Tfh1 cells were present at a frequency of 8.3 % (3.6–10.2 %) in HCMV-seropositive and 5.3 % (2.0–9.6 %) in HCMV-seronegative subjects, while the median frequency of activated Tfh1 cells was 1.4 % (0.4–5.7 %) in HCMV-seropositive and 1.1 % (0.6–2.0 %) in HCMV-seronegative subjects. The frequency of activated Tfh17 cell subsets was in the range of 30–40 % for activated Tfh17 cells and 10–20 % for activated Tfh17 cells in both HCMV-seropositive and HCMV-seronegative subjects, while activated Tfh12 cell frequency was 25.3 % (22.6–34.5 %) in HCMV-seropositive subjects and 46.3 % (24.0–54.6 %) in HCMV-seronegative subjects (data not reported). Taken together, these findings document that there is no difference for activated Tfh1 and Tfh17 between HCMV-seronegative and -seropositive subjects, while this difference may exist for Tfh12 cell frequency.

Differential virologic, immunologic and antibody response parameters in Ic and Tx patients during primary HCMV infection

As reported in Table 1, both Ic and Tx patients were followed-up for a median time of 634 (311–894) and 420 (203–811) days, respectively. As for viral DNA peak, the first peak detection occurred at a median time of 27 days after onset of infection in the Ic patients, and 41 days after transplantation in Tx patients, while the highest DNA peak consisted of a median value of 1.9 (0.6–2.4) x 10^6 /ml in Ic patients; whereas, in Tx patients the same parameters were reached much later, i.e. 56 (19–149) and 269 (59–455) days after transplantation in Tx patients, respectively.

Neutralizing (Nt) antibodies in epithelial cells appeared and reached a plateau at a median time of 24 (13–68) and 218 (28–797) days, respectively, after infection onset in the Ic patients; whereas, in Tx patients the same parameters were reached much later, i.e. 56 (19–149) and 269 (59–455) days after transplantation in Tx patients.
Table 1. Virologic, immunologic and antibody titre parameters in immunocompetent and immunocompromised transplant patients with primary HCMV infection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Immunocompetent (Ic)</th>
<th>Transplant (Tx)</th>
<th>Ratio (Tx/Ic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median years (range)</td>
<td>34 (22–63)</td>
<td>52 (13–75)</td>
<td>1.53</td>
</tr>
<tr>
<td>Follow-up, median days§‡ (range)</td>
<td>634 (311–894)</td>
<td>420 (203–811)</td>
<td>0.66</td>
</tr>
<tr>
<td>1st DNA peak, median days (range)</td>
<td>27 (13–88)</td>
<td>41 (27–65)</td>
<td>1.52</td>
</tr>
<tr>
<td>DNA viral load, median copies ml⁻¹ (range)</td>
<td>100 (24–6762)</td>
<td>183 (250 (16 600–2 750 800)</td>
<td>1.832</td>
</tr>
<tr>
<td>HCMV-spec CD4⁺, median days (range)</td>
<td>43 (13–94)</td>
<td>156 (28–330)</td>
<td>3.63</td>
</tr>
<tr>
<td>HCMV-spec CD8⁺, median days (range)</td>
<td>30 (13–94)</td>
<td>94 (28–177)</td>
<td>3.13</td>
</tr>
<tr>
<td>Nt Ab-ARPE appearance, median days (range)</td>
<td>24 (13–68)</td>
<td>56 (19–149)</td>
<td>2.33</td>
</tr>
<tr>
<td>Nt Ab-ARPE peak, median days (range)</td>
<td>218 (28–797)</td>
<td>269 (59–455)</td>
<td>1.23</td>
</tr>
<tr>
<td>Pentamer-IgG Ab appearance, median days (range)</td>
<td>10 240 (640–40 960)</td>
<td>163 840 (10 240–327 680)</td>
<td>16</td>
</tr>
<tr>
<td>Pentamer-IgG Ab peak, median days (range)</td>
<td>29 (10–68)</td>
<td>87 (44–223)</td>
<td>3.00</td>
</tr>
<tr>
<td>Pentamer-IgG Ab peak, median days (range)</td>
<td>218 (55–604)</td>
<td>340 (113–744)</td>
<td>1.56</td>
</tr>
<tr>
<td>gHgLG-IgG Ab peak (range)</td>
<td>12 800 (1 600–51 200)</td>
<td>51 200 (6 400–409 600)</td>
<td>4</td>
</tr>
<tr>
<td>gHgLG-IgG Ab peak (range)</td>
<td>6 400 (1 600–102 400)</td>
<td>25 600 (3 200–409 600)</td>
<td>4</td>
</tr>
<tr>
<td>gB-IgG Ab peak (range)</td>
<td>51 200 (12 800–409 600)</td>
<td>102 400 (6 400–819 200)</td>
<td>2</td>
</tr>
</tbody>
</table>

* n=40 (36 pregnant women, 4 non-pregnant patients).
† n=20 (10 kidney, 8 heart, 1 lung, 1 heart and lung).
‡ For immunocompetent patients, median days = days after infection onset.
§ For transplant patients, median days = days after transplant.

post-transplant. Similarly, IgG antibodies to the pentamer appeared and peaked at a median of 29 (10–68) and 218 (55–604) days after infection onset in the Ic patients, and 87 (44–223) and 340 (113–744) days post-transplant in Tx patients, respectively. Overall, this data documents a presumably earlier appearance of Nt antibody and peak in Ic subjects.

**Nt antibody and IgG antibody to viral glycoprotein complexes**

On the whole, the median peak ARPE-19-neutralizing antibody titre was 10 240 in Ic patients, and 163 840 in Tx patients, with a median titre ratio Tx/Ic of 16. On the other hand, the median antibody titre ratios (Tx/Ic) were 4 for peak IgG antibody to the pentamer, 4 for peak IgG antibody to gHgLgO, and 2 for peak IgG antibody to gB (Table 1). Thus, the highest median peak antibody titre ratio (16) is relevant to antibodies neutralizing infection of epithelial cells.

**Distribution of peak antibody titres and selection of cut-offs for differentiating higher from lower titres in the two patient groups according to different serological assays**

As shown in Fig. 1a and Fig. S1a (available in the online Supplementary Material), Nt antibodies preventing infection of epithelial/endothelial cells were distributed below the cut-off titre of 40 960 for more than 90% of Ic patients, and above this cut-off for more than 90% of Tx patients. A similar trend was observed for IgG antibodies to the pentamer gHgLpUL128L and gHgLgO for Ic patients, whereas peak titres observed with both ELISA assays were much more scattered for Tx patients (Fig. 1b and Fig. S1b, and Fig. 1c and Fig. S1c). As for IgG antibodies to gB, the highest peak titres were observed in both study populations (Fig. 1d and Fig. S1d). However, more than 90% of Tx patients had peak titres to gB above 51 200, while the number of Ic patients showing titres above and below this cut-off was comparable. On the whole, median gB-IgG antibody titres were fivefold higher than Nt titres in the Ic patients, and were the sole approaching median gB peak titres of Tx patients (Tx/Ic peak titre ratio=2) (Table 1).

**Primary HCMV infection in the Ic host and the Tx patient**

A representative flow cytometry analysis of activated T_{FH} cells in an Ic patient with primary HCMV infection at 41 and 111 days after onset of infection is shown in Fig. S2, where the initial rise, and subsequent drop in activated T_{FH} cells is documented.

The kinetics of HCMV DNA in blood, antibody and T-cell responses during primary infection of two Ic patients and two Tx patients are shown in Fig. 2. As shown in Fig. 2 (a, b) for patients NP 003 and NP 005, in the early convalescent phase of primary HCMV infection, HCMV DNAemia rapidly dropped concomitantly with the appearance of HCMV-specific CD4⁺ and CD8⁺ T-cells, then shortly thereafter disappeared. At the same time, activated (ICOS⁺PD-1⁺ "CCR7low") T_{FH} cells increased progressively in number. In parallel, the antibody titres started to increase for all the different antibody types. The peak antibody titre was reached rapidly for each type of antibody and a plateau was reached.
In all the Tx patients examined, HCMV infection (as revealed by the first detection of viral DNA in blood) started around 30 days after transplantation reaching a peak around 40 days (median 183 250 DNA copies ml\(^{-1}\)). In the meantime, activated T\(_{FH}\) cells started to increase in number until a median time of 175 days post-transplant. HCMV infection resolved rapidly following the appearance of HCMV-specific CD4\(^{+}\) T-cells at a median time of 156 days post-transplant (Table 1).

In conclusion, following the activated T\(_{FH}\) cell rise, antibodies (and particularly, both neutralizing and ELISA IgG to the pentameric complex) increased in titre at a median time of 50–90 days, reaching a peak at 250–350 days post-transplant. Prior to reaching the antibody titre peak, the activated T\(_{FH}\) cell rise generally stopped, often reaching the initial pre-transplant level. During follow-up, when HCMV-specific CD4\(^{+}\) T-cells dropped below the protective cut-off level, this caused a secondary rise in activated T\(_{FH}\) cells and sometimes a further rise in antibody titres. If follow-up is continued for up to 2 years, after the first year post-transplant, a drop in antibody titres may be observed concomitantly with the drop in activated T\(_{FH}\) cells (data not reported).

**Fig. 1.** Distribution (%) of patients with overall peak antibody titres (left) below and (right) above selected cut-offs according to different serological assays in a group of immunocompetent and a group of transplant patients. (a) neutralizing (Nt) antibody in ARPE-19 epithelial cells; (b) ELISA IgG antibody to the pentamer gHgLpUL128L; (c) ELISA IgG antibody to gHgLgO; and (d) ELISA IgG antibody to gB.

Kinetics of activated T\(_{FH}\) cells and Nt and IgG antibodies to envelope viral glycoproteins in primary infections of Ic and Tx patients

When activated T\(_{FH}\) cell frequencies were compared at different time points in the two groups of patients, a significantly higher frequency was observed in Ic patients only at 30 days post-infection (p.i.) (Fig. 3) The pathogenetic basis of this event resides in the sharp drop of activated T\(_{FH}\) cells occurring in Tx patients the first month after Tx due to immunosuppressive therapy.

As for the Nt antibody response, it reached a steady state in Ic patients starting 30 days p.i. and lasting until 6 months p.i., whereas in Tx patients it increased significantly from 30 days through 360 days post-Tx during the entire follow-up period, superseding the levels observed in Ic patients (Fig. 4a). Similarly, in Ic patients the IgG antibody titre to the pentamer increased significantly from 30 days through 90 days p.i., then reached a steady state whereas in Tx patients it reached a peak at 180 days, then remained substantially stable. However, the comparison of antibody titres from the two groups at different time points showed that pentamer IgG antibody titres were significantly higher in the Ic patients for the first 3 months p.i. (Fig. 4b), unlike Nt titres that were similarly higher in the Ic patients, but only during the first month (Fig. 4a). Taken together, both antibody types showed a trend towards overlapping at 3–4 months, then became higher in titre in Tx patients.

The kinetics of activated T\(_{FH}\) cells, HCMV-specific CD4\(^{+}\) T-cells and HCMV DNA in blood of 14 Tx patients with primary infection are shown in Fig. 4(c–e). In conclusion, at day 240, when the median level of activated T\(_{FH}\) cells reached the peak, the median level of HCMV-specific CD4\(^{+}\) T-cells...
Fig. 2. HCMV primary infection in (a) and (b), two immunocompetent non-pregnant patients, and (c) and (d), two solid organ transplant recipients (SOTR) during a 1–2-year follow-up. In each figure, the upper panel reports IFN-γ+ HCMV-specific CD4+ and CD8+ T-cells 10^6 ml⁻¹ blood, while the lower panel reports titres of the four antibody types determined together with the frequency of activated ICOS⁺PD-1⁺CCR7⁻ FH cells. VGCV (valganciclovir) and GCV (ganciclovir) boxes indicate the administration of antiviral therapy. The red arrow in panel (d) indicates the administration of a steroid bolus for treatment of an acute rejection episode.
among the 22 HCMV-seropositive SOTR analysed, 13 had no HCMV in blood or they developed a low DNA level self-resolving infection, while nine, after a decline in IFN-γ HCMV-specific CD4+ T-cells, developed a severe HCMV reactivation requiring antiviral treatment (see Gabanti et al., 2014). In both groups, there was an initial sharp drop in activated T_FH cell number from pre- to post-transplant due to immunosuppressive therapy (Fig. S3).

In Tx patients with no infection, in the absence of HCMV reactivation and presence of both HCMV-specific CD4+ and CD8+ T-cells, there was no substantial increase in antibody titres or acT_FH cells (Fig. S3a, SOTR 038). However, when a low-level DNA infection occurred, this induced a slight increase in both antibody level and activated T_FH cells (data not shown).

On the other hand, in pt SOTR 063 (Fig. 3b), who was suffering from a severe HCMV infection with a very high DNA peak and treated with ganciclovir (GCV), after an initial drop in activated T_FH cell number, a sharp rise in these cells followed HCMV reactivation and preceded a very rapid increase in all antibody titres. Peaks in antibody titres coincided with the appearance of IFN-γ+ HCMV-specific CD4+ T-cells. Afterwards, antibody titres remained stable or decreased slightly concomitantly with a slight decrease in activated T_FH cells, which rose again after a sustained second episode of HCMV reactivation associated with a low DNA level.

When the group of 13 Tx patients with no or self-resolving HCMV infection reactivation was compared with the group of nine Tx patients with severe HCMV infection reactivation, it was found that the frequency of activated T_FH cells was not significantly different in the two groups of patients at different time points (data not shown). However, while both the Nt and the pentamer gHgLpUL128L-IgG antibody titres were comparable at different times in the group with self-resolving infections (Fig. 5a, b), both types of antibody titre increased significantly between 30 days and 180 days only in the group of patients with severe reactivated infection (Fig. 5c, d). Taken together, these findings document that, even without a significant increase in activated T_FH cells, in patients with severe infection both antibody titres increased significantly at 6 months post-Tx.

**HCMV-specificity of the activated T_FH response in the acute and convalescent-phase of primary HCMV infection**

The immune response of activated T_FH cells to HCMV peptide pools of viral proteins IE, pp65, gHgLpUL128L and gB, as well as to control peptide pools of influenza (Flu) and respiratory syncytial virus (RSV), was investigated. CD4+ CXCR5+ (T_FH) and CD4+ CXCR5- (non-T_FH) cells were tested separately. As shown in Fig. 6, relevant to five cases (three Ic and two Tx patients) of primary HCMV infection, at the activated T_FH peak, T_FH specific for gHgLpUL128L and gB were more numerous than cells responding to pp65 and IE. The same trend, although to a lower level, was observed during the late convalescent-phase. CD4+ CXCR5+ (non-T_FH) cells displayed a similar drop in HCMV-specific reactivity between the acute and the convalescent phase of HCMV infection. In particular, while the response to IE was low in both T_FH and non-T_FH subsets, the frequency of pp65-specific with respect to gB- and gHgLpUL128L-specific T-cells was higher in 4/5 patients within non-T_FH as compared with T_FH cells.

In addition, in an Ic patient (NP29) we sorted activated (ICOS+PD1++) and quiescent (ICOS PD1) CD4+ CXCR5+ T_FH cells. HCMV-specific T-cells were found in the activated subset of T_FH cells, whereas their presence in the
Fig. 4. Comparison of (a) Nt antibody titre in ARPE-19 epithelial cells and (b) pentamer gHgLpUL128L-IgG antibody titre in IC and Tx patients during 1-year follow-up of primary HCMV infection. Significant differences in the comparison of Nt titres were found at 30 days (higher titres in IC) and at 360 days p.i. (higher titres in Tx patients). The comparison of the anti-pentamer IgG titre showed that antibody titres were significantly higher in IC patients for the first 3 months p.i., then the two populations showed a trend towards an overlap (Mann–Whitney U test; *, P<0.05; **, P<0.01; ***, P<0.001). Kinetics of peripheral blood: (c) activated ICOS$^+$PD-1$^{++}$CCR7$^+$ T$_{FH}$ cells; (d) HCMV-specific CD4$^+$ T-cells; and (e) HCMV DNA in 14 HCMV primary infections of Tx patients. The median level of activated T$_{FH}$ cells in blood reaches the peak at 240 days post-Tx, when the median level of HCMV-specific CD4$^+$ T-cells reaches the cut-off, and the median level of HCMV DNA in blood is undetected.
quiescent subset was negligible (Fig. 7). The frequency of HCMV-specific T-cells in the activated subset decreased markedly from the acute to the convalescent stage of infection (days after onset of symptoms). Although preliminarily, these findings indicate the HCMV specificity of the activated T<sub>FH</sub> response.

**DISCUSSION**

The initial idea for this manuscript was inspired by the observation that in primary HCMV infections the peak antibody level of Nt antibodies preventing the infection of epithelial cells was markedly higher in Tx patients during the post-transplantation period as compared to Ic patients during the convalescent phase. The Tx/Ic peak antibody titre ratio was 16 for Nt antibodies determined in epithelial cells, whereas it was 4 for ELISA antibodies to the pentamer gHgLpUL128L and gHgLqO, and 2 for ELISA antibodies to gB. Although all peak antibody levels were higher in Tx patients, the marked difference in the ratio levels between Nt and ELISA antibodies might be explained by the finding that the great majority of Nt antibodies indicate specific biological activity directed to the pentamer, while ELISA IgG antibodies to viral glycoproteins express mostly binding activity and exhibit a more scattered distribution (Kabanova et al., 2014).

Since it is now well established that T<sub>FH</sub> cells are specialized B-cell helpers and are essential for germinal centre (GC) formation and development of high-affinity antibodies and memory B-cells (Crotty, 2014), we decided to investigate the correlation of T<sub>FH</sub> cell activity and the magnitude and quality of the antibody response both in primary infections of Ic and Tx patients as well as in secondary infections of Tx patients. However, a major effort was dedicated to the comparative study of this correlation in primary HCMV infections of the two population groups.

It has been documented in mice, and to a minor extent in man, that acute and chronic infections trigger a five-step process regulating the formation, function and persistence of pathogen-specific T<sub>FH</sub> cells. The first step is T<sub>FH</sub> priming by unique subsets of antigen presenting cells (APCs) bearing the

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**Fig. 5.** In a group of 13 self-resolving HCMV reactivations of SOTRs, no significant increase in antibody titre was observed during 1-year follow-up for either (a) ARPE-19-Nt or (b) pentameric IgG antibody titre. On the other hand, a significant increase was observed between 30 days and 180 days post-Tx for both (c) Nt and (d) pentameric IgG antibody titres in nine Tx patients with severe HCMV reactivation requiring antiviral treatment. (Kruskal-Wallis test and Dunn’s post test with correction for multiple comparisons; *P<0.05; **P<0.01).
capacity to prime the $T_{FH}$ cell response. Priming may also be impacted by specific pathogen-associated molecular patterns (PAMPs) eliciting distinct cytokine profiles which can promote (IL-12 in humans) or limit (IL-2 or IFN-α/β) differentiation of $T_{FH}$ cells from naïve CD4$^{+}$ T-cell precursors. Furthermore, B-cells and macrophages may also contribute to initial $T_{FH}$ priming. The second step relates to cytokine-regulated localization and trafficking of $T_{FH}$ cells to or away from GCs, thus controlling the magnitude and quality of GC reactions. In the third step, $T_{FH}$ cells form stable conjugates with antigen-presenting GC B-cells, thus coordinating the bi-directional communication of $T_{FH}$ cells and GC B-cells, through the intervention of several cytokines and surface receptors and their ligands. Finally, the last two steps involve formation of memory $T_{FH}$ cells, and induction of suppressive Foxp3$^{+}$ follicular regulatory ($T_{FR}$) cells that impede the development of long-lived secreted antibody responses (Butler & Kulu, 2015).

In this study, the median peak antibody titre during the convalescent-phase of primary HCMV infection was determined in 40 Ic and 20 Tx patients. Of these patients, who evidenced a difference in antibody titre magnitude between the two population groups, only a portion could be prospectively investigated for the $T_{FH}$ cell kinetics during the 1–2-year follow-up period. However, the similar and somewhat reproducible results observed in the two population groups prompted us to draw some preliminary conclusions on the correlation between antibody response and $T_{FH}$ kinetics.

In the Ic patients, the first antibody detection occurred 20–30 days after onset of infection, upon first DNA peak detection and before HCMV-specific CD4$^{+}$ and CD8$^{+}$ T-cell appearance (limiting viral infection), and at least 1 month prior to detection of an activated $T_{FH}$ peak. This means that $T_{FH}$ priming, localization to GC and efficient T-B conjugate formation occur...
in the first few weeks after infection onset, with T_{FH} helper promotion of GC B-cell differentiation into plasma cells producing specific antibodies. On the other hand, in Tx patients, all the same steps occurred over a much longer time interval, i.e. first ARPE Nt antibodies occurred 50–90 days after transplantation, close to the first DNA peak detection and much later than the first HCMV-specific CD4^{+} T-cell appearance, and about 3 months prior to the activated T_{FH} peak. In determining the infection onset in the two populations, we considered that, in the Ic, (without knowledge of the infection source) the presence of HCMV-related clinical symptoms or the kinetics of the laboratory parameters [IgG seroconversion, specific IgM antibody, low Avidity Index (AI)] indicated onset of infection, while in Tx patients the day of transplantation was considered as the first exposure to the virus (which was mediated by the transplanted organ) in HCMV-seronegative patients. This discrepancy may have partially impacted on differential time parameters of this study. However, if we consider that in the early '60s, the post-perfusion syndrome occurred 3–6 weeks post-operation, we are confident that an interval higher than 40–50 days between the two patient groups is highly susceptible to reflect a real difference in time of the immune response.

While in the Ic patient, the kinetics of activated ICOS^{+}PD-1^{++} CCR7^{+} T_{FH} cells and HCMV-specific CD4^{+} T-cells appeared to proceed substantially in parallel, although sometimes acT_{FH} cells could be detected earlier than specific CD4^{+} T-cells, in the Tx patient, as a rule, during the extended lapse following transplantation in which HCMV-specific CD4^{+} T-cells were lacking and in the presence of a high viral load, activated T_{FH} cells increased rapidly in number in complete dissociation from HCMV-specific CD4^{+} T-cells. It has already been reported for human immunodeficiency virus (HIV) infection that CD4^{+} T-cell destruction could be associated with a T_{FH} accumulation in lymphnodes, which can affect the host's immune response not

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**Fig. 7.** HCMV specificity of quiescent (ICOS^{−}PD-1^{−}, left panels) and activated (ICOS^{+}PD-1^{++}, right panels) T_{FH} cells (CXCR5^{+} CD4^{+} T-cells) in an immunocompetent patient (NP29) examined 24 days (a, b) and 76 days (c, d) after onset of symptoms in a primary HCMV infection. In each subset, the frequency of T-cells specific for peptide pools of viral proteins IE, pp65, gHgLpUL128L, gB and peptide pool controls of influenza (Flu-pp) and respiratory syncytial virus (RSV-pp) is shown.
only against HIV, but also against other infectious agents (Lindqvist et al., 2012; Perreau et al., 2013; Cubas et al., 2013). Along this line, it is reasonable to hypothesize that the lack of specific CD4+ cells, while it does not limit HCMV infection (in conjunction with CD8+ T-cells), does not hamper activated T_HH cell survival and expansion (following the sharp drop in the first month post-Tx) and their stimulatory effect on GC B-cells, with subsequent differentiation of plasma cells and antibody production (Ueno et al., 2015). However, the frequency of activated T_HH cells was not significantly greater in Tx patients as compared with Ic patients, thus supporting their increased or sustained functional activity (rather than an increase in number) in the absence of HCMV-specific CD4+ T-cells. This event paralleled the progressive increase in Nt antibody titre, which reached levels significantly higher in Tx patients at 6–12 months post-Tx compared with Ic patients. Although they did not reach significance, a similar trend was observed for pentameric IgG antibody titres. These findings are consistent with some observations reported about 10 years ago, where the deliberate removal of CD4+ T-cell help in mice, experimentally infected with lymphocytic choriomeningitis virus, was shown to be associated with enhanced Nt antibody production without impairment of CD8+ T-cell activity (Recher et al., 2004).

This line of thought is supported by the data on the correlation of activated T_HH cells and numerous other parameters. While a weak, although significant, correlation was found between activated T_HH cells and both Nt and pentameric IgG antibody titres, a stronger correlation was detected between the time to activated T_HH cell peak and both the time to appearance of HCMV-specific CD4+ T-cells and the time to HCMV DNA clearance. This finding supports the conclusion that HCMV-specific CD4+ T-cells basically control the infection, thus reducing viral load with subsequent inhibition of T_HH cell activation.

Similarly, in the group of patients with severe HCMV reactivated infections requiring antiviral treatment, the lack of HCMV-specific CD4+ T-cells was associated with the expansion of activated T_HH cells (preceding reappearance of HCMV-specific CD4+ T-cells) and subsequent production of specific antibodies. As shown in this study, significantly increasing Nt-ARPE-19 and pentameric IgG antibody titres were observed in severe reactivations requiring treatment, but not in self-resolving infections. Again, this finding was associated with levels of activated T_HH cells that were comparable in the two patient groups at the sequential time points analysed. A recent work has shown that circulating memory T_HH cells potentiate a secondary immune response in vivo after interaction with dendritic cells (DCs) (Sage et al., 2014).

As for the preliminary analysis of the specificity of peripheral blood T_HH cell response to viral proteins, we found that T-cells responding to HCMV antigens were detected among the activated (ICOS+PD1++) subset and were virtually absent from the quiescent subset. This confirms that ICOS+PD1++ T_HH cells detected in blood during acute infection truly represent T_HH cells elicited by the pathogen involved in the infectious episode. Thus, monitoring the kinetics of ICOS+PD1++ T_HH cells in peripheral blood may represent a useful surrogate to analyse the T_HH response to infection or vaccination. Moreover, it was interesting to note that the frequency of T_HH cells specific for gHgLpUL128L and gB was higher in 4/5 patients as compared with non-T_HH cells, thus indicating that the two viral proteins eliciting the highest (Nt and gB) antibody titres in Tx patients were highly stimulatory for T_HH cells. This is similar to what was observed for Flu- or HIV-specific CD4+ T-cells, where T_HH cells are preferentially directed against the surface haemagglutinin or Env, while non-T_HH cells are preferentially directed against the internal nucleoprotein or Gag (Leddon et al., 2015; Schultz et al., 2016). These observations deserve further investigation.

In conclusion, the differential median peak antibody titre in primary HCMV infections of Tx patients as compared to Ic patients, with special reference to Nt antibodies, appears to be related to the sustained activity of activated T_HH cells in the absence of HCMV-specific CD4+ T-cells and presence of HCMV DNAemia. All these events occurred much more rapidly in Ic patients with primary infection, in whom the shorter duration of activated T_HH cells, earlier appearance of HCMV-specific CD4+ T-cells and earlier DNA disappearance, resulted in lower median peak antibody titre. Finally, in severe reactivated infections of Tx patients with high DNAemia level, the kinetics of antibody response was somewhat similar to that of Tx patients with primary infection.

**METHODS**

**Study population.** In the period 2012–2014, 40 Ic (36 pregnant women and 4 non-pregnant patients) and 20 solid-organ Tx patients (10 kidney, 8 heart, 1 lung, and 1 heart-lung transplant recipient) with primary HCMV infection were enrolled in the study for 1–2 year follow-up. Among them, 26 Ic and 16 Tx patients were preliminarily analysed in our previous study on the kinetics of the antibody response in primary HCMV infection (Gerna et al., 2015). In addition, 22 HCMV-seropositive Tx patients (8 heart and 14 kidney transplant recipients) with reactivated HCMV infection were included in the study. Ic patients were referred to the Virology and Microbiology Service, and Tx patients to Transplantation Centers of the University Hospital, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. The kinetics of Nt and ELISA IgG antibodies to HCMV glycoproteins as well as the kinetics of HCMV-specific CD4+ and CD8+ T-cell immunity were measured in all patients. T_HH cell kinetics was investigated in 15 Ic and 14 Tx patients with primary and 22 Tx patients with reactivated infection. Induction and immunosuppressive therapies for heart and kidney transplant recipients have been reported recently (Gabani, et al., 2014). Patients with organ rejection were treated with a daily bolus iv methylprednisolone (1 g or 500 mg) for 3 days. The study was approved by the Fondazione IRCCS Policlinico San Matteo Institutional Review Board (protocol 20100005459, Procedure 2010005459). Patients gave written informed consent prior to entering the study.

**Diagnosis of primary HCMV infection and monitoring of HCMV infection.** In both Ic and Tx patients, primary HCMV infection was diagnosed based on HCMV-related clinical symptoms (if present) and subsequently confirmed by viral/serological assays, or, in the absence of symptoms, based on the presence of at least two of the four following
parameters: IgG seroconversion, presence of IgM antibody, low IgG Avidity Index (AI), and presence of DNAemia (Revello et al., 2011). In the Ic, the infection onset was approximated as the inferred onset of the infectious event based on clinical symptoms and/or laboratory finding kinetics, whereas in Tx patients it was arbitrarily fixed at the time of transplantation.

In HCMV-seropositive Tx patients with HCMV reactivation/reinfec-
tion, HCMV infection and disease (infection in association with clinical symptoms and/or organ dysfunction) were diagnosed by real-time PCR, as reported previously (Furione et al., 2012). DNAemia was quantified weekly or bi-weekly upon occurrence of active HCMV infection. Pre-emptive antiviral therapy was given to Tx patients reaching a DNAemia cut-off of 300 000 DNA copies ml⁻¹ whole blood (Lilleri et al., 2004; Gerna et al., 2007, 2011) and consisted of iv ganciclovir (GCV) at a dosage of 5 mg kg⁻¹ twice a day, or oral valganciclovir (VGCV), 900 mg twice a day. Antiviral therapy was continued until the virus disappeared from blood.

Serological assays. Conventional ELISA (DiaSorin, Saluggia, Italy) diagnostic assays were used for determination of HCMV-specific IgG and IgM antibody by ETI-CYTOK-G and ETI-CYTOK-M, respectively. In addition, the HCMV-specific AI was measured with an in-house developed ELISA (Revello et al, 2010; Furione et al., 2013). The other unconventional in-house developed assays performed in this study were ELISAs for determination of IgG antibodies to the pentamerics hHgLUL128L, gHgLgO and gB, as previously reported (Lilleri et al., 2012).

Neutralizing (Nt) antibody assays were performed in duplicate on monolayers of ARPE-19 epithelial cells, using the HCMV isolate VR 1814, as reported previously (Gerna et al., 2008; Lilleri et al., 2013).

T-cell and Tfh cell monitoring: flow cytometry analysis. T-cell monitoring was performed (whenever feasible) on all subsequent medical visits in Ic patients, and at days 0, 30, 60, 90, 120, 180, 240, 300 and 360 after transplantation in Tx patients. Absolute CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T-cell counts were measured in whole blood samples by flow cytometry (TruCOUNT tubes, BD Biosciences, San Jose, CA, USA). For determination of HCMV-specific T-cells (defined as cells producing IFN-γ in response to the HCMV stimulus), PBMC were incubated with autologous monocyte-derived HCMV VR1814-infected dendritic cells (iDC) (Lozza et al, 2005), then washed and incubated with Live/Dead Fixable Violet Dye (Invitrogen, Frederick, MD, USA) and V500-conjugated anti-CD8 (clone RPA-T8) 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-conjugated anti-CD3 (clone UCHT1), APC-Cy7-conjugated anti-CD4 (clone RPA-T4), PE-Cy7-conjugated anti-IFN-γ (clone B27) (BD Biosciences). Finally, cells were washed, resuspended in 1 % (v/v) paraformaldehyde and analysed with a FACS-Canto II flow cytometer (BD Biosciences). As a routine, 1×10⁵ viable lymphocytes were collected and at least 2.5×10⁵ CD³⁺CD⁴⁺ and CD³⁺CD⁸⁺ T-cells were analysed.

The frequency of CD4⁺ and CD8⁺ T-cells producing IFN-γ in response to the iDC stimulus was determined by subtracting the frequency of control cells incubated with mock-infected DC (<0.05 %) from the test frequency. The total number of HCMV-specific CD⁴⁺ and CD⁸⁺ T-cells was calculated by multiplying the percentages of HCMV-specific T-cells by the relevant absolute CD⁴⁺ and CD⁸⁺ T-cell counts.

Cell surface staining of the blood aCTfh cell subsets was performed by incubating fresh or thawed PBMC with a mix of the following mAbs FITC (clone HI100)-conjugated anti-CD45RA, PE-Cy7 (clone EH12.1)-conjugated anti-CD279 (PD-1), BV-510 (clone RF8B2)-conjugated anti-ICOS, APC-Cy7 (clone RPA-T4)-conjugated anti-CD4 (BD Biosciences), APC (clone ISA-3)-conjugated anti-CD278 (ICOS) (eBioscience, San Diego, CA, USA), BV241 (clone G043H7)-conjugated anti-CD197 (CCR7), PerCP-Cy5.5 (clone G043E4)-conjugated anti-CD19 (CCR6), PE (clone G025H7)-conjugated anti-CD183 (CXCR3) (BioLegend Inc., San Diego, CA, USA). Finally, cells were washed, resuspended in 1 % (v/v) paraformaldehyde and analysed. The percentage of activated Tfh (CXCR5⁺ ICOS⁺ PD-1⁺⁺ CCR2⁺⁺⁺) among total CD⁴⁺ T-cells was calculated and reported.

Specificity of the activated Tfh response. The CD4⁺ T-cell response specific for HCMV (IE-1, pp65, gB and gHgLUL128L), influenza virus (Flu Matrix protein 1, Nucleoprotein and Neuraminidase) and respiratory syncytial virus (RSV: Fusion protein and Nucleoprotein) antigens was investigated with a novel high throughput method that allows measurement of low frequencies of antigen-specific T-cells in the human T-cell subset repertoire (Geiger et al., 2009). Briefly, PBMC were stained with anti-CXCR5-unlabelled mAb (R&D Systems, Minneapolis, MN, USA), followed by goat anti-mouse IgG2b-biotinylated antibody (Southern Biotech, Birmingham, AL, USA) and, then, streptavidin-PE-Cy7. In addition, PBMC were stained with the following mAbs anti-CXCR5-BV241, PD-1-BV711 (Biolegend), CD8-FITC (Immunootech, Marseille, France), CD4-FE-PE-Texas Red (Invitrogen, Frederick, MD, USA), CD45RA-Qdot655 (Life Technologies, Eugene, OR, USA), ICOS-APC (ebioscience). Then, CXCR5⁺ and CXCR5⁺ memory CD4⁺ T-cells or ICOS⁺PD1⁺⁺ and ICOS⁺PD1⁺CXCR5⁺ memory CD4⁺ T-cells, were sorted using FACSaria (BD Biosciences). T-cell samples were divided into several replicate cultures and expanded for 2–3 weeks in the presence of irradiated allogeneic feeder cells, IL-2 and phytohaemagglutinin. Aliquotes from each culture were then tested in parallel for their capacity to proliferate in response to overlapping peptides (15-mers) spanning entire HCMV proteins. The frequency of specific precursors was calculated according to response in proportion to overlapping peptides (15-mers) spanning entire HCMV proteins.

Statistical analysis. The Mann-Whitney U test was employed to compare activated Tfh cells and Nrg-ARPE-19/pentameric IgG antibody titres between Ic and Tx patients. The Kruskall–Wallis test was used to compare more than two groups, with Dunn’s post test and correction for multiple comparisons. The Spearman test was used to study the correlation of time to activated Tfh peak and time to HCMV-specific CD4⁺ T-cell appearance/time to HCMV clearance from blood, as well as activated Tfh cells and Nt pentameric IgG antibody titres.

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