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The lack of cytomegalovirus-specific cellular immune response may contribute to the onset of organ infection and disease in lung transplant recipients.

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Summary

Cellular immune response has been demonstrated to play a role in the control of human cytomegalovirus (HCMV) replication in organ transplant recipients. Herein, HCMV-specific T-cell response and association to the onset of organ infection/disease were prospectively evaluated by EliSPOT assay in a population of 46 lung transplant (LT) recipients at months 1, 3, 6, 9 and 12 posttransplantation. According to our centre's practice, a combined prolonged antiviral prophylaxis (HCMV-IG for 12 months and ganciclovir or valganciclovir for 3 weeks from postoperative day 21) was given to all LT recipients. HCMV-DNA was concomitantly detected on bronchoalveolar lavage (BAL) and whole blood by real-time PCR. Approximately one third of patients resulted HCMV persistently non-responder; the rate of HCMV infection, as evaluated by HCMV-DNA positivity, tended to be higher in non-responders. Mean viral load on BAL was significantly higher in non-responders vs other patients ($p < 0.001$). Temporal profile of infections appeared related to the HCMV responder status with a shorter time to onset of infection post-transplantation and a longer duration in non-responders. The occurrence of organ disease (i.e. pneumonia) tended to be higher in non-responders, with poor prognosis, as death occurred in one of three non-responder patients that developed HCMV pneumonia.

The lack of HCMV-specific cellular response can contribute to the onset of organ infection and disease also in patients in which antiviral prophylaxis was adopted; this could be due to the potential occurrence of incomplete control of replication in lungs or a delayed priming of T-cell reconstitution.

Keywords: Human cytomegalovirus; cellular immune response; lung transplantation.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous β-herpes virus that, following primary infection, establishes latency in host tissues and may reactivate in immunocompromised conditions. In solid organ transplant recipients, the incidence of HCMV infection and disease is variable according to the transplanted organ and is particularly high in lung transplantation (LT), being approximately 40% for both (1). Lung is a HCMV latency site and in LT, viral reactivation has been associated to direct (i.e. organ and systemic infection/disease) and indirect effects (including acute rejection and chronic allograft dysfunction), thus limiting the success of transplantation. Two strategies are usually adopted in the clinical management of transplant recipients: anti-viral prophylaxis and pre-emptive therapy, consisting in the administration of antiviral agents after reaching a predetermined cutoff in blood, but prior to the development of clinical symptoms. Therefore, virological monitoring is fundamental in the clinical decision-making process and, in LT, it is usually performed by evaluation of HCMV-DNA on bronchoalveolar lavage (BAL) and whole blood specimens. Beside this, in recent years, viro-immunological monitoring with evaluation of specific T-cell response has been introduced. Cell-mediated immune response has been demonstrated to play a role in the control of HCMV replication in the transplant setting, as early recovery following transplantation seems able to prevent and reduce the duration of infection, thus avoiding the onset of overt disease or recurrence, while a delayed/reduced response should represent the pathogenic basis for recurrent infections that may become symptomatic in the absence of treatment (2-4). Several assays are available for the evaluation of HCMV-specific cellular immune response, including intracellular cytokine staining, QuantiFERON®- CMV, EliSPOT assay, and MHC multimer staining, all of which

present advantages and limitations, as reported by the International Consensus Guidelines on the Management of Cytomegalovirus in solid organ transplantation (5). Among these, EliSPOT is an interferon (IFN)-γ-releasing assay (IGRA), that enumerates IFN-γ-secreting T-cells in response to stimulation with HCMV peptides and seems to represent a robust and reproducible tool for monitoring T-cell activity *ex-vivo* (6). Current evidence suggests that immunological monitoring tests can predict the risk of HCMV viremia and disease in both the post-prophylaxis and pre-emptive setting (5); while less is known about the impact of viro-immunological monitoring in the prophylaxis period. Anti-viral prophylaxis is effective in inhibiting viral replication, however is also associated to a delayed priming of T-cell immune reconstitution and higher incidence of late-onset HCMV disease. Herein we evaluated the clinical impact of viro-immunological monitoring of HCMV in a population of LT recipients having received anti-viral prophylaxis.

Materials and methods

Patient population included 46 lung transplant recipients followed-up in the first year post-transplantation; the main features of study population are reported in Table I. According to our centre's practice, LT recipients were submitted to surveillance visits at 1, 3, 6, 9, and 12 months; further visits are performed at 18 and 24 months post-transplantation and then annually, as well as in the presence of clinical signs and/or symptoms and/or rejection (7). At each visit whole blood and BAL were collected, accounting for a total number of 184 combined samples. Further BAL and whole blood specimens were collected within two weeks for checking-up a previous positivity; in these cases, no evaluation of cellular immune response was made. Long-term

immunosuppression was maintained with three drugs: tacrolimus or cyclosporine A (in patients with cystic fibrosis as underlying disease), mycophenolate mofetil and prednisone (to be tapered at low dosage or discontinued). According to our centre's practice, all LT recipients received a universal and combined anti-viral prophylaxis for HCMV consisting in the administration of ganciclovir (5 mg/kg twice daily) or valganciclovir (450 mg twice daily) from day 21 for 3 weeks associated to HCMV-IG (Cytotect Biotest) at days 1, 4, 8, 15, and 30 (1.5 ml/kg body weight) and every month up to 1 year post-transplantation (1 ml/kg body weight), irrespective of HCMV serostatus (8). Ganciclovir or valganciclovir were further administered based on clinical judgement and/or in case of HCMV-DNA load on BAL higher than 10^5 copies/ml and/or in case of HCMV-DNA load on whole blood higher than 10^5 copies/ml. As previously described (9), HCMV infection was defined as laboratory evidence of viral reactivation in the absence of clinical signs and/or symptoms; HCMV syndrome as viral detection in the presence of fever, leukopenia, thrombocytopenia, increased hepatic transaminases, malaise; and localized (organ) disease as viral detection in organ tissues or local secretions in association or not with viral detection in blood and symptoms/signs of organ involvement. Viral load was quantified by a commercially available real-time TaqMan PCR assay, as previously described (limit of detection, 1800 copies/ml BAL and 2400 copies/ml whole blood)(8). The EliSPOT assay was optimized and performed, as described elsewhere (7). Briefly, automated separation of total CD3+ cells was performed with the RoboSep® cell separator (StemCell Technologies, Vancouver, Canada) using the EasySep negative selection protocol, following the manufacturer's instruction. An aliquot of 2 x 10^5 cells were used for EliSPOT assay (EliSpot Interferon-γ Basis Kit; AID, Strassberg, Germany) and

incubated on anti-IFN-γ coated wells together with HCMV-specific peptide mix (including pp65 and IE-1 peptides [Nanogen Advanced Diagnostics, Milan, Italy]) for 20 hours in a CO_2 incubator; antigen-induced IFN-γ production was visualized by an enzyme-labelled detection antibody, with coloured spots representing one cell secreting IFN-γ. Results were analyzed using a computer-assisted system (AID EliSpot Reader System, AID). For negative and positive controls, cells incubated with RPMI -1640 medium (Sigma-Aldrich, St. Louis, MO, USA) alone and with phytohemagglutinin mitogen were used, respectively. Specific immune response was evaluated as previously described (4); in particular, a number of spot forming units <5/200,000 cells as no response (non-responders), 5-20 as a weak response, 20-100 as a good response, and >100 as a very good response (responders). Temporal profile of reconstitution of cellular response post-transplantation (i.e. early responder in the presence of a responder status at first evaluation at day 30 and late responder in its absence) was also evaluated. Data are expressed as raw numbers, percentages, and means, as appropriate. For statistical analysis, the chi square, the Fisher's exact and ANOVA tests was applied, as appropriate, using a commercially available software (MedCalc version 9.2.1.0, Mariakerke, Belgium). A p-value <0.05 was considered statistically significant.

Results

Results are summarized in Table II. Overall, 27 patients (58.7%) displayed an early responder status (good or very good response in all the cases), five (10.9%) were late or transiently (depending on the episode) responders (weak or good response), and 14 (30.4%) persistently non-responders (including the three individuals with D-/R- serological matching, as expected). An episode of HCMV infection (as evidenced by

positivity on whole blood and/or BAL specimens) occurred within the first year post-transplantation in 50% of study population (23/46 patients); in particular, in 13 of 27 (48.1%) early responders, two of five (40%) late or transiently responders, and eight of 14 (57.1%) non-responders. Repeated episodes of HCMV infection were detected in three of non-responder recipients and I no patient from other groups. Primary HCMV infection occurred in the D+/R- patient; this single episode of infection induced the development of a responder status. Although the rate of infections tended to be higher in non-responders, this does not reach the statistical significance. Mean viral load on BAL was significantly higher in non-responders vs other groups ($1.4 \times 10^5 \pm 2.4 \times 10^4$ copies/ml BAL vs $7.9 \times 10^3 \pm 1.4 \times 10^3$ and 2.4×10^3 in early responders and late or transiently non-responders, respectively)($p < 0.001$). No patient presented viral load on whole blood higher than 10^4 copies/ml and values were highly overlapping in the different groups. Interestingly, in transiently non-responder individuals the episode of infection coincided with the occurrence of non-responsiveness. Temporal profile of infection appeared related to HCMV-responder status of the patient, with a shorter time to onset of infection post-transplantation in non-responders (mean days post-transplantation: 68 in non-responders, 109 in late or transiently non-responders and 204 in early responders) and a longer duration of infection (>14 days to HCMV-DNA negativity on BAL in non-responders and late or transiently non-responders vs <14 in early responders). Similar data regarding onset of infection and duration of infection were found for HCMV detection of whole blood specimens.

The occurrence of HCMV organ disease (HCMV pneumonia confirmed at histopathology and immunohistochemistry [10]) tended to be higher (p = not significant) in non-responders: in particular, three of 14 (21.3%) patients vs two of 27

(7.4%) in early responders and no cases in late or transiently non-responders. The outcome was different according to HCMV immune response status: in fact, while pneumonia had a favourable outcome in responders (resolution following anti-viral administration in both cases), death for HCMV pneumonia occurred in one of three non-responders, despite of anti-viral treatment (mortality, 33.3%). No patient developed HCMV systemic syndrome. The kinetics of HCMV-specific T-cell reconstitution and HCMV replication in lung in different types of patients, that were representative of most common courses, are reported in Figure 1.

Discussion

HCMV is among the most important viral pathogens affecting the outcome of LT recipients, with direct effects being responsible for significant morbidity and mortality and several indirect effects with detrimental consequences on survival. Given the potentially severe impact of HCMV in LT, physicians must adopt effective strategies to prevent the onset of HCMV disease. Specific cellular immune response plays a crucial role in containing viral replication, thus potentially representing a determinant factor in the outcome of LT and a useful tool for the clinical decision-making process. This could be accomplished by evaluating the need to administer antiviral agents in relation to the responder status, as well as could represent the basis for the development of studies on the use of adoptive immunotherapy with generated HCMV T cells. Some considerations can be made based on the results of the present study. The onset of HCMV infection in LT recipients is quite common during the first year post-transplantation despite the adoption of a prolonged and universal prophylactic strategy, although the incidence of infection appears to be lower than that reported by other studies adopting pre-emptive

treatment (11). Considering specific cellular immune response, approximately one third of our population resulted persistently non-responder to HCMV up to 1 year post-transplantation. Interestingly, a small subgroup of patients displayed a delayed pattern of immune reconstitution (at >30 days, although all of them within 3 months posttransplantation) or a transient phase of non-responsiveness within a responder pattern. This subgroup evidenced no increased rate of infection as well as no need to treat; the only difference in comparison to early responder patients appeared to be a tendency to an earlier onset of infection posttranplantation (Table II) and/or the occurrence of infection in concomitance with the non-responsiveness phase, although viral load on BAL was low and HCMV-DNAemia was negative in all the cases. The lack of a specific cellular immune response seems to be associated to a tendency to a higher rate and a longer duration of infection, thus confirming that incomplete control of viral replication in lungs may occur during prophylaxis. The degree of local viral replication appears to be significantly higher in the absence of an efficient immune control, as evidenced by the evaluation of viral load on BAL from responder and non-responder patients and the lack of significant association to viral detection and load on whole blood. The occurrence of viral replication may lead to the onset of organ disease, in the absence of HCMV-specific cellular immune response, potentially leading to death as evidenced in the patient who developed HCMV-pneumonia and eventually died, in contrast to the favourable resolution of infectious episode in responder patients. The small size of our study population certainly represents a limit for statistical analysis, although this is due to the activity volume of lung transplantation; the collection of more data in the subsequent years will allow to further support these observation. Few studies have investigated HCMV-specific cellular response in LT. Sester and coll. found

that specific T-cell levels were significantly lower in LT in comparison to controls and showed a significant correlation with the frequency of infectious episodes (12). Similarly, Gerna and coll. found that HCMV-specific cellular response was associated to resolution of lung infection (11). At present, evaluation of T-cell immune response (viro-immunological monitoring) may complement virological monitoring and help to identify lung transplant recipients at risk of developing organ infection/disease. Further studies will allow to support these conclusions and to investigate the potential implications in terms of indirect effects of viral infection/disease, such as onset of acute or chronic rejection, particularly at >1 year post-transplantation when antiviral prophylaxis is discontinued and it is possible the development of late-onset HCMV-disease. The impact of different immunosuppressive protocols in the context of LT remains to be investigated, in that it is likely that tacrolimus levels play a role in determining the degree of suppression of HCMV-specific cellular immune response, as demonstrated for polyomavirus BK in renal transplantation (13), possibly due to its action on T cell proliferation. An interesting issue that should be further investigated is the role played by the underlying pathological background. Recent studies have suggested an inflammatory/autoimmune T cell-mediated component in the pathogenesis of chronic obstructive pulmonary disease/emphysema (14,15). In our population no significant differences of responder status was found according to the underlying disease that lead to transplantation, although the limited size of population should be taken into account.

In conclusion, the lack of HCMV-specific cellular response can contribute to the onset of organ infection and disease also in patients in which antiviral prophylaxis has been

adopted; this could be due to the potential occurrence of incomplete control of replication in lungs or a delayed priming of T-cell reconstitution.

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Table I. Main features of study population. SD, standard deviation; COPD, chronic obstructive pulmonary disease.

Features	**Patients** **N = 46**
Mean age (years +/- SD)	48.9 +/- 18.5
Gender (M/F)	25/21
Underlying disease	
Cystic fibrosis	22 (47.8%)
Idiopathic pulmonary fibrosis	4 (8.7%)
COPD/emphysema	13 (28.2%)
Alpha-1-antitrypsin deficiency	1 (2.2%)
Bronchiectasis	2 (4.3%)
Histiocytosis X	1 (2.2%)
Hyaline membrane disease	1 (2.2%)
Extrinsic allergic alveolitis	1 (2.2%)
Progressive systemic sclerosis	1 (2.2%)
Type of lung transplant	
Single	16 (34.8%)
Double	28 (60.9%)
Retransplantation	2 (4.3%)
HCMV Serostatus (D/R)	
D+/R+	34 (73.9%)
D+/R-	1 (2.2%)
D-/R-	3 (6.5%)
D-/R+	8 (17.4%)

Table II. Viro-immunological monitoring of HCMV-specific cellular response in study population. N, number; BAL, bronchoalveolar lavage; SD, standard deviation; tx, transplantation; n.s., not significant. Viral load is expressed as genome copies/ml BAL. *Occurrence of HCMV pneumonia confirmed by histopathological analysis and immunohistochemistry. Early responders: reconstitution of cellular immune response within 30 days post-transplantation; late responders: reconstitution of cellular immune response at >30 days post-transplantation.

Total **N = 46**	**Early responders** **N = 27 (58.7%)**	**Late or transiently responders** **N = 5 (10.9%)**	**Non-responders** **N = 14 (30.4%)**	**P**
Infections within 1st year post-tx	13 (48.1%)	2 (40.0%)	8 (57.1%)	n.s.
Viral load on BAL (mean ± SD)	$7.9x10^3 \pm 1.4x10^3$	$2.4x10^3$	$1.4x10^5 \pm 2.4x10^4$	<0.001
Onset of infection (mean days post-tx ± SD)	204 ± 51	109 ± 43	68 ± 29	n.s.
Duration of infection (days to negativity)	<14	>14	>14	-
HCMV pneumonia*	2 (7.4%)	0	3 (21.3%)	n.s.
Antiviral administration	4 (14.8%)	0	6 (42.9%)	n.s.
Outcome	Resolution	-	1 death (7.1%)	-

Figure 1. Kinetics of HCMV-specific T-cell reconstitution (continue line) and HCMV replication in lung (dotted line) in the first year post-transplantation in different types of patients. A, responder who did not develop infection; B, responder who developed infection with resolution following antiviral treatment; C; transiently non-responder who developed infection in coincidence with non-responsiveness period; D, non-responder who developed HCMV-pneumonia and eventually died at approximately 9 months post-transplantation. SFU, spot forming units, corresponding to the number of IFN-γ secreting cells/200,000 peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; tx, transplantation. Viral load is expressed as copies/ml BAL.
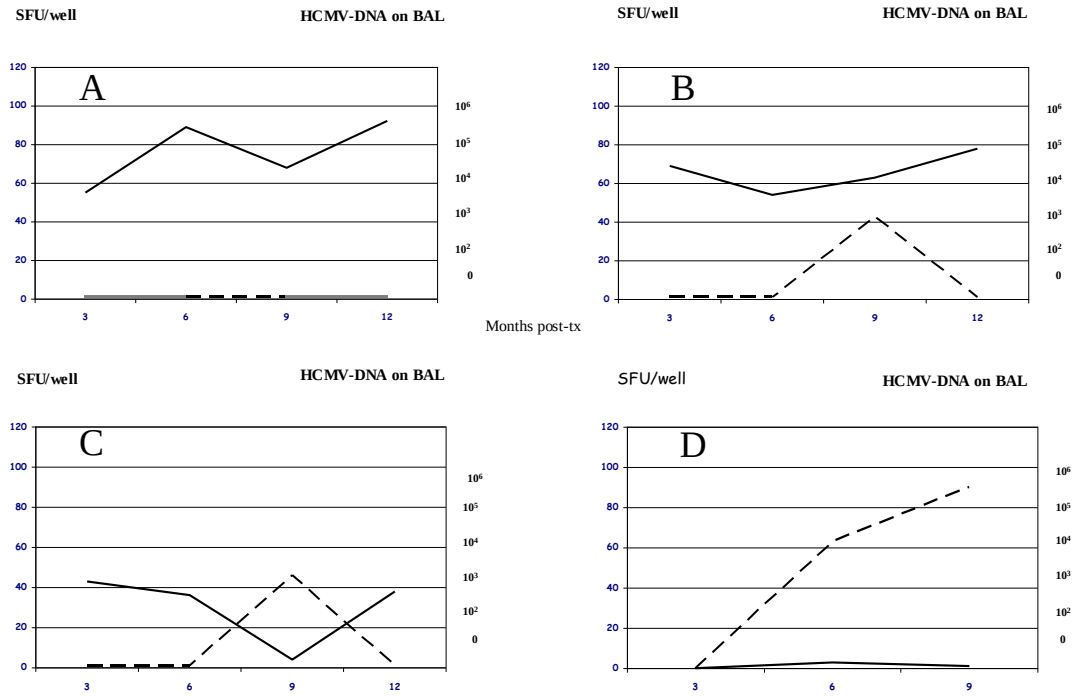
Figure 1.