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Cellular and humoral cytomegalovirus immunity changes in one-year combined prophylaxis after lung transplantation: suggestions from and for clinical practice

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Abstract

Background: Immune responses, both cellular and humoral, against cytomegalovirus (CMV) are used to predict CMV manifestations in solid organ recipients. The aim of this study is to evaluate CMV enzyme-linked immunospot (ELISPOT) assay and serology during CMV infections, their concordance and variations after lung transplantation (LTx).

Methods: We retrospectively analysed in one year the follow-up data of 43 patients receiving combined CMV prophylaxis with antiviral agents and CMV-specific immunoglobulin G (IgG). CMV infections were investigated by using molecular analyses on both 167 bronchoalveolar lavage and biopsy specimens and 1134 blood samples. Cellular CMV immunity was assessed with specific ELISPOT whereas the humoral one was assessed by quantifying specific immunoglobulins.

Results: At the first month after LTx the majority of patients were ELISPOT responders (52.3%) and 30.9% were non-responders. ELISPOT responders had a lower incidence of CMV viremia ($p = 0.047$), whereas neither effects on CMV pulmonary asymptomatic infection nor on acute rejection were observed. Responders had a higher CMV IgG titre ($p < 0.0001$) in particular at the first month after LTx ($p = 0.0001$). Concordance among CMV ELISPOT assay and IgG levels was moderate (Cohen's K 0.524), with an agreement of 89.8%. All ELISPOT responders maintained their status and almost all non-responders became responders during follow-up (92.3%); the percentage of IgG seropositive subjects increased from 74.4% at the first month of follow-up to 97.4% after 1 year.

Conclusions: Despite a moderate concordance with serology, ELISPOT response predicted a lower incidence of CMV viremia in LTx patients; no effects were reported on pulmonary clinical manifestations nor on acute rejection. The ELISPOT response as well as serology changed during the follow-up, not only after first CMV contact.

The reviews of this paper are available via the supplemental material section.

Keywords: CMV ELISPOT, CMV pulmonary infection, CMV viremia, cytomegalovirus, lung transplant, immunoglobulins

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Introduction

Cytomegalovirus (CMV) represents one of the most important pathogens affecting patients who undergo lung transplantation (LTx).[1,2] Up to now, donor (D) and recipient (R) serology status evaluation before transplantation is recommended for an efficacious assessment of CMV reactivation risk after transplantation.[3] More recently, monitoring CMV-specific cellular immunity has been investigated to predict its usefulness in detecting the risk of CMV infection among recipients.[4] In particular, promising data have been collected by employing an enzyme-linked immunospot (ELISPOT) assay that quantifies the number of

interferon-γ (INF-γ) producing CMV-specific effector T lymphocytes on *ex vivo* stimulation as spot-forming units.[5] Both the immunosuppressive regimen and induction therapy could decrease T lymphocyte activity during the early phases post-transplantation. Moreover, other factors could impact on immune response: acute rejection, early graft dysfunction, and other infections.[6] Previously, published studies demonstrated that pre-transplant kidney CMV ELISPOT response predicted the risk of post-transplant CMV infection.[7] Concerns regarding the use of the CMV ELISPOT assay in lung transplantation daily practice are represented by retrospective single-centre experiences;[8,9] our study group recently demonstrated the role of CMV ELISPOT response in predicting patients at risk of CMV viremia but not for CMV asymptomatic pulmonary infections.[10]

It is assumed that CMV-seropositive patients have a pre-existing immunity acquired against the virus that may contribute to control further viral replication.[11] Nevertheless, several studies have demonstrated that this assumption is not true for solid organ transplanted (SOT) patients: nearly one-third of SOT recipients with a pretransplant positive serology (R+), with a presumed specific immunological memory response, are lacking a T-cell-mediated response measured with ELISPOT or QuantiFERON-CMV assay.[12] Other studies evaluating CMV immunoglobulin G (IgG) serology during the follow-up of SOT recipients found that IgG seroconversion in pretransplant negative serology (R–), when CMV immunity is a primary response, occurred in 63.4% and 75.3% at 6 and 12 months, respectively; moreover, the authors demonstrated that IgG seronegativity was predictive of subsequent CMV disease (10.0% *versus* 1.3%).[13] The change in CMV-ELISPOT response could be the result of infective events occurring in non-responder patients, generating the specific immune response detectable with the ELISPOT assay.[10] This conversion, as previously demonstrated, is mainly guided by CMV viremia providing the correct stimulation for an immunological protective response.[6,14] This could be explained by both the shorter course of antiviral agents used in these patients and the concomitant immune stimulation leading to the specific response detected with the ELISPOT assay.[15,16] On the other hand, the monthly administration of high-titre CMV IgG provided a passive specific immunity, playing an important role in the immunomodulation of a specific protective response.[17]

Aim of the study

In a cohort of lung transplanted patients who received a combined CMV prophylaxis scheme we aimed to: (a) evaluate the potential use of a CMV ELISPOT assay to identify patients at risk of CMV infections after lung transplantation; (b) measure the concordance between CMV ELISPOT response and CMV serology; (c) describe concordance between CMV ELISPOT response and CMV serology during CMV infections; (d) describe changes in CMV ELISPOT response, CMV serology and titre during the observation period after lung transplantation.

Patients and methods

This retrospective, single centre, observational study was conducted at the Lung Transplant Centre of Turin (Città della Scienza e della Salute di Torino, Italy), includes all patients receiving LTx between 1 January 2014 and 31 December 2015. All patients were followed for 1 year after LTx. This study was conducted in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement for observational studies and it was approved by our institutional review board (protocol no. 0004577 – CS/416).[18]

Definitions

CMV systemic or local infection and disease as well as proved and probable CMV pneumonia were diagnosed in accordance with international guidelines.[3,19] Diagnosis of asymptomatic CMV pulmonary infection and significant CMV viremia were taken in accordance with previously published studies.[10]

Pulmonary allograft rejection was made in accordance with international guidelines; briefly, acute rejection is diagnosed in the case of perivascular and interstitial mononuclear cell infiltrates in lung biopsies obtained with transbronchial lung biopsies (TBLB). There are four grades of acute rejection: A1 minimal, A2 mild, A3 moderate, A4 severe. Other pathological features in TBLBs were also reported (i.e. organising pneumonia; OP).[20]

ELISPOT assay

ELISPOT measures an individual's CMV-specific cell-mediated immunity quantifying the number of IFN-γ produced by CMV-specific

effector T lymphocytes on *ex vivo* stimulation. The quantification of this response is measured in spot-forming units (SFUs).[5] An automated separation of total CD3+ cells was performed with the Robosep cell separator (Stemcell Technologies, Vancouver, Canada) using the EasySep negative selection protocol, as specified by the manufacturer. Then, an aliquot of 2×10^5 cells was used for the ELISPOT assay (Elispot Interferon-γ Basis Kit; Nanogen Advanced Diagnostics, Milan, Italy) and incubated in anti IFN-γ coated cells together with a CMV-specific peptide mix (including pp65 and IE-1 peptides; Nanogen Advanced Diagnostics) for 20 h in a carbon dioxide (CO_2) incubator. The antigen-induced IFN-γ production was displayed by an enzyme-labelled detection antibody; each coloured spot represents one cell-secreting IFN-γ. Finally, the results were analysed by using a computer-assisted system (AID Elispot Reader System, Strassberg, Germany).[8]

Patients' specific immune response was classified on the SFUs: responders ⩾20 SFUs, non-responders <20 SFUs.[10]

Treatment

CMV prophylaxis. In our centre we performed universal combined prophylaxis: intravenous administration of ganciclovir, followed by oral valganciclovir at a prophylactic dosage. The sequential administration scheme was detailed elsewhere.[10] In particular, the combined prophylaxis scheme consists of the intravenous administration of ganciclovir, followed by oral valganciclovir at a prophylactic dosage. The sequential administration scheme was as follows: acyclovir (400 mg) twice per day from postoperative day (POD) 5 to POD 14; intravenous ganciclovir (5 mg/kg) twice per day or valganciclovir (450 mg) twice per day from the POD 15 to POD 45; acyclovir (400 mg) twice per day from POD 46.

In addition, we included in our scheme the use of CMV-specific hyperimmune globulin (CMVIG), administered five times within 30 days after LTx (at a dosage of 0.75 ml/kg), and then monthly for 2 years (at a dosage of 0.5 ml/kg); 500 U of CMVIG (Cytotect Biotest) are composed of: IgG1 62%, IgG2 34%, IgG3 0.5%, IgG4 3.5%, immunoglobulin A (IgA) 5 mg.

Immunosuppressive regimens. Antithymocyte globulins (Fresenius, Munich, Germany) were used for the induction of immunosuppression; the immunosuppressive regimen consisted of a triple-drug therapy with a calcineurin inhibitor, an antiproliferative agent and corticosteroids.

Variables

Gathered data were divided into three phases:

1. Pre-transplant: age, disease leading to LTx, IgG CMV serology and their concentrations (qualitative and quantitative assessment in IU/mL).
2. Transplant (data collected during the LTx procedure hospital stay): age, type of procedure, CMV D/R serostatus, IgG CMV serology (qualitative and quantitative assessment in IU/mL), CMV ELISPOT, CMV DNA load in whole blood and bronchoalveolar lavage (BAL), CMV isolation from BAL and presence of CMV infection and acute rejection on TBLBs.
3. Post-transplant follow-up: same data collected at the transplant phase were gathered during each hospital stay for TBLB follow-up procedures (at 4th, 8th and 12th month after LTx).

Statistical analysis

Absolute and relative frequencies were reported for categorical data while mean and standard deviations were used to summarise continuous variables. For the comparison of the former we used Fisher's exact test; for the latter the two-sided Student's *t* test and analysis of variance (ANOVA) have been employed.

We calculated the sensitivity, specificity, positive and negative predictive values of CMV ELISPOT response for predicting CMV manifestation (asymptomatic pulmonary infection, significant viremia) and acute rejection. We then calculated the odds ratio to evaluate the association between CMV ELISPOT response and CMV manifestations and acute rejection.

To evaluate concordance between qualitative CMV ELISPOT assay response (responder and non-responder) and CMV serostatus (CMV IgG positive and negative), we reported the simple Cohen's K with their 95% confidence interval (CI). Cohen's K concordance was categorised as follows: <0.01 poor, 0.01–0.20 slight, 0.21–0.40

fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–1.00 excellent.[21]

A level of $p < 0.05$ was considered significant. Statistical analysis was performed using MedCalc Software (Ostend, Belgium).

Results

The study population consisted of 43 individuals all included in our analysis. Demographic data and clinical features are reported in Table 1 (Table 1). We collected 167 BAL samples, 167 TBLB specimens and 1134 whole blood samples. As represented in Table 1, the majority of patients were D+/R+ (27/43, 63%), followed by D+/R– (9/43, 21%), D–/R+ (6/43, 14%) and D–/R– (1/43, 2%).

As previously reported, in our cohort 51 cases of CMV had asymptomatic pulmonary infections (51/167 BAL, 30.5%) with a decreasing incidence through the observation period (Table 2) and higher incidence in the D+/R+ group (17/27 patients, 63.0%); considering CMV viremia, we reported 33 positive cases (33/1134 blood samples, 2.9%) with a higher incidence in the D+/R– group (8/9 patients, 88.8%). We recorded only two cases of CMV pneumonia with an incidence of 1.2% (2/167 BAL) and both were in D+/R+ patients (Table 2).

At pre-transplant evaluation, the majority of patients had a CMV IgG positivity (36/43, 83%), with a mean IgG titre of 215 AU/mL (±49.2 AU/mL); patients with CMV seronegativity (7/43, 17%) had a IgG mean titre of 8 AU/mL (±6 AU/mL).

During follow-up we recorded 158 CMV ELISPOT measurements and 167 CMV serology evaluations.

In our cohort we observed 27 cases of grade A1 rejection (27/167 biopsies, 16.1%), 20 A2 (20/167 biopsies, 11.9%) and eight A3 (8/167 biopsies, 4.8%). No cases of A4 rejection were reported. We recorded nine cases of OP.

CMV ELISPOT assay and CMV asymptomatic pulmonary infections

Among patients with positive ELISPOT we observed 42 cases of asymptomatic pulmonary infection (42/135 episodes, 31.1%) and three among non-responders (3/23 episodes, 13%) ($p = 0.085$, OR 3.010, 95% CI 0.84–10.68). Extrapolating data for each observation, we did not observe any statistically significant difference between responders and non-responders (Table 3). The sensitivity and specificity of ELISPOT response for predicting an asymptomatic CMV pulmonary infection were, respectively, 93.3% (95% CI 80.6–98.2%) and 17.6% (95% CI 11.3–26.2%), with negative and positive predictive values, respectively, of 86.9% (95% CI 65.3–96.5%) and 31.1% (95% CI 23.5–39.7%).

CMV ELISPOT assay and CMV viremia

We observed 23 cases of significant CMV viremia (23/158 episodes, 14.4%), 16 cases among ELISPOT responders (16/135 episodes, 11.8%) and seven among ELISPOT non-responders (7/23 episodes, 30.4%) ($p = 0.027$, OR 0.307, 95% CI 0.010–0.86). In particular, we observed that responders had a significantly lower incidence of CMV viremia at the first month and 4 months after LTx (respectively, $p = 0.043$ and $p = 0.047$) (Table 3). The sensitivity and specificity of ELISPOT response in predicting a significant CMV viremia were, respectively, 69.5% (95% CI 46.9–85.9%) and 11.8% (95% CI 7.1–18.8%), with a negative predictive value of 69.5% (95% CI 46.9–85.9%); however, the positive predictive value was 11.8% (95% CI 7.1–18.8%).

CMV ELISPOT and acute rejection

In our cohort, we observed 52 cases of all grade acute rejection: even if the incidence of all grade acute rejection was higher among ELISPOT non-responder patients, we did not observe any statistical difference with responders (respectively, responders 42/135, 31.1% and non-responders 10/23, 43.4%, $p = 0.336$, OR 0.587, 95% CI 0.23–1.44), either considering the overall population or looking to each post-transplant evaluation during the follow-up (Table 3). The sensitivity, specificity, negative and positive predictive values of ELISPOT in predicting an acute rejection (all grade) were, respectively, 80.7% (95% CI 67.0–89.9%), 12.1% (95% CI 6.9–20.4%), 56.5% (95% CI 34.8–76.1%), and 31.1% (95% CI 23.5–39.7%).

CMV ELISPOT and CMV IgG titre

CMV IgGs were dosed at each follow-up evaluation. We observed a significantly higher CMV IgG titre among ELISPOT responder patients

($p < 0.0001$); this difference was present already at the first month of follow-up ($p = 0.0001$) but was lost from the fourth month evaluation, and this is probably due to the fact that the number of non-responders progressively decreased during the observation period (Table 3).

Those patient non-responders who became responders increased their CMV IgG titre ($p < 0.0003$), tripling their mean IgG titre with an increase ranging from 52.4 (±70.5) IU/mL to 184.5 (±90.3) IU/mL.

CMV ELISPOT and CMV IgG serostatus concordance

At the first month of follow- up 22 patients were responders and all of them were CMV seropositive (100%); conversely, among non-responders only 38.4% were CMV seropositive (5/13 non-responder patients). During the follow-up, the vast majority of responders were IgG positive, with a percentage greater than 90% in all cases. On the contrary, among non-responders IgG seropositivity was highly variable. Concordance among CMV serology and ELISPOT assay response was moderate (Cohen's K 0.524) with a percentage of agreement of 89.8% (Table 4).

CMV IgG serostatus and CMV infections

In our cohort, 43.6% of CMV asymptomatic pulmonary infection episodes were recorded among CMV IgG seropositive patients, whereas only 14.1% of CMV viremia episodes were observed in CMV IgG seropositive patients. Compared to CMV ELISPOT assay, CMV IgG seropositivity correlates positively with CMV asymptomatic pulmonary infection episodes ($p = 0.026$); no correlations were found considering CMV viremia episodes (Table 5).

ELISPOT response and CMV serostatus variations

The assay response changed during the observation period: at the first month post-LTx 22 patients were classified, respectively, as responders (52.3%) and 13 as non-responders (30.9%). In eight cases specimens were not suitable for ELISPOT assay due to low cell viability, and consequently this group of patients was excluded from the one-year follow-up statistical analysis. All 22 responders maintained their CMV response status; after 1 year all but one (12/13) non-responder patients changed their status, being responders (Figure 1). Among the 12 non-responders becoming responders, we observed that four patients changed their status after an

Table 1. Demographics: patients' characteristics and evaluated samples.[10]

	Number	Percentage
Patients included	43	
Gender (female)	21	49%
Mean age at transplant ±SD (in years)	48.2 ± 15.4	
Type of transplant		
Bilateral lung transplant	37	86%
Single lung transplant	4	10%
Liver/bilateral lung transplant	1	2%
Heart/bilateral lung transplant	1	2%
Indication for transplantation		
COPD	14	33%
CF	9	21%
IPF	8	19%
Pulmonary arterial hypertension	3	7%
Lymphangioleiomyomatosis	2	4%
A1AT deficiency	2	4%
Other	5	12%
CMV serostatus		
D+/R+	27	63%
D+/R–	9	21%
D–/R+	6	14%
D–/R–	1	2%
Evaluated samples		
BAL	167	
TBLB	167	
Blood samples	1134	

A1AT, alpha-1-antitrypsin deficiency; BAL, bronchoalveolar lavage; CF, cystic fibrosis; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; SD, standard deviation; TBLB, transbronchial lung biopsy.

Table 2. Donor (D) and recipient (R) episodes of pulmonary asymptomatic infections, pneumonia and viremias.[10]

	D+/R+	D+/R−	D−/R+	D−/R−	Total
Patients	**27 (63%)**	**9 (21%)**	**6 (14%)**	**1 (2%)**	**43**
Pulmonary asymptomatic infections					
Patients	17/27 (63%)	9/9 (100%)	4/6 (66%)	0/1 (0%)	30/43 (69.8%)
Episodes	32/167 (19%)	13/167 (7%)	6/167 (3%)	0/167 (0%)	51/167 (30.5%)
Pneumonia					
Patients	2/27 (7%)	0/9 (0%)	0/6 (0%)	0/0 (0%)	2/43 (4.7%)
Episodes	2/167 (1%)	0/167 (0%)	0/167 (0%)	0/167 (0%)	2/167 (1%)
Viremia					
Patients	9/27 (33%)	8/9 (88%)	2/6 (33%)	0/1 (0%)	19/43 (44%)
Episodes	13/695 (1.8%)	16/248 (6.4%)	4/162 (2.4%)	0/29 (0%)	33/1134 (2.9%)

asymptomatic CMV pulmonary infection (4/11, 36.4%), three after a significant CMV viremia (3/11, 27.2%) and four after both an asymptomatic CMV pulmonary infection and a significant CMV viremia (4/11, 36.4%). Interestingly, we observed that the change in serology was associated with an increase of IgG titre: in fact, the mean CMV IgG titre changed both in those patients who experienced a CMV infection ($p = 0.009$) and in the only patients who did not report any infection at follow-up controls (CMV IgG non-responder 21 IU/mL, responder 250 IU/mL). Finally, we did not observe differences of mean CMV IgG titres after asymptomatic CMV pulmonary infection, CMV significant viremia or both ($p = 0.269$), neither considering the difference of CMV IgG titres before and after the ELISPOT status change (Table 3).

The IgG titre changed during the observation period: at the first month observation, 74.4% were IgG positive (32/43 patients) and the prevalence of seropositive increased during the year, achieving 97.4% at the 12-month evaluation; on the other hand, only one patient maintained his seronegative status for the whole observation period (Figure 2).

Considering cases of asymptomatic CMV pulmonary infection, we observed that the incidence was higher in seropositive patients during the whole period of observation, given that only two episodes occurred in seronegative patients ($p = 0.003$). As in the case of CMV viremia, we observed that the vast majority of significant CMV viremia episodes occurred in seropositive patients (only one case at the fourth month evaluation in a seronegative non-responder patient) (Table 6).

Discussion

This study aimed to describe and to evaluate the changes of CMV cellular and humoral immunity during 1 year of combined CMV prophylaxis treatment in lung transplanted patients. This retrospective analysis was designed to evaluate if the combined pharmacological and CMV hyperimmune globulin administration allowed the development of a full immunological response after the occurrence of CMV pulmonary asymptomatic infection and/or viremia.

We did not observe any statistically significant difference among both ELISPOT responders and non-responders with asymptomatic pulmonary infections, demonstrating that neither the cellular nor the humoral immune response can avoid CMV replication within the alveolar environment. On the other hand, our combined prophylaxis scheme seems to drive a 'controlled' CMV replication, as previously demonstrated, with a negligible incidence of pneumonia.[10] The higher incidence of CMV pulmonary infections detected

Table 3. Differences of CMV manifestations, acute rejection and CMV IgG titre among ELISPOT responders and non-responders.

Asymptomatic pulmonary infection	Responder	Non-responder	Total	*p* value	OR, 95% CI
I month	5/22 (22.7%)	1/13 (7.7%)	6/35 (17.1%)	0.377	0.283, 0.02–2.74
IV month	11/35 (31.4%)	1/7 (14.3%)	12/42 (28.5%)	0.651	2.750, 0.29–25.67
VIII month	13/40 (32.5%)	0/2 (0%)	13/42 (30.9%)	0.562	n.a.
XII month	13/38 (34.2%)	1/1 (100%)	14/39 (35.8%)	0.358	n.a.
Total	42/135 (31.1%)	3/23 (13%)	45/158 (28.4%)	0.085	3.010, 0.84–10.68
Viremia					
I month	0/22 (0%)	3/13 (23.1%)	3/35 (8.5%)	**0.043**	n.a.
IV month	3/35 (8.5%)	3/7 (42.8%)	6/42 (14.3)	**0.047**	0.125, 0.01–0.84
VIII month	8/40 (20%)	½ (50%)	9/42 (21.4%)	0.386	0.250, 0.01–4.44
XII month	5/38 (13.1%)	0/1 (0%)	5/39 (12.8%)	1.000	n.a.
Total	16/135 (11.8%)	7/23 (30.4%)	23/158 (14.5%)	**0.028**	0.307, 0.10–0.86
Acute rejection					
I month	10/22 (45.4%)	6/13 (46.1%)	16/35 (45.7%)	1.000	0.972, 0.24–3.84
IV month	14/35 (40%)	2/7 (28.5%)	16/42 (38.1%)	0.689	1.666, 0.28–9.82
VIII month	12/40 (30%)	1/2 (50%)	13/42 (30.9%)	1.000	0.428, 0.02–7.43
XII month	6/38 (15.7%)	1/1 (100%)	7/39 (17.9%)	0.179	n.a.
Total	42/135 (31.1%)	10/23 (43.4%)	52/158 (32.9%)	0.336	0.587, 0.23–1.44
Mean IgG (in IU/mL) ± SD					
I month	209.42 ± 52.93	60.92 ± 80.51		**0.0001**	
IV month	213.83 ± 68.80	168.57 ± 106.53		0.156	
VIII month	223.20 ± 55.16	240.50 ± 13.44		0.664	
XII month	222.73 ± 42.74	7		n.a.	
Total	218.24 ± 55.42	106.96 ± 105.71		<**0.0001**	

CI, confidence interval; CMV, cytomegalovirus; ELISPOT, enzyme-linked immunospot; IgG, immunoglobulin G; IU, international units; n.a., not applicable; OR, odds ratio; SD, standard deviation.

in seropositive patients (even if not statistically significant) could be due to the higher prevalence of seropositive patients, especially in the latest phases of the follow-up, and seems to step down the role of serostatus in predicting the CMV risk.

Conversely, we observed that responders had a significantly lower incidence of CMV viremia at the first and fourth month after LTx (the statistical significance was lost in later control because of the small number of non-responders) suggesting that cellular immunity can significantly reduce or stop the extra-alveolar spread-out of CMV in the bloodstream. Our data are similar to previous published data demonstrating that monitoring CMV-specific ELISPOT in the early phase of kidney transplant

Table 4. ELISPOT assay response and CMV IgG serology concordance.

		Responder	Non-responder	Total	Kappa, SE, 95% CI	Agreement
I month	CMV IgG positive	22/22 (100%)	5/13 (38.4%)	27/35 (77.1%)		
	CMV IgG negative	0/22 (0%)	8/13 (61.6%)	8/35 (22.9%)		
	Total	22	13	35	0.667, 0.137, 0.398–0.937	85.714%
IV month	CMV IgG positive	32/35 (91.4%)	5/7 (71.4%)	37/42 (88.1%)		
	CMV IgG negative	3/35 (8.6%)	2/7 (28.6%)	2/42 (11.9%)		
	Total	35	7	42	0.225, 0.246, 0–0.708	80.952%
VIII month	CMV IgG positive	39/40 (97.5%)	2/2 (100%)	41/42 (97.6%)		
	CMV IgG negative	1/40 (2.5%)	0/2 (0%)	1/42 (2.4%)		
	Total	40	2	42	n.a.	95.121%
XII month	CMV IgG positive	38/38 (100%)	0/1 (0%)	38/39 (97.4%)		
	CMV IgG negative	0/38 (0%)	1/1 (100%)	1/39 (2.6%)		
	Total	38	1	39	1.000, 0.000, 1.000–1.000	100.000%
Total	CMV IgG positive	131/135 (97.0%)	12/23 (52.1%)	143/158 (90.5%)		
	CMV IgG negative	4/135 (3.0%)	11/23 (47.9%)	15/158 (9.5%)		
	Total	135	23	158	**0.524, 0.112, 0.303–0.745**	89.873%

CI, confidence interval; CMV, cytomegalovirus; ELISPOT, enzyme-linked immunospot; IgG, immunoglobulin G; n.a., not applicable; SE, standard error.

Table 5. CMV manifestations episodes in CMV seropositive and seronegative patients.

Asymptomatic pulmonary infection	**IgG positive**	**IgG negative**	**Total**	***p* value**	**OR, 95% CI**
I month	6/32 (18.7%)	1/11 (9.1%)	7/43 (16.2%)	0.656	2.307, 0.24–21.65
IV month	12/37 (32.4%)	0/6 (0%)	12/43 (27.9%)	0.162	n.a.
VIII month	13/41 (31.7%)	0/1 (0%)	13/42 (30.9%)	1.000	n.a.
XII month	14/38 (36.8%)	0/1 (0%)	14/39 (35.8%)	1.000	n.a.
Total	45/103 (43.6%)	1/19 (5.2%)	46/167 (27.5%)	**0.026**	7.864, 1.01–60.71
Viremia					
I month	2/32 (6.25%)	1/11 (9.1%)	3/43 (6.9%)	1.000	0.666, 0.05–8.16
IV month	5/37 (13.5%)	1/6 (16.6%)	6/43 (13.9%)	1.000	0.781, 0.07–8.14
VIII month	9/41 (21.9%)	0/1 (0%)	9/42 (21.4%)	1.000	n.a.
XII month	5/38 (13.1%)	0/1 (0%)	5/39 (12.8%)	1.000	n.a.
Total	21/148 (14.1%)	2/19 (10.5%)	23/167 (13.7%)	0.748	1.405, 0.30–6.53

CI, confidence interval; CMV, cytomegalovirus; IgG, immunoglobulin G; n.a., not applicable; OR, odds ratio.

could identify those patients with a higher risk of CMV infection development.[22]

We also observed a significantly higher CMV IgG titre among responders at the first month of follow-up, and this result seems to be related to the development of a complete immune response, but once again the statistical significance was lost at subsequent controls, in this case probably because of immunosuppressive treatment effects and a reduction of non-responder patients. Indeed, at the first month evaluation all the 22 responders were CMV seropositive (100%); conversely, among 13 non-responders only 38.4% were CMV seropositive. This could probably be due either to an immunosuppressive effect on cellular immunity, or a far CMV contact before the lung transplantation with relevant reduction of cellular response.[23]

The ELISPOT responder status, even CMV seropositivity, seems to be efficacious in limiting the CMV viremia episodes: in our cohort only 14% of seropositive patients experienced CMV viremia during the follow-up; this incidence is similar to that reported among ELISPOT responder patients (11.8%) and the incidence trend is similar during each follow-up evaluation, confirming the tight connections between humoral and cellular response already demonstrated by other authors.[12]

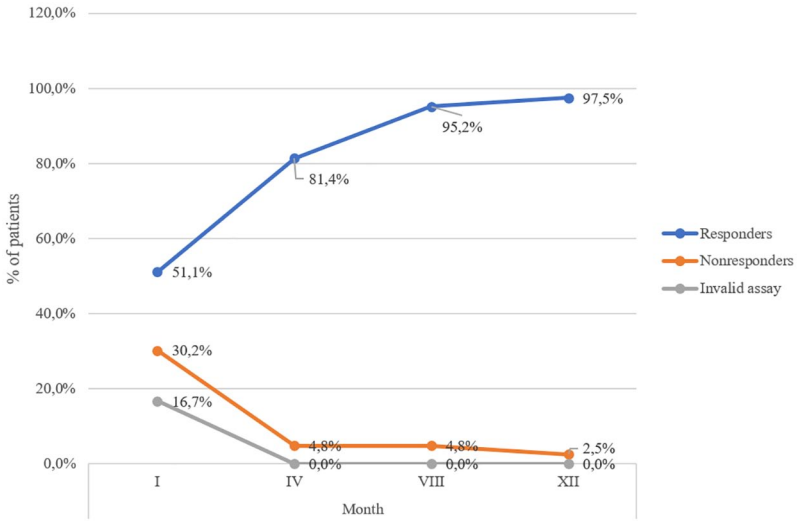

Figure 1. CMV ELISPOT assay response over time.
CMV, cytomegalovirus; ELISPOT, enzyme-linked immunospot.

It is well known that donor and recipient CMV serostatus is widely used for risk stratification and the management of patients undergoing solid organ transplantation;[23] however, a recently published survey demonstrated a heterogenous prevention practice across European centres.[24] CMV serological measurements and seroconversion evaluation during follow-up are predictive of subsequent CMV disease, with a protective effect of

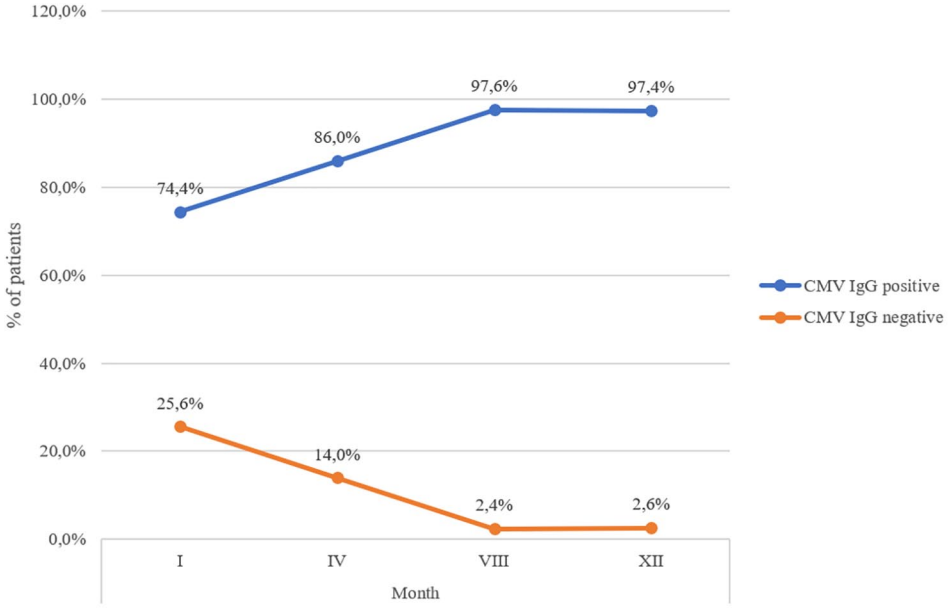

Figure 2. CMV IgG serostatus over time.
CMV, cytomegalovirus.

Table 6. CMV manifestation: ELISPOT assay response and CMV serology.

Asymptomatic pulmonary infection		Responder	Non-responder	Total	*p* value	OR, 95% CI
I month	CMV IgG positive	5/22 (22.7%)	1/5 (20%)	6/27 (22.2%)		
	CMV IgG negative	0/0 (0%)	0/8 (0%)	0/8 (0%)		
	Total	5/22 (22.7%)	1/13 (7.7%)	6/35 (17.1%)	n.a.	n.a.
IV month	CMV IgG positive	11/32 (34.3%)	0/5 (0%)	11/37 (29.7%)		
	CMV IgG negative	0/3 (0%)	1/2 (50%)	1/5 (20%)		
	Total	11/35 (31.4%)	1/7 (14.3%)	12/42 (28.5%)	0.083	n.a.
VIII month	CMV IgG positive	13/39 (33.3%)	0/2 (0%)	13/41 (31.7%)		
	CMV IgG negative	0/1 (0%)	0/0 (0%)	0/1 (0%)		
	Total	13/40 (32.5%)	0/2 (0%)	13/42 (30.9%)	n.a	n.a.
XII month	CMV IgG positive	13/38 (34.2%)	0/0 (0%)	13/38 (34.2%)		
	CMV IgG negative	0/0 (0%)	1/1 (100%)	1/1 (100%)		
	Total	13/38 (34.2%)	1/1 (100%)	14/39 (35.9%)	0.071	n.a.
Total	CMV IgG positive	42/131 (32.0%)	1/12 (8.3%)	43/143 (30.0%)		
	CMV IgG negative	0/4 (0%)	2/11 (18.2%)	2/15 (13.3%)		
	Total	42/135 (31.1%)	3/23 (13%)	45/158 (28.4%)	**0.003**	n.a.
Viremia						
I month	CMV IgG positive	0/22 (0%)	2/5 (40%)	2/27 (7.4%)		
	CMV IgG negative	0/0 (0%)	1/8 (12.5%)	1/8 (12.5%)		
	Total	0/22 (0%)	3/13 (23.1%)	3/35 (8.5%)	n.a.	n.a.
IV month	CMV IgG positive	3/32 (9.3%)	2/5 (40%)	5/37 (13.5%)		
	CMV IgG negative	0/3 (0%)	1/2 (50%)	1/5 (20%)		
	Total	3/35 (8.5%)	3/7 (42.8%)	6/42 (14.3)	n.a.	n.a.
VIII month	CMV IgG positive	8/39 (20.5)	1/2 (50%)	9/41 (21.9%)		
	CMV IgG negative	0/1 (0%)	0/0 (0%)	0/1 (0%)		
	Total	8/40 (20%)	1/2 (50%)	9/42 (21.4%)	n.a.	n.a.
XII month	CMV IgG positive	5/39 (12.8%)	0/0 (0%)	5/39 (12.8%)		
	CMV IgG negative	0/0 (0%)	0/1 (0%)	0/1 (0%)		
	Total	5/39 (12.8%)	0/1 (0%)	5/40 (10%)	n.a.	n.a.
Total	CMV IgG positive	16/132 (12.1%)	5/12 (41.6%)	21/144 (14.6%)		
	CMV IgG negative	0/4 (0%)	2/11 (18.2%)	2/15 (13.3%)		
	Total	16/136 (11.7%)	7/23 (30.4%)	23/159 (14.4%)	0.083	n.a.

CI, confidence interval; CMV, cytomegalovirus; ELISPOT, enzyme-linked immunospot; IgG, immunoglobulin G; n.a., not applicable; OR, odds ratio.

seropositive patients.[13] Nevertheless, a paired concordance evaluation between humoral and cellular CMV response was never evaluated. In our cohort we found a good concordance between ELISPOT assay results and CMV IgG serology: in particular, we found a moderate concordance (K 0.524) associated with an agreement of 89.8%. The agreement persists and is more than 80% during the whole observation period. These data are not surprising due to the conversion of the response (cellular and humoral) previously reported, but reflect a moderate concordance of results. In fact, 97% of ELISPOT responders are seropositive too; not so brilliant results were found among ELISPOT non-responders: seronegative patients showing a percentage lower than 50% (11 non-responder of 23 seronegative patients, 47.8%). These results suggest combining serostatus and ELISPOT response to understand better and predict the CMV infection risk, especially before the transplantation and during the first year of follow-up.

Interestingly, after 1 year all but one non-responder patients (12/13) changed status, becoming responders (Figure 1). A similar behaviour was observed in four patients after an asymptomatic CMV pulmonary infection, in three after a significant CMV viremia and in four after both an asymptomatic CMV pulmonary infection and a significant CMV viremia, without differences among CMV IgG mean titres. All but one of the patients changed their serostatus to IgG positive in 1 year. Finally, just one patient at 1 year was seronegative and a non-responder.

In our cohort seroconversion has a higher prevalence than previously reported (86% after 4 months and 97.4% after 1 year).[13] The change of ELISPOT response traces this seroconversion: the ratio increased from 51.1% of responders at the first month, to 97.5% after 1 year. First month ELISPOT evaluation results could be influenced by the presence of an invalid assay (16.7% of patients); notably, all these patients were ELISPOT responders at the fourth month evaluation. The conversion curves are quite similar even because the prevalence of seropositives among ELISPOT responders is high during the whole observation period.

Finally, we did not observe any statistical differences in acute rejection between responders and non-responders; this result could be related to the limited number of patients included in our study, even if we demonstrated a high sensitivity of ELISPOT responder status for acute rejection (sensitivity 80.7%). It should be carefully read: in fact, immune response to CMV primary infection and cellular acute rejection share multiple intracellular mechanisms, involving T-cell activation and IFN-γ production.[5,25]

This study has some limitations: first, its retrospective nature and the small number of subjects included in the statistical analysis do not allow definitive conclusions; therefore, this is one of the few published studies that systematically evaluated the ELISPOT assay through 1 year follow-up in lung transplanted patients. Moreover, at first, in our cohort we used a combined prophylaxis scheme with infusion of CMV-specific hyperimmune globulin; second, we do not have available data about patients' ELISPOT assay before lung transplantation. A study conducted on kidney transplanted patients demonstrated that monitoring the ELISPOT assay may be useful for predicting the post-transplantation risk of CMV infection and reactivation.[26] We had a qualitative ELISPOT assay response although the usefulness of these data in daily practice remains uncertain because the determination of cut-off values for qualitative assessment is still not clear for all solid organ transplantation.[5] Finally, in our centre we performed universal combined prophylaxis with the administration of CMV-specific hyperimmune globulins which could have influenced the CMV IgG titre of our patients, because during the follow-up we provide a monthly administration: the results concerning their effects on long-term survival and chronic lung allograft dysfunction are still not conclusive and further studies are needed.

On the other hand, the strength of this study is that this is the first study reporting 1 year changes in cellular and humoral CMV immunity of a lung transplantation cohort, in a real life setting, that points out that CMV immunoresponses have been developed in almost all patients who underwent a combined prophylaxis scheme with infusion of CMV-specific hyperimmune globulins.

Conclusion

From the clinical point of view our results suggest that CMV ELISPOT assay results defining the patients as responders and non-responders, during combined prophylaxis, could identify

non-responder patients at risk of CMV viremia independently of their donor/recipient serostatus. In particular, despite a moderate concordance with serology, the presence of a CMV ELISPOT positive response predicts a lower risk of early (at first and fourth month evaluation) CMV viremia after transplantation. On the contrary, the presence of a response at CMV ELISPOT assay does not seem to protect against asymptomatic pulmonary infections, allowing the development of a slow, 'controlled' immune response, with a 12-month seroconversion and a change in responder status in almost all the studied population. Even if our results are promising, up to now the ELISPOT assay cannot replace CMV serology for the stratification risk of CMV manifestations after lung transplantation.

Author contributions

PS and FP share the first authorship and contributed equally to the study. Design of the study: FP, PS, MB, MR, CC, RC, CA; acquisition of data: FP, PS, CA; interpretation of data: FP, PS, CAS, CA; drafting the manuscript: FP, PS, CAS, CA; critical revision of the manuscript: PS, CA, MB, MR, CC, RC.

Conflict of interest

The authors declare that there is no conflict of interest.

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Supplemental material

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