Development of an EliSPOT assay for HSV-1 and clinical validation in lung transplant patients.

[1,*]Cristina Costa, [1]Alessia Di Nauta, [1]Massimo Rittà, [1]Franca Sinesi, [1]Gabriele Bianco, [1]Francesca Sidoti, [2]Paolo Solidoro, [1]Rossana Cavallo.

[1]Microbiology and Virology Unit, Laboratory of Virology; [2]Division of Pneumology, Azienda Ospedaliero Universitaria Città della Salute e della Scienza di Torino, Corso Bramante 88 – 10126 Turin, Italy.

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***Corresponding author:**

Dr. Cristina Costa

Microbiology and Virology Unit

University Hospital Città della Salute e della Scienza di Torino

Corso Bramante 88 – 10126 Turin, Italy

Phone: +39(11)6335953 Fax: +39(11)6335194

E-mail: cristina.costa@unito.it

SUMMARY

Cellular immunity plays a relevant role in control of HSV-1 infection/reactivation with a potential impact on clinical-therapeutic management of immunocompromised patients, such as transplant recipients.

Herein, we quantitatively evaluated T-cell response directed at HSV-1 by a newly developed IFN-γ EliSPOT assay in 53 patients (including 45 lung transplant recipients and eight subjects in waiting list).

Overall, 62.2% of transplant patients and 62.5% of subjects in waiting list evidenced a response to HSV-1 with no significant difference in the level of virus-specific cellular immunity. Response tended to be lower in the first 3 months posttransplantation with progressive recovery of pretransplantation status by the second year and in the presence of HSV-1 DNA positivity in bronchoalveolar lavage. As expected, no response was found in seronegative patients. No significant difference in the level of response according to IgM and IgG status was found.

Further studies are required to define the role of HSV-1 specific immune response for the clinical-therapeutic management of lung transplant patients and in other clinical settings and to define cut-off levels discriminating between absence/low and strong response to be related to the risk of viral infection/reactivation.

Key words: herpes simplex virus type 1; cellular immune response; EliSPOT assay; lung transplantation.

Introduction

Herpes simplex virus type-1 (HSV-1) is a highly seroprevalent and ubiquitously distributed dsDNA virus belonging to the *Herpesviridae* family, α-herpesvirinae subfamily. Primary infection usually occurs early in the childhood and is followed by a lifelong latent infection in neurons of central ganglia, from which reactivation may occur. Whereas asymptomatic mucosal shedding is common and HSV-1 has been isolated from the saliva of 1-5% of healthy subjects (Tsakris and Pitiriga 2011), reactivation has been reported particularly in immunosuppressed and critical patients in which, beside classical presentation, visceral or disseminated disease can occur, including extensive mucocoutaneous involvement, hepatitis, meningoencephalitis, and pneumonitis (Tsakris and Pitiriga 2011; Simmoons-Smit et al. 2006; Costa et al. 2012c; Wilk et al. 2013; Preiser et al. 2003; Bonizzoli et al. 2016). As regards the lower respiratory tract, HSV-1 has been reported in 16 up to 32% of the cases (Bruynseels et al. 2003; van den Brink et al. 2004; Daubin et al. 2005; Luyt et al. 2007; Linssen et al. 2008) and has been increasingly associated to pulmonary diseases, with poor outcome and high mortality rates (Costa et al. 2012c; Luyt et al. 2007; Linssen et al. 2008; Ong et al. 2004; Engelmann et al. 2007; Gooskens et al. 2007; De Vos et al. 2009; Bouza et al. 2011; Scheithauer et al. 2010). Adaptive immunity plays a pivotal role in uncomplicated recovery from HSV infection, as evidenced by severe complications observed in immunocompromised individuals, although the kinetics and specificity of HSV-specific T-cells during primary infection are poorly unknown (Ouwendijk et al. 2013). After resolution of acute infection, memory T-cells are detected at moderate levels in blood of immunocompetent subjects, with a poly-specific T-cell response directed at distinct HSV-1 tegument and capsid proteins (Jing et al. 2012; Moss et al. 2012). Blood HSV-specific T-cells express high levels of cytolytic molecules and secrete IFN-γ upon antigenic recall (Ouwendijk et al. 2013); higher levels of IFN-γ production are associated with polyfunctionality of T-cells and better control of chronic viral infection (Merindol et al. 2012; Harari et al. 2006). Moreover, HSV-1-specific T-cells localize to sites of primary and recurrent infections, as well as latency sites, contributing to control viral latency and reactivation (Ouwendijk et al. 2013; Khanna et al. 2003; Gebhardt et al. 2009; Ariotti et al. 2012). Quantitative evaluation of HSV-1-specific T-cell response in blood compartment and the study of the relation between this and ability of controlling local reactivation in the lung could be relevant for the clinical management of immunocompromised patients at risk of severe pulmonary complications. At moment, no assay for evaluation of cellular immune response to HSV-1 is available, as well as no data on its potential

impact on clinical/therapeutic management of infection/reactivation in different categories of patients have been evaluated.

In the present study, we quantitatively evaluated T-cell responses directed at HSV-1 by an newly developed IFN-γ EliSPOT assay in a susceptible population such as lung transplant recipients and investigated the role of systemic virus-specific immunity in determining the risk of viral reactivation in the lower respiratory tract.

Materials and methods

Subjects and specimens

Cellular immune response to HSV-1 was evaluated in an observational, longitudinal and prospective study by IFN-γ EliSPOT assay on peripheral blood mononuclear cell (PBMC) specimens from all lung transplant recipients admitted to the University Hospital "Città della Salute e della Scienza di Torino", Turin, Italy (Regione Piemonte Transplant Centre) over a two-year period. The Lung Transplant Centre of the Piemonte region is the first in Italy for activity volume. Overall, 53 patients (M/F, 33/12; mean age ± standard deviation, 47.8 ± 15.2 years; range, 16-69), including 45 lung transplant recipients in the first two years posttransplantation and eight subjects in waiting list were prospectively evaluated. In this study population, one (in subjects in waiting list) or at least three (in transplant patients) PBMC specimens were collected, accounting for an overall number of 168 samples (160 from transplant recipients, including 81 from 27 patients with three evaluations, 64 from 16 patients with four evaluations, and 15 from three patients with five evaluations; eight specimens from individuals in waiting list). A pre-transplant evaluation of HSV-1 cellular immune response was also obtained for all the patients, but three. Pretransplant serological data for HSV-1/2 (IgG and IgM serostatus) were extrapolated from the local Transplant Registry and were available for all patients, in particular five individuals IgG-negative and 48 IgG–positive, with five subjects being IgM-positive. Baseline characteristics of the enrolled patients are reported in Table 1. Moreover, 42 healthy seropositive individuals (IgG-positive, IgM-negative), including 38 without recurrent HSV-1 infection and four with at least one episode of HSV-1 infection (herpes labialis) in the previous 12 months, were also studied by a single EliSPOT determination.

All subjects provided written informed consent and the study was approved by the institutional review board. According to our lung transplant center's practice, all patients received prophylaxis for HSV consisting in administration of acyclovir (400 mg twice daily; to be reduced in case of kidney failure or suspended in case of ganciclovir or valganciclovir treatment for CMV). In addition, all patients received a universal, prolonged and combined anti-viral prophylaxis for CMV, irrespective of serological matching donor/recipient, consisting in the administration of ganciclovir or valganciclovir (450 mg twice daily) from day 21 posttransplantation for 3 weeks associated to CMV-Ig (Cytotect Biotest) at days 1, 4, 8, 15, and 30 (1.5 ml/kg body weight) and monthly up to 2 years posttransplantation, according to local practice. Long-term immunosuppression was maintained with tacrolimus or cyclosporine A (in patients with cystic fibrosis as underlying

disease), mycophenolate mofetil and prednisone (to be tapered or discontinued). Follow-up surveillance bronchoscopies (with bronchoalveolar lavage [BAL] and transbronchial biopsy) were scheduled at 1, 3, 6, 9, 12, 18, and 24 months posttransplantation, for the evaluation of rejection and infections in the lower respiratory tract, as previously described (Costa et al. 2012a; Costa et al. 2008; Costa et al. 2011; Costa et al. 2012b). Therefore, virological data for HSV-1 were available on BAL specimens concomitantly collected with samples for EliSPOT assay in all the cases. HSV-1 was evaluated on BAL specimens by real-time PCR using a commercially available kit (HSV-1 ELITe MGB® kit, ELITechGroup) following automated extraction with the Qiasymphony (Qiagen, Hilden, Germany) instrument. Rapid shell vial isolation with indirect immunofluorescence for HSV-1 was also performed, as previously described (Costa et al. 2007).

IFN-γ EliSPOT assay

HSV-1 antigenic stimulus consisted of a freeze-thaw/sonicated viral lysate prepared from expanded long-term cultures of Vero cells (kidney epithelial cells from African green monkey, as previously described (Terlizzi et al. 2009), infected with the Human herpesvirus 1 ATCC® VR-260™ [American Type Culture Collection, Manassas, VA, USA]). Aliquots of viral preparation were stored at -80°C until use. For virus titration, 96-well plates at 60-80% confluence of Vero cells were inoculated with 100 µl of 10-fold diluted virus for $TCID_{50}$ assay, obtaining an end-point titer of 3.16×10^8 $TCID_{50}$/ml. Sonication included thawing of the virus in ice and 3 cycles at 20% intensity for 30 seconds using the Sonopuls Ultraschall-Homogenisatoren instrument (Bandelin electronic GmbH, Berlin, Germany). Subsequently, the virus underwent a through UV irradiation for inactivation, with two cycles per transilluminator set at 1.2 J/cm^2. UV inactivation was carried out also on the RPMI 1640-medium (Sigma-Aldrich, St. Louis, MO, USA), used for the EliSPOT assay (see below). In order to ascertain the effective inactivation of the virus, a rapid shell vial culture assay followed by indirect immunofluorescence was performed, as previously described (Costa et al. 2007), and resulted negative (Figure 1). For antigenic stimulus, serial dilutions from 10^6 up to 10^3 of the inactivated virus, starting at 3.16×10^8 $TCID_{50}$/ml were used. Dose response curves were performed with the lysate preparation to determine the amount of antigenic stimulus to use in the IFN-γ EliSPOT assay: in particular, on PBMCs obtained from four healthy controls and two lung transplant recipients.

Whole blood was collected directly into CPT Vacutainer tubes (BD, Franklin Lakes, NJ, USA) and PBMCs were separated by density gradient sedimentation according to manufacturer instructions, with minor modifications. Briefly, blood samples were centrifuged at 1800 g for 20 min at room temperature. The resulting mononuclear cell fraction was washed twice with phosphate buffered saline (PBS 1x, pH 7.4). Resulting PBMCs were cryopreserved in fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) with 10% dimethyl sulfoxide, placed into Nalgene Cryovials (Nalge Nunc, Rochester, NY, USA) at -80°C for ≥24 h prior to transfer to liquid nitrogen for long-term storage.

The IFN-γ EliSPOT assay was performed as described elsewhere (Costa et al. 2012b). Briefly, PBMCs were thawed in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum and 1% l-glutamine, washed twice and rested for at least 4 h in complete RPMI-1640 at 37°C, 5% CO_2, before assay. Subsequently, cell viability and count were assessed by trypan blue staining in Burker's chamber to a final concentration of 2 x 10^6 cells/ml. Peripheral blood mononuclear cells were plated at 2 x 10^5 cells/well onto a 96-well microplate pre-coated with anti-human IFN-γ monoclonal antibody (EliSPOT Interferon-γ Basis Kit; AID, Strassberg, Germany) and incubated with viral preparations, as described above. For negative and positive controls, cells incubated with supplemented RPMI -1640 medium alone and with 1 μg/ml phytohemagglutinin mitogen (supplied by ELITechGroup, Milan, Italy) were used, respectively. Following a 18-20 h incubation at 37°C, 5% CO_2, the microplates were washed 8-times with washing buffer and incubated with biotinylated anti-human IFN-γ mAb at 1 μg/ml in VP buffer at room temperature in a wet chamber, in the dark, for 2 h. Subsequently, the microplates were washed 8-times with washing buffer and incubated with streptavidin-horseradish peroxidase solution diluted 1:1000 in blocking buffer. Following another washing step, as before, substrate solution (tetramethylbenzidine) was added for colour development at room temperature in the dark for 12-15 min. The chromogenic reaction was stopped by extensive washing with tap water and microplates were allowed to completely dry before analysis. Results were analyzed using a computer-assisted system (AID EliSPOT Reader System, AID). Data were expressed as spot-forming units (SFU)/2 x 10^5 cells, with each spot representing a single cell that produces IFN-γ, calculated by subtracting the mean of SFU obtained in unstimulated negative control from the mean SFU obtained in the antigen-stimulated wells.

Statistical analysis

For descriptive statistics, data were expressed as raw number and percentage. For statistical analysis, chi square, t test, and analysis of variance (ANOVA, followed by Bonferroni post-test) were applied, as appropriate. A p value <0.05 was considered significant. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, USA).

Results

Validation of the EliSPOT assay

Based on dose-response curves on preliminary EliSPOT assays performed in triplicate on PBMCs from four IgG positive healthy controls and two IgG-positive lung transplant patients, serial HSV-1 lysate dilutions at 3.16 x 10^4 and 3.16 x 10^3 $TCID_{50}$/mL were associated to more robust and reproducible responses, even though not at statistical level (p = n.s.)(Figure 2). Therefore, these dilutions were used as antigenic stimuli for subsequent HSV1-EliSPOT evaluations on specimens from study population. The results evidenced that 3.16 x 10^3 $TCID_{50}$/mL HSV-1 stimulus was associated to higher responses in comparison to 3.16 x 10^4 $TCID_{50}$/mL (mean SFU/2 x 10^5 PBMCs ± SD; 3.765 ± 5.516 versus 2.662 ± 4.531, p=0.048), when considering samples from posttransplantation setting (peak value of response for each patient)(Figure 3).

Clinical evaluation

Overall, 28/45 (62.2%) transplant patients and 5/8 (62.5%) patients in waiting list evidenced a positive response to HSV-1 lysate, with level of response ranging from 1 to 211 and from 8 to 53 SFU/2 x 10^5 PBMCs, respectively. No significant difference of response was found between samples from pre- and post-transplant patients, considering both all specimens (mean SFU/2 x 10^5 PBMCs ± SD, 8.9 ± 15.9 versus 7.2 ± 25.6; p = 0.870) and specimens with peak value from each patient (10.4 ± 18.1 versus 5.15 ± 25.6; p = 0.120). Subsequently, as the main risk of HSV-1 infection is in the first period posttransplantation (up to 3 months, particularly the first 30 days), we compared the degree of specific cellular response in specimens collected in the first 3 months (overall, 10 specimens from as many patients) versus those collected at > 3 months and found no significant differences, although mean values of SFU/2 x 10^5 PBMCs tended to be lower in the early period in comparison to >3 months (mean ± SD, 2.7 ± 5.5 versus 7.4 ± 27.3; p = 0.638).

Among healthy seropositive individuals, 39/42 (83.3%) evidenced a positive response to HSV-1 lysate (37 with no episode of recent HSV-1 infection in the previous 12 months, two with two episodes of herpes labialis), with level of response ranging from 8 to 36 SFU/2 x 10^5 PBMCs; in three subjects no response was found (one with no episode of HSV-1 infection and two with an episode of herpes labialis in the previous 12 months). No significant difference of response was found between individuals with and without HSV-1 infection.

Considering serostatus, as expected, no response was found in seronegative patients, whereas a mean level of 11.2 SFU/2X10^5 PBMCs (range, 0-211; median, 3) was found in seropositive patients, with no significant difference in IgM-positive versus IgM-negative patients (mean SFU/2 x 10^5 PBMCs ± SD, 3.5 ± 4.7 versus 11.6 ± 33.6; p = 0.585). In Table 2, HSV-1 responses to different antigenic concentrations according to IgG serostatus are reported.

In order to investigate the kinetics of HSV-1-specific T-cell immunity after lung transplantation, EliSPOT data were evaluated at different time points, including prior to transplantation, at 1 month and at 6-month intervals posttransplantation. The pattern of HSV-1 specific cellular immune response evidenced a decrease in the first months posttransplantation in comparison to pretransplantation levels; this was seen with both 3.16 x 10^3 and 3.16 x 10^4 $TCID_{50}$/mL antigenic stimuli (mean SFU/2 x 10^5 PBMC ± SD, 2.889 ± 5.061 versus 9.5 ± 16.10, and 1.0 ± 2.0 versus 8.2 ±15.30, respectively), with progressive recovery of pretransplantation levels at the end of the second year posttransplantation (5.444 ± 8.819 versus 4.222 ± 6.685, for 3.16 x 10^3 and 3.16 x 10^4 $TCID_{50}$/mL HSV-1 stimuli, respectively)(Figure 4, A and B). This kinetics was observed also when excluding patients with HSV-1 DNA positivity on BAL specimens in concomitance with the EliSPOT determinations (n=7)(Figure 4, C and D).

Seven lung transplant recipients (15.6%) exhibited at least one episode of HSV-1 lower respiratory tract infection (as determined by molecular detection of HSV-1 DNA on BAL specimens [Costa et al. 2012c]), concomitant to the available EliSPOT assays. All cases of HSV-1 infection occurred in IgG-positive recipients, likely due to viral reactivation. In these patients, the level of HSV-1 cellular immunity tended to be lower in comparison to patients with no HSV-1 DNA positivity, even though not reaching statistical significance (mean EliSPOT values: 1.143 ± 0.5533 versus 3.967 ±0.7295; p=0.1986).

In order to assess the impact of pulmonary events of HSV-1 replication on subsequent virus-specific immunity induction, BAL determinations performed in a 6-month period prior to the available EliSPOT assays were retrospectively investigated. Six patients exhibited a history of at least one episode of pulmonary HSV-1 replication in this period; in these patients, HSV-1 EliSPOT response tended to be higher in comparison to patients with no evidence of lower respiratory tract infection in the same interval (mean SFU/2 x 10^5 PBMC, 8.167 ± 3.229 versus 3.568 ± 0.7953, p=0.0656, using 3.16x10^3 $TCID_{50}$/mL as antigenic stimulus). Moreover, no relation was found between HSV-1 EliSPOT responses and the occurrence of HSV-1 positivity in the subsequent 6-month period.

Discussion

In this study, cellular immune response to HSV-1 was evaluated by a newly developed IFN-γ EliSPOT assay. Whereas Posavad and colleagues described an EliSPOT assay for HSV-2 to be used in vaccine development (Posavad et al. 2011), an assay specifically designed for HSV-1 has not been reported in literature and its availability could be useful for defining the role of cellular immunity in the development and outcome of HSV-1 infection/reactivation, as well as in its clinical and therapeutic management. Immunocompromised patients, such as transplant recipients, present more frequent and severe clinical manifestations of HSV-1 infection, as well as decreased responses to anti-viral treatment (Wilk et al. 2013). In most of the cases, symptomatic HSV-1 disease in adult transplant recipients results from viral reactivation, particularly in the first month following transplantation (Fishman 2007). Among other clinical manifestations, including disseminated mucocutaneous disease, esophagitis and hepatitis, pneumonitis is described in all solid organ transplant patients, but most commonly in lung and heart-lung transplant patients (Smyth et al. 1990). The kinetics and specificity of HSV-1 T-cell immune response during primary infection are poorly known in humans. Following resolution of acute episode, specific memory T-cells are found at moderate levels of 0.1-1% in immunocompetent individuals (Ouwendijk et al. 2013; Jing et al. 2012; Moss et al. 2012). In healthy individuals, a complex and poly-specific CD4+ and CD8+ response towards more than 70 different proteins has been identified, including proteins abundantly present in the virion (e.g. viral envelope, tegument, capsid) and regulatory proteins (Jing et al. 2012; Merindol et al. 2012; Harari et al. 2006; Jing et al. 2013). HSV-1 specific T-cells localize to sites of primary, recurrent and chronic latent infections from which reactivation may occur in favoring conditions, such as immunosuppression. Several studies have demonstrated that the outcome of these infections depends on the efficacy of specific cellular immune response (Remakus and Sigal 2013; Sant and McMichael 2012; Calarota et al. 2015) and that the development of quantitative, sensitive and reproducible assays for evaluation and monitoring of virus-specific T-cell response is fundamental to investigate kinetics of HSV-1-specific immunity and in the clinical-therapeutic management of immunocompromised patients.

Among methods developed for evaluating virus-specific T-cell response, the EliSPOT assay allows for measurement of quantity and functionality of specific T-cells and can be used to define the whole repertoire of cellular responses without MHC-restriction. EliSPOT assay detects production of IFN-γ by PBMCs following stimulation with specific antigens and enumerates responsive cells using anti-IFN-γ monoclonal antibodies coated onto 96-well plates and a second enzyme-

conjugated monoclonal antibody; spots are counted using automated EliSPOT readers with each spot representing a single specific cell (Calarota et al. 2015).The most common antigenic stimuli used for EliSPOT assay are pools of overlapping peptides, peptide libraries spanning entire proteins or viral lysates. Given the antigenic complexity of herpesviruses which contain multiple potential protein targets recognized by CD4+ cells and the dose-response curves obtained on preliminary EliSPOT assays, in this study a viral lysate preparation at 3.16×10^4 and 3.16×10^3 $TCID_{50}$/mL dilutions was used.

By using these two dilutions of inactivated virus, we found that 3.16×10^3 $TCID_{50}$/mL HSV-1 stimulus was significantly associated to higher level of response in comparison to 3.16×10^4 $TCID_{50}$/mL. This difference was evidenced in almost all cases with very few exceptions and considering those collected in both the pre- and post-transplant settings; it could be hypothesized that this is due to the degree of saturation of binding sites.

As regards HSV-1 specific cellular immune response in study population, there was no significant difference in its level between the pre- and post- transplant period. As the higher risk (Fishman 2007) of HSV-1 reactivation is in the very first months (particularly up to 30 days), we evaluated whether this could be attributable, at least partly, to a lower degree of virus-specific cellular immune control. Although the difference was not significant, a tendency to lower levels of response in the first period was found; of course, it should be taken into account the small number of specimens that could have limited the statistical power of these data and the need for increasing the study group. Moreover, it has to be underlined that we consider cumulative data from all the specimens available for a certain period of time posttransplantation, giving the differne tnumebr of samples available at different time points.

Knowledge of HSV-1 serostatus, as well as of cell-mediated immune response, may be of great concern to stratify patients at major risk for primary HSV-1 acquisition – either from the allograft or from natural sources – after transplantation, which may be more clinically severe and prolonged due to lack of immunologic memory (Wilck and Zuckerman 2013; Nichols et al. 2003). As expected, no response was found in seronegative patients; on the other side, when considering seropositive patients, no significant difference in the degree of virus-specific response was found between IgG- and IgM-positive individuals, although values tended to be higher in patients with a serological status suggesting previous infection. This observation supports the hypothesis that a higher level of response is achieved following immunological boosting of memory T-cell, as already reported for

cytomegalovirus (Costa et al. 2014a; Rittà et al. 2015; Abate et al. 2010), Epstein-Barr virus (Rittà et al. 2015) and polyomavirus BK (Costa et al. 2014b).

Given the occurrence of HSV-1 infection/reactivation in the lower respiratory tract and the potential impact in the presence of impaired immune responses, as reported for other herpesviruses (Costa et al. 2007), a study population of lung transplant recipients was chosen for clinical validation of the developed HSV-1 EliSPOT assay and evaluation of kinetics of specific cellular immune response. As expected, a decrease (although not significant) in the level of response in the first months posttransplantation was found in comparison to pretransplantation levels, with progressive recovery of these levels along a period ranging from 3 months to 2 years posttransplantation.

As regards HSV-1 infection in the lower respiratory tract, as evidenced by positivity to HSV-1 DNA on BAL specimens, all the cases occurred in IgG-positive patients, thus representing viral reactivation. In terms of impact of the level of HSV-1 specific cellular immune response on viral reactivation, although not statistically significant, a tendency to lower levels in the seven patients with at least one episode of infection was observed, with values even lower in the presence of repeated episodes. Moreover, as these data referred to concomitant evaluation of HSV-1 DNA on BAL and EliSPOT assay, we also assessed the impact of pulmonary HSV-1 infection on subsequent level of virus-specific cellular immune response by retrospectively investigating BAL determinations in a 6-month period prior to the available EliSPOT assay. Interestingly, in patients with at least one episode of pulmonary HSV-1 infection in comparison to those with no infection, the degree of cellular immune response tended to be higher, thus supporting the boosting effect of viral replication on the development of HSV1-specific immunity.

In conclusion, we have evaluated T-cell responses directed at a HSV-1 in lung transplant patients by a newly developed, specific and quantitative IFN-γ EliSPOT assay and investigated the immunological status and kinetics. The availability of this assay could allow for a patient's tailored clinical-therapeutic management in terms of modulation of immunosuppressive therapy and use of antiviral agents in the presence of HSV-1 infection/reactivation in relation to the occurrence and level of virus-specific response. Further studies on larger and different populations of immunocompromised and immunocompetent patients are required to define the potential of quantitative evaluation of HSV-1 specific cellular immune response in different clinical settings and to define cut-off levels discriminating between absence/low and strong response to be related to the risk of viral infection/reactivation.

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Wilck MB, Zuckerman RA. (2013). Herpes simplex virus in solid organ transplantation. Am J Transplant 13(Suppl 4):121-127.

463 Table 1. Demographic and clinical features of study population. BAL, bronchoalveolar lavage;
464 COPD, chronic obstructive pulmonary disease; CSA, cyclosporin A; MMF, mycophenolate mofetil;
465 MPA, mycophenolic acid; TAC, tacrolimus; AZA, azathioprine; EVR, everolimus. Details on
466 antiviral prophylaxis are reported in the text.

Features	
Patients, total n	95
Male/female, n	56/39
Mean age (range), years	47.2 (16-69)
Healthy seropositive individuals, n	42
Mean age (range), years	37.5 (21-49)
N. of EliSPOT determinations per patient	1
Pre-transplant patients, n	8
Mean age (range), years	47 (22-65)
N. of EliSPOT determinations per patient	1
Post-transplant patients, n	45
Mean age (range), years	47.0 (16-69)
N. of EliSPOT determinations per patient (mean, range)	3.6 (3-5)
Time of EliSPOT determinations post-transplantation (months – mean, range)	21 (1-94)
Type of lung transplant	
Monolateral	9
Bilateral	36
Underlying disease	
Cystic fibrosis	37 (50.7%)
COPD/emphysema	22 (26.0%)
Idiopathic pulmonary fibrosis	6 (8.2%)
Bronchiectasis	7 (9.6%)
Extrinsic allergic alveolitis	1 (1.4%)
Antiviral prophylaxis (in all transplant patients)	
HSV	Acyclovir
CMV	Ganciclovir or valganciclovir + CMV-Ig
Immunosuppressive regimens	
CSA + MMF	25
CSA + MPA	1
TAC + MMF	15
TAC + MPA	2
TAC + AZA	1
TAC + EVR	1
HSV-1/2 serology at baseline	
IgM+	5
IgM-	48
IgG+	48
IgG-	5

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469 Table 2. HSV-1 EliSPOT responses to different antigenic concentration according to IgG serostatus
470 (mean ± standard deviation, spot forming units [SFU]/2x105 peripheral blood mononuclear cells
471 [PBMCs]).

	HSV IgG+ (n = 48)	HSV IgG- (n = 5)	p
$3.16x10^3$ $TCID_{50}$/mL	5.447 ± 1.543	0.2 ± 0.2	0.24
$3.16x10^4$ $TCID_{50}$/mL	3.929 ± 1.335	0.2 ± 0.2	0.32

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Figure 1. Rapid shell vial culture assay with indirect immunofluorescence using Vero cells infected with (A) human Herpesvirus 1 ATCC® VR-260TM, (B) UV-inactivated HSV-1 preparation (dilution 3.16 x 108 TCID50/mL), and (C) UV-treated RPMI-1640 complete medium alone at 24 h post-infection (Fluorescein isothiocyanate; counterstaining with Evans blue 1:10000). Magnification, 25X.

Figure 2. EliSPOT assay on peripheral blood mononuclear cells from a HSV-1 IgG-positive lung transplant patient stimulated with serial dilution of UV-inactivated HSV-1 preparation: (A) 3.16 x 104 TCID50/mL, (B) 3.16 x 103 TCID50/mL, (C) RPMI-1640 complete medium alone, and (D) phytohemagglutinin mitogen (PHA) 1 µg/mL. Results are reported as spot forming unit (SFU)/2x105 cells.

Figure 3. HSV-1 EliSPOT responses according to concentrations of HSV-1 antigenic stimulus in samples from posttransplantation patients (peak value of response for each patient).

Figure 4. Kinetics of HSV-1 EliSPOT responses according to concentrations of HSV-1 antigenic stimulus considering all specimens from transplant patients (A, 3.16 x 103 TCID50/mL; B, 3.16 x 104 TCID50/mL) and excluding specimens from patients with concomitant HSV-1 positivity on bronchoalveolar lavage (BAL) (C, 3.16 x 103; D, 3.16 x 104 TCID50/mL). Determinations are grouped as follows: at pre-transplant (n = 42), up to 1month (n = 7), at 1-6 months (n = 13), at 6-12 months (n = 79), at 12-18 months (n = 53), and at 18-24 months (n = 8) post-lung transplantation (LT).