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Optimization of interferon gamma ELISPOT assay to detect human cytomegalovirus specific T-cell responses in solid organ transplants

Davide Abate*, Alda Saldan, Gabriella Forner, Daniel Tinto, Alice Bianchin, Giorgio Palù

Department of Molecular Medicine, University of Padua School of Medicine, via Gabelli, 63-35121 Padova, Italy

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Assessing the CMV specific CMI in transplant subjects represents a promising strategy to determine the risk of infection on individual basis. In this study 61 adult CMV IgG seropositive solid organ transplant recipients were examined in order to improve the efficacy of CMI detection. For this purpose, pair-wise comparisons were conducted comparing positive control stimuli PWM and PMA/iono and CMV stimuli, pp65 peptide pool and whole CMV particle. Rosette pre-depletion of blood was also investigated for detecting CD4+ or CD8+ T-cell responses using the IFN-g ELISPOT assay. In the time-points 30–180 days after transplantation, PMA/iono produced statistically significant higher responses compared to PWM, probably because PMA/iono activation pathway is independent from the effect of immunosuppressive drugs. The data showed that 11% of transplant patients displayed very low or undetectable responses to pp65 peptide pool antigen while having sustained high responses to whole CMV particle. In addition, in all the subjects analyzed, CMI responses to CMV particle produced a statistically significant higher number of spots compared to pp65 peptide pool antigen. Rosette pre-depletion of whole blood proved to be effective in detecting CD4+ or CD8+ T-cell responses similarly to flow cytometry. Taken together, the following recommendations are suggested to optimize the CMV-ELISPOT for transplantation settings: (1) use PMA/iono as positive control; (2) whole virus particle should be used to avoid peptide-related false negative responses; (3) a rosette pre-depletion step may be useful to detect CD4+ or CD8+ T-cell responses.

1. Introduction

CMV represents one of the most common causes of morbidity and mortality in transplant subjects (Razonable, 2005; Fishman, 2007; Fishman et al., 2007; Emery et al., 2012; Kotton, 2013). Due to pharmacological immunosuppression, CMV infection often arises from the CMV seropositive recipient or graft. The antiviral treatments can control effectively the CMV infection and symptoms, however the drug-related toxicity discourages from prolonged treatments. The interferon gamma releasing assays have been used successfully to assess the (re)-acquisition of CMV specific CMI in transplant subjects (Sinclair et al., 2004; Gerna and Lilleri, 2006; Gerna et al., 2006; Walker et al., 2007; Kumar et al., 2009; Nickel et al., 2009a,b; Abate et al., 2010,2012a,b; Costa et al., 2012; Egli et al., 2012; Bestard et al., 2013; Clari et al., 2013; Kotton, 2013; Manuel et al., 2013). However, in transplant settings CMV interferon gamma releasing assays may present several pitfalls that may limit their clinical utility, including: (1) weak responses to mitogen-based positive control stimuli caused by presence of immunosuppressive drugs; (2) poor or no recognition of peptide pool stimuli by the immune system (Dunn et al., 2002; Sylwester et al., 2005; Walker et al., 2007; Abate et al., 2013); and (3) CMV-ELISPOT does not distinguish between CD4+ and CD8+ T-cell response.

In this report paired comparisons of CMI responses to positive controls and CMV stimuli were conducted in order to assess the most appropriate conditions of CMI detection in transplantation settings and the following recommendations are suggested: (1) the use as positive control of PMA/iono instead of lectin-based molecules; (2) the use as CMV stimulus of the whole UV irradiated CMV particle to overcome the peptide pool antigen–HLA misrecognition. In alternative, whenever CMV peptide pool antigen is used, it is strongly suggested to include as internal control in separate wells, the whole UV irradiated CMV particle; (3) the use of rosette CD4+ or CD8+ pre-depletion to detect CD4+ and CD8+ specific T-cell responses using the CMV-ELISPOT assay.

Abbreviations: AZA, azathioprine; CMI, cell-mediated immunity; CMV, human cytomegalovirus; ConA, concanavalin A; CsA, cyclosporine A; HLA, human leukocyte antigen; MMF, mycophenolic acid; PBMCs, peripheral blood mononuclear cells; PFU, plaque forming unit; PHA, phytohemoagglutinin; PKC, protein kinase C; PMA/iono, phorbol myristate acetate and ionomycin; PWM, pokeweed mitogen.

* Corresponding author. Tel.: +39 0498218940; fax: +39 0498272355.
E-mail address: davide.abate@unipd.it (D. Abate).

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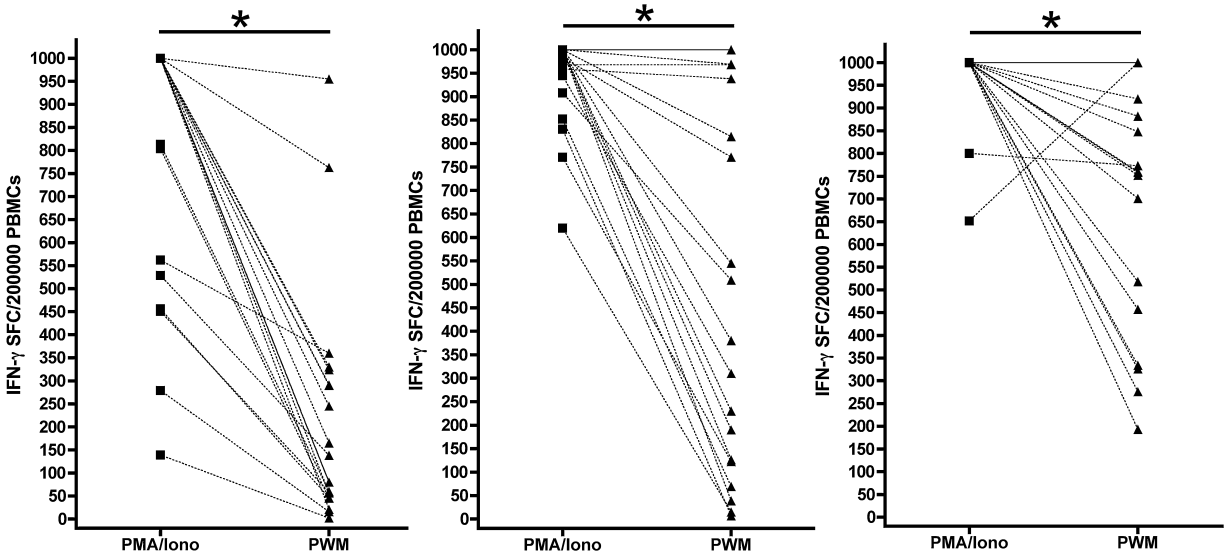

Fig. 1. IFN-g ELISPOT values in response to positive controls PMA/iono or PWM in a cohort of 61 solid organ transplants at (A) 1–30, (B) 31–90 and (C) 91–180 days after transplant. Asterisk indicates $p < 0.05$.

2. Materials and methods

2.1. Patients

This study was conducted on 61 CMV IgG seropositive Caucasian adult solid organ transplants including kidney, lung, liver and heart transplant recipients. The Padua General Hospital ethical committee approved the study. Study exclusion criteria were patients with any pre-existing or acquired immunodeficiency.

2.2. Virus preparation

pp65 mutant CMV (RVAd65) and MCMV K181+ were kindly provided by Bodo Plachter (University of Mainz, Germany) and Ed Mocarski (Emory University, USA), respectively. RVAd65 has a neomycin-resistance encoding cassette replacing the UL83 open reading frame. The pp65 mutant virus RVAd65 fails to produce and incorporate pp65 protein in the mature form of the virion (Schmolke et al., 1995). pp65 mutant virus was grown and propagated on human fibroblasts (HFs) and viral titers were calculated in standard plaque assays (Courcelle et al., 2001). MCMV K181+ (Stoddart et al., 1994) was grown, propagated and titrated on NIH-3T3 cells as described (Manning et al., 1992; Saederup et al., 1999). MCMV is host restricted and thus unable to replicate in human cells. MCMV shares multiple antigenic similarities with human CMV, including multiple ortholog and paralog viral gene products (Mocarski et al., 2007). After virus production and purification, pp65 mutant CMV and MCMV K181+ were UV irradiated and inactivated as described (Abate et al., 2004). Both UV inactivated viruses retain the ability of penetrating human cells but are incapable of initiating the viral lytic process (Abate et al., 2004). UV inactivated pp65 mutant CMV and MCMV K181+ were stored in −80 °C before use for at least 5 days in order to eliminate free radicals generated during the UV inactivation process (data not shown). UV inactivation was confirmed by absence of cytopathic effect in a standard plaque assay on HFs (CMV pp65 mutant) or NIH-3T3 (MCMV K181+). For IFN-g ELISPOT testing, UV inactivated pp65 mutant virus, and MCMV K181+ were used at a concentration of 1×10^4 PFU equivalent/200,000 PBMCs. Based on 10-fold serial dilution analysis we observed that $0.5–1 \times 10^4$ UV treated PFUs of MCMV were non-toxic for PBMCs (data not shown).

2.3. CD4+ and CD8+ cell depletion

RosetteSep (StemCell technology) was used to deplete selectively CD4+ or CD8+ cells from whole blood according to manufacturer instruction. Rosette CD4 or CD8 depletion was performed on whole blood prior to Ficoll-plaque (GE healthcare) gradient separation.

2.4. PBMCs extraction and IFN-g ELISPOT procedure

PBMCs were Ficoll-plaque extracted from 10 ml of peripheral blood collected in tubes containing sodium citrate as anticoagulant. In all the experiments shown, blood samples were stored at 4 °C and processed within 24 h upon collection. 200,000 PBMCs were seeded for each well in a 96-well IFN-g coated ELISPOT plate (AID, Autoimmun Diagnostika) as described (Abate et al., 2010). For each patient enrolled we tested as positive controls: 2 wells with PMA/iono (PMA/iono, 5 ng/ml and 500 ng/ml, respectively, Sigma) and 2 wells with PWM (10 μg/ml, AID); CMV stimuli: 2 wells with pp65 peptide pool (10 μg/ml, AID), 2 wells with pp65 mutant virus (1×10^4 PFU equivalent per well); as negative controls 2 wells with MCMV K181+ (1×10^4 PFU equivalent per well), 2 wells with RPMI medium supplemented with 10% human AB serum (Sigma). MCMV K181+ was used as control for spurious and non-specific responses for the pp65 mutant virus. IFN-g ELISPOT images were acquired using an automated spot detection device (AID) and mean spot value was calculated. IFN-g ELISPOT values of 1000 spots/200,000 PBMCs were considered the maximum countable number of spots. IFN-g ELISPOT < 10 spots/200,000 PBMCs were considered negative.

2.5. Flow cytometry

PBMCs were stimulated with pp65 peptide pool or with irrelevant non-specific peptide pool (negative control) and incubated at 37 °C in RPMI medium supplemented with 10% AB serum. After 1 h, Golgi stop (Becton Dickinson) was added and cells were incubated at 37 °C for additional 5 h. Then the PBMCs were fixed and permeabilized using FixPerm (Becton Dickinson) according to manufacturers instruction and stained for IFN-g, CD3, CD4, CD8, CD69 (Becton Dikinson). 200,000 PBMCs were acquired using an LSR II (Beckton Dickinson) and analyzed using FlowJo software (TreeStar).