Journal of Clinical Virology 115 (2019) 37–42

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

CMV-specific T-cells and CD27-CD28-CD4+ T-cells for assignment of cytomegalovirus (CMV) status in adults awaiting organ transplant

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ARTICLE INFO

Keywords:
Cytomegalovirus
Solid organ transplant
Passive antibody
Cell-mediated immunity
Viral-shedding

ABSTRACT

Background/objectives: Determination of Cytomegalovirus (CMV) status in solid organ transplant (SOT) candidates is essential to stratify risk of post-transplant CMV disease. Passive transfusion-acquired antibodies can make serologic determination of CMV status unreliable. We evaluated 3 assays, not affected by passive antibodies (PA), in assignment of CMV status: quantification of CMV-specific CD4 + T-cells (CMV-TC) and exhausted CD27-CD28- CD4 + T-cells, and detection of CMV DNA with Nucleic Acid Amplification Testing (NAAT).

Study design: We enrolled 50 adults awaiting SOT and 50 immunocompetent age-matched controls, and collected a throat swab, urine, saliva and blood sample on each. Using flow cytometry CD4 + T-cells were phenotypically analyzed for expression of CD27 and CD28 and CMV-specific CD4 + T-cells were identified by CD69 expression and intracellular IFN-γ quantification after stimulation with CMV-antigen lysate. CMV NAAT was performed on all specimens using real-time PCR. CMV serology (CMV IgG) was determined by enzyme immunoassay. Subjects were considered to have potential PA if they received blood products within 2 months of collection.

Results: The CMV-TC assay discriminated between CMV-seropositive and seronegative SOT candidates without PA well (sensitivity 79%, specificity 93%) while the CD27-CD28-CD4 + T-cell assay had good sensitivity (86%) but specificity of 74%. Detection of CMV DNA was uncommon in CMV-seropositive SOT candidates (2/21).

Conclusions: Given its high specificity, the CMV-TC assay is valuable in confirming true-positive CMV status in seropositive SOT candidates with PA, while use of CD27-CD28-CD4 + T-cell analysis is limited by moderate specificity. Detection of CMV DNA is of limited value in assignment of CMV status in adults.

1. Background and objectives

Determination of cytomegalovirus (CMV) infection status, using CMV serology, in solid organ transplant (SOT) donors and recipients is essential to stratify the risk of post-transplant CMV disease, a significant cause of morbidity in SOT recipients [1,2]. Unfortunately, serology may be falsely positive due to passive antibodies from recent transfusion, which is common in the pre-transplant population. In situations where CMV serology may be unreliable, guidelines suggest assigning the highest risk donor/recipient CMV category, but this strategy may result in unnecessary anti-viral use and/or monitoring for CMV [1,2]. Alternative methods are needed to assign CMV infection status in SOT candidates with potential passive antibodies.

Quantification of stimulation-induced CMV-specific CD4 + T-cells (CMV-TC), using flow cytometry, can reliably define CMV infection status in healthy adults and those awaiting renal transplant but has not been evaluated in other adult SOT candidates [3,4]. Previous studies have shown that CMV-TC have a unique “end-differentiated” T cell phenotype, characterized by the loss of expression of the co-stimulatory receptors CD27 and CD28 on CD4 + T-cells, thought to arise after

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https://doi.org/10.1016/j.jcv.2019.03.014
Received 12 February 2019; Received in revised form 20 March 2019; Accepted 22 March 2019

repeated cycles of antigenic stimulation [5–8]. Frequencies of CD27-CD28- CD4 + T-cells and stimulation-induced CMV-TC are highly correlated in healthy adults as well as those on hemodialysis and post-renal transplant, thus quantification of CD27-CD28- CD4 + T-cells may be a useful, rapid, stimulation-independent method of assigning CMV infection status in SOT candidates [9].

Detection of CMV DNA in oropharyngeal secretions or urine with nucleic acid amplification testing (NAAT) is useful to confirm true positive CMV infection status in infant SOT candidates with potential passive maternal antibodies, but the utility of CMV NAAT in assigning CMV infection status in adults with potential passive antibodies is uncertain [10,11]. CMV shedding is much less common overall in adults than in young children but CMV shedding may be more common in ill adult SOT candidates. Thus, CMV NAAT may be a rapid and inexpensive way to confirm true CMV infection in CMV-seropositive adult SOT candidates with unreliable serology [12,13].

We evaluated three assays un-affected by passive antibodies: flow cytometry-based detection of stimulation-induced CMV-specific CD4 + T-cells (CMV-TC assay), quantification of CD27-CD28-CD4 + T-cells, and direct detection of CMV DNA (with CMV NAAT), in assignment of CMV infection status in adults awaiting SOT across all allograft types, including those with potentially unreliable serology results due to the presence of passive antibodies.

2. Study design

2.1. Patients and samples

Following Institutional Ethics Board approval (Pro0042807), we enrolled adult subjects awaiting SOT (heart, lung, liver, small bowel, kidney) at the University of Alberta (Edmonton, Canada) and healthy controls, volunteers matched by gender and age (+/−5 years). Informed consent was obtained.

A throat swab, and samples of saliva, urine and blood were collected from all participants. Saliva samples were collected by sterile water gargle.

2.2. Detection of CMV-specific CD4 + T-cells

CMV-specific CD4 + T-cells were identified from whole blood *via* fluorescence-activated cell sorting (FACS) and activation was determined by activation marker CD69-PerCP expression and detection of intracellular IFN-γ-FITC following 6-hour stimulation with CMV-infected cell lysate as previously described [3,4,11,14,15]. Stimulation of blood samples was carried out within 24 h after venipuncture. *Staphylococcus aureus* enterotoxin-B and non-infected lysate served as positive and negative controls. Analysis was performed on a BD FACSCanto™. CMV-specific CD69 + IFN-γ + CD4 + T-cells were quantified by subtracting the percent in the negative control from the CMV-lysate stimulated cultures.

Samples were excluded from analysis if cell propidium iodide (PI)-viability was < 80%. Results were considered valid if the positive control elicited ≥0.035% CD69 + IFN-γ + CD4 + T-cells, and if there was ≥20% higher percentage of CD69 + IFN-γ + CD4 + T-cells obtained after CMV-specific stimulation compared to background reactivity after control stimulation, otherwise results were considered indeterminate [11].

2.3. Quantification of CD27-CD28-CD4 + T-cells

Peripheral blood mononuclear cells were surface stained for CD4-V450, CD28-PerCP-Cy5.5 and CD27-APC-EF780, and FACS analyzed on a BD FACSCanto™. Samples were excluded if PI-viability was < 80% [9,11].

2.4. Detection of CMV DNA and CMV serology

CMV DNA was detected and quantified directly in whole blood, throat, urine and saliva samples using our in-house real-time polymerase chain reaction assay (assay conversion factor is 5.65 genome copies/mL = 1 IU/mL) [16]. Results > 500 copies/mL (2.7 log10 copies/mL) were considered positive. CMV IgG antibodies were detected by enzyme immunoassay (Siemens Enzygnost Anti-CMV/IgG, Siemens Healthcare Diagnostics Products GmbH, Marburg/Germany).

2.5. Data analysis

CMV-seropositive individuals were considered to have potential passive antibodies (PPA) if they had received transfusion(s) of plasma, platelets, red cells, whole blood, cryoprecipitate, or IVIG within 2 months prior to study blood collection.

FlowJo™ (FloJo, LLC Ashland, OR) software was used for flow-cytometric analysis and analysis was performed blinded to CMV serology and NAAT results. Gates were placed as quadrants based on the respective negative controls of each individual and not modified based on visual appearance of distinct populations.

Categorical variables are presented as counts (percentages) and were analyzed using Chi-squared or Fischer's exact test. Continuous variables are presented as medians (25^{th}-75^{th} percentiles) or means (standard deviations). Kruskal-Wallis and Mann-Whitney U tests were used to compare distributions of continuous variables. Spearman's correlation coefficient (r_s) was used to compare frequencies of CMV-TC and CD27-CD28- CD4 + T cells. Receiver operator characteristics (ROC) analyses were performed for CMV-TC and CD27-CD28- CD4 + T cell phenotype data to determine cut-offs to distinguish CMV-seropositive from seronegative individuals. Tests were two-sided and a p-value of < 0.05 was considered statistically significant. When multiple pairwise comparisons were made, p values were multiplied by the number of comparisons and reported as an adjusted p value. Data analysis was performed with STATA 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

3. Results

We enrolled 50 subjects awaiting SOT (15 kidney, 14 liver, 1 liver + small bowel, 11 lung, 9 heart) and 50 controls (Table 1) who were similar with respect to median age (subjects 49.1 yrs., controls 48.6 yrs.) gender (64% male subjects and controls), and CMV-seropositivity (42% subjects, 54% controls). Of the 21 CMV-seropositive subjects, 7 (33.3%) had potential passive antibodies. Saliva, blood and urine samples were collected from all participants and throat swabs were collected for 99/100.

3.1. CMV-TC assay and CD27-28- CD4 + T-cells

Results for the CMV-TC assay and CD27-CD28- CD4 + T cell analysis were available for 98/100 and 97/100 participants, respectively. Two samples (1 CMV-seronegative subject, 1 CMV-seropositive control) with poor cell viability were excluded from both assays. One sample of a CMV-seronegative subject was excluded from CD27-CD28-CD4 + T-cell analysis for technical reasons.

The frequency of CMV-TC by CMV-serostatus is presented in Fig. 1. ROC analysis using CMV-TC results from controls (26 seropositive, 23 seronegative), determined that a cut-off of 0.075% CMV-TC distinguished CMV-seropositive from seronegative controls with a sensitivity of 92% and specificity of 100% (AUC 0.96). Among subjects, one sample of a CMV-seronegative individual had an indeterminate result due to high background reactivity, and was excluded from analysis. When the cut-off of 0.075% was applied to the remaining subjects without passive antibodies (n = 41), CMV-seropositive were distinguished from seronegative subjects with a sensitivity of 79% (11/14)

Table 1
Characteristics of Subjects and Controls.

Characteristics	Subjects n = 50	Controls n = 50
Age in years median (25^{th},75^{th})	49.1 (32.4,59.9)	48.6 (35.0, 60.4)
Sex Male, n (%)	32 (64.0)	32 (64.0)
CMV-seropositive, n (%)	21 (42.0)	27 (54.0)
Potential passive antibody[a], n	7	0
Organ and Wait-list status[b]		
Kidney, n (%)	15 (30.0)[c]	
Liver, n (%)	15 (30.0)[d]	
status 0	3	
status 1	5	
status 2	7	
status ≥3	0	
Lung, n (%)	11 (22.0)	
status 1	3	
status 2	8	
Heart, n (%)	9 (18.0)	
status 0	1	
status 1	1	
status 2	2	
status ≥3	5[e]	
Hospitalized, n (%)	17 (34.0)	
ICU, n (%)	2 (4.0)	

[a] Potential passive antibody due to transfusion in the previous 2 months.
[b] Waitlist status in Canada: higher numbers reflect more urgent status. For heart and liver, status ≥3 considered high status, for lung status 2 reflects rapidly deteriorating condition.
[c] 8/15 (53.3%) pre-renal transplant candidates on dialysis.
[d] Average Model for End Stage Liver Disease (MELD) score: 22.9 (SD 6.12). 1/15 awaiting a liver and small bowel transplant.
[e] 4/5 on ventricular assist device.

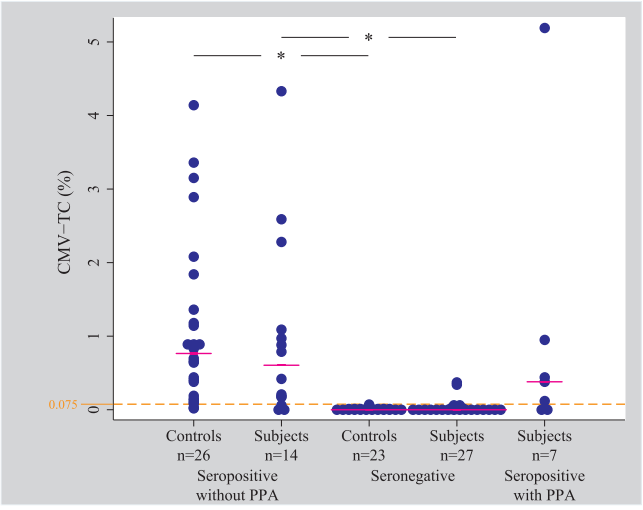

Fig. 1. Comparison of CMV-specific CD4 + T-cells frequencies between CMV seropositive and seronegative subjects and controls.
CMV-TC frequencies are similar between CMV seropositive controls and subjects without potential passive antibody (p = 1.0) and between CMV seronegative controls and subjects (p = 1.0). CMV-TC frequencies in CMV seropositive subjects with potential passive antibodies did not differ significantly from those in CMV seropositive subjects without passive antibodies (p = 1.0) or from CMV seronegative subjects (p = 0.071). *p < 0.0001.Dashed line (0.075%) represents the detection limit for CMV-TC. Solid lines represent median CMV-TC frequencies. CMV, cytomegalovirus; CMV-TC, CD4+ CMV-specific T-cells; PPA, potential passive antibody.

and specificity of 93% (25/27). Three CMV-seropositive subjects without potential passive antibody did not have detectable CMV-TC: 2 renal transplant candidates on dialysis (ages 44 and 47 years) and a lung transplant candidate (age 63 years) listed as status 2 (Fig. 2A). Two CMV seronegative subjects had detectable CMV-TC: a 21 year old liver and small bowel transplant candidate and a 36 year old renal transplant candidate who was not on dialysis (Fig. 2A). Two CMV seropositive controls (ages 35 and 63 years) did not have detectable CMV-TC (Fig. 2B). Among the 7 CMV-seropositive subjects with potential passive antibodies, 5 had detectable CMV-TC (Fig. 3).

Fig. 4 illustrates the distribution of CD27-CD28-CD4 + T-cell frequencies by CMV-serostatus. ROC analysis using CD27-CD28- CD4 + T-cell frequencies from controls (26 seropositive, 23 seronegative), determined that a cut-off of 0.46% CD27-CD28- CD4 + T-cells distinguished CMV-seropositive from seronegative controls with a sensitivity of 88% and specificity of 83% (AUC 0.86). When applied to subjects without passive antibodies, the 0.46% CD27-CD28- CD4 + T cell cut-off distinguished CMV seropositive from CMV seronegative subjects with a sensitivity of 86% (12/14) and specificity of 74% (20/27). Two CMV seropositive subjects without passive antibodies had CD27-CD28- CD4 + T-cell frequencies below the 0.46% cut-off: renal transplant candidates ages 46 and 76 years. The seven CMV-seronegative subjects with CD27-CD28- CD4 + T-cell frequencies above the 0.46% cut-off ranged in age from 21 to 59 years and included all organ groups (Fig. 2A). Among the 7 CMV-seropositive subjects with potential passive antibodies, the 5 individuals with detectable CMV-TC also had CD27-CD28- CD4 + T-cell frequencies above the 0.46% cut-off (Fig. 3).

Overall, there was a moderate positive correlation between frequencies of CD27-CD28- CD4 + T-cells and CMV-TC in adult controls (r_s = 0.68, p < 0.001) and in adult subjects (r_s = 0.52, p < 0.001).

3.2. Detection of CMV DNA by NAAT

Among all CMV-seropositive individuals (n = 48), only two (4.2%) had a positive CMV NAAT result (a 23 yr. old and a 59 yr. old patient awaiting lung and liver transplants). In both cases, DNA was detected from urine samples. No seronegative individual had a positive CMV NAAT result.

Among the 7 seropositive subjects with potential passive antibodies, only one had detectable CMV NAAT (Fig. 3).

4. Discussion

Using a cut-off of 0.075% CMV-TC, CMV-TC levels had excellent sensitivity and specificity in assigning CMV infection status in healthy adults (92%, 100%). Our results were comparable to results from a previous study in adults who were healthy, in chronic renal failure or post-renal transplant, where CMV serostatus was predicted with 100% sensitivity and 98% specificity; they used the assay from which ours was adapted and a cut-off of 0.05% CMV-TC [4]. In the current study of adults awaiting SOT of all organ types, including patients listed at a high-status, the CMV-TC assay discriminated between CMV-seropositive and seronegative adults well (sensitivity 79%, specificity 93% in subjects without passive antibody). The lower sensitivity in our SOT-candidates is likely due to the high severity and chronicity of illness in our pre-SOT subjects which may impair their cell-mediated immune responses. There are available commercial assays, including QuantiFERON®-CMV, and enzyme-linked immunosorbent spot (ELISpot) assays such as T-SPOT®.CMV, for detection of CMV-specific T cell immunity. While many studies have investigated the use of QuantiFERON®-CMV and ELISpot assays to predict the risk of CMV infection and to guide CMV prophylaxis strategies post-transplant, knowledge regarding the use of T-cell assays as an alternative or adjunct to serology in patients with potential passive immunity is limited to a few studies [2,3,11,15,17]. In assignment of pre-transplant CMV infection status, both assay sensitivity and specificity are critical. CMV cell mediated immunity assays using antigens with broad stimulatory capacity, such as CMV lysate used in our study, have higher sensitivity, and thus are preferable to assays using individual antigens for stimulation, such as the commercially available QuantiFERON®-CMV and T-

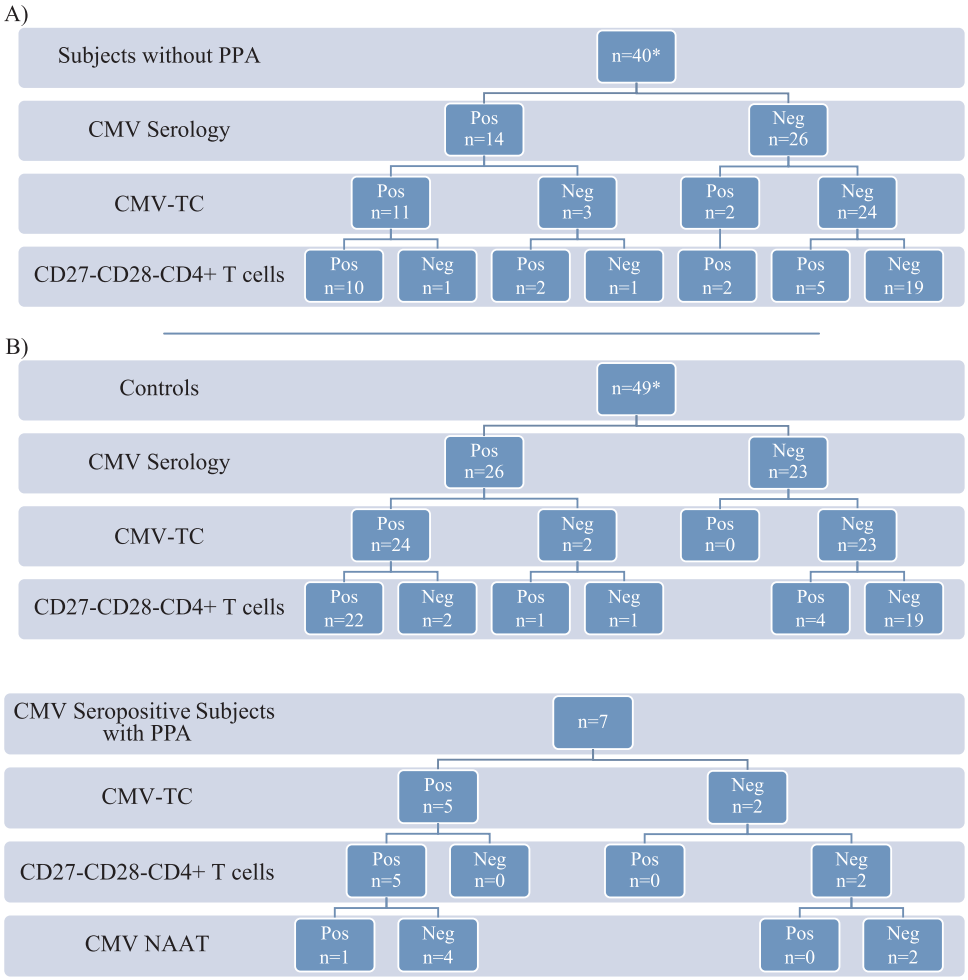

Fig. 2. CMV-specific CD4 + T-cells and CD27-CD28-CD4 + T cells in Subjects (A) and Controls (B), without potential passive Antibody* had valid results from both the CMV-TC assay and CD27-CD28-CD4 + T cells. CMV-TC considered positive if ≥ 0.075%. CD27-CD28-CD4 + T cells considered positive if ≥ 0.46%. CMV, cytomegalovirus; CMV-TC, CD4+ CMV-specific T-cells; Pos, positive; Neg, negative.

Fig. 3. CMV-specific CD4 + T-cells, CD27-CD28-CD4 + T cells and CMV-NAAT in CMV Seropositive Subjects with Potential Passive Antibody.
There was 100% agreement between the CMV-TC assay and CD27-CD28- CD4 + T cell phenotype in the 7 subjects with potential passive antibodies, (classified 5 as positive and 2 as negative). CMV-TC considered positive if ≥ 0.075%. CD27-CD28-CD4 + T cells considered positive if ≥ 0.46%. CMV, cytomegalovirus; CMV-TC, CD4+ CMV-specific T-cells; NAAT, nucleic acid amplification testing; Pos, positive; Neg, negative.

SPOT®.CMV assays [2,4,17–19].

Consistent with previous studies, we identified individuals without passive antibodies whose serology and CMV-TC results were discordant; the presence of CMV-TC may be more useful than serology in predicting the transplant candidate's ability to control CMV post-transplant, but this requires further investigation [4,17,20,21]. Our study suggests that this CMV-TC assay is useful in CMV risk-stratification in adults awaiting SOT, across multiple organ types, whose serology may be unreliable due to potential passive antibodies. This assay could have other applications where identifying the correct CMV infection status is vital, including recruitment into CMV vaccine trials.

Quantification of CD27-CD28- CD4 + T-cells as a marker of CMV infection-status is attractive as, compared to stimulation-based CMV-TC assays, determination of CD27-CD28- CD4 + T-cell frequencies requires a lower blood volume, is less expensive, more rapid (24 h *versus* 48–72 h reporting time), and does not require a tissue culture facility. A prior study determined that a frequency of ≥0.44% CD27-CD28- CD4 + T-cells discriminated between CMV-seropositive and seronegative adults with 93% sensitivity and 97% specificity and found that frequencies of CD27-CD28- CD4 + T cells and stimulation-induced CMV-TC were strongly positively correlated (r_s = 0.73) [9]. In our cohort we found that a similar frequency of ≥0.46% CD27-CD28- CD4 + T cells discriminated between CMV-seropositive and seronegative subjects without passive antibodies with good sensitivity (86%) and reasonable specificity (74%). There was moderate positive correlation between the frequency of CD27-CD28- CD4 + T cells and CMV-TC, with higher correlation in controls (r_s = 0.68) than in subjects (r_s = 0.52). Although not significantly different, compared with controls seropositive subjects had higher CD27-CD28-CD4 + T cell frequencies and lower CMV-TC frequencies, perhaps related to premature immune aging and waning cellular immunity in patients with chronic or critical illness, which may explain the poorer correlation between the two assays in subjects awaiting SOT [7]. Among the 7 CMV-seropositive adults with potential passive antibodies, there was perfect agreement between the CMV-TC and CD27-CD28-CD4 + T cell assays with 5/7 having detectable CMV-TC and CD27-CD28-CD4 + T cell frequencies above the cut-off and 2/7 having undetectable CMV-TC and CD27-CD28-CD4 + T cell frequencies below the cut-off, which may be considered as a sign of true passive immunity in a CMV non-infected subject.

Our study confirms that detection of CMV DNA is uncommon in asymptomatic CMV-seropositive adults, even in chronically ill patients awaiting SOT, thus is likely of limited value as an adjunct to CMV serology in SOT candidates with potential passive antibody.

Our study has several limitations. As this study was performed at a single institution, it had a limited sample size and was not powered to detect specific differences in assay performance by organ group or waitlist status. In the two CMV seropositive subjects with potential passive antibodies, the absence of detectable T cells may indicate absence of infection with CMV. Nevertheless, we were unable to confirm false positive passive CMV serology as we did not have repeat CMV serology more than 2 months after transfusion to determine if there was sero-reversion. Our gold standard of CMV serology to establish CMV infection status has limitations. Apart from variabilities among different serology assays as a reason for discordant results with T-cell analyses,

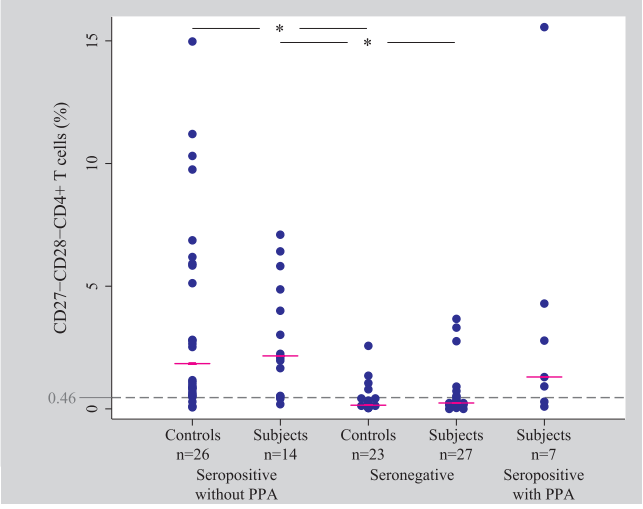

Fig. 4. Comparison of CD27-CD28- CD4 + T cell frequencies between CMV seropositive and seronegative subjects and controls.
CD27-CD28- CD4 + T cell frequencies are similar between CMV seropositive controls and subjects without potential passive antibodies (p = 1.0) and between CMV seronegative controls and subjects (p = 1.0). CD27-CD28- CD4 + T cell frequencies in CMV seropositive subjects with potential passive antibodies did not differ significantly from those in CMV seropositive subjects without passive antibodies (p = 1.0) or from CMV seronegative subjects (p = 0.12). *p < 0.001. Dashed line (0.46%) represents the cut-off determined to distinguish CMV seropositive from seronegative individuals. Solid lines represent median CD27-CD28- CD4 + T cell frequencies. CMV, cytomegalovirus; PPA, potential passive antibody.

previous studies have shown discordance between CMV serology and CMV-specific T-cell responses in SOT candidates even in situations where passive antibody is not an issue [17,20–22].

5. Conclusions

The high specificity of the CMV-TC assay makes it a valuable adjunct to CMV serology to confirm true positive infection status in CMV seropositive SOT candidates with potential passive antibodies. While a negative CMV-TC assay in a CMV seronegative individual after transplantation may indicate lack of individual immune control towards CMV, the implications of a negative CMV-TC assay in stable CMV seropositive individuals, with or without potential passive antibodies, requires further investigation. Although more rapid and potentially more accessible, the use of CD27-CD28-CD4 + T-cell analysis to resolve unreliable positive CMV serology in SOT candidates with potential passive antibodies, may be limited by its moderate specificity.

Funding

This work was supported by an Alberta Innovates Health Solutions (AIHS) Collaborative Research and Innovation Opportunity (CRIO) Project Award [grant number 201201224] and a Canadian Blood Services Research and Development Award [grant number 00539]. The funding sources had no involvement in study design, writing the manuscript or in the decision to submit the article for publication.

Competing interests

None of the authors have any conflicts of interest to disclose.

Ethical approval

Obtained from the University of Alberta Health Research Ethics Board (pro00042807).

CRediT authorship contribution statement

Catherine E. Burton: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Visualization. **Martina Sester:** Conceptualization, Methodology, Writing - review & editing. **Joan L. Robinson:** Writing - review & editing, Visualization. **Dean T. Eurich:** Methodology, Writing - review & editing, Visualization. **Simon Urschel:** Conceptualization, Methodology, Writing - review & editing, Resources, Supervision. **Jutta K. Preiksaitis:** Conceptualization, Methodology, Writing - review & editing, Resources, Supervision, Funding acquisition.

Acknowledgements

The authors thank Dr. Yupin Tong and Fred Wong for performing the virology and immunologic assays, Dr. Jan Dirks for sharing his expertise with the CMV-TC and CD27-CD28- CD4 + T cell assay, and Candice McEachren for her assistance in participant recruitment and sample collection.

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