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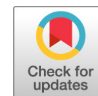
MINIREVIEW



Utility of Cytomegalovirus Cell-Mediated Immunity Assays in Solid Organ Transplantation

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ABSTRACT Cytomegalovirus (CMV) is one of the most important viral complications after solid organ transplantation (SOT). Current preventive and management strategies rely primarily on serologic and viral load testing and remain suboptimal. To address these issues, multiple techniques to measure CMV-specific cell-mediated immunity (CMI) have been developed and evaluated in clinical studies over the past two decades. These assays show significant promise for the personalization of CMV management. For example, CMI assays can be used to help determine the optimal duration of antiviral prophylaxis or whether antiviral therapy is indicated in patients with low levels of CMV reactivation. However, despite numerous studies showing potential utility, these assays are not yet in widespread routine clinical use. Barriers to adoption include variations in test complexity, standardization, and thresholds for positivity and insufficient interventional clinical trials. Here, we provide an updated assessment of commonly available tests and the clinical utility of CMV-specific CMI testing in SOT recipients.

KEYWORDS cellular immunity

Cytomegalovirus (CMV) remains one of the most important pathogens after solid organ transplantation (SOT), with significant morbidity and occasional mortality (1–4). Following primary infection, CMV establishes lifelong latency and can reactivate in an immunosuppressed host. Primary CMV infection can also occur after transplantation when a CMV seropositive donor organ is transplanted into a seronegative recipient (D+/R−). The interplay between host and virus posttransplant is complex, involving both the innate and adaptive immune systems, viral replication dynamics, and immune modulation (2). In SOT recipients, factors influencing the balance between viral replication and control include treatment with T-cell-depleting antibodies, such as antithymocyte globulin (ATG), prolonged immunosuppressed state, and human leukocyte antigen (HLA) mismatch between the graft and host immune cells (1, 3). Manifestations of CMV in an SOT recipient span from asymptomatic viremia, a viral syndrome with fever and leukopenia, and/or subsequent tissue invasive disease (e.g., pneumonitis and enteritis). CMV infection is thought to also result in so-called "indirect effects" which are thought to occur as a result of virally induced immune dysregulation and may include the development of acute and chronic rejection states as well as a predisposition to bacterial, fungal, and other viral infections (2, 3, 5, 6).

Pretransplant donor and recipient CMV serology (as measured by CMV IgG) remains the main measurement that defines the risk of posttransplant CMV disease. The constellation of donor and recipient serology coupled with the type of organ transplanted and the immunosuppressive regimen help guide management decisions related to the optimal preventive strategy for CMV. The two primary strategies are pre-emptive therapy (PET) and antiviral prophylaxis. The PET strategy involves regular monitoring of viral load and initiation of antiviral therapy at a specific threshold of viremia in order to

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prevent progression. In the antiviral prophylaxis approach, the therapy is typically administered for a prolonged period of time, such as 3 to 6 months posttransplant or sometimes longer (1, 4). Although significant practice variation exists, SOT recipients who are donor seropositive, recipient seronegative (D+/R−) usually receive antiviral prophylaxis, whereas seropositive recipients (R+) are either given prophylaxis or undergo pre-emptive monitoring, with therapy commenced at a predefined cutoff or increasing viral load (1, 4). Both strategies have significant limitations. For example, despite 3 months of prophylaxis, 20% to 40% of D+/R− patients still develop CMV disease occurring typically shortly after the discontinuation of prophylaxis; in addition, adverse effects related to current antivirals, such as leukopenia, are common (7–9). For pre-emptive therapy, viral load thresholds to initiate treatment are not well understood, and different thresholds may be required for different patients. In addition, CMV loads must be monitored at least weekly for pre-emptive therapy to be effective. In high-risk patients (D+/R−), however, rapid viral-doubling times as short as 1 to 2 days may limit the utility of a pre-emptive strategy (10).

The limitations of the current approaches to CMV prevention have led to interest in including an immunologic profile to help further refine prediction and to individualize management. Both humoral and cellular immune response may be assessed. Humoral assessment (serology) is very useful (as outlined above) when applied in the pretransplant setting to donors and recipients. However, posttransplant measurement of CMV IgG seems to have limited clinical utility. For example, in one study, seroconversion in previously seronegative recipients was correlated poorly with reactivation of CMV in the posttransplant setting (11). The explanation for this result likely relates to the fact that the control of herpesviruses, including CMV, in the posttransplant setting is based primarily on pathogen-specific T cells. This reasoning is well illustrated from CMV adoptive T cell studies which show that the infusion of CMV-specific T cells can lead to the control of CMV viremia (12). Thus, in the last two decades, there has been an impetus to develop T cell assays that can more accurately predict CMV reactivation (of either donor- or recipient-derived latent virus) in the posttransplant setting. The rationale is that a more accurate way to define and monitor the host immunological response to CMV could lead to a better prediction of which patients will develop clinically significant CMV reactivation and allow for more tailored prevention. Here, we review the current status of CMV CMI assays, their advantages and limitations, and their potential use in clinical practice.

GENERAL PRINCIPLES AND TYPES OF CMV CMI ASSAYS

The principle of CMV-specific CMI assays is based typically on the measurement of interferon gamma (IFN-γ), or other cytokines, produced by CD4$^+$ and/or CD8$^+$ T cells in response to the stimulation of peripheral blood mononuclear cells (PBMCs) or whole blood with CMV antigens, lysate, or overlapping peptides (2, 3, 13). In general, high CMV-CMI indicates adequate CMV-specific CD4$^+$ and/or CD8$^+$ T cell immunity, predicting protection against CMV disease, whereas low CMI increases the likelihood of CMV reactivation or progression (13). The *in vitro* response can be elicited by single peptides, peptide libraries, or whole-virus lysate (2). Assays commonly use "immunodominant" peptides that are able to stimulate T cells of patients with specific HLA backgrounds (2). The use of single peptides, however, may exclude certain HLA types and therefore, in selected patients, the test may exhibit no stimulation (2). For example, the Quantiferon-CMV (QFN-CMV) assay contains a combination of over 20 peptides for cell stimulation; however, patients who have CMV immunity but have uncommon HLA types may still be negative by this assay (2). The whole-virus lysate has several CMV proteins and therefore may be more sensitive; however, it is generated from infected fibroblasts, resulting in variability and difficult standardization. For quality control, all CMI assays generally have a negative and positive control. The positive control is typically a mitogen (e.g., staphylococcal enterotoxin B or phytohemagglutinin) that helps identify patients whose T cells are globally unresponsive. This may be related to immunosuppression, preanalytic errors or low overall lymphocyte number (2). If there is a low or

undetectable positive-control value, the test result can be more difficult to interpret (2). A negative control, usually cell medium or a mock antigen, helps to distinguish those patients who demonstrate background reactivity (2).

Various CMV-CMI assays have been commercialized although none are FDA approved at the time of this writing. The QuantiFERON-CMV (Qiagen Inc.) is an enzyme-linked immunosorbent assay (ELISA)-based IFN-γ release assay that uses a CMV peptide pool to stimulate whole blood. It is meant to primarily stimulate $CD8^+$ T cells due to the short peptide length. The peptides cover >98% of the HLA types found in the human population. The manufacturer's cutoff value for a positive result is 0.2 IU/mL of IFN-γ. ELISpot-based assays include the T.Spot.CMV (Oxford Diagnostics) and T-Track CMV (Lophius Biosciences now acquired by Mikrogen GmBH). They are highly sensitive immunoassays that measure the frequency of both $CD4^+$ and $CD8^+$ T cells producing IFN-γ in response to CMV (1, 2). In this assay, PBMCs are stimulated with CMV-specific peptides or whole-antigen lysates. IFN-γ is then quantified using a labeled antibody (1, 2). There is currently no defined manufacturer's cutoff for positivity, although observational studies have suggested some potential cutoffs (14, 15). In another commonly used assay, a detailed analysis of CMV-specific T cell responses can be obtained by intracellular cytokine staining (ICS) for IFN-γ using flow cytometry (Table 1). In this technique, whole blood or isolated PBMCs are stimulated with CMV peptides or CMV lysate and stained with monoclonal antibody against IFN-γ. When a whole-antigen lysate is used, the assay is not HLA restricted and therefore knowledge of patient HLA type is not required (2). In comparison to other modalities, it is a somewhat more versatile technique that can be expanded to include other cytokines and cell surface molecules, to provide quantitative and qualitative measurements of CMV-specific T cells, and to distinguish between $CD4^+$ and $CD8^+$ responses (2). With recent advances, more than one marker can be tested at the same time, providing a more comprehensive understanding of immune control. A commercial assay T cell immunity panel using a flow cytometry technique (Eurofins Viracor) is available for clinical use in the United States. In addition, various other laboratory-developed tests are emerging, including those that use major histocompatibility complex (MHC) multimer technology. An additional method of CMV CMI testing uses MHC-multimer-based assays that directly stain peptide-specific T cells using peptide-conjugated MHC class I tetramers or pentamers (Table 1). When combined with surface markers, this assay has been shown to be predictive of CMV viremia. It is able to determine $CD8^+$ T cell responses; however, it is limited by being epitope specific, requiring knowledge of the HLA type. Both ICS and MHC-multimer staining need a fluorescence-activated cell sorting facility, which may limit widespread use.

Although these assays are based on IFN-γ release, other markers have also been found to correlate CMV-specific T cell responses with the risk of CMV infection. These markers include interleukin-2 (IL-2), tumor necrosis factor-alpha, CD107, programmed death-1 (PD-1), and CD154 (1, 2). In SOT recipients with CMV viremia, the expression of the chemokines CCL8 and CXCL10 was shown to be associated with the control of viral replication (16). CCR6 expression, a chemokine receptor involved in the homing of memory T cells to sites of mucosal inflammation, predicted late-onset CMV reactivation at the time of discontinuation of anti-CMV prophylaxis in a small single-center study (17). However, these markers are currently measured only in the research setting and have not been used in commercial assays.

CLINICAL SCENARIOS FOR USE OF CMI ASSAYS

CMI for postprophylaxis prediction of CMV. Several observational studies that include high-risk (D+R−) and intermediate-risk (R+) patients have shown that a positive CMV-CMI assay predicts protection from subsequent CMV reactivation and clinical disease development, thereby being potentially useful for personalizing the duration of CMV antiviral prophylaxis (18, 19). One of the earlier studies using a CMI assay (QFN-CMV) in high-risk SOT recipients showed that CMV disease occurred in only 2/38 (5.3%) of patients with a positive CMI response at the end of antiviral prophylaxis compared with 16/70 (22.9%) of patients with a negative CMI response (20). This finding was later

TABLE 1 Cell-mediated immunity assays to detect cytomegalovirus-specific T cell immunity

CMV-CMI assay type	Method	Equipment requirement	Commercial test available (manufacturer)[b]	Cutoff value for positivity	Clinical scenario in organ transplantation (selected references)	Available interventional studies (reference)[a]
ELISA	Enzyme-linked immunosorbent assay (ELISA)-based IFN-gamma release assay, capable of detecting CD8+ T cell response after peptide stimulation	ELISA plate reader	QuantiFERON-CMV (Qiagen Inc.)[c]	0.2 IU/mL	Pre-transplant risk stratification (14, 29, 33, 35–37); adjunct to pre-emptive monitoring (26, 31); assessment at time of discontinuation of prophylaxis (18–22, 28, 38)	Several (14, 21, 25–27, 30)
ELISpot	Highly sensitive immunoassay that measures the frequency of both $CD4^+$ and $CD8^+$ T cells producing IFN-gamma in response to CMV-specific peptides or whole proteins	ELISpot reader	T.Spot.CMV (Oxford Diagnostics); laboratory-developed test in the United States T-Track (Lophius Bioscience/ Mikrogen GmBH)[c]	20–40 spot forming units after stimulation with either IE-1 or pp65	Pretransplant risk stratification (29, 32, 39, 40); adjunct to pre-emptive monitoring (15, 41, 42); assessment at time of discontinuation of prophylaxis (6, 43)	Few interventional studies (32)
Intracellular cytokine staining	Intracellular cytokine staining for IFN-gamma using flow cytometry; whole blood or isolated PBMCs are stimulated with CMV peptides or CMV lysate and stained with monoclonal antibody against IFN-gamma; other cytokine markers can now be tested at the same time; provides quantitative and qualitative assessment	Flow cytometer and intracellular and extracellular markers	CMV Insight T cell immunity testing (Eurofins Viracor); laboratory-developed test in the United States	>0.2% for the commercial assay (multiple research assays)	Pretransplant risk stratification (34, 44, 45); pre-emptive monitoring (46–48); assessment at time of discontinuation of prophylaxis (49, 50)	None
MHC multimer staining	Whole-blood assay that directly stains peptide-specific T cells using peptide-conjugated MHC class I tetramers or pentamers		CMVC8 (Mayo Clinic Laboratories)	None established	Observational studies for posttransplant monitoring and risk assessment (51)	None

[a]Interventional study denotes a prospective study where CMV-CMI is used in clinical decision-making.
[b]Laboratory Developed Test in the United States describe the test that they follow.
[c]CE-marked in Europe, Conformité Européenne Mark (French) - declaration that the product meets regulatory body statndards and is licensed for use in Europe.

confirmed in a multicenter study of CMV D+/R− SOT recipients (21). Interestingly, this study and another also found that patients with an indeterminate result (low mitogen reactivity) appear to have an even greater risk of CMV than those with negative results, suggesting that patients with global T cell anergy are at a high risk for CMV (19, 21). The ELISpot (T.Spot.CMV) assay has also been studied in a large, multicenter observational study of 583 kidney transplant recipients (260 D+/R− and 277 R+). Patients underwent ELISpot testing at the end of antiviral prophylaxis (15). The primary outcome was clinically significant CMV defined as site-determined viremia or disease that necessitated a change in antiviral therapy. The authors found that a cutoff value of >40 spot-forming units (sfu)/250,000 cells for either IE-1 or pp-65 antigens was optimal as a threshold for positivity, with a negative predictive value (NPV) of >97% for subsequent CMV-related events (15). Other studies have noted that the cutoff value may be lower (i.e., 20 sfu/300,000 cells) and that only stimulation with IE-1, and not pp65, was predictive with this method (14). Interestingly, in a subgroup analysis of the larger multicenter study, the assay was noted to have clinical utility in seropositive patients but was not predictive of CMV in D+/R− patients primarily because very few D+/R− patients showed a positive CMI (15). Other studies have also found that CMI is unlikely to develop in CMV seronegative transplant recipients during prophylaxis but may be generated after viremia has occurred (22–24).

Although many observational studies have shown that a positive CMI after prophylaxis can predict protection from subsequent CMV, the next step is to determine whether regular monitoring of CMV-specific CMI can be used to modify the duration of prophylaxis. With a positive CMI, a shorter prophylaxis course could be given and could save on antiviral costs and reduce toxicity. With a negative CMI, prophylaxis could be continued or more intensive viral load monitoring could be instituted. Paez-Vega et al. (25) performed a randomized trial of an early discontinuation of primary prophylaxis in CMV R+ kidney transplant patients receiving ATG induction. In this trial, there was no difference in CMV disease outcomes in patients that discontinued prophylaxis early based on a positive CMV-CMI result, suggesting that CMI measurements could be used to tailor the duration of prophylaxis (25). A study in heart transplant recipients showed that the duration of prophylaxis could be guided successfully using the QFN-CMV assay (26). Similarly, a study in lung transplant recipients randomized patients to receive 5 months of antiviral prophylaxis versus variable-length prophylaxis depending the results of the QFN-CMV assay (22). There was a significant reduction of CMV infection as measured within the lung allograft in the CMI guided group. In a modification of this principal, our group performed a study using the QFN-CMV assay to determine whether to initiate secondary antiviral prophylaxis in 27 SOT patients that had been treated successfully for CMV viremia (27). Of these patients, 14/27 had a positive CMV-CMI response and had antivirals discontinued, with only 1 patient experiencing an asymptomatic, low-level viremia after discontinuation. The remaining 13/27 patients had a negative CMV-CMI response and received 2 months of secondary prophylaxis (27). In the CMI-negative patients ($n = 13$), the rate of CMV recurrence was 9/13 (69.2%) ($P = 0.001$) (27). These recurrences occurred either due to breakthrough viremia while on prophylaxis ($n = 2$), after premature discontinuation of prophylaxis for adverse effects ($n = 5$), noncompliance ($n = 1$), or after the 2 months of prophylaxis was complete ($n = 1$). This finding illustrates the difficulty in ongoing prophylaxis for CMI-negative patients.

However, not all studies show clinical utility. In a recently published single-center observational cohort study of 120 CMV seropositive kidney transplant recipients who received ATG induction therapy and valganciclovir prophylaxis (median, 92 days), QFN-CMV was monitored posttransplant from months 2 to 5 (28). There was suboptimal accuracy for the prediction of protection from CMV viremia with the use of QFN-CMV (28). There was no difference in 1-year CMV infection rates between patients with negative (nonreactive or indeterminate) or reactive results (45.8% versus 36.1%, $P = 0.24$) (28). By increasing the IFN-γ cutoff values, specificity and positive predictive value

(PPV) to predict protective CMV-CMI improved, however, this improvement was at a detriment to the negative predictive value (NPV) (28). This study highlights the predictive variability of CMI assays which may be confounded by the patient population studied (R+ versus R−), the type of immunosuppression used, and what exactly one is attempting to predict (clinically significant CMV disease or low-level viremia) (13, 28). In addition, the measurement of CMV responses in the early posttransplant period (within 1 month posttransplant) may be overly influenced by highly potent immunosuppression used at the time of transplantation and may negatively impact clinical utility (19, 29).

CMV-CMI as an adjunct to CMV viral load monitoring. Pre-emptive strategies are a commonly used alternative to antiviral prophylaxis and rely on regular (typically weekly) viral load monitoring and initiation of antivirals at a predefined (but uncertain) threshold, such as 1,000 IU/mL. Studies have shown that allowing DNAemia to occur at a low level may have a beneficial effect by contributing to the development of CMV-CMI (23, 24) and thereby leading to more robust long-term viral control as opposed to patients who receive antiviral prophylaxis. Since viral load thresholds for the initiation of pre-emptive antiviral therapy are poorly defined, it has been hypothesized that longitudinal CMV-CMI measurements may be a useful adjunct to virologic monitoring in order to determine when to initiate antivirals. In 67 lung transplant recipients, both plasma CMV load and CMV-specific $CD8^+$ T cell responses were measured frequently in the first year posttransplant (30). High-level CMV DNAemia requiring pre-emptive antiviral therapy occurred more frequently when CMV-specific T cell responses were undetectable or fluctuated or if they were detected only after the occurrence of DNA detection. The QFN-CMV assay has also been assessed in the setting of low-level CMV DNAemia (31). In a prospective study of 37 SOT patients with low-level DNAemia ($<$1,000 copies/mL), QFN-CMV was measured at the first detection of CMV (31). In this study, the institutional threshold for starting treatment was 15,000 copies/mL, and therefore, low-level DNAemia was followed with repeated viral load measurements. A positive CMV-CMI was associated with spontaneous clearance of DNAemia over a median of 21 days (interquartile range [IQR], 10 to 42 days), suggesting that in many patients, low-level DNAemia does not require antiviral therapy (31). Moreover, CMV-CMI could be used to guide the choice of whether a patient at risk of CMV is assigned to a pre-emptive or prophylactic strategy. Jarque et al. used the result of a pretransplant CMV-CMI to assign 160 D+/R+ kidney transplant recipients to a low-risk or high-risk group (14). They further randomized each group to receive prophylaxis or pre-emptive therapy. The authors found that CMV-CMI was predictive of CMV DNAemia in both risk groups, especially the CMV-CMI value at 15 days posttransplant, regardless of whether the patients received prophylaxis or pre-emptive therapy.

CMV-CMI in the pretransplant setting. There has also been significant interest in measuring CMI responses in candidates prior to transplant in order to better risk stratify patients. While this process is useful primarily in seropositive candidates, some patients who are seronegative also appear to have detectable cellular responses pretransplant. In a cohort study by Bestard et al. of mixed serostatus kidney transplant recipients receiving either routine prophylaxis or pre-emptive therapy, CMV-specific T cell responses against IE-1 and pp65 were measured pretransplant and at 6 months posttransplant using ELISpot (32). Patients developing CMV infection showed significantly lower anti-IE-1-specific T cell responses than those who did not ($P < 0.05$), with low pretransplant anti-IE-1-specific T cell responses predictive of both primary and late-onset CMV infection (32). A study of 55 transplant recipients showed that pretransplant CMV-CMI was negative in CMV seronegative patients; however, only 68% of CMV seropositive patients had CMV-CMI (33). Those lacking CMV-CMI had a 10-fold greater risk of CMV replication posttransplant (33). Other studies have found similar results in that approximately one-third of CMV seropositive patients lack CMV-CMI in the pretransplant setting and that it could be used to predict CMV outcomes in the posttransplant period (34–36). It has been hypothesized that the use of antithymocyte globulin may dampen T cell responses in the first month posttransplant such that the pretransplant T cell response may no longer be protective. However, longitudinal studies have shown that patients receiving

ATG recover CMV-specific CMI by the first month posttransplant (37); as noted previously, immediate posttransplant measurements (e.g., at day 15) may also predict CMV. Overall, however, it is still uncertain whether a transplant recipient's pretransplant CMI measurement in combination with donor serology is a better predictor of posttransplant CMV replication than serology alone.

SUMMARY

There are many clinical scenarios where CMV-CMI assays could be potentially useful (1, 2, 4, 13). These scenarios include using CMI to determine the duration of either primary or secondary prophylaxis or using it as an adjunct test with frequent viral load measurements to refine antiviral use in a pre-emptive strategy. More specifically, in CMV D+/R− patients or R+ patients with risk factors who receive antiviral prophylaxis, it could be used as a monitoring tool performed at regular intervals. If CMI is positive, prophylaxis could be discontinued earlier, and if CMI is negative, either prophylaxis could be prolonged or more careful viral load monitoring could be initiated. A similar strategy could be used to decide about the initiation of secondary prophylaxis after treatment of an episode of CMV infection. In a pre-emptive strategy, patients with low-level DNAemia could have CMI testing performed as an adjunctive tool to decide if antiviral therapy should be initiation or await spontaneous clearance. Finally, it is possible that CMI could replace or be used with serology to help risk stratify candidates in the pretransplant settings.

Before CMV-CMI testing becomes part of the routine clinical care of SOT recipients, multiple issues need to be addressed. An ideal assay should have the ability to be performed in any center's immunology or microbiology laboratory and have a well-defined cutoff for positivity that is applicable to most patients and in most clinical settings. An ELISA-based assay would be simple to implement especially in a lab with expertise performing other Quantiferon assays (e.g., Quantiferon-TB). Currently, the need for specialized equipment for ELISpot and flow cytometry assays may be a limiting factor to their broader implementation. Specialized laboratories may continue to offer these tests as laboratory-developed tests. Nevertheless, regulatory approval will be an advantage for broad acceptance of these assays. Cost-effectiveness considerations are also important, although the cost of CMI testing may be balanced by a reduced need for viral load monitoring as well as a reduction in the need for antiviral prophylaxis and treatment (1, 2). Eventually, the goal of these assays is to personalize the management of CMV to avoid excess treatment and toxicity. Further interventional studies that clearly demonstrate this benefit would further help with clinical adoption.

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