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INVITED REVIEW

Human CMV-specific T-cell responses in kidney transplantation; toward changing current risk-stratification paradigm

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Summary

Despite the great efficacy of current antiviral preventive strategies, hCMV infection is still a major complication after renal transplantation, significantly challenging patient and graft survival. This issue seems to be explained because of the rather poor immunologic monitoring of the antiviral immune response. An important body of evidence has shown that monitoring the hCMV-specific T-cell response, at different time points of the transplant setting, seems to add crucial information for predicting the risk of viral infection, thus potentially helping individualization of therapeutic decision-making in clinical transplantation. While several immune-cellular assays have shown its capability for accurately monitoring hCMV-specific T-cell responses, only few such as the IFN-γ ELISPOT and the ELISA based technology assays might be reliable for its application in the clinic. Nonetheless, an important effort has to be made among the transplant community to standardize and validate such immune assays. Noteworthy, large-scale prospective randomized trials are highly warranted to ultimately introduce them in current clinical practice as a part of the highly desired personalized medicine.

Introduction

Human cytomegalovirus (hCMV) infection is still a major complication after kidney transplantation. While primary infection in immunocompetent hosts is normally asymptomatic, transplant recipients are at increased risk to develop hCMV infection short time after transplantation, critically challenging both graft and also patient survival [1,2]. Indeed, hCMV infection may negatively impact on kidney transplantation by two main mechanisms; on the one hand, hCMV may directly lead to persistent post-transplant viral replication and tissue-invasive injury such as pneumonitits, enteritis, or retinitis, and on the other, indirectly-related hCMV effects, either by bystander immune activation or by T-cell cross-reaction with donor alloantigens, have also been associated with facilitate acute and chronic allograft rejection as well as new onset diabetes (NODAT) and accelerated coronary artery atherosclerosis [3,4].

It is well known that hCMV is a potent immunogenic virus triggering strong immune responses from all the effector mechanisms of the immune system. Despite that humoral immunity through the presence of hCMV-specific IgG antibodies is considered the gold-standard biomarker determining the history of viral infection, it is well accepted that cellular immunity, particularly memory/effector $CD4^+$

and $CD8^+$ T cells, is considered to be crucial for protection from hCMV infection. In fact, in the human system, there are relevant examples showing the predominance of T-cell responses for the control of hCMV; both T-cell lymphopenia and impaired lymphoproliferative responses to hCMV have been demonstrated as risk factors for hCMV disease [5,6], and more illustrative, adoptive transfer of hCMV-specific T-cell clones after allogeneic stem cell and solid organ transplantation (SOT) has provided reasonable indirect evidence demonstrating the importance of hCMV-specific T-cell responses for protection against viral replication [7,8].

Importantly, although outstanding progress has been made in terms of reduction in hCMV-related morbidity and mortality, with the advent of preventive antiviral strategies, using either universal prophylaxis or pre-emptive treatment initiated after viral detection in peripheral blood [9,10], hCMV infection still remains a frequent and unpredictable complication in an important number of transplant patients. Therefore, important efforts are currently being made among the transplant community to find more accurate biomarkers defining the risk for hCMV infection. Therefore, as all transplant recipients may display diverse hCMV-specific T-cell function predisposing to hCMV replication, a main area of research has focused on the evaluation of protective hCMV-specific cellular responses at different time points of the transplant setting.

In this review, we discuss the major role of hCMV-specific cellular immunity for controlling hCMV replication, the potential of hCMV-specific T-cell monitoring using different cellular-based immune assays and its relevant clinical implications for ultimately helping guiding therapeutic decision-making after kidney transplantation.

Caveats and controversies of current serological immune-risk stratification

Today, the immune-risk stratification for hCMV infection in SOT is exclusively based on the hCMV-specific antibody (IgG+) serostatus of donor (D) and transplant recipient (R), as it has been considered a surrogate marker of the hCMV-specific T-cell immunity [11]. Therefore, hCMV-seronegative recipients (R−) considered that lack of any hCMV-specific immunity, antiviral prophylaxis treatment is strongly recommended when receiving an organ from a hCMV-seropositive donor (D+/R−). Conversely, for hCMV-seropositive recipients (R+), thought to be effectively immunized against hCMV, a pre-emptive protocol with periodical viral replication monitoring is more likely proposed. However, important discrepancies may be observed when evaluating the impact of the different preventive antiviral strategies after transplantation. On the one hand, although recent reports have shown that routine prophylaxis may reduce the incidence of post-transplant hCMV infection and improve long-term kidney graft survival as well as cost-effective [12–16] and even anticytomegalovirus drug resistance, especially among D+R− KTR with high hCMV loads [17], others have also reported that pre-emptive therapy is consistently able to decrease the incidence of hCMV disease with the advantages of avoiding development of antiviral resistance, drug toxicity [18,19], and appearance of late-onset hCMV infection [20,21]. Altogether, it suggests that current serological risk stratification for hCMV infection has important limitations: first, although R(+) recipients receiving a seropositive allograft (D+) are considered to have only an "intermediate risk" of hCMV replication, hCMV may reactivate in some recipients after transplantation producing hCMV-related complications [22]; second, despite only few R(+) will develop hCMV disease, most of them are currently followed with a thorough and expensive viral-monitoring protocol [23,24] and in addition, although most kidney transplant patients receiving antiviral prophylaxis will never develop hCMV replication after discontinuation, the extension of the prophylaxis period or continuation with pre-emptive therapy is also being proposed [25].

Therefore, the analysis of hCMV-specific T-cell responses and function using novel immune assays might potentially allow direct quantification of the patient's ability to control hCMV replication, thus helping an appropriate individualization of the type and duration of preventive antiviral treatment. Importantly, this would not be trivial, but because an accurate immune-monitoring of the risk of hCMV infection would also impact in other relevant medical issues such as the avoidance unnecessary drug-related toxicity exposure in some patients and to note, it would also directly influence in the overall cost savings, as the costs of unnecessary drug prophylaxis and serial testing for pre-emptive therapy would significantly be reduced.

Immune-biology against hCMV infection

After transplantation, it is well accepted that both innate and adaptive immune responses play a relevant role in the control of hCMV replication. However, and although it seems that there is a predominant role of the adaptive immune response, it is most likely that interactions between several arms of the innate and the adaptive immune system might contribute to the protection or increased susceptibility of hCMV infection, each of them contributing at different time periods of the disease.

Innate immune responses

Although the exact mechanisms by which hCMV is subject to innate immune control after transplantation still remain

not clear, there are interesting reports suggesting its importance for hCMV control, namely the presence of some specific single nucleotide polymorphisms (SNPs) of Toll-like receptors (TLR2) [26–28] and other immune genes such as the dendritic cell–specific ICAM3-grabbing nonintegrin (DCSIGN) [29], the deficiency of the complement pathway product mannose-binding lectin (MBL) [30] or natural killer cell (NK) dysfunction through their activating killer-cell immunoglobulin-like receptor (KIR) genes [31–35] all of them have been associated with increase in the individual susceptibility to hCMV infection.

Adaptive immunity against hCMV infection

The crucial role of the adaptive immunity against hCMV infection through its two main effector mechanisms (the humoral and cellular) in the transplant setting has been more accurately identified.

Humoral immune response

While the advent of long-lasting humoral immunity toward a primary viral infection is universally accepted, the contribution of antibodies for protection against and control of hCMV replication in transplant recipients is still a matter of debate. However, data coming from experimental models suggesting the importance of the humoral response, particularly in restricting viral dissemination and in limiting the severity of the disease [36,37]. HCMV-specific neutralizing antibodies appear during the first 4 weeks after primary infection and are mainly directed against hCMV glycoprotein B, but also H, L, and pUL128-131, all of them involved in cell attachment, penetration, and fusion of the viral envelope to the cell membrane of the host [38]. In fact, the association shown between the former use of hCMV-specific immunoglobulins as prophylaxis and better transplantation outcome among liver transplant recipients also suggests a protective role of humoral immunity against viral replication [39].

In human transplantation, some hCMV-seropositive transplant individuals are at risk of hCMV infection despite detectable humoral immunity, suggesting either a low avidity or poor neutralizing activity of the antibody response. Interestingly, post-transplant IgM and IgG antibody seroconversion has been shown to not be a reliable predictor of hCMV disease [40]. Furthermore, while most of R−/D+ are at significantly higher risk, some of them (20–30%) do not develop hCMV infection after transplantation, suggesting either an optimal antibody seroconversion early after transplantation or the presence of preformed hCMV-specific memory B cells prior to transplantation even though no detection of circulating hCMV-specific IgG antibodies.

Cellular immune response

The cellular immune response is the major mechanism by which hCMV replication may be controlled (Fig. 1). Both the $CD8^+$ and $CD4^+$ T-cell compartments are crucial for controlling and restricting viral replication [32,41]. Nevertheless, while it is suggested the preponderant role of $CD8^+$ T cells for the control of hCMV replication [42], it appears that $CD4^+$ T cells would be fundamental for conferring long-lasting protection [43], either through the provision of T-cell help in maintaining virus-specific antibody responses [44] and expanding the $CD8^+$ T-cell populations [45] or by directly killing virus-infected cells [46–48]. A highly diverse virus-specific T-cell response develops between 4 and 6 weeks after primary antigen exposure. The memory compartment is generated, based upon the amount of antigen, the replication pattern, and the type of infected tissue. The proportion of both $CD4^+$ and $CD8^+$ T cells committed to the anti-hCMV response is extraordinarily large, ranging from 10% to even 40% in peripheral blood among elderly patients [49,50]. Moreover, the viral proteins to which T cells are directed are considerably diverse, with recognition of a variety of structural, early, and late antigens in addition to hCMV-encoded immunomodulatory antigens [51,52]. To note, these different hCMV-specific T-cell responses are directed toward these hCMV-encoded proteins expressed at different stages of viral replication (immediately-early, early, early-late, and late) and also proteins associated with diverse functions (capsid, matrix/tegument, glycoprotein, DNA/regulatory, and immune evasion), revealing a strong hierarchy among virus-encoded proteins, being the most immunodominant antigens UL123 (immediately early-1, IE-1), UL122 (IE-2), and the UL83 tegumen ones (phosphoprotein 65, pp65).

Even though T-cell responses may target multiple hCMV-specific proteins [52,53], it appears that protective cellular immunity is mainly directed against the lower matrix tegument protein pp65 (encoded within the UL83 gene locus) and to the immunodominant immediately-early proteins (encoded within the UL123 gene locus) [54–57]. Importantly, IE-1 is the initial protein expressed upon hCMV reactivation [58], thus IE-1-specific T-cell clones would be the first to be activated and directed to sites of replication [59–61]. Moreover, in experimental models, it has been shown that IE-1 epitope-specific $CD8^+$ T cells are extremely protective upon adoptive transfer [54].

HCMV-immunity in immunocompentent and immunocompromised transplant individuals

As it is well known, in immunocompetent individuals, primary hCMV infection is usually asymptomatic. However, in few cases, it may result in a mononucleosis-like

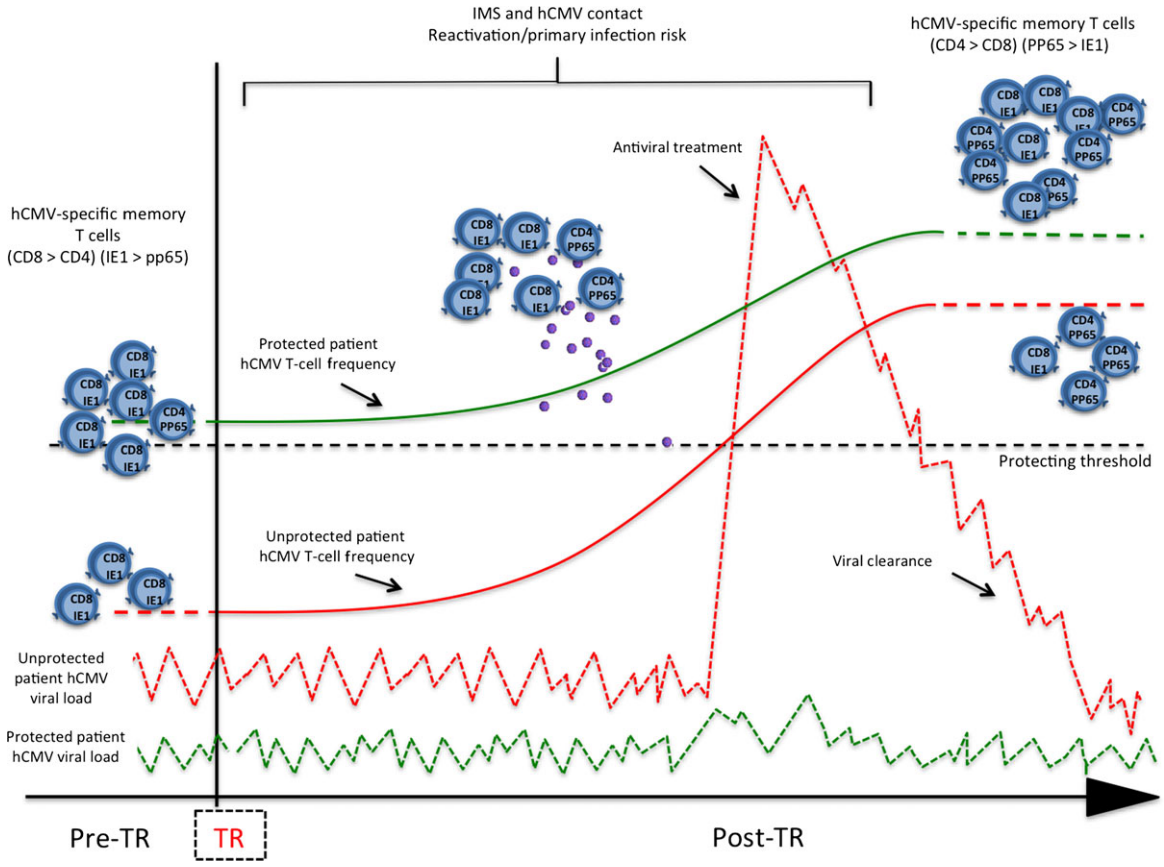

Figure 1 Patterns of hCMV-specific T-cell responses during the transplant setting.

syndrome, similar to that originated by Epstein–Barr virus (EBV). Very rarely, tissue-invasive hCMV infection might be observed among individuals with a preserved immune function. Noteworthy, as solid organ transplant individuals can be considered as predominantly T-cell immunocompromised hosts, due to chronic immunosuppressive treatment, fundamentally targeting T cells, transplant patients are at significantly higher risk than immunocompetent individuals. This fact is even more relevant among nonsensitized individuals against hCMV (i.e, serologically (IgG) negative and with low frequency of hCMV-specific memory/effector T cells) that receive an organ from a seropositive donor. In this regard, hCMV infection can be a frequent and serious complication, in which its presentation may range from a mononucleosis-like syndrome to a severe tissue-invasive disease if not efficiently and rapidly treated.

Impact of current immunosuppressive agents on antiviral immune responses

Importantly, type and amount of immunosuppression may significantly influence the likelihood of hCMV infection after transplantation by delaying hCMV-specific immune responses. To note, the use of T-cell depleting agents such as antithymocyte globulin, alemtuzumab, or OKT3 antibodies, has been associated with a significantly increased risk of hCMV infection [62,63], either due the direct depletion of functional hCMV-specific T cells or by the induction of large amounts of proinflammatory cytokine release, directly involved in the activation of latent hCMV [64]. Classically, mycophenolate mofetil by inhibiting *de novo* guanosine synthesis, targeting activated B and T lymphocyte, has been shown to facilitate hCMV infection, especially at high dosages (higher than 2 g/day) [65]. Regarding calcineurin inhibitors (CNI), cyclosporine A (CsA)-based strategies have been postulated to increase the risk of hCMV infection as compared to tacrolimus-based regimens [66]. Conversely, mTOR inhibitors (both sirolimus and everolimus) have been shown to have a protective effect against hCMV disease as compared to other maintenance immunosuppressants [65–67]. While it is still not that clear which are the main mechanisms by which mTOR inhibitors display such antiviral effect, it has been pointed out that the blockade of the protein complex mTORC, which is crucial for cell-cycle progression, might account

for the inhibition of hCMV to successfully propagate viral protein translation into cells [68,69]. In addition, other reports have also shown that mTOR inhibitors are capable of regulating hCMV-specific $CD8^+$ memory T cells, enhancing its effector functionality [70,71].

Immune-monitoring hCMV-specific T-cell responses in human transplantation

An increasing body of evidence is now showing the feasibility of immune monitoring the hCMV-specific T-cell compartment using different cell-based assays in humans. These studies have allowed a comprehensive analysis of the kinetics and function of the cellular immune response against hCMV, evaluated at different time points of the transplant setting, thus providing an accurate information in terms of prediction of the hCMV disease. Nevertheless, an important limitation of such studies relies in the fact that most of them have evaluated different SOT at the same time, not taking into account the relevant differences in terms of type and amount of immunosuppression used between different organs, thus potentially leading to confusing results. Nonetheless, the relatively homogenous reports, even though evaluating different SOT patients at the same time, suggest a strong correlation between detection of hCMV-specific effector T-cell responses and risk of viral infection. These studies have evaluated the hCMV-specific T-cell immunity using diverse *in vitro* immune assays. Some techniques may directly identify hCMV-specific T cells using peptide–MHC multimers or tetramer-based staining. Others, such as the flow cytometry intracellular cytokine staining, the IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT), or the enzyme-linked immunosorbent (ELISA)-based assays (Quantiferon-CMV) provide a more dynamic or functional information by enumerating cytokine-producing T cells at a single-cell level after hCMV-derived stimuli. Furthermore, T-cell proliferation assays have also been used to measure hCMV-specific T-cell activation *in vitro*. As explained in Table 1, there are main differences between the different assays; while the ELISPOT is more sensitive and robust than flow cytometry, the latter is more capable to provide simultaneous information on functionality (conventional, regulatory, single cytokine producers, multifunctional cells), differentiation (central memory, effector memory, effectors), and phenotype (CD4/CD8) on a single-cell level. Nevertheless, none of these hCMV-specific assays have been approved by the Drug and food administration (FDA) yet, but only the Quantiferon-CMV test has been accepted and commercialized by the European Union. Despite that all of them have shown to accurately reproduce antiviral T-cell responses, the most reliable assays eventually been used in the clinic are the Quantiferon and

Table 1. Main immune assays to assess hCMV-specific T-cell responses.

Immune assay	Sample material/ Amount	Frequency	Time	Protective threshold frequencies	Stimuli/Antigens	Advantages	Disadvantages
Intracellular cytokine staining (flow cytometry)	Whole blood or PBMC 1 ml/1 × 10^6	% CMV-specific CD8 or CD4/IFN-γ cells of the respective reference population	48 h	0.2–0.4%	15aa short peptide pool spanning the whole antigen or whole virus lysate	High sensitivity No HLA restriction Allows CD4 and CD8 independent analysis	Expert personnel required Equipment required (flow cytometer) Research tool
ELISPOT (IFN-γ, IL2)	PBMC 1 × 10^6	IFN-γ spots/3 × 10^5 stimulated PBMC	36 h	8–11 IFN-γ spots	15aa short peptide pool spanning the whole antigen or whole virus lysate	High sensitivity No HLA restriction Reproducibility May allow multiple effector information	Expert personnel required Equipment required (elispot reader)
Tetramer staining (flow cytometry)	PBMC 1 × 10^6	% CMV-specific CD8 or CD4/IFN-γ cells of the respective reference population	48 h	Unspecified	15aa short peptide pool spanning the whole antigen or whole virus lysate	Single epitope-specific clone staining	Expert personnel required Equipment required (flow cytometer) Research tool HLA restriction
Quantiferon (ELISA-based)	Whole blood 3/4 ml	IFN-γ detection (IU)/ml	24 h	0.1–0.2 IU/ml	15aa single peptide collection	Mid-expert personnel required High sensitivity Approved for use in EU	HLA restriction (rare HLA types excluded) might lead to indeterminate result

the IFN-γ ELISPOT assays. Interestingly, while both assays are capable of measuring CMV-specific T-cell responses, both are sustained on different concepts, namely the stimulus peptide composition is designed to selectively stimulate $CD8^+$ T cells in an HLA-restricted manner (Quantiferon) or both $CD4^+$ and $CD8^+$ T cells (ELISPOT), the Quantiferon test evaluates the IFN-γ production in a volume of 1 ml of whole blood, while the ELISPOT test considers the IFN-γ production in a given number of PBMCs isolated from blood, and the Quantiferon-CMV assay quantitatively measures IFN-γ as international units (IU), while the ELISPOT test quantifies the spot-forming colonies (SFC) produced by a given number of PBMCs. Therefore, all these differences may eventually lead to some discrepancies. In this regard, a recent relevant published study compared the ability of these two tests to predict hCMV-specific T-cell responses in 221 kidney transplant recipients [72]. While among seropositive healthy individuals, some discordance was observed between both techniques, among transplant recipients tests displayed similar robustness, sensitivities, specificities, and an inverse correlation with the development of CMV viremia. However, while the IFN-γ ELISPOT has been cross-validated among different centers for monitoring T-cell alloimmune responses [73,74], there is an urgent need for standardization of these assays across different laboratories for accurately establish clear cutoff values predicting the risk for hCMV infection. Indeed, the majority of currently existing assays, but the Quantiferon-CMV assay, have no well-validated cut off for defining positivity. Indeed, a positive value of an IFN-γ level ≥0.2 IU/ml has been defined for the Quantiferon-CMV assay, although this has not been well validated in the transplant population.

To note, different hCMV-derived stimuli have been used to evaluate T-cell responses *ex vivo,* namely whole virus lysates [75–77], hCMV-infected immature dendritic cells [78,79], single peptides, or peptide pools of short peptides spanning the main hCMV antigens (essentially pp65 and IE-1) [80]. To note, all of them may directly affect the efficiency and sensitivity of the *in vitro* tests for the detection of hCMV-specific T cells. Importantly, as the amino acid sequence and length of the peptide may significantly influence the type of the immune response through the restriction of HLA-I presentation on $CD8^+$ T cells, the evaluation of both $CD4^+$ and $CD8^+$ T cells using a pool of peptides spanning the main hCMV antigens is able to avoid the HLA-I presentation restriction *in vitro*. Conversely, using single peptides might be an important disadvantage as might potentially exclude certain HLA types, thus the test may shown no stimulation. Therefore, as hCMV proteins have different roles in the infection process and the pathogenesis of the disease, some particular of them might more clearly illustrate the potential cellular protection at the different stages of the disease. Thus, since immediately-early antigens as compared to tegument-derived antigens appear to play a major role during the first stages of hCMV infection, the former should preferentially be more commonly used before or during the first periods of the transplant, whereas the later should be more likely analyzed later on after transplantation. Nevertheless, immune-monitoring hCMV-specific T-cell responses should include a spectrum of viral proteins to reflect this variability.

Clinical scenarios for monitoring hCMV-specific cellular immunity in the transplant setting

Attempts to immune-monitor hCMV-specific T-cell responses in the transplant setting have been performed at different time points of the transplant evolution with the aim of investigating the kinetics of the hCMV-specific cellular responses either during or after viral infection and furthermore, to evaluate its predictive value as a risk/protective biomarker for developing hCMV viremia or disease (Table 2). While most studies have primarily focused at the post-transplant period, thus taking into account the influence of immunosuppression on the immune response, more recently, some other groups have also assessed the antiviral T-cell immunity before transplantation to potentially predict the likelihood of hCMV infection after transplantation in an earlier time point.

Assessment of hCMV-specific T-cell responses before transplantation

As commented all along the review, current prediction of the risk of developing hCMV infection in the transplant setting is exclusively fundamented on the presence or absence of humoral immunity against the virus before transplantation. Alternatively, a very attractive approach has been recently proposed; as all transplant patients may display an intrinsic baseline functionality of hCMV-specific T-cell responses, thus predisposing to viral replication after transplantation, its assessment would add crucial information for stratifying the risk of hCMV infection already before the transplant (Table 3).

First observations pointing to this direction were found by Bunde and colleagues [55] evaluating a group of lung and heart transplant patients. Using flow cytometry intracellular IFN-γ staining, they showed that frequencies of IE-1, but not pp65-specific $CD8^+$ T cells already at day 0, clearly discriminated patients who did not develop CMV disease from patients at risk. This effect was reproducible for any time point after transplantation. Furthermore, two recent reports have shown similar data although using different T-cell immune assays. On the one hand, Cantisan and coworkers using the Quantiferon-CMV assay against a

Table 2. Suitable clinical settings to immune-monitoring hCMV-specific T-cell responses in kidney transplant patients.

Clinical setting	Main goal	Guided therapeutic strategy
Before transplantation		
All R+ transplant recipients	Discriminate patients at risk of hCMV infection	Assign a 3-months antiviral prophylaxis
	Identify patients at low risk of hCMV infection	Avoid systematic viral monitoring
All R− transplant recipients	Detect measurable protecting antiviral T-cell responses	Allow safe pre-emptive treatment
After transplantation		
At the end of 3-months primary prophylaxis	Identify patients at risk of late-onset hCMV infection	Assign a longer prophylaxis course (6 months)
At the end of treatment of hCMV viremia/disease	Identify patients at risk of viral relapse	Continue with on-going prophylaxis
Patients requiring significant immunosuppression	Discriminate over-immunosuppressed patients at high risk of hCMV infection	Continue on-going prophylaxis
In cases of low levels of hCMV viremia	Identify patients with effective anti-hCMV T-cell responses and low risk of hCMV disease	Avoid antiviral treatment

Table 3. Pretransplant assessment of hCMV-specific T-cell responses to predict hCMV infection after transplantation.

Author/ Reference	Number & type SOT/Serostatus	Type preventive strategy	Immune assay/CMV stimuli	Main result
Bunde *et al.* [55]	*N* = 27 (23 heart, 4 lung TR) 27 R+ (13/27 D+)	Prophylaxis	$CD4^+/CD8^+$ intracellular IFN-γ staining Pp65 peptide pool IE-1 peptide pool	Higher PreTR IE-1-specific CD8 T-cell frequencies negatively associated with CMV disease 6 /15 patients with IE1 T-cell frequencies below the protective threshold did not develop disease
Nickel *et al.* [56]	*N* = 36 kidney TR 24/36 R+ (14/36 R+/D+) (5/36 R−/D+)	36 pre-emptive	Elispot IFN-γ Pp65 peptide pool IE-1 peptide pool	High T-cell responses to IE1 correlated with 6-month graft function Association between CMV disease with low IE-1-specific T-cell frequencies
Bestard *et al.* [82]	*N* = 137 kidney TR 109/137 R+ (28/137 R−/D+)	98 pre-emptive 39 prophylaxis	Elispot IFN-γ Pp65 peptide pool IE-1 peptide pool CMV lysate	Low PreTR IE-1-specific T-cell frequencies independently predicts postTR hCMV infection (antigenemia and disease) Low levels of CMV T-cell frequencies might be detected in few CMV IgG patients Patients lacking IE-1-specific T-cell clones preTR experiencing hMCV infection, reach the same protective IE-1-specific T-cell frequencies at 6 months as those patients never experiencing hCMV infection
Cantisán *et al.* [81]	*N* = 55 (23 lung, 32 kidney) 44 R+ (8/53 R−/+) (3/53 R−/D−)	23 pre-emptive 31 prophylaxis	Quantiferon pp65, IE-1, IE-2, gB	Combining Quantiferon reactivity PreTR and donor serostatus strong association with risk of hCMV infection Quantiferon test performed close to end of prophylaxis did not predict late-onset hCMV replication
Shabir *et al.* [42]	*N* = 38 kidney TR (19/38 R−/D+) (19/38 R+/D+)	38 pre-emptive	$CD4^+/CD8^+$ intracellular IFN-γ staining /MHC-tetramer staining Pp65 peptide pool IE-1 peptide pool	PreTR frequencies of 0.16% IE-1-specific CD8 T cells or 0.08% pp65-specific CD8 T cells independently predict hCMV replication in R−/D+ Detection of PreTR T-cell frequencies did not predict as accurately hCMV replication in the R+/D+ cohort

mix of 22 hCMV peptides in a group of lung and kidney transplant patients showed that pretransplant nonreactive hCMV-specific $CD8^+$ T-cell recipients receiving an organ from a seropositive donor displayed a significantly increased risk of hCMV replication compared with pre-transplant reactive hCMV-specific $CD8^+$ T-cell recipients

[81]. Similarly, our group using the highly sensitive IFN-γ ELISPOT assay in 137 kidney transplant recipients prior to transplant surgery showed that transplant recipients displaying high frequencies of IFN-γ producing T cells against IE-1 antigens were protected from either hCMV replication or disease, regardless the type of preventive strategy used. To note, both immune tests showed a relatively high sensibility and negative predictive value [82]. Another important point raised in this study is the potential to predict the likelihood of hCMV infection, despite receiving T-cell depleting agents after transplantation. To note, none of the two mentioned previous studies found any influence of dialysis treatment with the baseline hCMV-specific T-cell immunity. While this might be a really useful approach to differentiate those seropositive individuals with a "true" effective antiviral immune response, its assessment among hCMV-seronegative patients seems to eventually be able to identify some few individuals already immunized despite no detection of humoral immunity in peripheral blood. Therefore, the knowledge of such information already before transplantation would help on the one hand to identify patients deserving prophylaxis treatment after transplantation and on the other hand to avoid unnecessary serial viral replication monitoring and use of antiviral treatment in an important number of transplant recipients.

Assessment of hCMV-specific T-cell responses after transplantation

Most studies assessing the hCMV-specific T-cell immune response for stratifying the risk of viral infection have focused at the post-transplant setting. Monitoring anti-hCMV T-cell responses after transplantation would be clinically useful for both high-risk seronegative transplant recipients (R−/D+) as well as for seropositive patients (R+) (Table 4).

On the one hand, in seronegative transplant recipients, the presence of hCMV-specific cellular responses after or during an initial 3-month course of antiviral prophylaxis would help to identify those individuals at significantly lower risk of developing late-onset viral infection. In this setting, it seems that hCMV-specific $CD4^+$ T cells and specifically those directed against pp65 antigens would have the main role for controlling hCMV replication. In this regard, a first report among 17 seronegative liver transplant recipients [83] evaluating $CD4^+$ and $CD8^+$ T-cell responses against a pp65 and IE-1 immunodominant hCMV antigens after prophylaxis discontinuation did not show any prediction of hCMV disease or viremia development despite the presence of a relevant T-cell response reconstitution in all patients. Conversely, Kumar and colleagues [84] using the Quantiferon assay evaluated a larger cohort of different SOT patients after a standard course of antiviral prophylaxis the risk of late-onset hCMV infection after prophylaxis treatment. Interestingly, low levels of anti-hCMV IFN-γ T-cell response were predictive of late-onset disease, regardless type of recipient serostatus. Similarly, but in a smaller group of lung transplant recipients ($n = 22$), Pipeling and colleagues [85] reported that high frequencies of pp65 but not IE-1-specific $CD8^+$ effector responses after primary infection were protective of hCMV viral relapse during early chronic infection. To note, in a recent multicenter prospective clinical trial evaluating the predictive value of the Quantiferon assay for protection from late-onset hCMV disease, it was shown the relatively high positive predictive value of the test predicting the risk of development of subsequent hCMV infection [86].

On the other hand, monitoring anti-hCMV T-cell responses after transplantation among seropositive (R+) transplant recipients would also be useful to identify those patients with protective antiviral T-cell reconstitution, thus avoiding the use of prophylaxis treatment as well as the implementation of unnecessary periodical viral monitoring. In this regard, Abate *et al.* investigated the frequency of hCMV-specific IFN-γ-secreting T cells using the ELISPOT assay, in a different cohort of seropositive kidney, heart, and small bowel transplant recipients and observed that those low T-cell responder patients were at significantly lower risk of developing subsequent hCMV infection [87–89]. Similarly, but using the Quantiferon assay, among kidney transplant recipients, non-T-cell responders were at significantly increased risk of hCMV reactivation [84,90]. Furthermore, Egli and colleagues [91] using the intracellular IFN-γ staining flow cytometry reported the importance of pp65-specific $CD4^+$ T cells protecting from hCMV replication. However, some others did not observe any association between early post-transplant antiviral responses and the advent of hCMV reactivation [22,92].

To note, prediction of hCMV replication using different *in vitro* assays might potentially be misleading, especially among R+/D+, as hCMV peptides used as stimulators are presented by recipient HLA, thus *in vivo* viral presentation through donor cells could be underestimated [42].

Importantly, the kinetics of hCMV-specific T-cell responses during ongoing viral replication has also been deeply investigated. First relevant reports conducted in bone-marrow transplant recipients correlated hCMV-specific cytotoxic T-cell responses with recovery of hCMV replication [93]. Among solid organ transplant recipients, a dominant hCMV-specific $CD8^+$ T-cell response has been suggested in the early response to primary hCMV infection in seronegative recipients receiving a seropositive donor [22,94]. Likewise, in a group of kidney transplant recipients, Mattes and colleagues [95] showed that functional impairment of hCMV-specific $CD8^+$ T cells is associated with a significant increased risk of progression to high-level

Table 4. Post-transplant assessment of hCMV-specific T-cell responses to predict hCMV infection.

Author/ Reference	Number & type SOT/Serostatus	Type preventive strategy	Immune assay/CMV stimuli	Main results
Sester *et al.* [76]	*N* = 76 kidney TR 76 R+	Pre-emptive	$CD4^+/CD8^+$ intracellular IFN-γ staining Viral lysate	Low hCMV-specific $CD4^+$ T cells (>0.25%) significantly correlates with CMV viral load Asymptomatic KTR with no CMV replication showed median hCMV T-cell frequencies similar to healthy controls
Sester *et al.* [75]	*N* = 96 (68 kidney, 14 heart, 14 lung) 96 R+	Pre-emptive	$CD4^+/CD8^+$ intracellular IFN-γ staining Viral lysate	Lung TR recipients displayed significantly lower frequencies of hCMV-specific $CD4^+$ T cells than kidney TR or healthy controls Low levels of hCMV-specific $CD4^+$ T cells correlate well with hCMV viral load
Radha *et al.* [97]	*N* = 39 kidney TR 33 (24R+/9R−) 6 CMV+Viremia (3R+/3R−)	R−/D+ prophylaxis R+ pre-emptive	$CD4^+/CD8^+$ intracellular IFN-γ staining Viral lysate pp65 peptide mix	Healthy controls and kidney TR patients showed a strong correlation between serostatus and $CD8^+$ hCMV-specific cell frequencies
Gerna *et al.* [78]	*N* = 38 (20 heart, 9 lung, 9 kidney) 38 R+	Pre-emptive	$CD4^+/CD8^+$ intracellular IFN-γ staining hCMV-infected immature dendritic cells	A threshold of 0.4 hCMV-specific T cells/μl detected at 1 month post-TR predicted protection from hCMV infection
La Rosa *et al.* [83]	*N* = 17 liver TR 17 R−/D+	Prophylaxis	$CD4^+/CD8^+$ intracellular IFN-γ staining Viral lysate Pp65 peptide pool IE-1 peptide pool	No evidence of higher hCMV-specific T-cell counts in asymptomatic patients
Egli *et al.* [91]	*N* = 73 kidney TR 25/73 R−/D+ 48/73 R+	Pre-emptive	$CD4^+/CD8^+$ intracellular IFN-γ staining Viral lysate pp65 peptide mix pp72 peptide mix	Inverse association between hCMV-specific T-cell counts and viral replication Pp65 $CD4^+$ cell counts higher than 0.03% showed a positive predictive value of 95%
Kumar *et al.* [84]	*N* = 108 (32 kidney, 48 lung, 19 liver, 4 kidney-pancreas, 5 other TR) 73 R + 35 R−D+	Prophylaxis	Quantiferon pp65, IE-1, IE-2, gB	Detection of hCMV-specific T-cell frequencies at the end of prophylaxis predicted risk of hCMV disease
Abate *et al.* [87]	*N* = 85 kidney TR 70/85 R+ 13/85 R−/D+ 2/85 R−/D−	70 pre-emptive 13 prophylaxis	Elispot IFN-γ Pp65 peptide pool Scramble peptide mix	Significantly lower hCMV-specific T-cell frequencies were detected, 2 months before the increase of hCMV load, in patients experiencing viral replication Similar hCMV-specific T-cell reconstitution in ATG-treated and not treated patients Highly heterogenous pattern of immune recovery in R + was found after transplantaion
Eid *et al.* [92]	*N* = 44 kidney TR 33/44 R+, 11/44 R−/D+	Prophylaxis	$CD4^+/CD8^+$ intracellular IFN-γ staining Viral lysate Pp65 peptide mix IE1 peptide mix	No association between hCMV-specific T-cell counts and viral load, in the high-risk group (R−/D+)
Sund *et al.* [77]	*N* = 17 (17 kidney TR, 1 kidney-pancreas) 17 R+/D+	Pre-emptive Prophylaxis	$CD4^+/CD8^+$ intracellular IFN-γ staining MHC-tetramer staining Pp65 short peptide pool Viral lysate	Proportion of $CD4^+$ IFN-γ T cells at 2 months as compared to baseline, strongly correlated with hCMV viremia

Table 4. continued

Author/ Reference	Number & type SOT/Serostatus	Type preventive strategy	Immune assay/CMV stimuli	Main results
Gerna *et al.* [79]	*N* = 134 (58 heart, 24 lung, 52 kidney TR) (117R+, 13R−)	Preemptive	$CD4^+/CD8^+$ intracellular IFN-γ staining hCMV-infected immature dendritic cells	High frequencies of hCMV-specific T cells (>0.4 cells/μl) conferred protection to CMV disease
Lisboa *et al.* [96]	*N* = 37 (17 kidney, 4 liver, 3 lung, 2 heart, 3 combined) 26 R+ 3R−/D+	Pre-emptive and prophylaxis	Quantiferon pp65, IE-1, IE-2, gB	Patients with a positive test (0.2 IU/ml) displayed a significantly higher spontaneous viral clearance than patients with a negative test
Manuel *et al.* [86]	*N* = 127 (68 Kidney, 10 kidney-pancreas, 27 liver, 14 lung, 4 heart, 2 other) All R−/D+	Prophylaxis	Quantiferon pp65, IE-1, IE-2, gB	Patients with a positive result (0.2 IU/ml) had a subsequent lower incidence of CMV disease than patients with a negative and an indeterminate result PPV and NPV for predicting CMV disease were 90%, 27%, respectively

viral replication as compared to patients maintaining high antiviral T-cell frequencies keeping hCMV replication suppressed to undetectable levels. Furthermore, spontaneous clearance of hCMV viremia might be observed in those highly T-cell-reactive transplant recipients at the onset of viremia [96]. Interestingly, and trying to further analyse this issue, Gerna and coworkers [79] accurately showed that hCMV-specific $CD8^+$ T cells alone do not seem to consistently control hCMV replication, whereas reconstitution of both hCMV-specific $CD4^+$ and $CD8^+$ T-cell immunity is needed. Taken together, it seems that while a dominance of hCMV-specific $CD8^+$ T-cell immunity is required during the early response to hCMV infection, a relatively predominant hCMV-specific $CD4^+$ T-cell response is necessary in long-term protection in persistent or latent infections [76,97], which at the same time would potentially correlate with optimal neutralizing antibodies against hCMV [79]. To note, whether central rather than effector/memory antigen-specific T-cell responses would better predict longlasting antiviral immunity still remains to be answered.

Summary

In parallel with the other arms of the immune response, cellular immunity through both effector $CD4^+$ and $CD8^+$ T cells play a critical role for controlling hCMV replication after transplantation. As all kidney transplant patients display an intrinsic functionality of CMV-specific T-cell responses depending on different factors such as previous antigenic contact, type, and amount of given immunosuppression, monitoring hCMV-specific T-cell effector responses beyond current serostatus assessment between recipient and donor seems to add crucial information to discriminate patients at increased risk for post-transplant hCMV infection. Several immune-cellular assays have shown its capability for accurately monitoring hCMV-specific T-cell responses, among them, the IFN-γ ELISPOT and the Quantiferon assays seem to be most reliable for its application in the clinic. However, standardization and validation of such immune assays preferentially through large-scale, statistically powered prospective trials in which random allocation of patients to different CMV-preventive strategies by their hCMV-specific T-cell immune-response stratification is highly warranted in order to ultimately bring them in current clinical practice as part of the highly desired personalized medicine.

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