Assessment of human cytomegalovirus (HCMV)-specific memory T and B-cell responses as predictors of HCMV infection after kidney transplantion

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ASSESSMENT OF HUMAN CYTOMEGALOVIRUS (HCMV)-SPECIFIC MEMORY T AND B-CELL RESPONSES AS PREDICTORS OF HCMV INFECTION AFTER KIDNEY TRANSPLANTION

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CERTIFY

That Marc Lúcia Pérez, graduated in Biotechnology, has carried out under our direction the research work to elaborate his doctoral thesis untitled "Assessment of Human HCMV-Specific Memory T and B-cell responses as Predictors of HCMV infection after Kidney Transplantation", and through this writing they authorize its presentation to achieve the degree of Doctor in Biomedicine.

This is made evident to all effects in Barcelona, the 9th of December 2014.

Oriol Bestard Matamoros — Thesis Director

Josep Ma Grinyó i Boira — Thesis Co-director

‘Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice, never at least fatally’.

Thucydides, History of the Peloponnesian War. 430 B.Ch.E.

Al meu pare, qui sense esperar res de mi, ho ha esperat tot.

A la meva mare, qui m'ha ensenyat a estimar i a somiar.

Al meu germà, qui m'ha servit de referència i de mirall.

A la Laura, la més gran de les meves inspiracions.

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I. Introduction.

Kidney transplantation is the elective treatment of choice for any kind of patient suffering from end-stage chronic disease, offering greater patient survival (1) , better quality of life and lower cost than other renal replacement therapies (2).

After the introduction of potent immunosuppressants such as calcineurin-inhibitors (CNI), anti-metabolites and novel monoclonal or polyclonal antibodies during the late 80s early 90s, one-year survival rates for renal allografts improved from approximately 60 percent to between 80 and 90 percent (3,4) mainly because of a significant reduction in acute rejection. However, long-term allograft survival has not improved as expected (5), being chronic allograft rejection and death of patient with a functioning graft the leading causes of the late loss of renal allografts (more than one year after transplantation), resulting in an annual rate of loss of 3 to 5 percent (6). Importantly, Cardiovascular and Infectious complications account as main causes of morbidity and mortality in recipients of kidney and also other solid organ transplantation (SOT).

Human Cytomegalovirus (hCMV) infection is still the most significant opportunistic infection after kidney transplantation, having a direct impact on both morbidity and mortality and is also associated with diminished allograft survival (7,8). Over the last decades, HCMV has been linked to premature aging of the immune system ("immunosenescence") as well as premature clinical manifestations of vascular pathology, particularly in the context of T-cell immunosuppressed individuals (9–11) . HCMV is noted for sometimes inducing very large T cell responses but at the same time for possessing multiple immune evasion mechanisms (12). As an ubiquitous virus from the herpesvirus family, it can be transmitted in many ways such as via saliva, sexual contact, placental transfer, breastfeeding, blood transfusion, solid-organ

transplantation (SOT), and hematopoietic stem cell transplantation (SCT) (13) and it is carried by a vast majority of human population with a seroprevalence ranging from 30 to 90% in developed countries (14). HCMV infection has multiple manifestations depending on the site of infection and the nature of the host response. The reservoir for latent infection appears to include cells of the monocyte lineage, but viral replication may occur in multiple differentiated cell types, including fibroblasts, epithelial and endothelial cells, and other parenchymal cells.

1.Effects of hCMV infection in transplant recipients

Primary Infection in immunocompetent hosts very rarely presents any symptomatology, as the hCMV immune responses are usually capable of controlling viral replication and confining the virus permanently into a latency stage.

Conversely, when infecting an immunocompromised individual, hCMV infection becomes a major complication. Likewise, transplant recipients, due to the effects of chronic immunosuppressive drugs aimed at preserving the graft, are particularly susceptible to both primary infection and hCMV reactivation short time after transplantation, critically challenging both graft and patient survival. . As a consequence of the absence of an effective immune response, it is well-recognized that hCMV infection may lead to two main effects; on the one hand, persistent hCMV viral replication may *directly* spread and induce severe tissue-invasive injury such as pneumonitis, enteritis or retinitis, and on the other hand, *indirect*- hCMV effects based on interactions between low levels of viremia and the host immune response have also been associated to acute and chronic allograft rejection by either bystander immune activation or T-cell cross-reaction with donor-alloantigens through heterologous

immunity (15,16). Furthermore, more recently, hCMV infection has also been reported to favor the occurrence of new-onset diabetes mellitus as well as accelerated artherosclerosis within renal transplant patients (15,16).

2.Impact of hCMV infection in clinical transplantation before and after prophylaxis regimens

Noteworthy, with the advent of more accurate and expeditious methods to diagnose active viral disease (17,18) as well as with the introduction of novel and more potent preventive antiviral strategies, the incidence and the severity of hCMV infection after solid organ transplantation has considerably been reduced. Indeed, with the introduction of 2 main types of preventive therapeutic strategies after transplantation, either by the use of universal *prophylaxis* that is, a systematic long–lasting anti-viral treatment during the first 3 to 6 or even 12 months after transplantation irrespective of the serological donor/recipient combination or conversely, through a *preemptive* treatment initiated on the basis of the detection of viral replication in peripheral blood, has significantly helped to reduce morbidity and mortality of solid organ transplant patients.

Intravenous ganciclovir, long the mainstay of therapy, has been partially replaced by oral therapy with valganciclovir, based on recent data demonstrating its therapeutic non inferiority (19). Indeed, while recent reports have shown that routine prophylaxis with valganciclovir may reduce the incidence of post-transplant hCMV infection and improve long-term kidney graft survival (20–23), other groups have also shown that preemptive therapy is also able to decrease the incidence of hCMV disease, avoiding

development of antiviral resistance, drug toxicity (22,24,25) and the advent of late onset hCMV infection (26,27).

Nonetheless, despite the significant improvement achieved, it is still not possible to accurately individualize anti-viral therapy to those patients that are actually at increased risk of hCMV infection after transplantation thus, suggesting that current immune assessment of the hCMV risk before kidney transplantation, which is exclusively relying on circulating hCMV IgG titers is not accurate and informative enough to predict the risk of hCMV infection in all transplant recipients.

3.Challenge of current immunosuppression on hCMV infection

Importantly, type and amount of immunosuppression seems to also 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 (rATG), alemtuzumab, or OKT3 antibodies, has been associated with a significantly increased risk of hCMV infection (28,29), either due the direct depletion of functional hCMV-specific T cells or by the induction of large amounts of pro-inflammatory cytokine release, directly involved in the activation of latent hCMV (30). Classically, mycophenolate mofetil by inhibiting *de novo* guanosine synthesis, targeting activated B and T lymphocytes, has been shown to facilitate hCMV infection, especially at high dosages (higher than 2g/day) (31). Regarding CNI drugs, cyclosporine A (CsA)-based strategies have been postulated to increase the risk of hCMV infection as compared to tacrolimus-based regimens (32). Conversely, mTOR inhibitors (both sirolimus and everolimus) have been shown to have a protective effect against hCMV infection as compared to other maintenance immunosuppressants (31–33). 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 (34,35). In addition, other reports have also shown that mTOR inhibitors are capable of regulating hCMV-specific C8+ memory T cells, enhancing its effector functionality (36,37) .

II. Immune biology of hCMV infection.

As a result of more than a hundred million years of coevolution with its vertebrate hosts, hCMV has developed a multitude of strategies to modulate the host immune defenses, thus facilitating establishment of lifelong persistence through the achievement of a balance between the host immune responses (both innate and adaptive) and the viral pathogenesis mechanisms.

Figure 1. The virus-host balance.

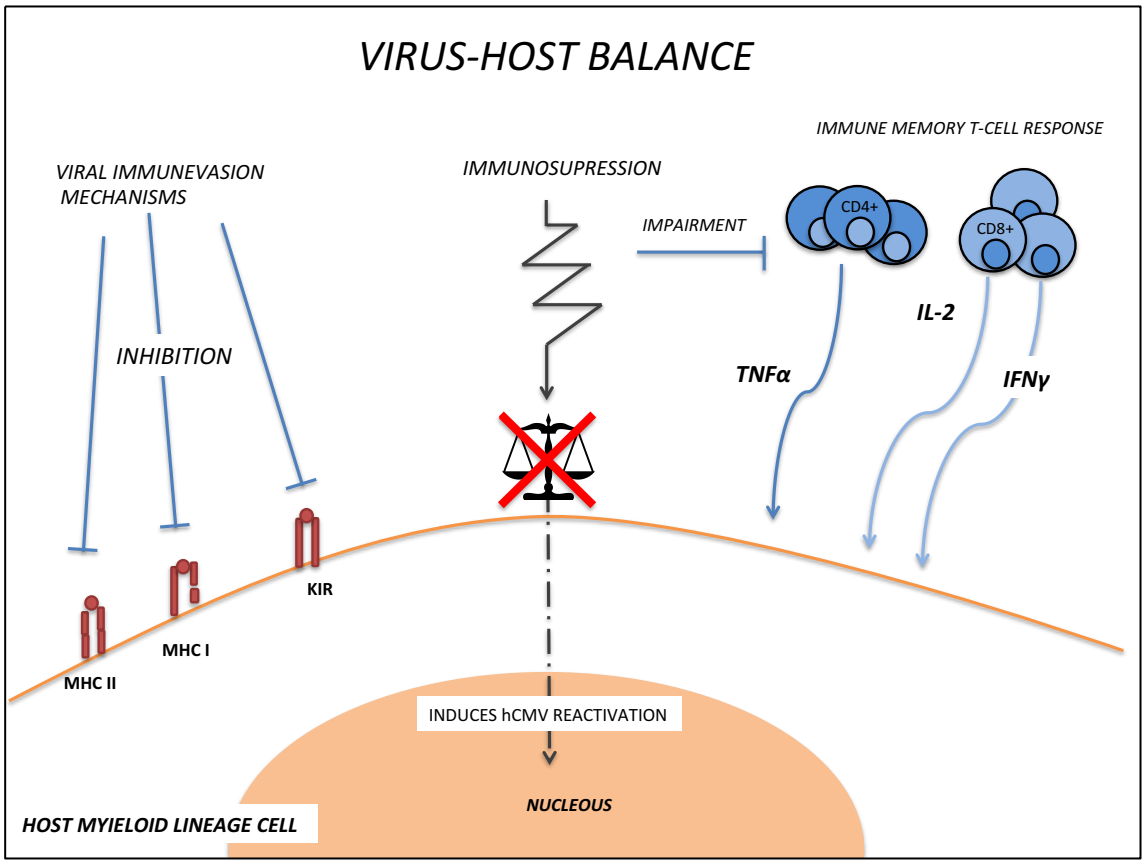
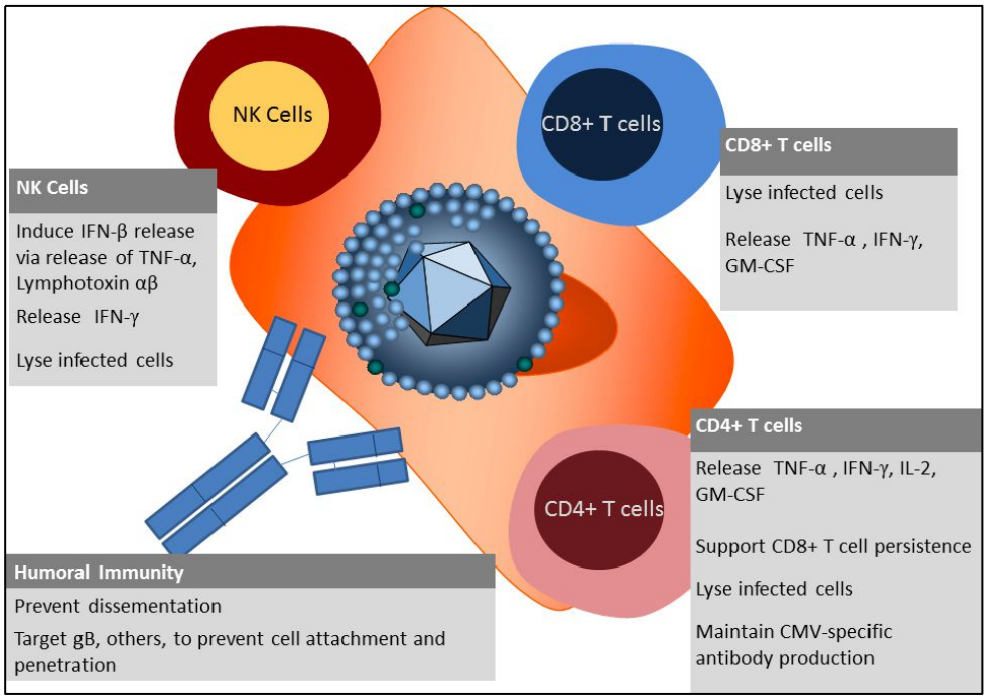


Figure I. '*Given the capacity of unrestrained infection to cause disease, the biology of hCMV infection in immunocompetent populations can be conceptualized as an evolutionary 'negotiated' balance between viral mechanisms of pathogenesis, persistence, an immune evasion and the host cellular immune response'.* (38).

Even though it is likely that the overall protection to hCMV is based on complex interactions between the different arms of the innate and adaptive immune responses, each of them contributing at different levels and time-periods of the disease, the role of the adaptive immune response, based on cytotoxic (CD8+) and helper (CD4+) T cells, is pivotal to protect effectively against hCMV infection and replication.

Therefore in the post-transplant setting, where the adaptive immune system results partially impaired due to the effects of Immunosuppressive drugs, the balance between viral pathogenesis, immune evasion and the host immune response is disrupted leaving transplanted patients at a high risk to contract disease (7,8).

Figure 2. (modified from Hanley et al. (39)**) NK, cellular and humoral immunity to hCMV.**



1. Mechanisms of viral immune-evasion

As many other members of the herpesvirus family, hCMV has developed multitude of mechanisms to hinder the host immune defense. There are a wide number of evasive mechanisms and they may take place at different times during hCMV life cycle affecting both the innate and adaptive arms of the immune system.

In fact, hCMV disposes of a large array of strategies to avoid an efficient immune response and these are thought to be the reason for its successful survival in both

human and animal populations. Among several described mechanisms, *latency* appears to be a particularly important part of the ability of HCMV to hide from the immune system. Some specific viral genes, such as US2, US6, US11, US3, *interfere in antigen processing* and presentation to prevent recognition by cytotoxic CD8+ T-cells, by decreasing cell surface expression of MHC class I (40), although it might not completely deplete cellular recognition and cytotoxic effect on infected cells, depending on its antigenicity (41). Similarly, in order to intefere with CD4+ T-cell responses, hCMV disrupts the MHC II up-regulation process by inhibiting JAK1 as well as class II transactivator mRNA. US2 expression also inhibits MHC II expression by eliciting the HLA-DR α and HLA-DM α MHC class II subunits degradation in the cytosol (41). Interestingly, a viral homologue of the anti-inflammatory IL-10 cytokine (cmv-IL10) (42) encoded in the viral genome and expressed during the latency period, may as well down-regulate the expression of both, MHC class I and II molecules and exert other anti-inflammatory effects (43,44).

Furthermore, favoring the non-classical MHC class I molecule HLA-E expression in the cell surface, hCMV has been shown to inhibit natural killer (NK) cell-mediated lysis by interacting with CD94/NKG2A receptors (45,46).

Importantly, although hCMV seems to be capable, through different intricate molecular mechanisms, to interfere with its recognition by each of the innate and adaptive components of the immune system, ensuring its persistence in the host cells, these immune evasive mechanism are of limited efficiency and cannot ultimately prevent the immune recognition of a broad range of epitopes from cytomegalovirus-encoded proteins after natural infection (47).

2. HCMV-specific immunity

2.1. Innate Immune Response to hCMV

Although the importance of innate immunity in relation to hCMV protection is often over-shaded by the adaptive effector arm of the adaptive immune response, it has been shown that innate immunity directly contributes to the initial defense against the virus and also primes the adaptive immune response through different mechanisms which that promote a more efficient activation of antibody-presenting cells (APC) and T lymphocytes. (45,46,48).

Interesting reports have made valid points linking the presence of certain single nucleotide polymorphisms in genes such as TLR2 (47) and in the promoter of the dendritic-cell specific ICAM3-grabbing non-integrity (DCSIGN) to an increased risk of hCMV reactivation(49). In addition, the stimulation via innate mechanisms such as TLR activation by hCMV, may activate signal transduction pathways which will induce the secretion of inflammatory cytokines that will in turn recruit cells of the innate and adaptive immune system by up-regulating costimulatory molecules such as CD80 and CD86 (50).

Noteworthy, Natural Killer (NK) cells are a main effector mechanism in the innate control of the hCMV. In fact, relevant evidence suggests that patients with NK cells defects on their activating killer-cell immunoglobulin-like receptors (KIR) genes may have an increased susceptibility to hCMV infection leading to recurrent episodes of hCMV infection (51–54). In vitro studies have demonstrated that IL-2 activated human NK can inhibit hCMV replication in hCMV-infected fibroblasts through the secretion of IFN γ (55), and inducing TNFb secretion by the infected fibroblast(56). Moreover,

research conducted on experimental animal models have shown how NK cells participate directly in the MCMV replication control and, how protection to MCMV can be restored in mice with deficient NK subset and susceptible to MCMV infection by transferring NK from normal mice (57,58) .

In addition, γ / δ T cells have also been shown to play a certain role in hCMV replication control. Among γ / δ T cells, the V δ 2- population is of particular interest in HCMV infection because its long-term expansion is almost like a signature of HCMV infection (59). Recently, major insights into the cognate ligands of this population in the context of HCMV infection were made (60). While these cells are not strictly related to HCMV-specific, they can effectively lyse HCMV-infected fibroblasts and endothelial cells as a result of a cellular stress response that leads to the upregulation of endothelial protein C receptor (EPCR) plus co-stimulatory molecules like CD54 (ICAM-1).

2.2. Adaptive Immunity to hCMV

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

2.2.1. 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 suggest the importance of the humoral response,

particularly in restricting viral dissemination and in limiting the severity of the disease (61,62). 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 (63). 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 (64)

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 (65). Furthermore, while most of seronegative recipients (R-) receiving a seropositive donor (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.

2.2.2. Cellular Immune Response

HCMV triggers an overwhelming response to all immune system components in order to control the virus, but an effective cellular immune response is mandatory to control latency and impede viral replication in latently infected individuals (66), where an

extraordinary percentage of up to 10% of all circulating CD8+ T cells can be dedicated towards recognition of hCMV epitopes (67).

Early evidence of the critical role of hCMV-specific CD8+ T-cells in restricting and controlling viral replication was observed on the Bone Marrow transplant setting (68) and in murine models of MCMV infection (65), where both CD4+ and CD8+ T-cells compartments have been shown to play pivotal and complementary roles in controlling hCMV (65,69).

Cytotoxic CD8+ hCMV-specific T cells are thought to exert a direct an immediate control of viral replication, not only by the secretion of cytotoxic molecules such as Granzyme B and Perforin, but also a wide range of cytokines such as IFN γ , TNF$_{\alpha}$ and MIPb (70–72). On the other hand, helper CD4+ hCMV-specific T-cells are necessary to initiate and maintain long lasting protection (73) either by providing T-cell help (Th2) to in maintaining virus-specific antibody responses (74), and expanding the CD8+ effector T-cell populations (75) or by directly killing virus-infected cells (76–78).

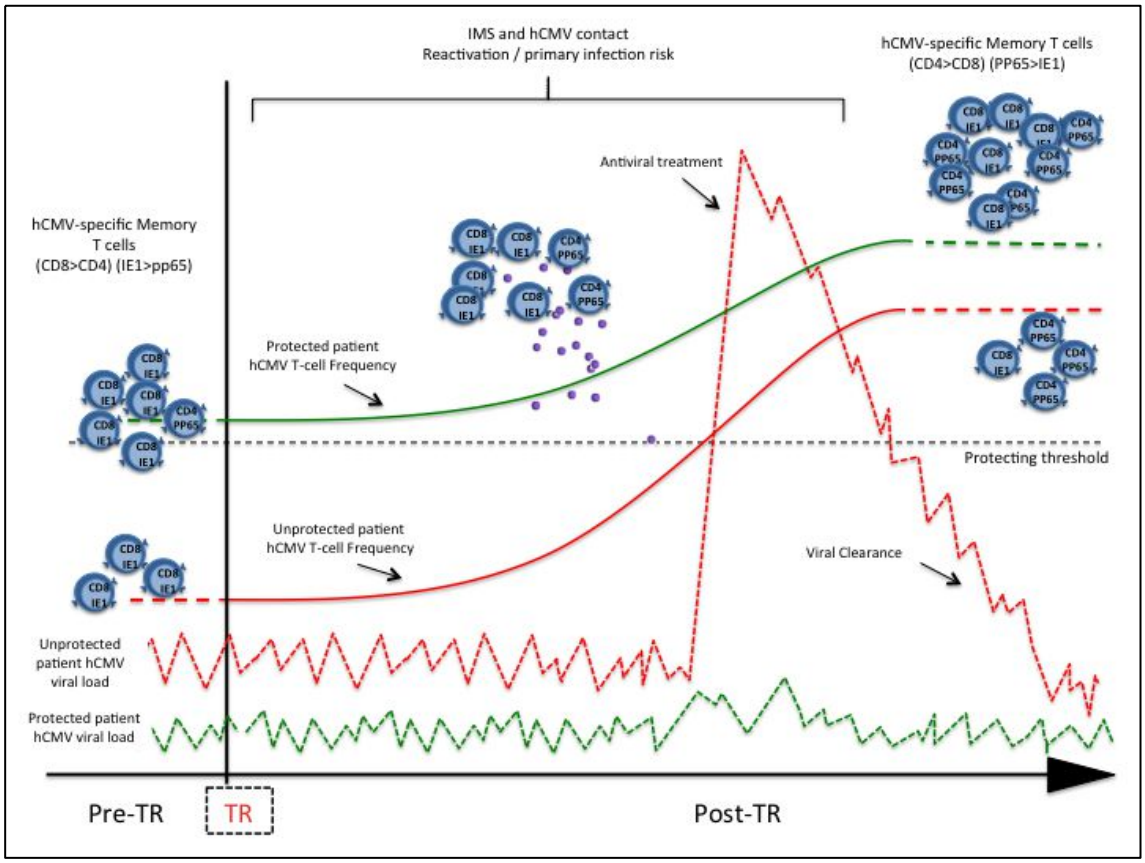
Clinical evidence of the T-cell protective role may be observed as impaired lymphoproliferative responses to hCMV and T-cell lymphopenia are demonstrated risk factors for hCMV disease(79,80). Moreover, adoptive transfer protocols in the SOT and SCT setting, consisting in harnessing the patient's cellular effector arm by transfusing either hCMV lysate–stimulated or hCMV-peptides-stimulated donor T-cells, has yielded reasonable indirect evidence of its effectiveness in both prophylactic and therapeutic use, as in most cases resulted in replication control and resolved infection (81–83).

As the hCMV has a large genome of 230Kb, encoding for more than 200 different proteins, distributed in 3 different stages of viral replication (Immediate-Early, Early

and Late), it is not unlikely that the population of T-cell clones targeting hCMV antigens is equally extraordinarily large and diverse, reaching up to 40% in peripheral blood in elder individuals (84,71). In addition, the repertoire of hCMV-encoded proteins targeted by T-cell responses, includes proteins expressed at the different stages of viral replication as well as proteins associated to the different viral components (capsid, matrix/tegument, glycoprotein) and functions (DNA regulatory and immune evasion)(38,85).

The most immunodominant antigens described so far are UL123 (Immediately Early-1, IE-1), UL122 (IE-2), and UL83 (phosphoprotein 65, pp65), comprising at least 40% of the whole hCMV-specific T-cell clonality, from which the great majority of hCMV-specific T cells are directed against the lower matrix tegument protein pp65 (38). In the last years, the study of T-cell responses directed against immediately-early expressed proteins such as IE-1 has raised particular interest, as they are the very first expressed proteins right after viral reactivation and therefore, a main potential target by T cells to abrogate the spread of viral replication. In this regards, in experimental models it has been shown that IE-1-specific CD8+ T cells are extremely protective upon adoptive transfer (86).

Figure 3. *Schematic kinetics of hCMV-specific T-cell population patterns during the transplant setting.*



III. Immune monitoring hCMV infection in clinical practice; Caveats and Controversies.

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 (87). 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 (21–23,88,89) and even anti-cytomegalovirus drug resistance, especially among D+R- KTR with high hCMV loads (90), 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 (24,25), and appearance of late-onset hCMV infection (26,27).

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 (91); second, despite only few R(+) will develop hCMV disease, most of them are currently followed with a thorough and expensive viral-monitoring protocol (92,93) 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 (94).

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 preemptive therapy would significantly be reduced.

IV. Hypothesis.

Monitoring preformed frequencies of circulating hCMV-specific memory T and B cells using an IFNγ and IgG-enzyme-linked immunosorbent spot assay (ELISPOT) assays, may allow a more accurate characterization of the anti-viral immune sensitization state of kidney transplant patients regardless their hCMV-specific serological profile, ultimately illustrating the potential risk for hCMV infection after transplantation.

V. Objectives.

- The first objective of this thesis is to evaluate whether immunenomitoring hCMV-specific T-cells circulating on peripheral blood using IFNγ-ELISPOT assay on kidney transplant recipients before transplantation could be a reliable biomarker to discriminate patients at increased risk of hCMV infection after kidney transplantation.

- The second objective of this thesis is to characterize the hCMV-specific adaptive immune response of kidney transplant recipients by means of assessing the hCMV-specific memory T and B-cell responses using the IFNγ- and IgG-ELISPOT assays within both hCMV IgG-seropositive and IgG-seronegative kidney transplant recipients.

- The third objective of this thesis is to evaluate whether baseline frequencies of circulating hCMV-specific memory T-cells in hCMV serologically negative could identify hCMV-immunized kidney transplant patients at lower risk of hCMV infection after kidney transplant patients.

- The fourth objective of this thesis is to investigate the kinetics of circulating hCMV-specific memory T -cells after kidney transplantation in relation to the occurrence of hCMV infection.

VI. Studies.

1. Article American Journal of Transplantation:

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Pretransplant Immediately Early-1-Specific T Cell Responses Provide Protection For CMV Infection After Kidney Transplantation

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Cytomegalovirus (CMV) infection is still a major complication after kidney transplantation. Although cytotoxic CMV-specific T cells play a crucial role controlling CMV survival and replication, current pretransplant risk assessment for CMV infection is only based on donor/recipient (IgG)-serostatus. Here, we evaluated the usefulness of monitoring pre- and 6-month CMV-specific T cell responses against two dominant CMV antigens (IE-1 and pp65) and a CMV lysate, using an IFN-γ Elispot, for predicting the advent of CMV infection in two cohorts of 137 kidney transplant recipients either receiving routine prophylaxis ($n = 39$) or preemptive treatment ($n = 98$). Incidence of CMV antigenemia/disease within the prophylaxis and preemptive group was 28%/20% and 22%/12%, respectively. Patients developing CMV infection showed significantly lower anti-IE-1-specific T cell responses than those that did not in both groups ($p < 0.05$). In a ROC curve analysis, low pretransplant anti-IE-1-specific T cell responses predicted the risk of both primary and late-onset CMV infection with high sensitivity and specificity (AUC > 0.70). Furthermore, when using most sensitive and specific Elispot cut-off values, a higher than 80% and 90% sensitivity and negative predictive value was obtained, respectively. Monitoring IE-1-specific T cell responses before transplantation may be useful for predicting posttransplant risk of CMV infection, thus potentially guiding decision-making regarding CMV preventive treatment.

Key words: Cytomegalovirus infection, Elispot, kidney transplantation, T cell response

Abbreviations: BPAR, biopsy-proven acute rejection; CMV, cytomegalovirus; CNIs, calcineurin-inhibitors; Elispot, enzyme-linked immunosorbent spot assay; IE-1, immediately early-1; mTor-i, mTor-inhibitors; PBMCs, peripheral blood mononuclear cells; PP65, phosphoprotein 65; rATG, rabbit antithymocyte globulin; ROC, receiver operating characteristic

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Introduction

Human cytomegalovirus (CMV) infection is still a major complication after kidney transplantation. Because of T cell immunosuppression, transplant recipients are at increased risk to develop CMV infection a short time after transplantation, critically challenging both graft and also patient survival (1,2). On the one hand, CMV infection may *directly* lead to persistent viremia and tissue-invasive injury such as pneumonitis, enteritis or retinitis, and on the other, *indirect*-related CMV effects have also been associated to acute and chronic allograft rejection, diabetes and accelerated atherosclerosis (3,4).

Noteworthy, the advent of preventive strategies using either universal prophylaxis or preemptive treatment initiated on the basis of viral detection in blood has significantly helped to reduce morbidity and mortality. Indeed, while recent reports have shown that routine prophylaxis with valganciclovir may reduce the incidence of posttransplant CMV infection and improve long-term kidney graft survival (5–8), other groups have also shown that preemptive therapy is also able to decrease the incidence of CMV disease, avoiding development of antiviral resistance, drug toxicity (7,9–10) and the advent of late-onset CMV infection (11,12). Furthermore, some CMV seronegative patients receiving a kidney allograft from a CMV seropositive donor never develop CMV infection despite not receiving any prophylaxis treatment (13–15). Altogether, it suggests that current immune assessment of the CMV risk before kidney transplantation exclusively

evaluating detectable circulating CMV IgG titers is not accurate and informative enough to predict the risk of CMV infection in all transplant recipients.

Cytotoxic CMV-specific T cells play a crucial role controlling CMV survival and replication (16,17). While host CD8+ T cells may target a wide range of CMV immunogenic proteins, particular dominant T cell responses against immediately early-1 (IE-1) antigens and to phosphoprotein 65 (pp65) seem to be essential for CMV control (18–20). Recent relevant reports using different T cell immune-monitoring tools have shown the importance of such CMV-specific T cell responses for controlling CMV infection after transplantation. However, most of them have mainly focused on the posttransplant period, gathering different solid organ transplants and assessing rather low numbers of kidney transplant recipients (21–25).

Since all kidney transplant patients display an intrinsic baseline functionality of CMV-specific T cell responses, thus predisposing to CMV replication after transplantation, we aimed to evaluate the clinical usefulness of monitoring prior to transplantation CMV-specific T cell responses against dominant CMV antigens (IE-1 and pp65) and a CMV lysate, using an IFN-γ Elispot assay, for predicting the advent of posttransplant CMV infection in two cohorts of kidney transplant recipients either receiving routine prophylaxis (n = 39) or preemptive treatment (n = 98). Furthermore, changes in 6-month posttransplant CMV-specific T cell responses were also analyzed in both groups of patients.

Methods

Patients and study groups

This is a single-center retrospective study performed at our Renal Transplant Unit at Bellvitge University Hospital in Barcelona, Spain. Between June 2009 and June 2011, consecutive kidney adult renal transplant recipients were enrolled to the study if pretransplant peripheral blood mononuclear cells (PBMCs) were available. The study was approved by the Ethics Committee of our center.

Patients were divided in two groups, depending on the CMV preventive strategy performed; either prophylaxis or preemptive therapy was done following the clinical protocol established in our Transplant Unit during the study time period. Until June 2010, prophylaxis treatment posttransplantation was restricted to CMV seronegative transplant recipients receiving a seropositive donor (R−/D+), and preemptive therapy was carried out in all CMV positive recipients either receiving a positive or a negative donor allograft (R+/D+ and R+/D−, respectively), including those receiving T cell depleting antibodies. Subsequently, from July 2010 on, a prophylaxis policy was also extended to all CMV positive transplant recipients (R+) receiving T cell depleting antibodies. In addition, six R− patients because of either hypersensitivity history to acyclovir or showing posttransplant absolute leukocyte count <2000 cells/μL, platelet count <100 000 cells/μL or hemoglobin levels lower than 8.0 g/dL, preemptive therapy was assigned.

CMV preventive strategies

In the *prophylaxis* group, including those transplants recipients receiving T cell depleting agents such as rATG, patients received 900 mg (2 × 450 mg) per day oral valgancyclovir tablets starting within 14 days after transplantation until Day 100 posttransplantation, and in the *preemptive* group, quantitative CMV monitoring by means of antigenemia was performed once weekly at weeks 1–4; every 2 weeks at weeks 6–12; every 4 weeks at months 4–6; and every 3 months at months 9 and 12, or additionally as clinically indicated.

Patients in either group who tested positive (detectable CMV antigenemia higher than 20 positive cell/2 × 10^5 PBMC) at any time after transplantation received 1800 mg (2 × 900 mg) per day oral valganciclovir for at least 14 days, until CMV antigenemia became negative on two consecutive assessments within 1 week. Thereafter, secondary prophylaxis was given using 900 mg (2 × 450 mg) per day oral valganciclovir for 1 month. In case of CMV disease or if the patient was unable to take oral medication, intravenous ganciclovir at 2 × 5 mg/kg body weight per day was permitted. In all cases, doses of all antiviral regimens were adjusted by kidney allograft function.

Clinical data and definitions

CMV *antigenemia* was defined as a positive antigenemia for CMV with no symptoms. CMV *disease* included both viral syndrome and tissue invasive disease. Identification of the viral syndrome caused by CMV required the following: (1) positive antigenemia for CMV; (2) temperature of >38°C with no other source to account for it and (3) one of the following findings: leukocyte count of <4000/mm^3, atypical lymphocytes of >3%, elevation of transaminases and platelet count of <100 000/mm. Tissue invasive disease required histopathological evidence of CMV, with or without virus culture of the tissue. This included identification of inclusion bodies or viral antigens in biopsy material or in bronchoalveolar lavage specimen cells by immunocytochemistry (3,22–25).

Microbiological studies

Surveillance by means of CMV antigenemia was routinely performed (approximately every 1–2 weeks) during the first 3 months after transplantation in both preemptive and prophylaxis strategies. CMV antigenemia was determined in polymorphonuclear Leukocytes, obtained by dextran sedimentation, formaldehyde fixed, stained and read under a fluorescence microscope (rapid antigenemia anti-human CMV ppUL83, Argene, Varilhes, France; Ref 14-002). The maximum sensitivity of the method in our laboratory was 1 positive cell/2 × 10^5 PBMCs.

ELISA for CMV-IgG

CMV serostatus was determined using a commercial CMV IgG ELISA Kit (BioCheck, Inc., Burlingame, CA) according to the manufacturer's instructions.

CMV peptides

Pools of peptides derived from a peptide scan (15 mers with 11aa overlap), covering the whole antigen length through the immediate-early protein 1 (IE-1) and through the 65 kDa phosphoprotein (pp65; Jerini Peptide Technologies, Swiss-Prot ID: P13202 and Swiss-Prot ID: P06725, respectively) of Human CMV (HHV-5), as well as a CMV lysate (Autoimmune Diagnostik®, Strasberg, Germany), were used as stimuli for the IFN-γ Elispot assay, allowing us to avoid HLA restrictions.

Anti-CMV T cell immune response assessment

IFN-γ Elispot assay: A multiscreen, 96-well filtration plate (AID®, Strasberg, Germany) coated with antihuman IFN-γ antibody (AID®, Autoimmune Diagnostika) was used. Cryopreserved PBMCs from either pretransplantation and/or 6 months after transplantation were thawed and

incubated for at least 3 h at 37°C before peptide stimulation. Thereafter, 3×10^5 of lymphocytes (in a 100 μL volume) were added to each well together with each different peptide, medium alone as a negative control and with PHA (Sigma–Aldrich®, Madrid, Spain) as a positive control. All Elispot assays were carried out in triplicate. After 18 h incubation at 37°C/5% CO_2, cells were removed by washing the plates four times with PBS containing 5% Tween 20 and twice with PBS. Fifty microliters of biotinylated anti-IFN-γ antibody was added (1:1000 dilution, 7-B6-1-biotin; Mabtech) and incubated for 3 h at room temperature. The Elispot plate was washed a further six times with PBS/Tween 20 and incubated for 2 h with streptavidin-ALP substrate (AID®) followed by the addition of an alkaline phosphatase conjugate substrate (50 IL; AID®, Autoimmune Diagnostika). The resulting spots were counted semi-automatically with an Elispot reader (AID® Elispot Reader HR, 4th generation). Results were expressed as percentage of cells secreting IFN-γ after subtracting the number of spots due to spontaneous IFN-γ release (measured in the control wells) from the number of spots obtained in the wells incubated with each peptide.

IFN-γ flow cytometry: Following incubation with respective peptides, PBMC were tested for intracellular IFN-γ production by the cytokine flow cytometry assay in five transplant recipients showing relevant anti-viral T cell responses against all three evaluated stimuli in the Elispot assay. PBMC were washed and stained for 30 min in ice with APC-conjugated mAb anti-CD3 (clone HIT3a; BD®, Madrid, Spain), PE-conjugated mAb anti-CD4 (clone RPA-T4; BD®) and PERP-CY.5-conjugated mAb anti-CD8 (clone RPA-T8; eBioscience®, Barcelona, Spain) in PBS + 5% FBS, containing 5% human immunoglobulin and 0.01% sodiumazide. Cells were then washed with PBS + 5% FBS, fixed and permeabilized using the FIX and PERMJ kit (BD®), according to the manufacturer's instructions, and stained for 45 min with FITC-conjugated mAb anti-IFN-γ (clone 4s.B3; BD®).

Statistical analysis

All data are presented as mean ± SD. Groups were compared using the χ^2 test for categorical variables, the one-way ANOVA or t-test for normally distributed data and the nonparametric Kruskal–Wallis or Mann–Whitney U test for non normally distributed variables. Both CMV antigenemia and disease were considered the outcome variables of the study. Bivariate correlation analyses were done using Pearson or Spearman test for non-parametric variables. A sensitivity/specificity ROC curve test was done to investigate the value of the Elispot test for predicting the advent of posttransplant CMV infection. The statistical significance level was defined as 2-tailed $p < 0.05$.

Results

Patient demographics

As shown in Figure 1, 137 consecutive kidney transplant recipients were assessed for their anti-CMV T cell response before transplantation. Of these, 39 patients received posttransplant CMV prophylaxis and 98 followed a pre-emptive protocol. Six-month CMV-specific T cell responses could be evaluated in 58 patients, 21 receiving prophylaxis and 37 preemptive therapy. Mean follow-up of the study was 25 months (range 37–15 months).

Main demographic and baseline characteristics of all patients are depicted in Table 1. Incidence of posttransplant antigenemia or CMV disease was not different between patients receiving prophylaxis or preemptive therapy. No CMV infection events were observed beyond 6 months after transplantation. Among prophylactic-treated patients, the advent of CMV antigenemia appeared in all but one patient after completing valganciclovir treatment with a median of 45 days after stopping treatment. Within preemptive-followed patients, most CMV infection episodes occurred during the first 3 months after transplantation with a median of 38 days after transplantation. Incidence of CMV recurrence after treatment was equally distributed between both groups (three within preemptive and two among prophylaxis). Among patients receiving preemptive therapy, the advent of CMV antigenemia was significantly more common in older recipients (54.8 ± 9 vs. 48.3 ± 13, $p < 0.005$) and in those experiencing delayed graft function (DGF; 45.5% vs. 14.4%, $p < 0.005$). To note, T cell depletion induction treatment was associated to a significantly increased risk of both posttransplant antigenemia and CMV disease (63.6% vs. 38% and 80% vs. 40% for antigenemia and disease, respectively, $p < 0.005$). Conversely, type of maintenance immunosuppression was not associated with CMV infection. At 6 months, allograft function was significantly worse among those patients experiencing either CMV antigenemia or disease as compared to those that did not.

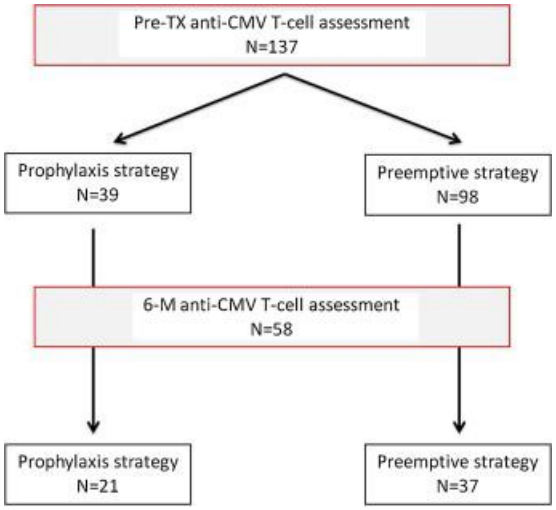

Figure 1: Illustration of the study course.

Main clinical data of patients with and without CMV antigenemia/disease within preemptive and prophylactic-treated patients are displayed in Tables 2 and 3, respectively. Most CMV infections in both cohorts of patients were asymptomatic CMV-detected antigenemia (28% and 22% in prophylactic and preemptive, respectively) and clinical disease was observed in 20% and 12% of prophylactic and preemptive groups, respectively. To note, the majority of clinical diseases were diagnosed as viral syndromes (11/18) whereas tissue invasive diseases were observed in seven patients, located in the gastrointestinal tract and two in the pulmonary tract.

Table 1: Main demographic and baseline characteristics of the entire study group

	All patients (N = 137)	Prophylaxis (N = 39)	Preemptive (N = 98)
Gender (male/female)	91/46	26/13	65/33
Age (years, mean ± SD)	48.9 ± 13.2	46.4 ± 14.7	49.8 ± 12.7
Type of kidney TX (living/deceased)	63/74	20/19	43/55
Pre-TX CMV donor (D)/recipient (R) serostatus			
R−/D+ (%)	28 (20.4)	22 (56.4)*	6 (6)
R+/D+ (%)	83 (60.6)	12 (30.8)	71 (72.5)
R+/D− (%)	26 (19)	5 (1.2)	21 (21.5)
Maintenance immunosuppression			
CNI-based (CsA/TAC; %)	8 (6)/112 (82)	3 (8)/3 (8)	5 (5)/81 (83)
CNI-free (mTor-i; %)	17 (12)	5 (13)	12 (12)
Mycophenolate mofetil (%)	137 (100)	39 (100)	98 (100)
No Induction therapy (%)			
Induction immunosuppression	10 (7.3)	1 (2.5)	9 (9.2)
rATG (%)	68 (49.7)	25 (6)	43 (43.8)
Anti-CD25 monoclonal Ab (%)	59 (43)	13 (33.4)	46 (47)
DGF (yes/no)	30/107	9/30	21/77
BPAR (%)	18 (13)	6 (15)	12 (12)
Allograft function (eGFR; mL/min)			
Month 6	40.6 ± 25	45.8 ± 26	38.4 ± 24
Month 12	52.6 ± 15	53.4 ± 17	52.2 ± 14
Month 18	52.5 ± 16	53.8 ± 14	52 ± 16
Pre-TX anti-CMV IgG titers (UA/mL)	166.7 ± 99	99.9 ± 116*	190.7 ± 81
Pre-TX anti-CMV T cell response (spots/3 × 10^5 PBMC)			
CMV lysate	128.9 ± 183	61.9 ± 112*	155.5 ± 198
Pp65 antigen	101.7 ± 168	39.4 ± 65*	126.5 ± 189
IE-1 antigen	39.8 ± 86.1	21.5 ± 29*	47 ± 99
CMV infection (antigenemia/disease)	33 (24)/18 (13)	11 (28)/8 (20)	22 (22)/10 (10)
Exitus (%)	8 (5.8)	2 (5)	6 (6)

*$p < 0.05$.

Pp65- and IE-1-specific T cell responses are predominantly provided by the CD8+ T cell compartment

While T cell responses against both pp65 and IE-1 CMV peptides were predominantly CD8+, CD4+ T cell responses could also be detected against the CMV lysate (Figure 2).

Low pretransplant IE-1-specific T cell responses is associated with posttransplant CMV infection

All anti-CMV T cell responses within prophylactic patients were significantly lower as compared to patients with preemptive therapy (Table 1). Pretransplant pp65 and CMV lysate but not anti-IE-1-specific T cell responses positively correlated with pretransplant CMV IgG titers ($r = 0.298$, $p = 0.001$ and $r = 0.325$, $p < 0.001$, respectively). Although pretransplant CMV-specific T cell responses could be detected among some seronegative transplants recipients (12/28), they were significantly lower than within seropositive recipients (Figure 3).

Patients receiving either preemptive or prophylaxis therapy developing CMV infection showed significantly lower anti-IE-1 T cell responses as compared to patients that did not. No association was observed between pretransplant anti-pp65 and CMV lysate T cell responses and incidence of CMV infection (Figure 4). Similar findings were observed among those patients receiving rATG (Figure 5). Furthermore, prophylaxis-treated transplant recipients developing CMV disease, did also show lower pretransplant pp65-specific T cell responses as compared to those that did not. When all patients of the study were assessed together, those with posttransplant CMV infection showed significantly lower pretransplant anti-IE-1 T cell responses than patients not experiencing CMV infection (data not shown). Patients under mTor-i did not show a different CMV-specific T cell immunity as compared to those receiving CNI-based regimens.

Frequencies of pretransplant anti-IE-1 T cell responses independently predict the risk of posttransplant CMV infection

Receiver operating characteristic curve (ROC) analysis for predicting either posttransplant antigenemia or disease in patients receiving prophylaxis and preemptive therapy is depicted in Figure 6. As shown, considerably high AUC, ranging from 0.635 up to 0.760, were obtained for pretransplant anti-IE-1 T cell responses for prediction of both CMV antigenemia and disease in the different treatment groups, respectively. Sensitivity and specificity of anti-IE-1 T cell IFN-γ Elispot is summarized in Table 4. No additive effect for predicting posttransplant CMV infection

Table 2: Main demographic and baseline characteristics of preemptive-treated patients

	CMV antigenemia		CMV disease	
Preemptive strategy (N = 98)	Yes (N = 22)	No (N = 76)	Yes (N = 10)	No (N = 88)
Gender (male/female)	17/5	48/28	7/3	58/30
Age (years, mean ± SD)	54.8 ± 9*	48.3 ± 13	53.3 ± 10	49.5 ± 13
Type of kidney TX (deceased/living)	6/16	37/39	1/9	42/46
DGF (yes/no)	10/12*	11/65	3/7	18/70
BPAR (%)	3 (14)	9 (12)	2 (20)	10 (11)
Pre-TX CMV donor (D)/recipient (R) serostatus				
R−/D+ (%)	1 (4.5)	5 (6.5)	1 (10)	5 (5.5)
R+/D+ (%)	17 (77)	60 (79)	7 (70)	72 (82)
R+/D− (%)	4 (18)	11 (14.5)	2 (20)	11 (12.5)
Pre-TX anti-CMV IgG titers	216.6 ± 59	183.4 ± 85	177.9 ± 85	192 ± 81
Maintenance immunosuppression				
CNI-based (CsA/TAC; %)	0/19	5/62	0/9	5/72
CNI-free (mTor-i; %)	3	9	1	11
Mycophenolate mofetil (%)	22	76	10	88
Mycophenolate acid tough levels (mean ± SD)				
Month 1	2.9 ± 1.9	2.9 ± 1.9	2.9 ± 2.1	2.9 ± 1.9
Month 3	2.9 ± 2	3.3 ± 2	2.7 ± 1.8	3.2 ± 1
Month 6	3.5 ± 2	2.7 ± 1.7	3.2 ± 1	2.8 ± 1.7
No induction therapy (%)	1 (4.4)	8 (10.5)	0 (0)	9 (10)
Induction immunosuppression				
rATG (%)	14 (63.6)*	29 (38)	8 (80)*	35 (40)
Anti-CD25 monoclonal Ab (%)	7 (32)	39 (51.5)	2 (20)	44 (50)
Allograft function (eGFR; mL/min)				
Month 6	28.3 ± 23*	41.2 ± 23	12.3 ± 14*	41.2 ± 22
Month 12	47.1 ± 17	53.8 ± 12	48.1 ± 19	53 ± 13
Month 18	50.1 ± 19	52.7 ± 15	53 ± 22	52 ± 15
Exitus (%)	3 (13)	3 (4)	1 (10)	5 (5.6)

*$p < 0.05$.

was observed when using pp65 and IE-1 T cell responses together (data not shown).

When risk of CMV infection was categorized as a binary variable, taking into account most sensitive and specific cut-off values of pretransplant IE-1-specific T cell IFN-γ Elispots for each group of transplant recipients (7 and 8 IFN-γ spots per 3×10^5 stimulated PBMCs, for preemptive and prophylaxis-treated patients, respectively) a higher than 80% and 90% sensitivity and negative predictive value were obtained, respectively (Table 4).

Anti-CMV T cell responses at 6 months after transplantation between CMV infected and noninfected transplant recipients

As all CMV infection events appeared before the first 6 months after transplantation, changes in 6-month CMV-specific T cell responses were evaluated. In general, anti-CMV T cell responses significantly increased after transplantation despite that patients were receiving immunosuppression (128.9 ± 183 vs. 278 ± 433, $p = 0.012$; 101.7 ± 168 vs. 127 ± 183, $p = 0.006$ and 39.8 ± 86.1 vs. 126 ± 454, $p < 0.001$, for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively). There were no differences between 6-month anti-CMV T cell responses among patients having received prophylaxis or preemptive therapy, CNI or non-CNI-based immunosuppression or different type of induction therapy (data not shown). However, when patients with or without posttransplant CMV infection were compared regarding their change in the CMV-specific T cell response at 6-month, patients having experienced CMV infection showed a significantly increase in pp65 and IE-1-specific T cell responses as compared to those that did not (Figure 7).

Discussion

While current clinical immune assessment of the CMV risk of infection before transplantation exclusively relies on donor and recipient CMV IgG-serostatus, our study shows that CMV-specific T cell response, particularly against the IE-1 dominant CMV antigen, may improve the identification of those kidney allograft recipients at high-risk for CMV infection. Importantly, our approach is capable to discriminate such patients already before transplantation, with high sensitivity and specificity, regardless the type of preventive strategy used. Furthermore, the high negative

Table 3: Main demographic and baseline characteristics of prophylactic-treated patients

	CMV antigenemia		CMV disease	
Prophylaxis treatment (N = 39)	Yes (N = 11)	No (N = 28)	Yes (N = 8)	No (N = 31)
Gender (male/female)	7/4	19/9	6/2	20/11
Age (years, mean ± SD)	44.7 ± 15	47.1 ± 14	38.8 ± 13	48.5 ± 13
Type of kidney TX (deceased/living)	3/8	17/11	3/5	17/14
DGF (yes/no)	5/6	4/24	2/6	7/24
BPAR (cellular/humoral)	3/0	2/1	1/0	4/1
Pre-TX CMV donor (D)/recipient (R) serostatus				
R−/D+ (%)	5 (45.5)	1 (3.5)	4 (50)	18 (58)
R+/D+ (%)	4 (36.4)	8 (28.5)	3 (37.5)	9 (29)
R+/D− (%)	2 (18.2)	3 (11)	1 (12.5)	4 (13)
Pre-TX anti-CMV IgG titers	90 ± 117	104 ± 118	50.1 ± 92	115 ± 120
Maintenance immunosuppression				
-CNI-based (CsA/TAC)	0/10	3/21	0/7	3/24
-CNI-free (mTor-i)	1	4	1	4
-Mycophenolate mofetil	11	28	8	31
Mycophenolate acid tough levels (mean ± SD)				
Month 1	3.1 ± 1.6	3.2 ± 1.8	3.1 ± 1.4	3.2 ± 1.8
Month 3	3.7 ± 2	3.6 ± 2	3.4 ± 2	3.7 ± 2
Month 6	3.1 ± 2	3.6 ± 2.4	3.2 ±	3.1 ± 2
No induction therapy (%)	0 (0)	1 (3.5)	0 (0)	1 (3.2)
Induction immunosuppression				
-rATG (%)	8 (73)	17 (61)	7 (87.5)	18 (58)
-Anti-CD25 monoclonal Ab (%)	3 (27)	10 (35.5)	1 (12.5)	12 (38.8)
Allograft function (eGFR; mL/min)				
Month 6	41.7 ± 23	47.5 ± 28	48.2 ± 21	45.1 ± 28
Month 12	50.1 ± 11	55.3 ± 20	53.4 ± 8	53.4 ± 20
Month 18	48.6 ± 12	57 ± 16	53.8 ± 13	54.6 ± 15
Exitus (%)	1 (9)	1 (3.5)	0 (0)	2 (6)

*$p < 0.05$.

predictive value of the test highlights the usefulness of such approach.

Noteworthy, our study shows that monitoring IE-1 CMV-specific T cell frequencies before transplantation would help transplant physicians on the one hand to better discriminate those patients with no need of CMV prophylactic treatment from those in whom prophylaxis should preferentially be indicated and on the other to better predict those patients in whom prophylaxis treatment could safely be discontinued. Interestingly, intrinsic impairment of the IE-1-specific T cell response was not only associated with the advent of posttransplant CMV infection but also with the development of CMV disease, thus reinforcing the importance of such functionally active CMV-specific T cell precursors for achieving CMV control under immunosuppression.

The observation that patients receiving T cell depleting antibodies experiencing CMV infection were those with significantly lower pretransplant IE-1-specific T cell frequencies, suggests that the increased susceptibility for CMV infection after T cell depletion is particularly facilitated by the impairment of IE-1-specific T cell precursors already before transplantation rather than to a generalized T cell subset depletion after rATG therapy. Differently from what has been shown among normal individuals (26), within our seronegative group of chronic kidney disease patients, CMV-specific T cell responses were also detectable in a group of them, though at significantly lower frequencies than among seropositive patients. Nevertheless, only patients with adequate pretransplant anti-IE-1-speciifc T cell frequencies were at significant low-risk for CMV infection. This finding supports the notion that although CMV triggers both humoral and cellular responses, only the latter and particularly that directed to IE-1 CMV antigens seem to be crucial for posttransplant viral replication control, therefore being the former of limited utility in the clinical practice (27,28). Nonetheless, whether the detection of CMV-specific peptide T cell responses among CMV-seronegative patients could result from cross-reactive recognition of CMV epitopes by memory T cells originated from distinct (e.g. non-HCMV) antigenic exposures or if a more accurate assessment of CMV-specific memory B-cell IgG frequencies would increase the sensitivity to detect patients already sensitized to CMV antigens deserves further evaluation.

To date, studies in transplant recipients evaluating the impact of CMV-specific cellular responses have mainly

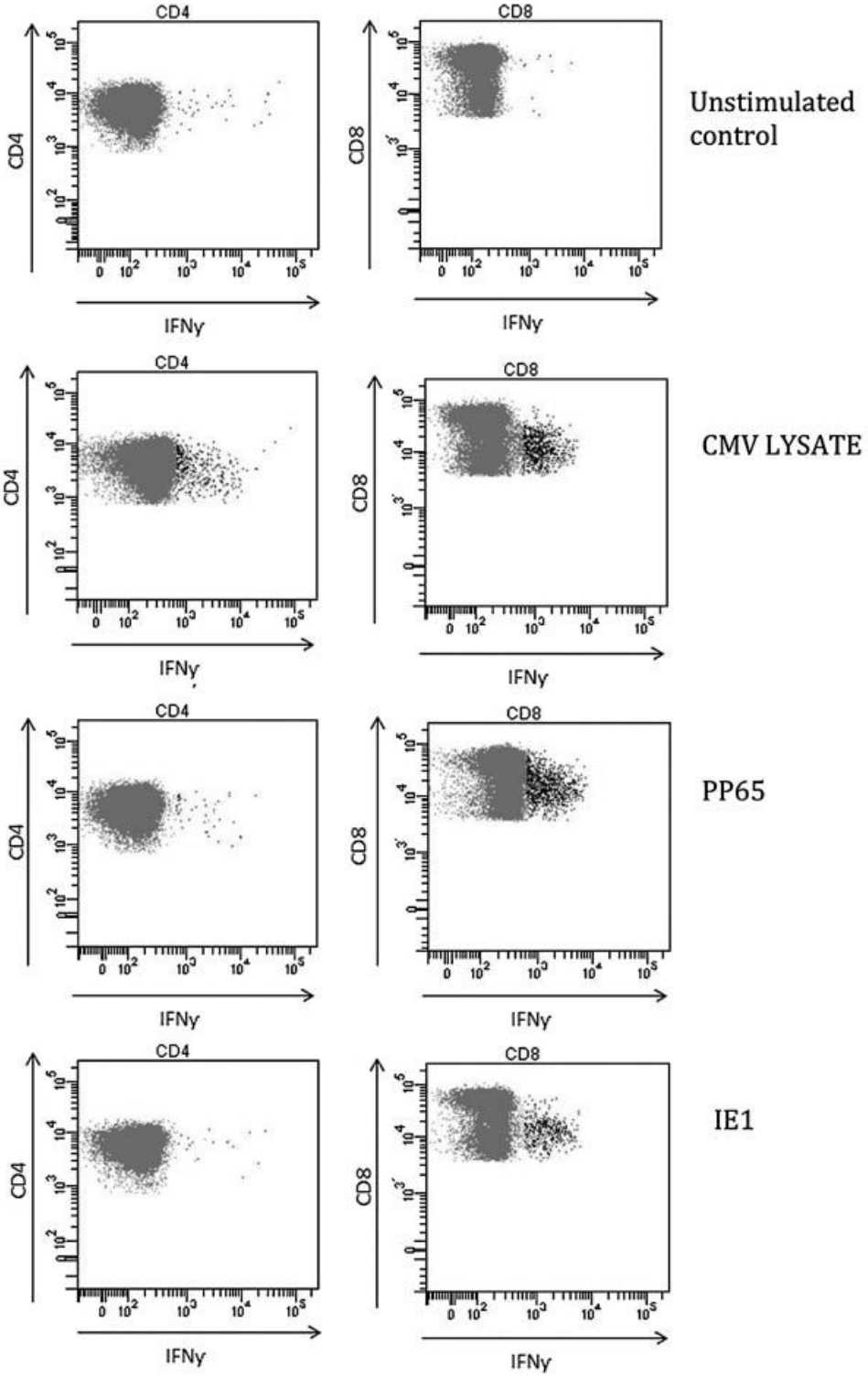

Figure 2: Intracellular IFN-γ FACS analysis on CD4+ and CD8+ T cells stimulated with three different CMV stimuli in a representative kidney transplant patient with high frequencies of IFN-γ producing T cells assessed by the Elispot assay before transplantation. CD8+ T cell subsets accounted for the most predominant anti-IE-1 and pp-65 T cell responses. CD4+ T cell responses were also detected against the CMV lysate stimuli.

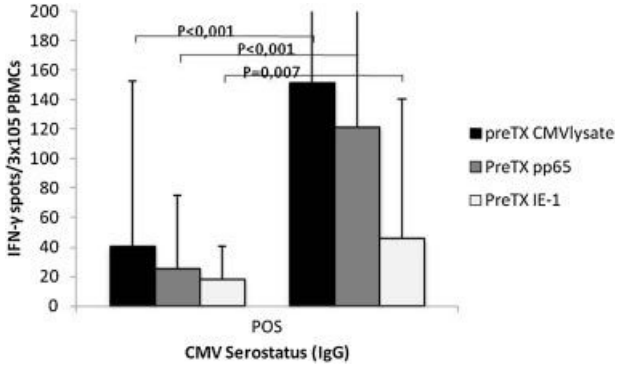

Figure 3: CMV-specific T cell responses between CMV-seropositive and seronegative transplant recipients. Pretransplant CMV-specific T cell responses were significantly lower among seronegative patients than within seropositive transplant recipients (40.3 ± 112 vs. 151.6 ± 191, $p < 0.001$; 25.2 ± 50 vs. 121.3 ± 181, $p < 0.001$ and 17.6 ± 23 vs. 45.5 ± 95.1, $p = 0.007$ for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively).

focused at the posttransplant period and used different cellular immune assays with distinct CMV stimuli (21–26,29–32). Our study is in consonance with these previous reports, but also shows that the increased risk to develop posttransplant CMV infection (even after a course of prophylactic treatment) seems to rely in an individual immune susceptibility already manifested prior to transplantation. Likewise, but in lung and heart transplant patients, Bunde et al. (21) showed that frequencies of IE-1 but not pp65-specific CD8+ T cells already at Day 0, discriminated patients who did not develop CMV disease from patients at risk. Although focusing on the association between allogeneic and CMV-specific effector T cell responses, Nickel et al. (33) reported similar findings in a group of 36 kidney transplant patients.

Although different studies have suggested a preponderant role of CD8+ T cells for CMV control (21,24,26,29,30), others have also shown the concomitant key function of CD4+ T cells, which seem to even have a preferential role conferring long-lasting protection (29,32). In our study, we

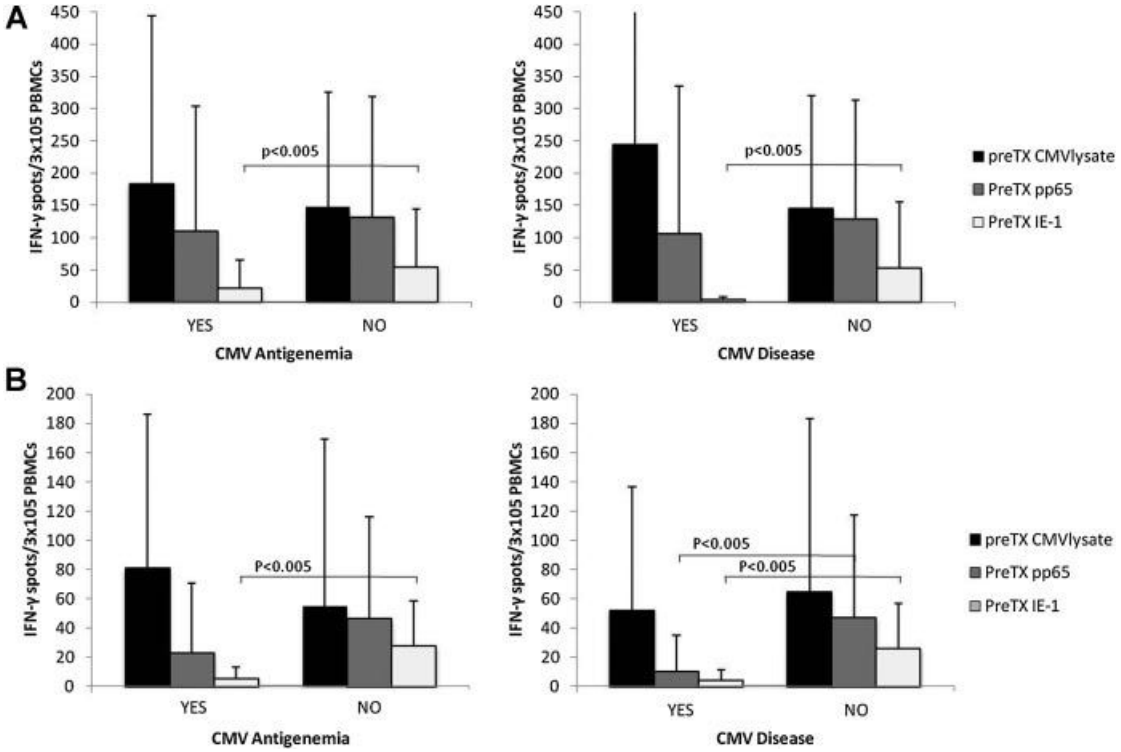

Figure 4: Pretransplant anti-CMV T cell responses and risk of CMV infection. (A) CMV-specific T cell responses between patients developing CMV viremia and those that did not, in patients receiving preemptive treatment (182.7 ± 262 vs. 147.6 ± 178, 109.4 ± 194 vs. 131.4 ± 188, 21.5 ± 44 vs. 54.4 ± 90 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses), and between patients developing CMV disease and those that did not (244.3 ± 342 vs. 145.4 ± 175, 105.9 ± 229 vs. 128.8 ± 185, 4 ± 4.8 vs. 52 ± 103 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses). (B) CMV-specific T cell responses between patients developing CMV viremia and those that did not, in patients receiving prophylaxis treatment (80.5 ± 106 vs. 54.6 ± 115, 22.7 ± 48 vs. 46 ± 70, 5.4 ± 8 vs. 27.8 ± 31 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses), and between patients developing CMV disease and those that did not (51.8 ± 85 vs. 64.5 ± 119, 10.2 ± 25 vs. 47 ± 70, 4.2 ± 7 vs. 26 ± 31 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ for both pp65 and IE-1 responses).

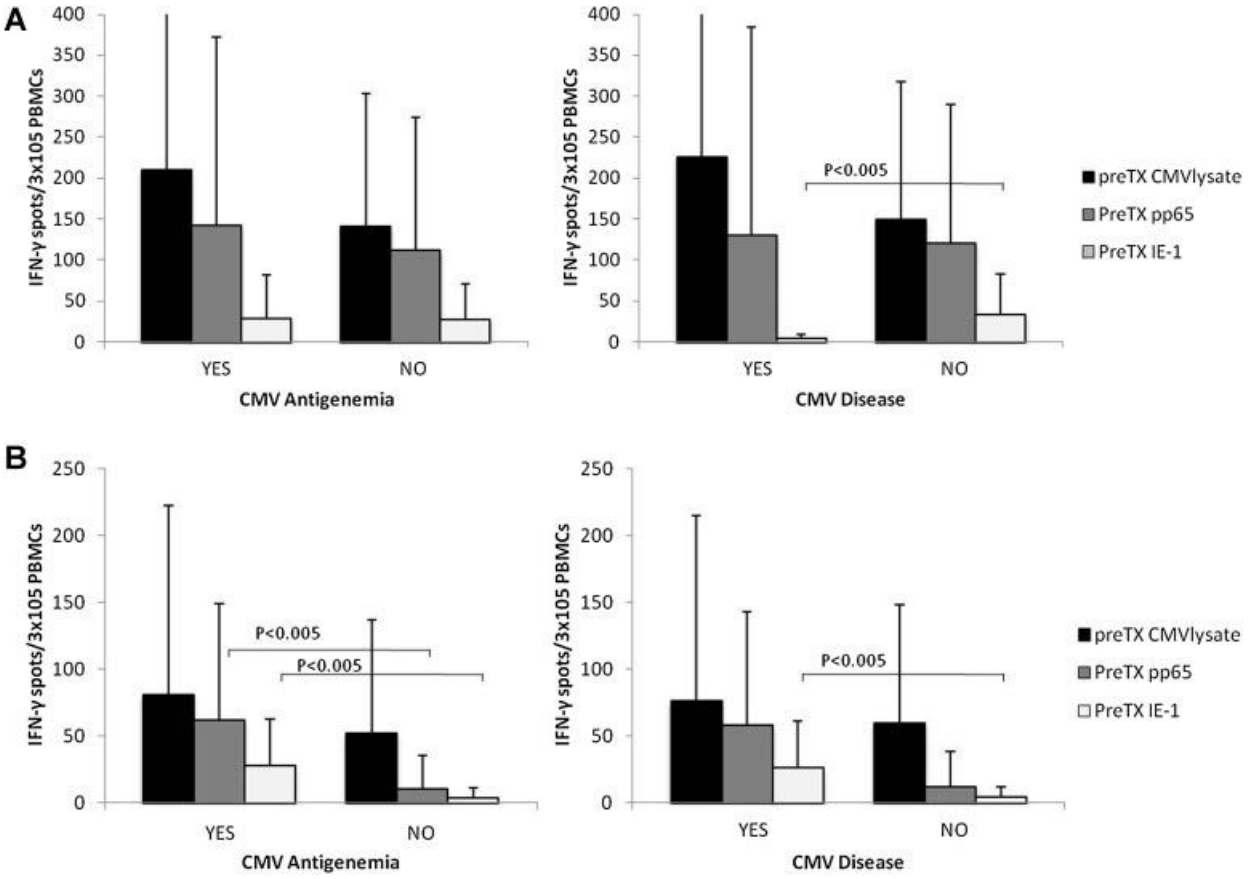

Figure 5: CMV-specific T cell responses and development of CMV viremia and disease within rATG-treated patients receiving preemptive and prophylaxis therapy. (A) CMV-specific T cell responses between patients developing CMV viremia and those that did not, in patients receiving rATG and preemptive treatment (209.4 ± 292 vs. 141.5 ± 162, 142.6 ± 230 vs. 112 ± 162, 28.3 ± 53 vs. 27.4 ± 44 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. P = NS for any sCMV stimuli), and between patients developing CMV disease and those that did not (226.2 ± 3456 vs. 149.3 ± 168, 129.8 ± 254 vs. 120.2 ± 170, 4.8 ± 4 vs. 33 ± 50 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ only for IE-1 responses). (B) CMV-specific T cell responses between patients not developing CMV viremia and those experiencing CMV viremia, in patients receiving rATG and prophylaxis treatment (80.5 ± 142 vs. 52 ± 85, 61.6 ± 87 vs. 10.2 ± 25, 27.7 ± 35 vs. 3.5 ± 7.6 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ for both pp65 and IE-1 responses), and between patients developing CMV disease and those that did not (76 ± 139 vs. 59.2 ± 89, 58.2 ± 85 vs. 11.7 ± 27, 26.2 ± 35 vs. 4 ± 8 IFN-γ spots for CMV-lysate, pp65 and IE-1 for 3×10^5 stimulated PBMCs, respectively. $p < 0.05$ for IE-1 responses).

found that pp65 and IE-1-specific T cell responses are predominantly but not exclusively restricted to CD8+ thus, CD4+ T cells responses could similarly be required to confer long-term protection against CMV infection.

Even though T cell responses may target multiple CMV-specific proteins (18,34,35), it appears that protective cellular immunity is mainly directed against the tegument protein ppUL83 and to the immediately early protein ones (19,21,33,36). To note, IE-1 is the first protein expressed upon CMV reactivation (37), thus IE-1-specific T cells would be the first to be activated and directed to sites of replication (38,39). Hence, this mechanism could explain why high levels of IE-1 but not other CMV-specific T cells would be associated with protection from CMV disease. Some other groups have shown lack of correlation with exclusive IE-1-specific T cell responses and risk of CMV disease (40,41). To note, most of them focused at the posttransplant setting and evaluated a rather low number of transplant recipients. In our study, at 6 months while there was a general increase of all CMV-specific T cell responses (both against IE-1 and also pp65), this feature was specifically observed within those having recovered from CMV infection, suggesting that broader CMV-antigen

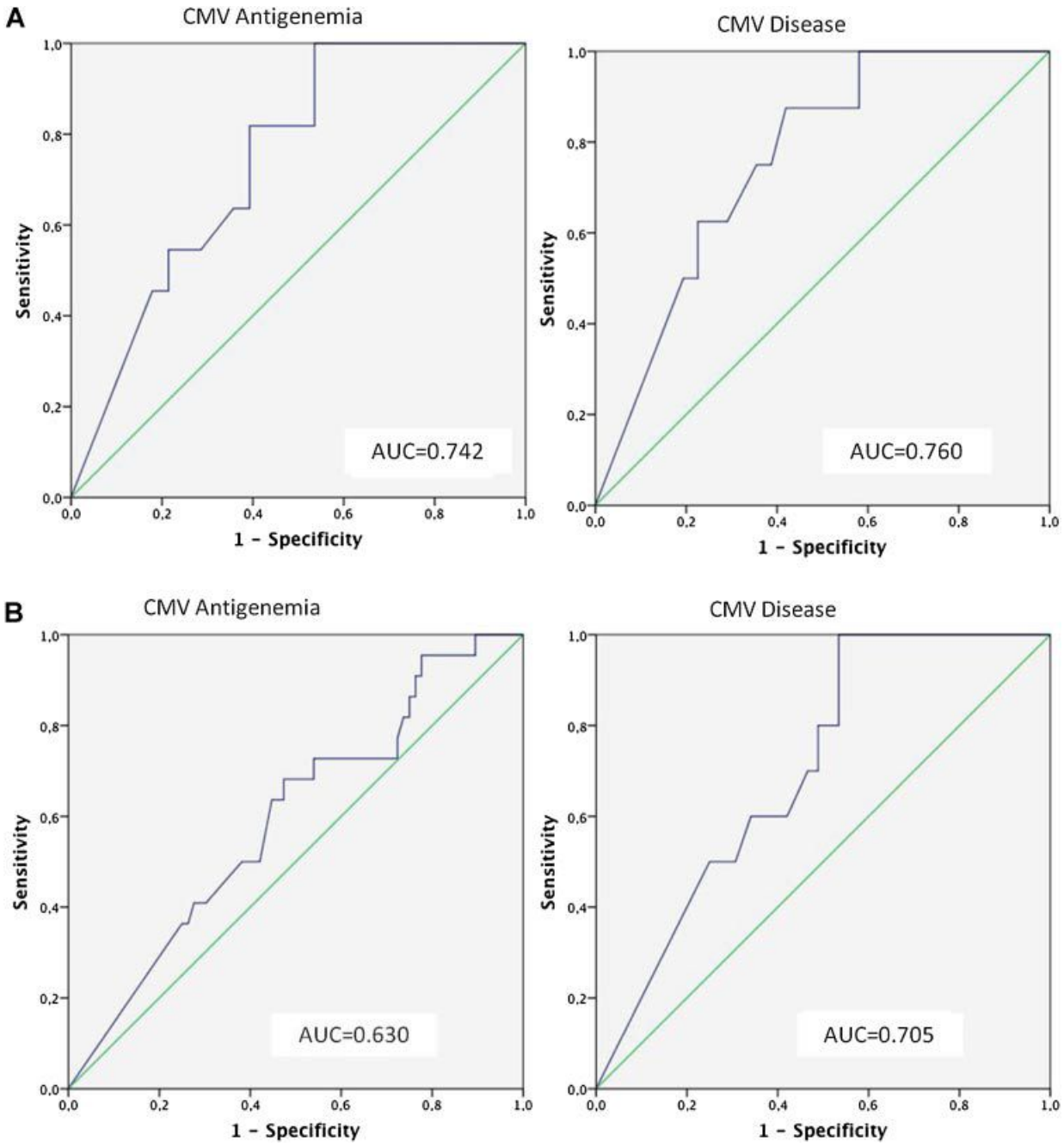

Figure 6: ROC curve analysis estimates sensitivity and specificity of pretransplant anti-IE-1 T cell frequencies for predicting the risk of both CMV viremia and disease in prophylactic and preemptive-treated patients.

specific T cell responses might be also necessary for controlling CMV replication. This data reinforces the potential value of preventive strategies using recombinant CMV proteins as vaccines, preferentially containing immunogenic IE-1 antigens already before transplantation.

There are some limitations in this study. First, although we used a non-standardized immune assay, the IFN-γ Elispot has already been shown to be highly reproducible for measuring antigen-specific cellular responses in other relevant fields of medicine (42–44), allowing a comprehensive quantitative-dynamic idea of the antigen-specific cellular strength at a single cell level. Another limitation is the lack of PCR-CMV viremia monitoring in our study that could have induced misleading diagnosis. Nonetheless, although PCR-CMV viremia has shown higher sensitivity as

Table 4: Predictive value of anti-IE-1 T cell response for posttransplant CMV infection

			Predictive value		
Treatment group	Variables	Cut-off values (IFN-γ spots)	Specificity (%)	Sensitivity (%)	NPV (%)
Prophylaxis	Pre-TX anti-IE-1 (CMV infection)	8 spots 3×10^5 PBMCs	65	82.5	89.5
Preemptive	Pre-TX anti-IE-1 (CMV infection)	7 spots 3×10^5 PBMCs	55	80	95.7

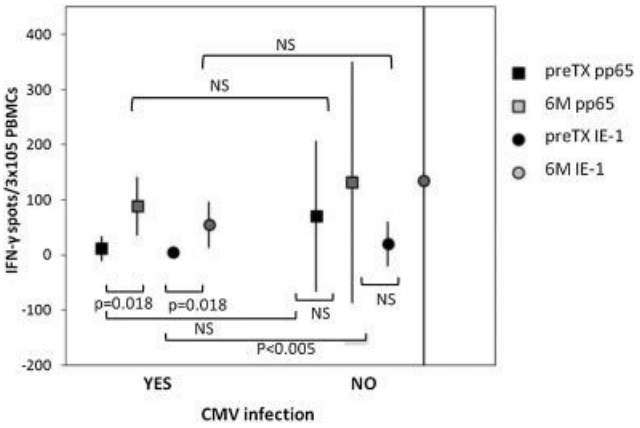

Figure 7: Anti-CMV T cell response changes between infected and non-infected transplant recipients. Patients having experienced CMV infection showed a significantly increase in their anti-pp65 and anti-IE-1 T cell responses as compared to those that did not (4.8 ± 2 vs. 54.4 ± 42, $p = 0.018$; 11.7 ± 22 vs. 88.8 ± 53 $p = 0.018$ for IE-1 and pp65, respectively among patients having experienced CMV infection, and 20 ± 41 vs. 135 ± 532, $p =$ NS and 70.2 ± 137 vs. 131.3 ± 219 $p =$ NS for IE-1 and pp65 for 1.5×10^5 stimulated PBMCs, respectively among non-infected transplant recipients).

compared to CMV antigenemia (45), the incidence of CMV antigenemia or disease among our two cohorts of kidney transplant recipients fitted with that reported in the literature using PCR-based assays (46,47).

In conclusion, we have shown that monitoring frequencies of IE-1-specific T cell responses before transplantation may be useful for predicting posttransplant risk of CMV infection, thus being potentially valuable for guiding decision-making regarding CMV preventive treatment. To further support this result and validate its potential clinical utility, large-scale prospective randomized trials are highly warranted and should be preferentially performed in the context of multicenter cooperative networks.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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2. Article Clinical Infectious Disease journal:

“Preformed Frequencies of Cytomegalovirus (CMV)–Specific Memory T and B Cells Identify Protected CMV-Sensitized Individuals Among Seronegative Kidney Transplant Recipients”

MAJOR ARTICLE

Preformed Frequencies of Cytomegalovirus (CMV)–Specific Memory T and B Cells Identify Protected CMV-Sensitized Individuals Among Seronegative Kidney Transplant Recipients

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Background. Cytomegalovirus (CMV) infection remains a major complication after kidney transplantation. Baseline CMV risk is typically determined by the serological presence of preformed CMV-specific immunoglobulin (Ig) G antibodies, even though T-cell responses to major viral antigens are crucial when controlling viral replication. Some IgG-seronegative patients who receive an IgG-seropositive allograft do not develop CMV infection despite not receiving prophylaxis. We hypothesized that a more precise evaluation of pretransplant CMV-specific immune-sensitization using the B and T-cell enzyme-linked immunospot assays may identify CMV-sensitized individuals more accurately, regardless of serological evidence of CMV-specific IgG titers.

Methods. We compared the presence of preformed CMV-specific memory B and T cells in kidney transplant recipients between 43 CMV IgG–seronegative (sR^-) and 86 CMV IgG–seropositive (sR^+) patients. Clinical outcome was evaluated in both groups.

Results. All sR^+ patients showed a wide range of CMV-specific memory T- and B-cell responses. High memory T- and B-cell frequencies were also clearly detected in 30% of sR^- patients, and those with high CMV-specific T-cell frequencies had a significantly lower incidence of late CMV infection after prophylactic therapy. Receiver operating characteristic curve analysis for predicting CMV viremia and disease showed a high area under the receiver operating characteristic curve (>0.8), which translated into a high sensitivity and negative predictive value of the test.

Conclusions. Assessment of CMV-specific memory T- and B-cell responses before kidney transplantation among sR^- recipients may help identify immunized individuals more precisely, being ultimately at lower risk for CMV infection.

Keywords. kidney transplantation; CMV infection; T- and B-cell ELISPOT assay; adaptive immunity.

Despite the outstanding progress made with the advent of preventive antiviral strategies, cytomegalovirus (CMV) infection remains the most common opportunistic infection in kidney transplant recipients. Although primary Infection in immunocompetent hosts is usually asymptomatic, transplant recipients are at increased risk of developing CMV infection in the period immediately after transplantation. This poses a critical challenge to both graft and patient survival [1, 2].

The T-cell immune response to CMV is known to be of primary importance in controlling viral infection [3–5]. However, the humoral adaptive immune response, evaluated by serological CMV-specific immunoglobulin (Ig) G titers, is the only marker currently available for immune-risk stratification in clinical practice. Unfortunately, this surrogate approach does not entirely help identify all truly immune-sensitized transplant recipients

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Table 1. Clinical and Demographic Characteristics of Kidney Transplant Recipients by CMV IgG Serostatus

Characteristic	sR^- Patients (n = 43)	sR^+ Patients (n = 86)
Sex, male/female, No.	30/13	56/30
Age, mean ± SD, y	47.9 ± 17.3	51.7 ± 11.4
Type of kidney transplant, living/deceased donor, No. (%)	34 (79)/9 (21)	25 (29)/61 (71)
Donor CMV IgG serostatus		
Seronegative, No. (%)	6 (14)	15 (17.5)
Seropositive, No. (%)	37 (86)	71 (82.5)
Preventive therapy, prophylaxis/preemptive, No. (%)	37 (86)/8 (14)	11 (12.8)/75 (87.2)
Maintenance IS, No. (%)		
CNI-based (TAC/CsA)/other	39 (90.7)/4 (9.3)/0 (0)	79 (92)/6 (7)/1 (1)
MMF/mTor-i	41 (95.3)/2 (4.7)	78 (90.7)/8 (9.3)
Induction IS, No. (%)		
No induction/rATG/basiliximab	3 (7)/20 (46.5)/20 (46.5)	5 (6)/35 (40.5)/46 (53.5)
DGF, yes/no, No. (%)	11 (25.6)/32 (74.4)	22 (25.6)/64 (74.4)
BPAR, yes/no, No. (%)	7 (16.3)/36 (83.7)	12 (14)/74 (86)
Allograft function (eGFR), mean ± SD, mL/min		
Month 6	40.6 ± 28	45.8 ± 21
Month 12	40.8 ± 23	52.4 ± 16
CMV infection, yes/no, No. (%)		
Viremia	11 (25.6)/32 (74.4)	25 (29)/61 (71)
Disease	8 (18.6)/35 (81.4)	12 (14)/74 (86)
Pretransplant anti-CMV T-cell ELISPOT count, mean ± SD (range), IFN-γ spots/3 × 10^5 PBMCs		
CMV lysate	33.61 ± 97.7 (0–448)	150.2 ± 190 (0–856)
pp65 Antigen	20.5 ± 42.8 (0–259)	120.24 ± 181 (0–765)
IE-1 antigen	26.78 ± 92.5 (0–604)	45.1 ± 95 (0–539)

Abbreviations: BPAR, biopsy-proved acute rejection; CMV, cytomegalovirus; CNI, calcineurin-inhibitors; CsA, cyclosporin A; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; ELISPOT, enzyme-linked immunospot assay; IE-1, immediate-early protein 1; IFN, interferon; Ig, immunoglobulin; IS, immunosuppression; MMF, mycophenolate mofetil; mTor-i, mTor-inhibitors; PBMCs, peripheral blood mononuclear cells; pp65, 65-kDa phosphoprotein; rATG, rabbit anti-thymocyte globulin; SD, standard deviation; sR^-, CMV IgG–seronegative transplant recipient; sR^+, CMV IgG–seropositive transplant recipient; TAC, tacrolimus.

at lower risk of CMV infection after transplantation. Indeed, there is clear clinical evidence for this position; although most CMV IgG–seropositive (sR^+) transplant recipients receiving a seropositive allograft are unlikely to develop CMV infection after transplantation, up to 20%–30% may experience CMV disease without antiviral prophylaxis [6, 7]. Furthermore, even though most seronegative recipients of seropositive allografts will develop CMV infection if not treated with antiviral prophylaxis, a considerable proportion (30%–40%) will never experience CMV infection [8].

An important body of evidence suggests that monitoring CMV-specific T-cell responses, at different times before and after transplantation, may allow a more accurate characterization of the immune risk profile against CMV infection [9–13]. Measuring circulating CMV-specific IgG antibodies is the most common method of assessing the CMV-specific B-cell sensitization status. However, this approach may underestimate the true magnitude of the humoral immune response, because it excludes the whole memory B-cell pool. In fact, memory B cells can exist in the absence of detectable serum antibody levels [14, 15], but are able to rapidly differentiate into antibody-secreting cells (ASCs), which may be highly relevant for an effective humoral response. Therefore, a direct assessment of the CMV-specific memory T and B cells in transplant recipients could provide a more complete picture of their adaptive memory immune response against CMV.

This study aimed to investigate the baseline CMV-specific memory T- and B-cell compartments using highly sensitive enzyme-linked immunospot (ELISPOT) assays in a cohort of seronegative and seropositive kidney transplant recipients. We measured the frequency of CMV-specific interferon (IFN) γ– and IgG–producing memory T and B cells and determined whether it could illustrate the immune sensitization status against CMV more accurately than circulating CMV IgG titers. These observations could be relevant to clinical CMV risk stratification, and they provide new insights into the mechanisms of the adaptive immune response against CMV infection in kidney transplantation.

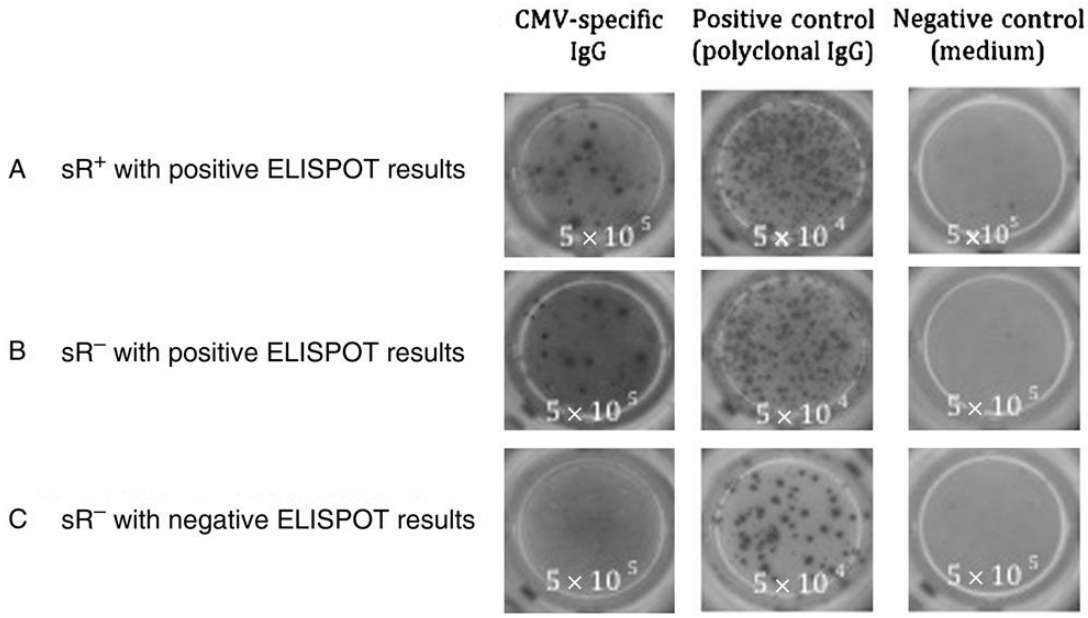

Figure 1. Representative images of cytomegalovirus (CMV)–specific immunoglobulin (Ig) G B-cell enzyme-linked immunospot (ELISPOT) assay results from CMV IgG seronegative (sR^-) and seropositive (sR^+) kidney transplant recipients with positive or negative CMV-specific interferon (IFN) γ T-cell ELISPOT results; CMV-specific IgG-producing memory B cells were detected in sR^+ (*A*) and sR^- (*B*) patients with positive CMV-specific IFN-γ T-cell ELISPOT results but not in sR^- patients with negative results (*C*).

METHODS

Study Patients

This was a retrospective study case-control study. Between February 2010 and January 2013, a total of 50 consecutive CMV IgG–seronegative (sR^-) kidney transplant recipients from our renal transplant unit were eligible to participate in the study. To confirm a stable pretransplant CMV IgG serostatus, 2 serial serology tests were performed during the year before transplantation, followed by another at the time of transplantation. We excluded 7 patients from the study: 5 patients without pretransplant blood samples, 1 with low but detectable CMV IgG titers 6 months before transplantation that were not detectable at transplantation, and 1 who received numerous blood transfusions during the 6 months before transplantation. Therefore, we evaluated 43 kidney transplant recipients. We included 86 contemporary and consecutively paired sR^+ transplant recipients in a 2:1 ratio with the sR^- group.

CMV Preventive Strategies

A preemptive strategy was used, with all sR^- patients receiving a seronegative allograft (sR^-/sD^-) and all sR^+ patients not receiving T-cell depleting agents (eg, rabbit anti-thymocyte globulin [rATG]). Prophylaxis with valgancyclovir over 100 days was restricted to sR^- patients receiving a seropositive allograft (sR^-/sD^+) and or rATG induction therapy.

Clinical Data and Definitions

The definition of CMV infection was based on the criteria recommended by the American Society of Transplantation for use in clinical trials [16]. Briefly, CMV viremia was defined as the detection, by either quantitative nucleic acid testing or the 65-kDa phosphoprotein (pp65) antigenemia assay, of replicating CMV in blood without symptoms, and CMV disease was defined as evidence of CMV replication/antigenemia with compatible symptoms, including both viral syndrome and invasive tissue disease.

Microbiological Studies

Surveillance of CMV antigenemia was determined in polymorphonuclear leukocytes obtained by dextran sedimentation and formaldehyde fixed, stained, and read under a fluorescence microscope (rapid antigenemia anti-human CMV ppUL83; Argene; reference 14-002). Surveillance quantitative CMV DNA detection was evaluated in plasma using a real-time CMV kit (Abbott). The cutoff value for CMV DNA detection was 1000 copies/mL assessed in plasma.

Enzyme-Linked Immunosorbent Assay for CMV IgG Detection

CMV serostatus was determined using a commercial CMV IgG enzyme-linked immunosorbent assay Kit (BioCheck) according to the manufacturer's instructions. Two serial serological tests were performed during the year before transplantation, followed

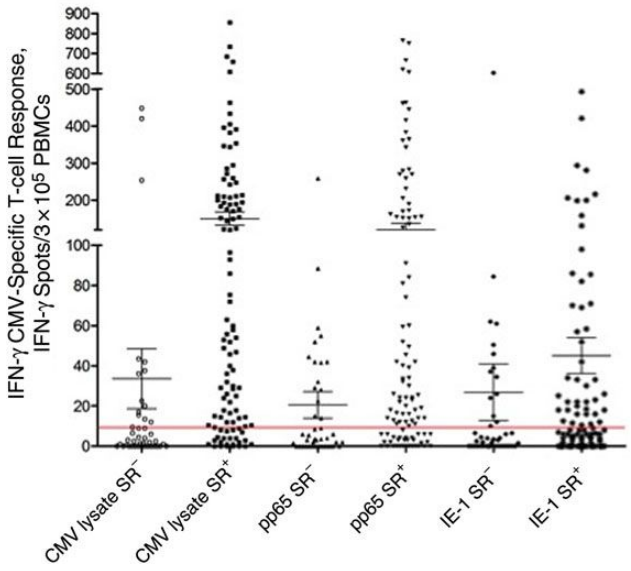

Figure 2. Preformed cytomegalovirus (CMV)–specific interferon (IFN) γ–producing T-cell responses between CMV seropositive (sR^+) and seronegative (sR^-) kidney transplant recipients before transplantation. In sR^+ versus sR^- patients, the mean (± SD) CMV-specific T-cell responses to CMV antigens (given as IFN-γ spots per 3×10^5 stimulated peripheral blood mononuclear cells [PBMCs]) were 150.2 ± 190 versus 33.6 ± 198 ($P < .001$) for CMV lysate, 120.24 ± 181 versus 20 ± 42 ($P < .01$) for 65-kDa phosphoprotein (pp65), and 45.1 ± 95 versus 25.7 ± 42 ($P = .03$) for immediate-early protein 1 (IE-1). The red line represents the most sensitive and specific IFN-γ cutoff value predicting the development of CMV infection after transplantation.

by another evaluation just at the time of the transplant surgery. The CMV IgG cutoff value for seronegativity was <1.1 IU/mL.

CMV Peptides

As stimuli for the IFN-γ ELISPOT assay, we used pools derived from a peptide scan (15mer overlapping by 11 amino acids) covering the whole antigen length through the immediate-early protein 1 (IE-1) and the pp65 (Jerini Peptide Technologies; Swiss-Prot ID P13202 and P06725, respectively) of human CMV (Human Herpes Virus-5), plus a CMV lysate (Autoimmune Diagnostik). This allowed us to avoid human leukocyte antigen restrictions. We used Human CMV (AD169 strain) viral lysate (Advanced Biotech; 10-144-000) to detect CMV-specific IgG-secreting B cells.

CMV-Specific Memory/Effector T-Cell Assessment

CMV-Specific T-Cell ELISPOT

CMV-specific T-cell ELISPOT assays were performed as described elsewhere [13]. Briefly, 3×10^5 peripheral blood mononuclear cells (100 µL) were stimulated in triplicate with a CMV antigen peptide pool (1 µg/mL) for 18 hours, which exclusively assessed memory immune responses. We detected IFN-γ spots using a biotinylated anti-human IFN-γ antibody developed by the addition of alkaline phosphatase conjugate substrate (AID). The resulting spots were counted semiautomatically with an ELISPOT reader (AID ELISPOT Reader HR, fourth generation).

CMV-Specific Memory/Effector B-Cell Assessment

Memory B-Cell Stimulation Assay

To induce and differentiate memory B cells to ASCs, peripheral blood mononuclear cells were cultured (1.5×10^6 cells/mL; at 37°C in 5% carbon dioxide) for 6 days in Roswell Park Memorial Institute medium (supplemented with 2 mmol/L L-glutamine), 10% fetal calf serum, 0.1 mg/mL penicillin G (Britannia Pharmaceuticals), 0.1 mg/mL streptomycin (Sigma-Aldrich, 10 ng/mL recombinant human interleukin 2 (Mabtech), and 1 µg/mL Toll-like receptors 7/8 agonist R848 (Mabtech) [17]. As shown in Supplementary Figure 1, a significant proportion of memory B cells proliferated and differentiated into ASCs. After thorough washing, the cells were used in IgG ELISPOT assays.

CMV-Specific IgG B-Cell ELISPOT Assay

A detailed description for the IgG B-cell ELISPOT assay can be found in the supplementary data. Briefly, we seeded 5×10^5 cells from the memory B-cell stimulation assay in 100 µL triplicates on a CMV purified viral lysate (Advanced Biotech; 10-144-000) coated 96-well ELISPOT plate (MAIPSWU10 MultiScreen, Millipore) after 18 hours of incubation at 37°C. The IgG spots were detected using a biotinylated human anti-IgG antibody and developed by the addition of streptavidin-conjugated alkaline phosphatase substrate (Mabtech). The resulting spots were counted semiautomatically with an ELISPOT reader (AID ELISPOT Reader HR, seventh generation).

Statistical Analysis

All data are presented as means and SDs. Groups were compared using the χ^2 test for categorical variables, the 1-way analysis of variance or *t* test for normally distributed data, and the nonparametric Kruskal–Wallis or Mann–Whitney *U* test for nonnormally distributed variables. Both CMV antigenemia and disease were considered outcome variables of the study. Bivariate correlation analyses were done using Pearson or Spearman tests for nonparametric variables. A sensitivity/specificity receiver operating characteristic analysis was done to investigate the value of the ELISPOT test for predicting posttransplant CMV infection. The 2-tailed statistical significance level was $P < .05$.

RESULTS

Baseline Patient Demographic Characteristics

Table 1 summarizes the main clinical and demographic characteristics of the 43 sR^- patients and the 86 sR^+ patients. Most

Table 2. Clinical Variables in 43 sR$^-$ Kidney Transplant Recipients With CMV Viremia or Disease

Variable	CMV Viremia		CMV Disease	
	Yes (n = 11)	No (n = 32)	Yes (n = 8)	No (n = 35)
Sex, male/female, No. (%)	9 (82)/2 (18)	21 (66)/11 (34)	5 (62.5)/3 (37.5)	25 (71)/10 (29)
Age, mean ± SD, y	45 ± 19.4	48.9 ± 16.7	56.5 ± 17.4	46 ± 16.9
DGF, yes/no, (%)	5 (45)/6 (55)	6 (19)/26 (81)	5 (62.5)/3 (37.5)[a]	6 (17)/29 (83)
BPAR, yes/no, (%)	3/ (27)/ 8 (73)	4 (12.5)/28 (87.5)	1/ (12.5)/7 (87.5)	6 (17)/29 (83)
CMV serostatus, No (%)				
sR$^-$/sD$^-$	0 (0)	6 (18.7)	0 (0)	6 (17.1)
sR$^-$/sD$^+$	11 (100)	26 (81.3)	8 (100)	29 (82.9)
Preventive therapy, No. (%)				
Prophylaxis	10 (91)	25 (78)	8 (100)	27 (77)
Preemptive	1 (9)	7 (28)	0 (0)	8 (23)
MPA trough level, mean ± SD, µg/mL				
Month 1	3.9 ± 3.2	3.6 ± 2.1	4.7 ± 3.5	3.4 ± 2.1
Month 3	3 ± 1.4	3.6 ± 2.8	3.2 ± 1.9	3.5 ± 2.6
Month 6	3.2 ± 3	3 ± 2.87	2 ± 1.5	3.3 ± 3
Induction IS (rATG), yes/no, No. (%)	7 (64)/4 (36)	13 (41)/19 (59)	4 (50)/4 (50)	46 (16)/19 (54)
Graft function (eGFR), mean ± SD, mL/min				
Month 6	39 ± 25[a]	41.2 ± 27	22.9 ± 25.9[a]	44.7 ± 27
Month 12	34.5 ± 24	43.1 ± 22.5	19.9.1 ± 22[a]	46 ± 20.3
Patient death, yes/no, (%)	0 (0)/11 (100)	2 (6)/30 (94)	1 (12.5)/7 (87.5)	1 (3)/34 (97)
Anti-CMV T-cell ELISPOT count, mean ± SD, IFN-γ spots/3 × 10^5 PBMCs				
CMV lysate	7.8 ± 12.8	42.4 ± 112	6.6 ± 6.5	39.7 ± 107
pp65 Antigen	2.6 ± 3[a]	26.7 ± 48.2	1.7 ± 1.8[a]	24.8 ± 48.5
IE-1 antigen	1.36 ± 2.1[a]	35.5 ± 106	0.25 ± 0.7[a]	47 ± 99

Abbreviations: BPAR, biopsy-proved acute rejection; CMV, cytomegalovirus; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; ELISPOT, enzyme-linked immunospot; IE-1, immediate-early protein 1; IFN, interferon; IS, immunosuppression; MPA, micophenolyc acid; PBMCs, peripheral blood mononuclear cells; pp65, 65-kDa phosphoprotein; rATG, rabbit anti-thymocyte globulin; SD, standard deviation; sD$^-$, seronegative allograft; sD$^+$, seropositive allograft; sR$^-$, CMV immunoglobulin G–seronegative transplant recipient.

[a] $P < .05$.

patients (86%) received a kidney allograft from a CMV IgG–seropositive donor (sD$^+$). Most sR$^-$ patients received anti-CMV prophylaxis, whereas sR$^+$ patients were followed up with the preemptive strategy. All but 1 patient in the sR$^+$ group who received belatacept were treated with a calcineurin inhibitor–based immunosuppressive regimen. Induction therapy was used in most patients with either anti-CD25 monoclonal antibodies or T-cell depletion (rATG). We observed CMV viremia and disease in 11 (25.6%) and 8 (18.6%) of the 43 sR$^-$ patients, respectively; the corresponding rates in the 86 sR$^+$ patients were 25 (29%) and 12 (14%). All late-onset CMV infections in the sR$^-$ group were observed within the sR$^-$/sD$^+$ combination and appeared a median of 33 days after prophylactic treatment; most patients were asymptomatic or had viral syndromes diagnosed (5 of 8). The 3 cases of invasive tissue disease were located in the gastrointestinal tract. Two patients experienced CMV recurrence after valganciclovir treatment.

Preformed T- and B-Cell CMV Sensitization Among sR$^-$ Kidney Transplant Recipients

First, we evaluated the frequency of CMV-specific IFN-γ–producing T cells against 2 specific CMV antigens (pp65 and IE-1) and a CMV lysate. As shown in Table 1 and Supplementary Figure 2, 13 (30%) and 15 (34%) of the 43 sR$^-$ patients, respectively, displayed different detectable IE-1 (26.78 ± 92.5) and pp65 (20.5 ± 42.8) CMV-specific IFN-γ spots / 3×10^5 stimulated peripheral blood mononuclear cells (PBMCs) T-cell frequencies.

Subsequently, we analyzed CMV-specific IgG-secreting memory B cells using the B-cell ELISPOT assay in sR$^-$ and sR$^+$ patients. As shown in Figure 1, sR$^+$ patients showed high frequencies of both CMV-specific IFN-γ and IgG-producing memory T and B cells, respectively (Figure 1*A*), and sR$^-$ transplant recipients without detectable CMV-specific T-cell responses showed no evidence of CMV-specific IgG-producing memory B cells (Figure 1*C*). Notably, sR$^-$ individuals with

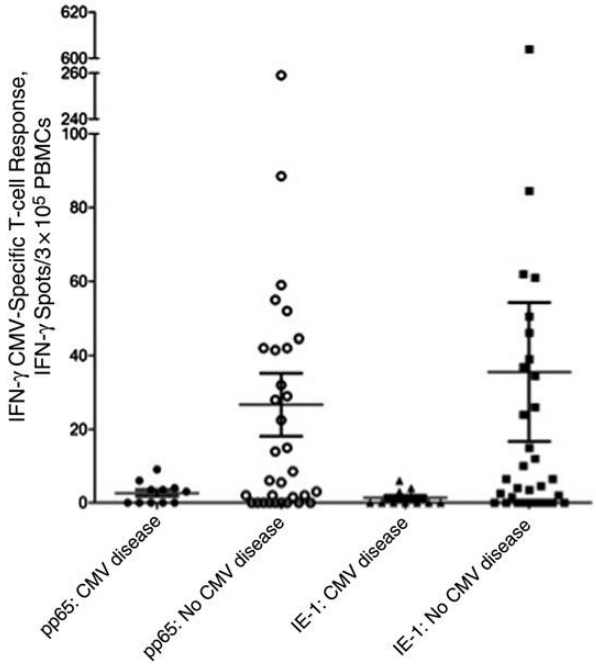

Figure 3. Preformed cytomegalovirus (CMV)–specific interferon (IFN) γ–producing T-cell responses were significantly lower among CMV immunoglobulin (Ig) G–seronegative (sR^-) kidney transplant recipients with CMV disease and viremia than among those without CMV disease and viremia. In sR^- patients who developed CMV infection versus those who did not, the mean (± SD) CMV-specific IFN-γ–producing T-cell responses to CMV antigens (given as IFN-γ spots per 3×10^5 stimulated peripheral blood mononuclear cells [PBMCs]) were 2.23 ± 3 versus 28.4 ± 49.4 ($P = .007$) for 65-kDa phosphoprotein (pp65) and 1.15 ± 2 versus 37.9 ± 56 ($P = .04$) for immediate-early protein 1 (IE-1).

detectable CMV-specific IFN-γ–producing T-cell frequencies also showed circulating CMV-specific IgG-secreting memory B cells (Figure 1*B*).

Preformed CMV-Specific IFN-γ T-Cell Frequencies in sR^+ and sR^- Patients

Next, we compared the strength of preformed IFN-γ–producing T cells against different CMV-specific antigens between sR^- and sR^+ patients. As shown in Figure 2, the mean preformed CMV-specific T-cell responses were significantly weaker among sR^- than among sR^+ patients, although a number of sR^- patients displayed high IFN-γ T-cell frequencies, similar to those observed in some sR^+ kidney transplant recipients.

Preformed CMV-Specific T-Cell Responses and CMV Infection in Both sR^- and sR^+ Patients

The main demographic and clinical variables were evaluated with regard to the advent of CMV infection after kidney transplantation (Table 2). No statistically significant associations were found between such variables as the type of CMV preventive therapy, the type of induction immunosuppression, the donor IgG serostatus (sD^+ vs sD^-), micophenolyc acid trough levels, the incidence of acute rejection, and the development of either CMV viremia or disease. Conversely, patients who experienced delayed graft function showed higher CMV disease incidences after transplantation. Of note, those with detectable preformed CMV-specific T-cell responses (against both IE-1 and pp65 CMV antigens) displayed significantly lower rates of CMV infection (both viremia and disease) than those with no evidence of CMV-specific T-cell sensitization before transplantation (Figure 3). Likewise, preformed CMV-specific IFN-γ–producing T-cell frequencies (both pp65 and IE-1 specific) were significantly lower among sR^+ patients who developed CMV disease or viremia than among those who did not (Supplementary Figure 3).

Pretransplant Anti-CMV T-Cell Responses and Prediction of CMV Infection Risk

Receiver operating characteristic curve analysis of CMV-specific IFN-γ ELISPOT assay results for IE-1 and pp65 CMV antigens showed high sensitivity and specificity for the prediction of both CMV viremia and disease (Figure 4). The most sensitive and specific IFN-γ ELISPOT threshold against IE-1 and pp65 antigens were evaluated to establish the optimal threshold to define the CMV ELISPOT result as a binary variable (positive or negative) capable of predicting posttransplant CMV infection (both viremia and disease). As shown in Table 3, low specificities and positive predictive values were obtained for both CMV viremia and disease, but consistently high negative predictive values and sensitivities were observed for both tests, particularly for IE-1 T-cell responses. When these cutoff values were applied to the sR^+ transplant group, similar low pretransplant CMV-specific T-cell frequencies predicted the development of CMV infection with high sensitivity and specificity (Supplementary Figure 4).

Discussion

The precise identification of a kidney transplant recipient's immune susceptibility to CMV infection is a crucial goal for establishing guided preventive therapeutic strategies. Currently, the only criterion available to determine the patient's immune status is the presence of preformed IgG antibodies against the virus. However, this is merely a surrogate of the complete humoral adaptive immunity expected to confer protection. Here, we report that evaluating the frequency of both CMV-specific IFN-γ and IgG-producing memory T and B cells allows a more precise assessment of immune-sensitized individuals without serological evidence of CMV-specific humoral immunity. Furthermore, we showed that transplant recipients with high frequencies of preformed CMV-specific IFN-γ–producing

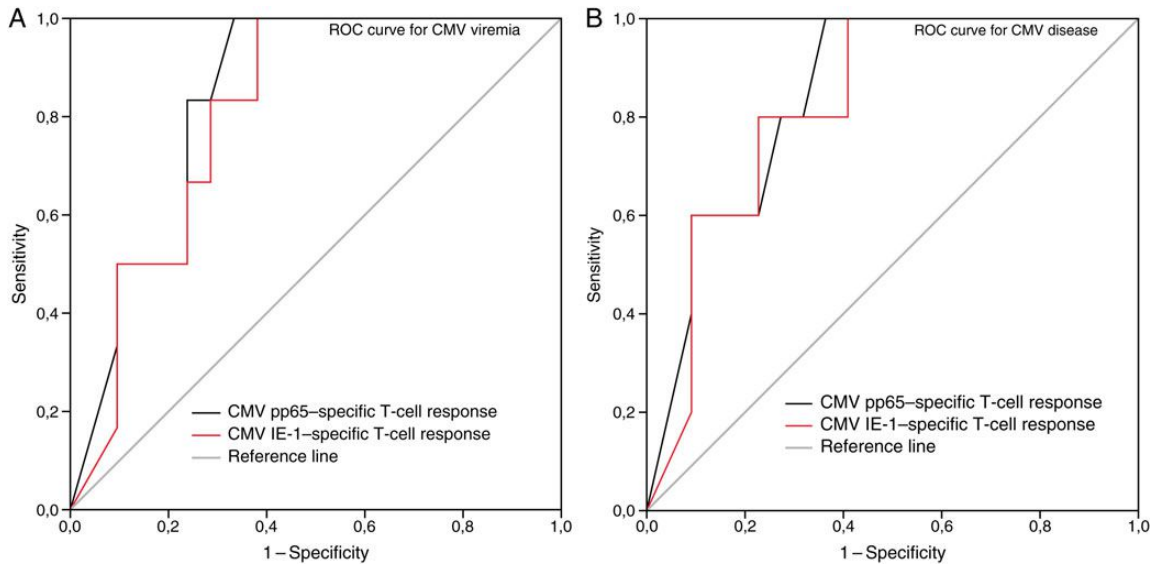

Figure 4. Receiver operating characteristic (ROC) curves for the sensitivity and specificity of pretransplant anti–immediate-early protein 1 (IE-1) and anti–65-kDa phosphoprotein (pp65) T-cell frequencies for predicting the risk of cytomegalovirus (CMV) viremia and disease. Area under the curve (AUC) values were 0.837 ($P = .01$; 95% confidence interval [CI], .687–.988) and 0.810 ($P = .02$; 95% CI, .646–.974) for pp65 and IE-1, respectively, for the prediction of CMV viremia and 0.845 ($P = .02$; 95% CI, .690–1.000) and 0.827 ($P = .03$; 95% CI, .659–.995) for the prediction of CMV disease.

memory T cells demonstrate clinical protection. They were unlikely to develop CMV infection after kidney transplantation compared with patients without cellular immune responses, which is consistent with our recent report on sR^+ kidney transplant recipients [13].

Kidney transplantation waiting lists may contain to 20%–30% sR^- individuals, and it is well known that sR^-/sD^+ recipients are at considerably higher risk of CMV infection. However, a significant proportion will never develop clinical infection, despite not receiving preventive treatment [8, 18], suggesting either that primary CMV-specific effector T-cell responses recover quickly and effectively immediately after transplantation, thereby providing sufficient protection and control of CMV replication [19, 20], or that these individuals may be appropriately sensitized before transplantation, despite no evidence of CMV IgG titers in their serum samples.

It is important to note that, although B-cell responses are commonly evaluated by the serological measurement of specific antibodies [21], analysis limited to this level may not provide a sufficient assessment of the absolute memory repertoire, because it excludes the memory B-cell subset [22, 23]. Indeed, memory B cells may exist in the absence of detectable serum antibody levels in different biological settings [14, 15], and their rapid differentiation into ASCs with antibody production may have high relevance for a protective humoral response [24]. To this end, the highly sensitive B-cell ELISPOT assay allows accurate detection of antigen-specific IgG ASCs at a single-cell level [17]. Using this, we observed that sR^+ patients have concomitantly high frequencies of CMV-specific IgG ASCs, and some sR^- patients may also have detectable frequencies of CMV-specific IgG ASCs. This suggests that these patients were already sensitized against CMV despite not showing circulating antibodies.

The T-cell compartment is thought to play a key role in viral replication and control [25–28]. Therefore, we aimed to investigate the CMV-specific memory T-cell response against the 2 dominant immunogenic CMV antigens (IE-1 and pp65) in the sR^- patients. First, we observed that the sR^- patients with detectable CMV-specific IgG-ASC also showed high CMV-specific IFN-γ–producing memory T-cell frequencies, reinforcing the fact that these individuals had had previous contact with CMV despite no serological evidence of IgG antibodies. Second,

Table 3. Predictive Values of pp65 and IE-1 IFN-γ T-Cell ELISPOT Tests for Predicting CMV Viremia or Disease

ELISPOT Test	Sensitivity, %	PPV, %	NPV, %
CMV viremia			
pp65	90	37	94
IE-1	100	35	93
CMV disease			
pp65	100	30	100
IE-1	100	28	100

Abbreviations: CMV, cytomegalovirus; ELISPOT, enzyme-linked immunospot assay; IE-1, immediate-early protein 1; IFN, interferon; NPV, negative predictive value; pp65, 65-kDa phosphoprotein; PPV, positive predictive value.

although significantly lower frequencies were observed in sR^- than in sR^+ patients, robust T-cell responses occurred in both groups, suggesting some degree of immune sensitization variability between individuals that may appropriately be detected using a sensitive tool such as the IFN-γ ELISPOT assay.

Together with other investigators, we have recently reported that the presence of CMV-specific T-cell frequencies among sR^+ patients seems to provide protection against CMV infection after kidney transplantation [11–13]. Interestingly, in our current study, sR^- patients not developing CMV viremia or disease showed significantly higher detectable IFN-γ–producing memory T-cell frequencies, especially against the IE-1 CMV antigen, than patients developing CMV infection after prophylactic treatment. Likewise, the same protective effect was observed among our control sR^+ patients. Moreover, when the most sensitive and specific T-cell ELISPOT cutoff value was used as a binary variable (positive or negative) to define the risk for late posttransplant CMV infection, very high sensitivity and negative predictive values for both CMV disease and viremia were obtained. This illustrates the usefulness of the assay for identifying immunized kidney transplant recipients without detectable serum CMV IgG titers.

A limitation of our study is the relatively small sample size, which precludes multivariate analysis of whether preformed T- and B-cell sensitization are independent protective variables against CMV infection after transplantation. Nonetheless, our data should be used to guide future prospective randomized interventional trials of these immune assays in the context of kidney transplantation and other solid organ transplant settings.

In summary, our observations may have relevant clinical implications, because 25%–30% of adult sR^- transplant recipients may have robust undetected preformed CMV-specific memory B and T-cell memory clones, ultimately conferring sufficient immune protection to avoid CMV infection after kidney transplantation.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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

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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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human cytomegalovirus, immune-monitoring, immunosuppression, kidney transplantation, T-cell immune response.

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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

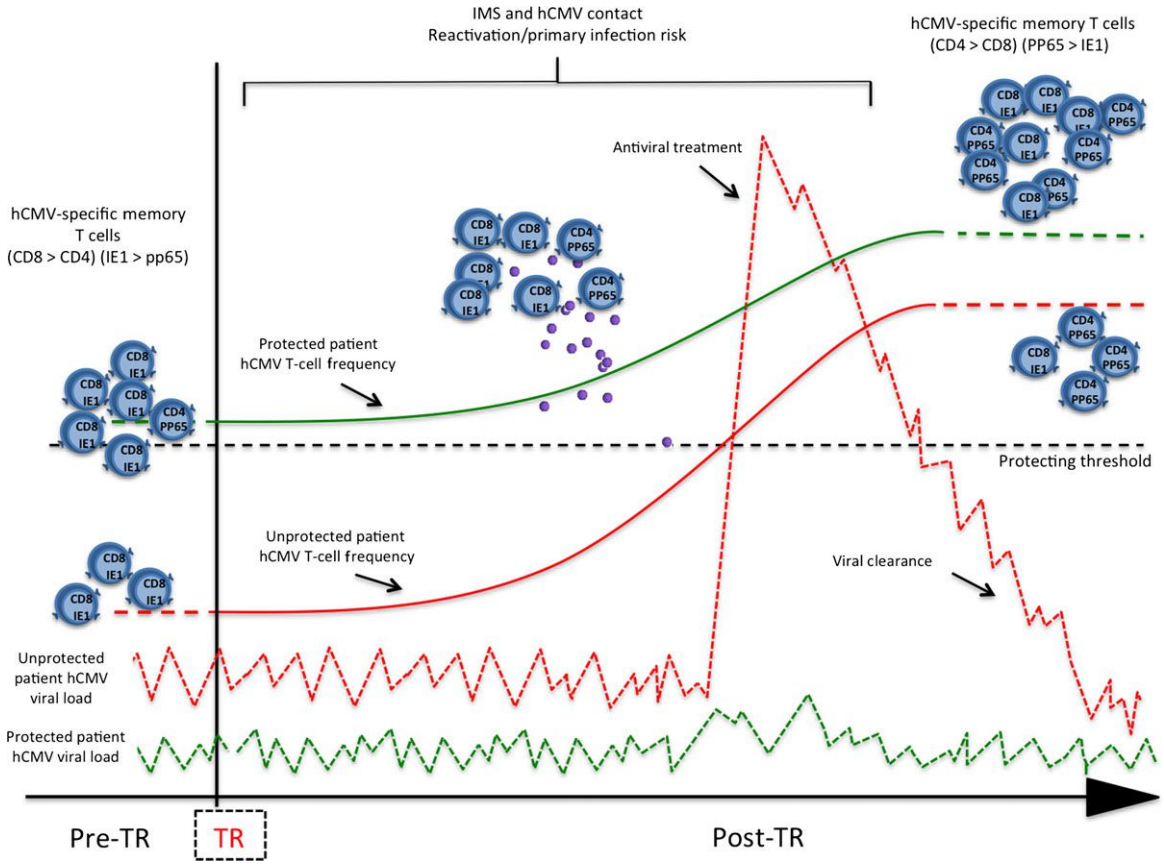

Figure 1 Patterns of hCMV-specific T-cell responses during the transplant setting.

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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VII. Results

RESULTS STUDY 1

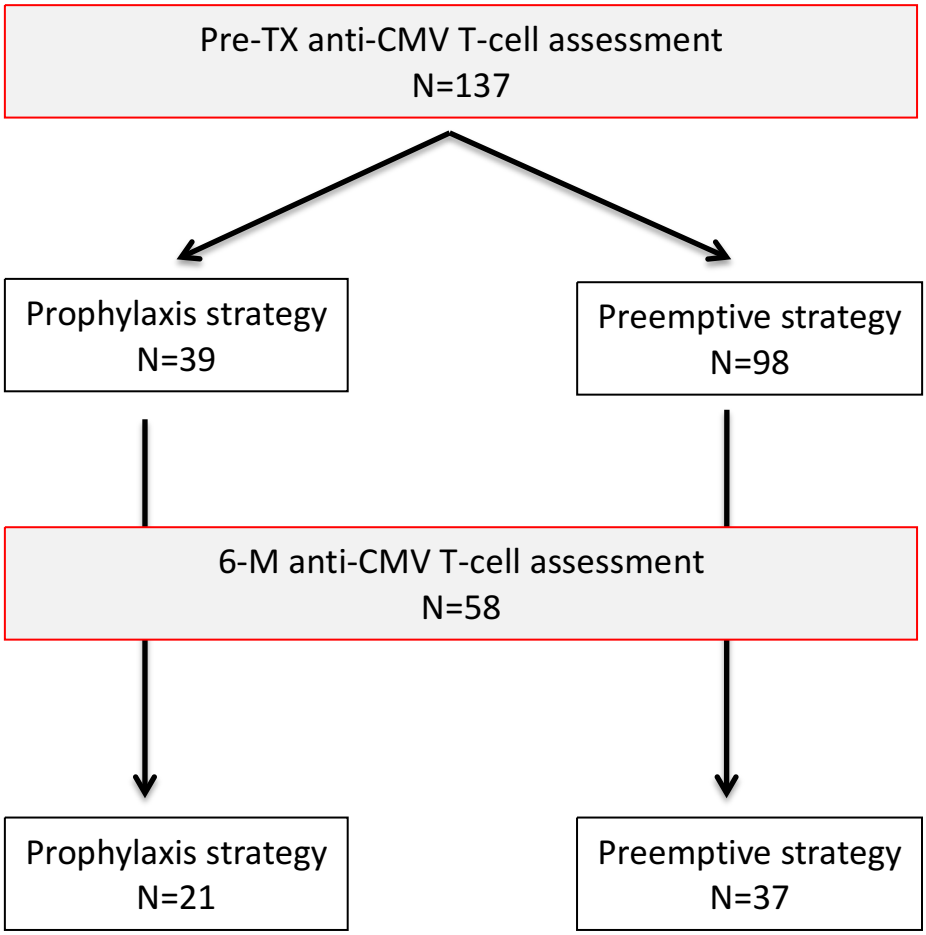
Immediately Early-1-Specific T Cell Responses Provide Protection For hCMV Infection After Kidney Transplantation.
Am J Transplant. 2013 May 24; 13(7):1–13.

One hundred and thirty-seven consecutive kidney transplant recipients with more than one year's evolution were retrospectively assessed for their baseline anti-hCMV T-cell response using the IFNγ-ELISPOT assay. Of these, 39 patients received post-transplant hCMV prophylaxis and 98 followed a preemptive protocol.

Also, six-month hCMV-specific T-cell responses were evaluated in 58 patients, 21 receiving prophylaxis and 37 preemptive therapy.

Mean follow-up of the study was 25 months (range 37–15 months). The presence or absence of such cellular responses was correlated with the appearance of both hCMV antigenemia and disease.

Figure 1. Distribution of the study population.



Clinical variables within Preemptive and Prophylactic-treated patients

Main demographic characteristic of all patients of the study are shown in table 1 of study number 1. Among prophylactic-treated patients, the advent of CMV antigenemia appeared in all but one patient after completing valganciclovir treatment with a median of 45 days after stopping treatment. Within preemptive-followed patients, most CMV infection episodes occurred during the first 3 months after transplantation with a median of 38 days after transplantation. Incidence of CMV recurrence after

treatment was equally distributed between both groups (3 within preemptive and 2 among prophylaxis).

Among patients receiving preemptive therapy, the advent of CMV antigenemia was significantly more common in older recipients (54,8±9 vs 48,3±13 years old, p<0,005) and among those experiencing delayed graft function (DGF) (45,5% vs 14,4%, p<0,005). To note, T-cell depletion induction treatment was associated to a significantly increased risk of both post-transplant antigenemia and CMV disease (63,6 vs 38% and 80 vs 40% for antigenemia and disease, respectively, p<0,005). Conversely, type of maintenance immunosuppression was not associated with CMV infection. At 6 months, allograft function was significantly worse among those patients experiencing either HCMV antigenemia or disease as compared to those that did not.

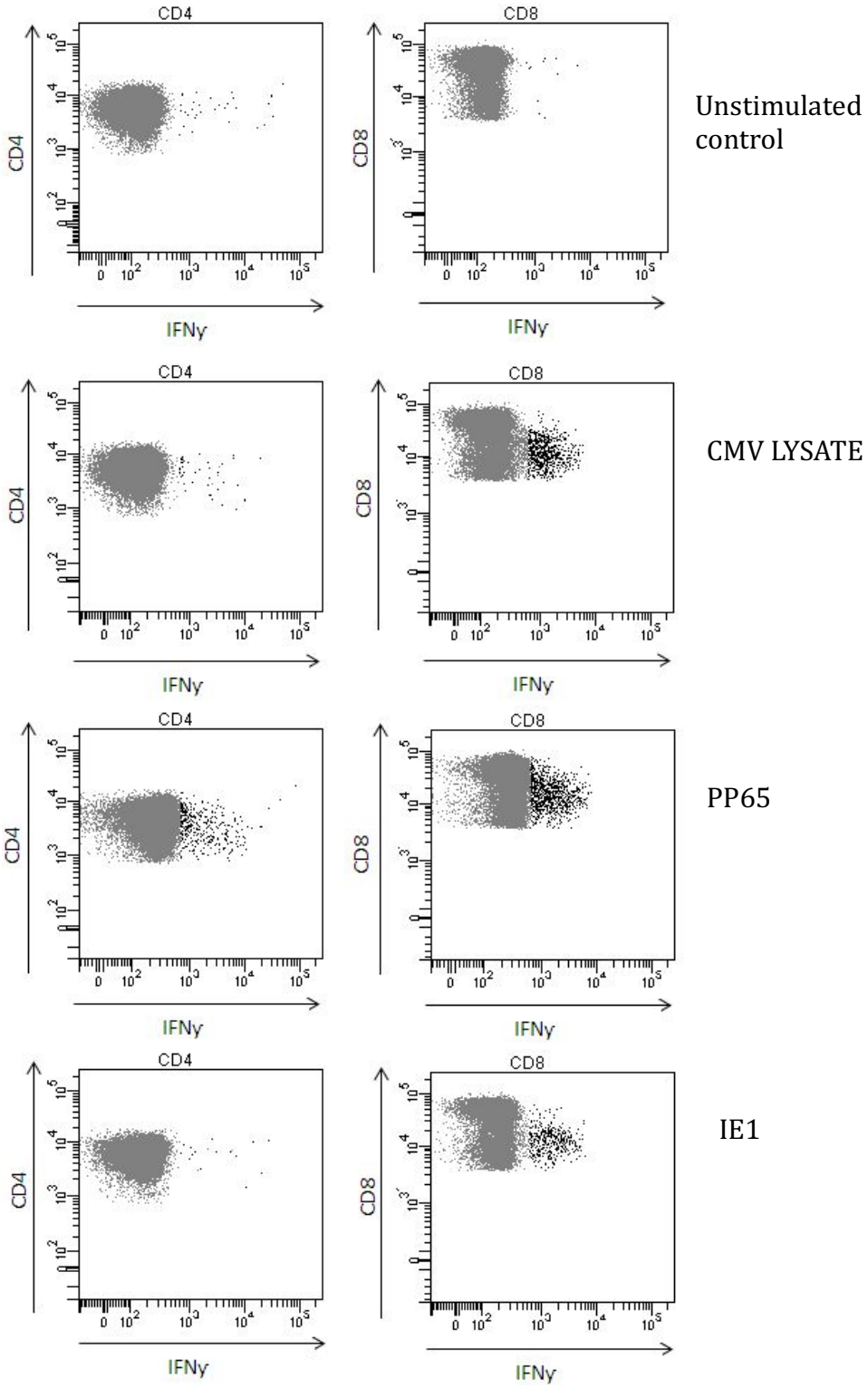
Main clinical data of patients with and without CMV antigenemia/disease within preemptive and prophylactic-treated patients are displayed in tables 2 and 3 of article number 1, respectively. Most hCMV infections in both cohorts of patients were asymptomatic CMV-detected antigenemia (28% and 22% in prophylactic and preemptive, respectively) and clinical disease was observed in 20% and 12% of prophylactic and preemptive groups, respectively. To note, the majority of clinical diseases were diagnosed as viral syndromes (11/18) whereas tissue invasive diseases were observed in 7 patients, located in the gastro-intestinal tract and 2 in the pulmonary tract).

A. *Immune-monitoring hCMV-specific T-cell responses before kidney transplantation*

1. Pp65- and IE-1-specific T cell responses are predominantly provided by the CD8+ T-cell compartment.

It was first analyzed which predominant T-cell subset population was accounting for the anti-hCMV immune response assessed by the IFNγ-ELISPOT assay against different CMV stimulations. While T-cell responses against both pp65 and IE-1 hCMV peptides were predominantly CD8+, CD4+ T cell responses was also detected against the hCMV lysate stimuli used.

Figure 2. Intracellular IFN$^{-}_{\gamma}$ FACS analysis on CD4+ and CD8+ T cells stimulated with three different CMV stimuli in a representative kidney transplant patient with high frequencies of IFNγ producing T cells by the ELISPOT assay before transplantation.



2. Low pre-transplant IE-1-specific T-cell responses is associated with post-transplant hCMV infection

All anti-hCMV T cell responses within prophylactic patients were significantly lower as compared to patients with preemptive therapy (table 1, Study 1).

Pre-transplant pp65 and HCMV lysate but not anti-IE-1-specific T-cell responses positively correlated with pre-transplant hCMV IgG titers (figure 3, Study 1). No association was observed between pre-transplant anti-pp65 and hCMV lysate T-cell responses and incidence of hCMV infection (figure 4, Study 1).

3. HCMV-specific T-cell responses in patients receiving T-cell depletion induction therapy

Interestingly, similar findings wee observed in patients receiving T-cell depletion induction therapy; those patients with lower anti-IE-1 T-cell responses displayed significantly higher incidence of hCMV infection after kidney transplantation as compared to patients showing higher baseline IE-1-specific T-cell responses (figure 5, study 1).

4. Frequencies of pre-transplant anti-IE-1 T-cell responses independently predict the risk of post-transplant HCMV infection

Receiver operating characteristic curve (ROC) analysis for predicting either post-transplant antigenemia or disease (figure 6, study 1) showed a considerably high AUC (0,635-0,760) for pre-transplant anti-IE-1 T-cell responses in both treatment groups. A high sensitivity (>80%) and negative predictive value (≥90) were obtained of IE-1-specific T-cell responses for post-transplant hCMV infection (either antigenemia or disease)

			Predictive values		
Variable	**Outcome**	**Cut-off**	**Specificity (%)**	**Sensitivity (%)**	**NPV (%)**
Pre-TX IE-1 T-cell response	Late hCMV infection (**Prophylaxis**)	8 spots / 10^5 PBMC	65	82.5	89.5
Pre-TX IE-1 T-cell response	Early hCMV infection (**Preemptive**)	7 spots / 10^5 PBMC	55	80	95.7

B. Immune-monitoring hCMV-specific T-cell responses after kidney transplantation

Changes in CMV-specific T-cell responses were also evaluated at 6 months after transplantation and were compared between kidney transplant patients that had been infected by the virus and those that did not.

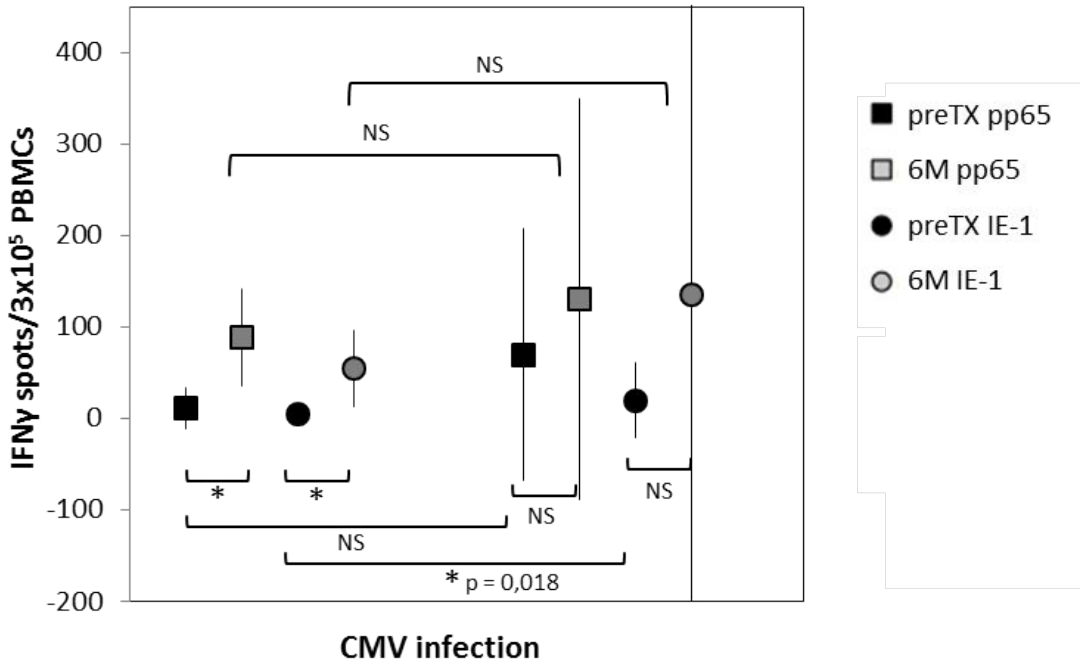
Overall, anti-hCMV T-cell responses significantly increased after transplantation despite the effect of chronic immunosuppression exposure (128,9±183 versus 278±433, p=0,012; 101,7±168 versus 127±183, p=0,006 and 39,8±86,1 versus 126±454 spots, p<0,001, for hCMV-lysate, pp65 and IE-1 for $3x10^5$ stimulated PBMCs both before and after transplantation, respectively).

Furthermore, no differences in 6-month hCMV-specific T-cell responses were observed between either types of hCMV preventive strategies (preemptive or prophylaxis), maintenance immunosuppression (CNI-based or CNI-free regimens), or different type of induction therapy (data not shown).

Nevertheless, when patients with or without post-transplant hCMV infection were compared regarding their change in the hCMV-specific T cell response at 6-month,

patients having experienced hCMV infection showed a significantly increase in pp65 and IE-1-specific T cell responses as compared to those that did not, suggesting an optimal anti-viral cellular immunization, particularly patients with low pre-transplant T-cell responses.

Figure 3. Changes in anti-hCMV T-cell responses among kidney transplant patients prior and at 6 month after transplantation.



RESULTS STUDY 2.

Preformed Frequencies of Cytomegalovirus (hCMV)-Specific Memory T and B Cells Identify Protected hCMV-Sensitized Individuals Among Seronegative Kidney Transplant Recipients.

Clin Infect Dis. 2014 Dec 1;59(11):1537-45. doi: 10.1093/cid/ciu589. Epub 2014 Jul 21.

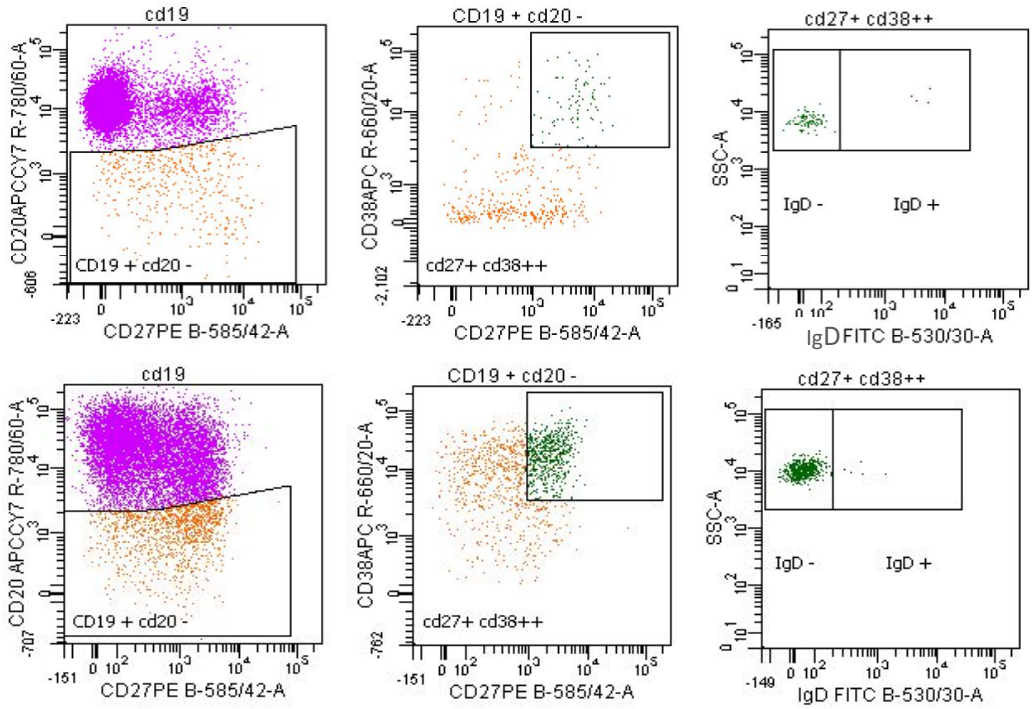
Human CMV-specific humoral and cellular memory immune responses were evaluated among kidney transplant patients before undergoing transplant surgery. Forty-three hCMV IgG-seronegative (sR-) kidney transplant recipients were first evaluated and results were further validated in an independent cohort of 86 hCMV IgG-seropositives (sR+) transplant recipients for its relationship with the risk of developing post-transplant hCMV infection.

1. Development of a novel hCMV-specific memory B-cell Elispot assay to enumerate hCMV-specific IgG-producing antibody-secreting cells (ASC)

For this study and with the aim of enumerating the presence of circulating hCMV-specific memory B cells, we developed a new B-cell Elispot assay. Firstly, to induce and differentiate memory B cells to (IgG) antibody-secreting cells (ASCs), peripheral blood mononuclear cells were cultured (1.5×10^6 cells/mL; at 37° C in 5% carbon dioxide) for 6 days in Roswell Park Memorial 160 Institute medium (supplemented with 2 mmol/L L-glutamine), 10% fetal calf serum, 0.1 mg/mL penicillin G (Britannia Pharmaceuticals), 0.1 mg/mL streptomycin (Sigma-Aldrich, 10 ng/mL recombinant human interleukin 2 (Mabtech), and 1 μg/mL Toll-like receptors 7/8 agonist R848 (Mabtech). As shown below, a significant proportion of memory B cells proliferated and differentiated into ASCs. After thorough washing, the cells were used in an IgG B-

cell ELISPOT assay in order to enumerate the frequency of hCMV-specific IgG-antibody-secreting cells.

Figure 1. Expansion and differentiation of memory B cells into ASC.



2. Patient baseline demographic characteristics

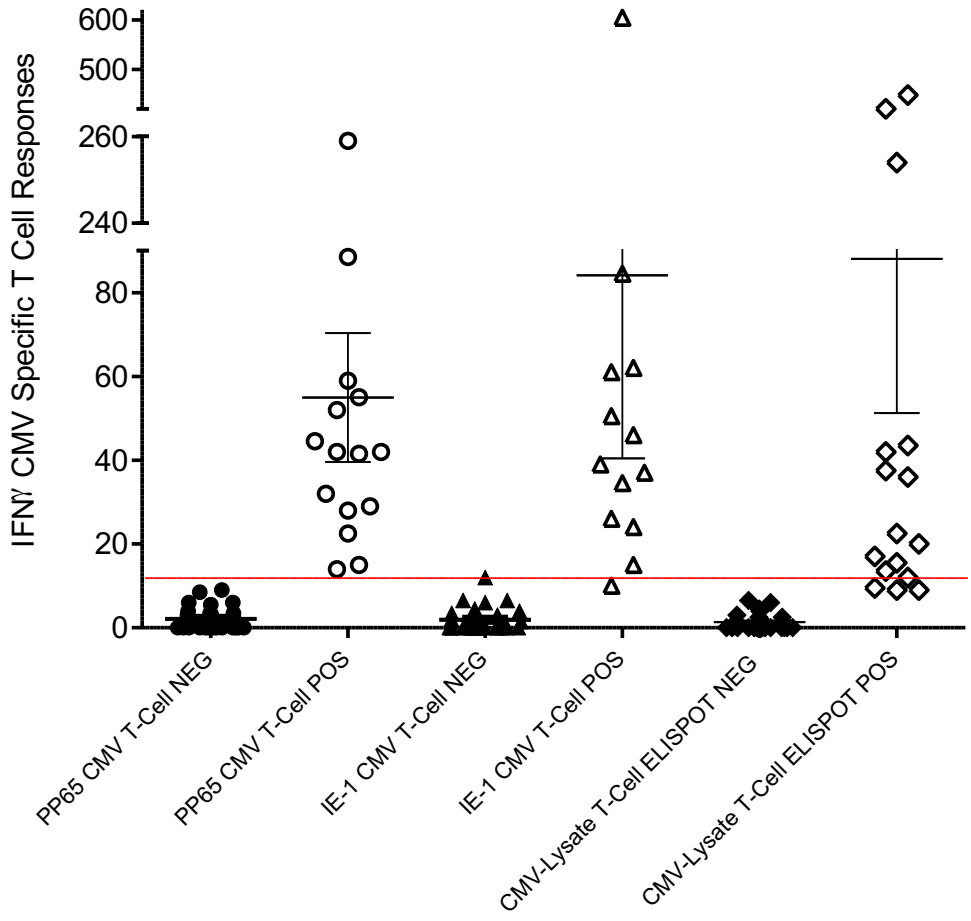
Main demographic characteristics of all patients of the study are shown in table 1 of article number 2. Eighty-six and 82.5% of sR- and sR+ patients received a kidney allograft from a hCMV IgG-seropositive donor (sD+), respectively. Most sR- patients received anti-hCMV prophylaxis, whereas sR+ patients were followed with a preemptive strategy. All but one patient in the sR+ group that received belatacept were treated with a CNI-based immunosuppressive regimen. Induction therapy was used in most patients with either anti-CD25 monoclonal antibodies or T-cell depletion (rATG).

HCMV viremia and disease was observed in 11 (25.6%) and 8 (18.6%) of the 43 sR- patients, respectively; the corresponding rates in the 86 sR+ patients were 25 (29%) and 12 (14%). All late-onset hCMV infections among the sR- group were observed within the sR-/sD+ combination and appeared a median of 33 days after prophylactic treatment; most of them were asymptomatic or diagnosed with viral syndromes (5/8). The three cases of invasive tissue disease were located in the gastrointestinal tract. Two patients experienced hCMV recurrence after valganciclovir treatment.

3. Preformed T and B-cell hCMV sensitization can be found in some hCMV sR- kidney transplant patients

We initially evaluated the frequency of CMV-specific IFN- γ –producing T cells against 2 specific CMV antigens (pp65 and IE-1) and a CMV lysate. This analysis revealed that 13/43 (30%) and 15/43 (34%) of the sR- patients, displayed detectable IE-1 (26.78 ± 92.5) and pp65 (20.5 ± 42.8) hCMV-specific IFN-ɣ T-cell frequencies, respectively.

Figure 2. High and low frequencies of hCMV-specific IFN-ɣ T-cell responses among some sR- transplant patients



Next, assessment of hCMV-specific memory B cells using the B-cell Elispot was also conducted. As expected, sR+ patients displayed high frequencies of hCMV-specific IgG-producing ASC as well as hCMV-specific IFN-ɣ-producing memory T cells(figure 1A, study 2). Conversely, sR- transplant recipients without detectable hCMV-specific IFN-ɣ T-cell responses showed no evidence of hCMV-specific IgG-producing memory B cells (figure 1B, study 2). Notably, sR- individuals with detectable hCMV-specific IFN-ɣ-producing T-cell frequencies also displayed circulating hCMV-specific IgG-ASCs (figure 1C, study 2).

4. Preformed hCMV-specific IFN-γ T-cell frequencies within sR+ are significantly higher than in sR- patients

Preformed hCMV-specific T-cell responses were significantly weaker among sR- than among sR+ patients, although a number of sR- patients displayed similarly high IFN-γ T-cell frequencies than those observed in some sR+ kidney transplant patients (figure 2, study 2).

5. Detectable preformed hCMV-specific T-cell responses are associated with lower hCMV infection rates among both sR-

No statistically significant associations were found between main clinical variables such as the type of hCMV preventive therapy, the type of induction immunosuppression, the donor IgG-serostatus (sD+/sD-), MPA trough levels, the incidence of acute rejection, and the development of either hCMV viremia or disease. Conversely, patients that experienced delayed graft function (DGF) showed higher hCMV disease incidences after transplantation (table 2, study 2).

Noteworthy, patients with detectable preformed hCMV-specific T-cell responses (against both IE-1 and pp65) displayed significantly lower rates of hCMV infection (both viremia and disease) than patients with undetectable hCMV responses (figure 3, study 2).

6. High frequencies of pre-transplant anti-hCMV T-cell responses predicts hCMV infection risk with high sensitivity and specificity

Moreover, receiver operating characteristic (ROC) curve analysis of hCMV-specific T-cell respones to IE-1 and pp65 hCMV antigens showed high sensitivity and specificity for the prediction of both hCMV viremia and disease (figure 4, study 2).

The most sensitive and specific hCMV-specific T-cell response values for IE-1 and pp65 were evaluated to establish the optimal threshold to define the hCMV-ELISPOT test as a binary variable (positive or negative) capable of predicting post-transplant hCMV infection (both viremia and disease).

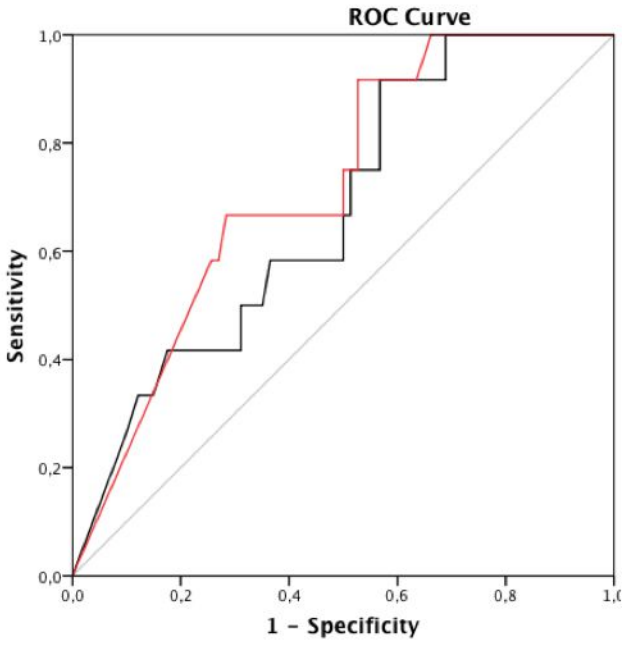
Low specificities and positive predictive values (PPV) were obtained but consistently high negative predictive values (NPV) and sensitivities were observed, particularly when evaluating IE-1 T-cell responses to evaluate risk of hCMV viremia and disease.

		Predictive values		
Outcome	**Variable**	**Sensitivity (%)**	**PPV (%)**	**NPV (%)**
HCMV viremia	Pre-TX **Pp65** T-cell response	90	37	94
	Pre-TX **IE-1** T-cell response	100	35	93
HCMV disease	Pre-TX **Pp65** T-cell response	100	30	100
	Pre-TX **IE-1** T-cell response	100	29	100

7. Validation of pre-transplant hCMV-specific T-cell responses as a protective biomarker of post-transplant hCMV infection in sR+ kidney transplant patients.

The analysis of preformed anti-hCMV T-cell responses within the new independent 86 sR+ cohort of kidney transplant patients was used as a validation set for the prediction of hCMV infection. As shown, using the same cut-off Elispot values, similar low pretransplant CMV-specific T-cell frequencies predicted the development of CMV infection with high sensitivity and specificity

Figure 3. ROC curve analysis for the prediction of hCMV infection among an independent sR+ cohort of kidney transplant patients (n=86)



Variables	(AUC)	P value	95% CI
Pp65 T-cell Elispot	0,673	0,05	0,526-0,821
IE-1 T-cell Elispot	0,718	0,016	0,583-0,854

VIII. Discussion.

Despite the outstanding progress made in the understanding of the immune response against viral pathogens as well as on the care-management of transplant patients since the beginning of times of organ transplantation, hCMV infection still represents a major adverse complication among solid organ and hematopoietic cell transplant patients directly threatening both allograft and patient survival.

While the advent of new and more potent anti-viral therapies used either as anti-viral treatments during active infection or as part of a preventive strategy, has lead to a significant reduction of the incidence of hCMV infection and its related complications, the occurrence of viral infection after transplantation is still considered as a rather unpredictable event. Certainly, this is the reflection of the considerably poor clinical monitoring of the viral immune susceptibility of each individual, which is merely based on the serological immune status combination between recipient and donor IgG-antibody levels in sera and the direct assessment of the virus itself replicating in peripheral blood.

The work constituting this doctoral thesis, researches further into how assessing hCMV-specific memory T and B-cell subsets, using a highly sensitive technique such as the ELISPOT assay, which allows an accurate enumeration of antigen-specific immune responses at the single cell level, may help to better identify cellular and humoral immunized patients against the hCMV and thus, ultimately helping identifying kidney allograft recipients at high risk of hCMV infection after kidney transplantation. Importantly, an accurate and reliable knowledge of the immune-protection level

against hCMV of transplant patients would allow individualization for anti-viral decision-making, thus personalizing this therapy.

To date, an important body of evidence has been generated within the transplant scientific community, demonstrating the key role of the adaptive immunity, and particularly the cellular immune response in preventing, controlling and restricting viral replication. In contrast to previous reported data, in which have analyzed different types of organ transplant patients, receiving distinct type of anti-viral therapies and have fundamentally focused on the post-transplant setting, we here focused for the first time on the evaluation of hCMV-specific memory T and B cells against different immunogenic hCMV antigens prior to kidney transplantation in very clean and homogenous cohorts of kidney transplant recipients. In the first part of this thesis, it is shown that high frequencies of hCMV-specific T-cell responses, particularly against the IE-1 dominant hCMV antigen, may significantly improve the identification of those kidney allograft recipients at high-risk for hCMV infection. Importantly, this approach was capable to discriminate such patients already before transplantation with high sensitivity, regardless the type of preventive anti-viral strategy used. Furthermore, the high negative predictive value of the test highlights the usefulness of such non-invasive diagnostic tool among the kidney transplant population. In the second part of this study, we provide further insight about the cross-talk between the humoral and cellular memory adaptive immune response against hCMV, by monitoring baseline hCMV-specific memory T and B-cell responses, which significantly increases the capacity to discriminate “true” immunized kidney transplant recipients against hCMV as compared to current evaluation of hCMV-specific antibody titers in the sera.

Our study points to the direction that monitoring IE-1 hCMV-specific T-cell frequencies before transplantation would help transplant physicians on the one hand to better discriminate patients with no need of hCMV prophylactic treatment from those in whom prophylaxis should preferentially be indicated and on the other, to better predict those patients in whom prophylaxis treatment could safely be discontinued. Interestingly, intrinsic impairment of the IE-1-specific T cell response was not only associated with the advent of post-transplant hCMV infection but also with the development of hCMV disease, thus reinforcing the importance of such functionally active hCMV-specific T cell precursors for achieving hCMV control under chronic immunosuppression. Hence, our study suggests that the increased risk to develop post-transplant hCMV infection (even after a course of prophylactic treatment) seems to rely in some patients in an individual immune susceptibility already manifested prior to transplantation.

Another relevant clinical observation found here, is that patients receiving T-cell depleting antibodies experiencing hCMV infection were predominantly those with significantly lower pre-transplant IE-1-specific T-cell frequencies, suggesting that the increased susceptibility for hCMV infection after T-cell depletion is particularly facilitated by the impairment of IE-1-specific T cell precursors already before transplantation rather than to a generalized T cell subset depletion after rATG therapy. This point is of especial interest as it may provide additional information to transplant clinicians about the type of induction therapy to be indicated in certain clinical situations.

To date, studies in transplant recipients evaluating the impact of CMV-specific cellular responses have mainly focused at the post-transplant period and used different cellular immune assays with distinct CMV stimuli (21–26,29–32). Our study (95) is in consonance with these previous reports, but also shows that the increased risk to develop posttransplant CMV infection (even after a course of prophylactic treatment) seems to rely in an individual immune susceptibility already manifested prior to transplantation.

Likewise, but in lung and heart transplant patients, Bunde et al.(96) showed that frequencies of IE-1 but not pp65-specific CD8þ T cells already at Day 0, discriminated patients who did not develop CMV disease from patients at risk. Although focusing on the association between allogeneic and CMV-specific effector T cell responses, Nickel et al. (33) reported similar findings in a group of 36 kidney transplant patients.

Of note, in our study at 6 months after transplantation while there was a general increase of all hCMV-specific T cell responses (both against IE-1 and also pp65), this feature was specifically observed within those having recovered from hCMV infection, suggesting that broader hCMV-antigen specific T-cell responses might be also necessary for controlling hCMV replication. Hence, our results reinforce the potential value of preventive strategies using recombinant hCMV proteins as vaccines, preferentially containing immunogenic IE-1 antigens already before transplantation.

Although different studies have suggested a preponderant role of CD8+ T cells for hCMV control.(97–99) , others have also shown the concomitant key function of CD4+ T cells, which seem to even have a preferential role conferring long-lasting protection (100,101). In our study, we found that pp65 and IE-1-specific T cell responses are

predominantly but not exclusively restricted to CD8+ thus, CD4+ T cells responses could similarly be required to confer long-term protection against hCMV infection.

Even though T cell responses may target multiple hCMV-specific proteins (38,85,102), it appears that protective cellular immunity is mainly directed against the tegument protein ppUL83 and to the immediately early protein ones (86,96,103,104). To note, IE-1 is the first protein expressed upon hCMV reactivation (105), thus IE-1-specific T cells would be the first to be activated and directed to sites of replication (106,107). Therefore, this mechanism could explain why high levels of IE-1 but not other hCMV-specific T cells would be associated with protection from hCMV disease when assessed prior to transplantation. Nevertheless, although some other groups have shown lack of correlation with exclusive IE-1-specific T cell responses and risk of hCMV disease (108,109), most of them focused at the post-transplant setting and evaluated a rather low number of transplant recipients.

A second main point investigated in this thesis, is the relationship between the humoral and cellular anti-viral memory immune responses and the presence of circulating hCMV-specific IgG titers as biological markers of anti-viral immunization. Currently, the only criterion available to determine the patient's immune status is the presence of preformed IgG antibodies against the virus. However, this is merely a surrogate of the complete humoral adaptive immunity expected to confer protection. In this study, we report that evaluating the frequency of both hCMV-specific IFN-γ and IgG-producing memory T and B cells allows a more precise discrimination of actually immunized individuals, even without serological evidence of hCMV-specific humoral immunity in a subset of patients.

Kidney transplantation waiting lists may contain 20% to 30% hCMV IgG-seronegative (sR−) individuals, and it is well known that sR−/sD+ recipients are at significantly higher risk of hCMV infection. However, the clinical experience have shown that a considerable proportion of these patients will never develop clinical infection, despite not receiving any preventive treatment (23) thus, suggesting either that primary hCMV-specific effector T-cell responses recover quickly and effectively immediately after transplantation, thereby providing sufficient protection and control of hCMV replication, or that these individuals may be appropriately immunized before transplantation, despite no evidence of circulating hCMV IgG titers.

It is important to note that, although B-cell responses are commonly evaluated by the serological measurement of specific antibodies (110), analysis limited to this level may not provide a sufficient assessment of the absolute memory repertoire, because it excludes the memory B-cell subset (111,112). Indeed, memory B cells may exist in the absence of detectable serum antibody levels in different biological settings (113,114), and their rapid differentiation into antibody-secreting cells (ASCs) with antibody production may have high relevance for a protective humoral response (115). To this end, we used a highly sensitive B-cell ELISPOT assay allowing an accurate detection of hCMV-specific IgG-ASCs at a single cell level (116). Using this assay, we observed that sR+ patients have high frequencies of hCMV-specific IgG-ASCs and that some sR−, may also have detectable frequencies of hCMV-specific IgG-ASCs thus, suggesting that these patients had previously recognized these hCMV antigens despite not showing circulating antibodies.

Similarly to what was shown in the first study, sR- patients showing hCMV-specific memory B-cell responses in peripheral blood, did also display high frequencies of

hCMV-specific T-cell immune responses, particularly against IE-1 hCMV antigen, that provided consistent immune protection against hCMV infection after kidney transplantation, as shown by the significantly lower incidence of both hCMV viremia and disease as compared to those transplant recipients with absence of hCMV-specific T-cell immune responses.

The hCMV-specific T-cell compartment may be evaluated using different cell-based *in-vitro* assays in humans (table 1, Annex). While most of them are capable to screen for such type of anti-viral immune responses, some particular assays have demonstrated a more reliable and reproducible results, particularly in the transplant setting. 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 IFNγ-ELISPOT, or the enzyme-linked immunosorbent (ELISA)-based assays (Quantiferon-hCMV) 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. 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-hCMV and the IFNγ-ELISPOT assays. Interestingly, while both assays are capable of measuring hCMV-specific T-cell responses, both are sustained on different concepts, which confer main differences between them. Importantly, a main advantage of the ELISPOT over the other assays is the lack of HLA restriction as it uses an extensive peptide collection spanning the whole hCMV-antigenic determinants, targeting both CD4+ and CD8+ T-cells and thus,

avoiding any HLA restriction. Interestingly, using the Quantiferon-hCMV assay both before and after transplantation, interesting associations between absence of hCMV-specific T-cell response and an increase susceptibility to hCMV infection has also been reported (117,118). Nevertheless, as an important constraint of this immune assay is that up to 30% of transplant recipients may show an inconclusive result thus, impeding a reliable generalized use for the entire transplant population.

The data reported in this thesis has relevant clinical implications as it strengthens the urgent need for an accurate immunemonitoring against the hCMV, in order to ultimately help transplant clinicians individualize decision-making regarding the type of hCMV preventive strategies. In this regard, there are different potential suitable clinical scenarios in which immune-monitoring hCMV-specific T-cell immune response could provide key information to the clinician to make a directed therapeutic intervention (table 4, Annex).

Before kidney transplantation, the assessment of hCMV-specific cellular immunity could help to discriminate patients at high or low risk of hCMV infection and thus assign or not a long-course of anti-viral prophylaxis as well as avoid a systematic costly viral monitoring after transplantation, respectively. Furthermore, the detection of hCMV-specific T and B-cell responses among sR- individuals on the waiting list could allow a safe pre-emptive treatment strategy.

On the other hand, the assessment of hCMV-specific T-cell responses at different time points after kidney transplantation would help identify patients that would benefit of a short course of prophylaxis treatment instead of longer one or conversely, discriminate those that would really need of a long-lasting treatment period due to the

risk of late hCMV infection or relapse. In addition, post-transplant hCMV-immune evaluation could potentially also help discriminate over-immunosuppressed patients at higher risk of hCMV infection.

In conclusion, we have shown that monitoring frequencies of hCMV-specific T and B-cell responses before transplantation, particularly against IE-1 hCMV antigen, may be useful for predicting post-transplant risk of hCMV infection irrespective of the hCMV serological immune status, thus being of potentially high value for guiding decision-making regarding hCMV preventive treatment.

However, standardization and validation of such results among large-scale powered prospective interventional trials are highly warranted and currently on-going in order to ultimately bring them in current clinical practice as part of the highly desired personalized medicine.

IX. Conclusions

1. The presence of high frequencies of hCMV-specific T-cell responses assessed in peripheral blood, particularly against IE-1 hCMV antigens, using the highly sensitive IFNγ-ELISPOT assay prior to transplantation, is able to differentiate consistently immunized kidney transplant recipients at low risk of post-transplant hCMV infection.

2. Low hCMV-specific hCMV-specific T-cell frequencies before kidney transplantation is able to discriminated patients at risk of early or late hCMV infection (both antigenemia or disease), regardless the type of anti-viral preventive treatment (either preemptive or prophylaxis) followed.

3. The baseline anti-viral T-cell immune response seems to also be crucial to discriminate patients at increased risk f developing HCMV infection after transplantation, despite receiving induction therapy with polyclonal T-cell depleting antibodies

4. The recovery after kidney transplantation of optimal hCMV-specific T-cell responses in peripheral blood seems to illustrate the relevance of an active anti-viral immunization to avoid the development of hCMV infection among kidney transplant patients receiving chronic immunosuppression.

5. The anti-hCMV serological status assessed in peripheral blood before kidney transplantation does not accurately reflect the degree of effective adaptive immunization of kidney transplant patients. Rather, the evaluation of hCMV-specific

IgG-producing memory B cells seems to more precisely illustrate the anti-viral immunization state of a given individual.

6. A substantial proportion of (hCMV) IgG-serologically negative kidney transplant recipients may display detectable hCMV-specific memory T and B-cell responses, which would be sufficiently effective to induce a protective immune state against hCMV infection.

7. Immune-monitoring hCMV-specific T and B-cell immune responses before and also after transplantation seems to be a reliable and promising tool for accurately identify patients at higher or lower risk for post-transplant hCMV infection and thus, help transplant clinicians to individualize selective anti-viral preventive strategies in kidney transplant patients.

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XI. Tables and Figures

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 1mL/1x10^6	% CMV-specific CD8 or CD4/IFN-γ cells of the respective reference population	48h	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 1x10^6	IFN-γ spots/3x10^5 stimulated PBMC	36h	8-11 IFN-γ spots	15aa short peptide pool spanning the whole antigen or Whole virus lysate	- High sensitivity - No HLA restriction - Allows multiple effector information	- Expert personnel required - Equipment required (elispot reader)
Tetramer Staining (Flow cytometry)	PBMC 1x10^6	% CMV-specific CD8 or CD4/IFN-γ cells of the respective reference population	48h	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	24h	0,1-0,2IU/mL	15 aa 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

Table 2. Pre-transplant 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 results
Bunde et al. 2005 (46)	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	1- Higher PreTR IE-1 specific CD8 T-cell frequencies negatively associated with CMV disease. 2- 6 /15 patients with IE1 T cell frequencies below the protective threshold did not develop disease.
Nickel et al. 2009 (47)	N=36 Kidney TR 24/36 R+ (14/36 R+/D+) (5/36 R-/D+)	36 Preemptive	Elispot IFN-γ Pp65 peptide pool IE-1 peptide pool	1- High T cell responses to IE1 correlated with 6mo graft function 2- Association between CMV disease with low IE-1 specific T-cell frequencies
Bestard et al. 2013 (73)	N=137 Kidney TR 109/137 R+ (28/137 R-/D+)	98 Preemptive 39 Prophylaxis	Elispot IFN-γ Pp65 peptide pool IE-1 peptide pool CMV lysate	1- Low PreTR IE-1 specific T cell frequencies independently predicts postTR hCMV infection (antigenemia and disease) 2- Low levels of CMV Tcell frequencies might be detected in few CMV IgG- patients. 3-Patients lacking IE-1 specific T-cell clones preTR experiencing hMCV infection, reach the same protective IE-1 specific T-cells frequencies at 6mo as those patients never experiencing hCMV infection
Cantisán et al. 2013 (72)	N=55 (23 lung, 32 Kidney) 44 R+ (8/53 R-/+) (3/53 R-/D-)	23 Preemptive 31 Prophylaxis	QuantiFERON pp65, IE-1, IE-2, gB	1- Combining QuantiFERON reactivity PreTR and Donor serostatus strong association with risk of hCMV infection 2-Quantiferon test performed close to end of prophylaxis didn't predict late-onset hCMV replication
Shabir et al. 2013 (37)	N=38 Kidney TR (19/38 R-/D+) (19/38 R+/D+)	38 Preemptive	CD4+/CD8+ Intracellular IFN-γ staining /MHC-tetramer staining Pp65 peptide pool IE-1 peptide pool	1- 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+. 2- Detection of PreTR T-cell frequencies did not predict as accurately hCMV replication in the R+/D+ cohort.

Table 3. 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 M. et al. 2001.(61)	N=76 Kidney TR 76 R+	Preemptive	CD4+/CD8+ Intracellular IFN-γ staining Viral lysate	1- Low hCMV-specific CD4+ T cells (>0,25%) significantly correlates with CMV Viral load. 2- Asymptomatic KTR with no CMV replication showed median hCMV T cell frequencies similar to healthy controls
Sester U.et al. 2005 (60)	N=96 (68kidney,14heart,14 Lung) 96 R+	Preemptive	CD4+/CD8+ Intracellular IFN-γ staining Viral lysate	1- Lung TR recipients displayed significantly lower frequencies of hCMV specific CD4+ T cells than Kidney TR or healthy controls. 2- Low levels of hCMV specific CD4+ T cells correlate well with hCMV viral load.
Radha et al. 2005 (70)	N=39 kidney TR 33 (24R+/9R-) 6 CMV+Viremia (3R+/3R-)	R-/D+ Prophyaxis R+ Preemptive	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. 2006 (63)	N=38 (20 Heart, 9 Lung, 9 Kidney) 38 R+	Preemptive	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. 2007 (75)	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. 2008 (82)	N=73 Kidney TR 25/73 R-/D+ 48/73 R+	Preemptive	CD4+/CD8+ Intracellular IFN-γ staining viral lysate pp65 peptide mix pp72 peptide mix.	1- Inverse association between hCMV-specific T-cell counts and Viral replication. 2- Pp65 CD4+ cell counts higher than 0.03% showed a positive predictive value of 95%.

Kumar et al. 2009 (75)	N=108 (32 Kidney ,48 Lung,19 Liver,4 Kidney-pancreas,5 Other TR.) 73 R+ 35 R-/D+	Prophhylaxis	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. 2010 (78)	N=85 Kidney TR 70/85 R+ 13/85 R-/D+ 2/85 R-/D-	70 Preemptive 13 Prophylaxis	Elispot IFN-γ Pp65 peptide pool scramble peptide mix.	1- Significantly lower hCMV-specific T-cells frequencies were detected,2months before the increase of hCMV load, in patients experiencing viral replication. 2- Similar hCMV-specific T-cell reconstitution in ATG-treated and not treated patients . 3- Highly heterogenous pattern of immune recovery in R+.was found after transplantaion
Eid et al. 2010 (83)	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. 2010 (62)	N=17 (17 Kidney TR,1 Kidney-pancreas) 17 R+/D+	Preemptive 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.
Gerna et al. 2011 (64)	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. 2012 (69)	N=37 (17 Kidney,4 Liver,3 Lung, 2 Heart,3 Combined) 26 R+ 3R-/D+	Preemptive 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.

Table 4. 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-mo anti-viral prophylaxis
	Identify patients at low risk of hCMV infection	Avoid systematic viral monitoring
All R- transplant recipients	Detect measurable protecting anti-viral T-cell responses	Allow safe preemptive treatment
AFTER Transplantation		
At the end of 3-mo primary prophylaxis	Identify patients at risk of late-onset hCMV infection	Assign a longer prophylaxis course (6-mo)
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 anti-viral treatment