Determinants of Cytomegalovirus Infection Outcomes in Solid Organ Transplant Recipients

by

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ABSTRACT

Cytomegalovirus (CMV) is the most common opportunistic post-transplant infection. Although important advances have been made in the management and prevention of post-transplant CMV, current strategies for monitoring and prevention still show significant limitations. Such gaps create opportunities to understand the determinants of viremia and disease episodes that occur despite the use of antiviral prophylaxis and pre-emptive antiviral therapy. Straddling the boundaries between clinical and basic science, in this PhD thesis I explore the potential utility and limitations of both molecular and immunological biomarker monitoring of Cytomegalovirus in predicting viral replication/disease and assessing therapeutic response. Additionally, I investigate the biological relevance of selected viral and host biomarkers as determinants of viral infection outcomes. This thesis is comprised of a series of 5 studies in which I examine the use of: viral DNA monitoring to assess responses to antiviral therapy during CMV disease (Chapter 2); viral DNA monitoring to predict viral replication/disease post antiviral prophylaxis (Chapter 3); interferon-gamma monitoring in the virus-specific immune response during viral replication (Chapter 4); host chemokine monitoring in the virus-specific immune response during viral replication (Chapter 5); and virus microRNA monitoring to assess the response to antiviral therapy during Cytomegalovirus disease (Chapter 6). Together, these studies highlight novel aspects of the virus-host interaction that contribute to the regulation, or loss thereof, of CMV replication in solid organ transplant recipients.

PREFACE

This thesis is an original work by Luiz Lisboa. The research projects, of which the studies in this thesis are a part, received research ethics approval from the University of Alberta Research Ethics Board as follows: Project "A retrospective chart review of the value of CMV surveillance after antiviral prophylaxis in D+R- solid organ transplant recipients", Study ID Pro00000724, 10/15/2008; Project "Cell mediated immunity and the prediction of cytomegalovirus disease in solid organ transplant recipients", Study ID Pro00002140, 07/04/2009; and Project "Cytomegalovirus (CMV) microRNA expression in vivo and immune evasion correlates", Study ID Pro00002175, 09/06/2009.

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"Remember the tale of 'The Three Billy Goats Gruff'? The transplant patient, like the billy goats, initially is on rocky ground and wants to cross the bridge over the rushing river to greener pastures on the other side. Cytomegalovirus is the troll under the bridge, hidden in shadows and often undetectable even by the most sophisticated diagnostic techniques. As we immunosuppress patients to help them cross the bridge, the troll comes out and threatens to devour them. Like the two smaller billy goats in the story, we clinicians are passing the buck to stall for time, hopeful that in the near future our patients, armed with either a vaccine or an effective antiviral agent, will be strong enough to throw the voracious CMV troll off the bridge and back into obscurity."

Henry H. Balfour, Jr., MD

In "Cytomegalovirus – The Troll of Transplantation"

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CHAPTER 1: Introduction

Transplantation is considered to be the therapeutic modality of choice for irreversible organ failure. Allograft rejection is the first barrier that must be overcome in order to preserve normal function of the transplanted organ; therefore, life-long pharmacologic modulation is fundamental to enabling the recipient's immune system to accept the graft. Achieving a balance between suppressing the immune system enough to ensure organ acceptance, and maintaining the immune system's ability to keep infection in check is often challenging to achieve. Replication of Cytomegalovirus (CMV or human herpesvirus 5), a member of the Herpesviridae family, is facilitated in an immunosuppressive environment. CMV infection normally presents as a mild, self-limited infection in a healthy host; however, the relative lack of immune containment of viral dissemination in transplant recipients allows for a spectrum of clinical manifestations, including life-threatening, tissue-invasive disease. Not surprisingly, CMV became a significant early obstacle to the widespread use of organ transplantation as a life-saving therapy (1-3), and it was soon dubbed "the troll of transplantation" (4). Over the past few decades, meaningful progress has been made in diagnosis, treatment and prevention of CMV in transplantation. However, there is room for improvement in the clinical management of the infection. This thesis addresses gaps in prevention of CMV infection in the solid organ transplantation immune suppression model.

In this chapter, I offer a brief overview of the main developments in the field prior to the start of the present thesis work in 2009. The subsequent chapters complement this Introduction by presenting the findings from additional work since then. The studies that comprise this thesis had been published in manuscript form (Chapters 2 - 4) or submitted for publication (Chapters 5 - 6) as of the Summer of 2014, when this thesis was finalized. For the purposes of standardatization, the definitions of CMV disease utilized in this thesis are in line with those recommended by the American Society of Transplantation for use in clinical trials ({Humar, 2006 #125}), which are reproduced in Appendices – Table S1-1.

In the early days of transplantation, CMV infection was most commonly diagnosed post-mortem, when giant cells containing inclusion bodies were observed under conventional tissue microscopy (1). The isolation (5) and identification (6) of CMV years earlier, in the context of complications caused by its congenital transmission, had led to the development of reference

strains. These allowed for the development of the first serologic test for CMV – the complement fixation test. The clinical syndromes in transplantation associated with complement-fixing antibodies against CMV antigens were then characterized (7-12). Retrospective serologic testing performed on donors and recipients generated invaluable insight into CMV transmission and reactivation, delineating a CMV-naïve population (13) in whom transplanted organs likely were the source of infection (14-18). CMV antibody screening soon expanded (19) to guide allocation of seronegative organs to seronegative recipients, which was the only available strategy to prevent primary CMV infection in transplantation at the time (19-22). Although they no longer serve that purpose, pre-transplant donor and recipient CMV serostatuses – typically expressed as CMV D+/-/R+/- in the literature – still largely determine CMV disease risk stratification and the preventative strategies to be adopted post-transplant. Observations of the presence of CMV in leukocytes and the development of a monoclonal antibody (23) led to the development of the CMV antigenemia test.(24-31). This was the first assay used for large scale monitoring of transplant recipients, and soon antigenemia replaced viral culture and serial CMV IgM in clinical monitoring (32, 33). Later, polymerase chain reaction (PCR) methods, which are robust and more sensitive than preceding techniques, replaced antigenemia tests and began to be used to detect circulating DNA of viral origin (34-37). PCR techniques are now fundamental in the screening (34) and therapeutic monitoring of CMV infection in transplant patients (38).

The management of CMV infection was initially restricted to reduction of therapeutic immunosuppression. This strategy, however, exposed patients to risks of allograft loss through immune-mediated rejection, and alternatives were desperately sought. Treatment options for CMV were tested through trial and error; initially, immunomodulation using interferon and antiviral therapy with idoxuridine and vidarabine were attempted. Acyclovir, a highly effective drug against herpes simplex and varicella zoster viruses, was shown not to be efficacious for treatment of CMV disease, especially among those who were the most immunosuppressed (39). A modification of its acyclic side chain led to the development of ganciclovir, a drug with much lower inhibitory concentration against CMV and significantly improved pharmacokinetics (40-45). Ganciclovir has since been substituted by its oral prodrug, valganciclovir, as the preferred drug for treatment of CMV infection, with bioavailability that is 10-times higher than oral ganciclovir (46).

Initial knowledge accumulated from the first generation of PCR assays and antigenemia-based monitoring of ganciclovir therapy established the basis of modern management of CMV disease. Episodic clinical and/or virological recurrence following discontinuation of antiviral drug therapy led to the current practice of treating the infection until CMV is no longer detectable in the blood (47-50). In Chapter 2, this treat-until-negative paradigm is re-examined for its validity in light of widespread use of newer in-house real-time PCR technologies with increased CMV detection sensitivity (51).

New preventative strategies emerged as monitoring tests and antiviral drugs became more widely available. Antiviral prophylaxis was shown to be efficacious in reducing the risk for CMV disease and associated mortality (52), and valganciclovir became the preferred drug (53, 54). However, when prophylaxis was discontinued, CMV replication and disease recurred (55, 56), along with increased mortality (57). Therefore, for some high risk groups, prolongation of prophylaxis is justified (58-60), despite concerns about the toxic effects of ganciclovir on leukocyte populations (61-64), which limit its long-term use. Some experts use pre-emptive CMV therapy as an alternative to prophylaxis. The goal of pre-emptive therapy is to detect infection early in order to prevent the development of symptomatic infection (65). However, for this strategy to be effective, patients must be monitored regularly at short intervals utilizing adequate cut-offs to trigger the start of antiviral therapy (66, 67). Relative to prophylaxis, pre-emptive therapy may not offer the additional benefits of reducing the indirect effects of CMV, including bacterial and fungal sepsis and death (68). Our center adopts a hybrid approach involving prophylaxis followed by pre-emptive therapy to prevent CMV disease in D+/R- patients. In Chapter 3, a critical appraisal of the efficacy of this hybrid strategy is offered, determining limits and shortcomings of its use with high-risk patients.

Inspired by progress in the clinical management of CMV infection, researchers in the basic sciences have investigated the complex host-virus interactions in this immunosuppression model. It is now evident that the resolution of CMV primary infection and protection from recurrent disease is associated with the development of CMV-specific T-cell responses (69-71). Conversely, the impairment or absence of CMV-specific T-cell response facilitates viral replication (72-74). These virus-specific responses are crucial to the successful clearance of CMV viremia, which is treated with antiviral drugs in transplant recipients (75). CMV cell-

mediated immunity testing has been increasingly studied as an adjunct tool for CMV disease risk assessment (76). This has been greatly facilitated by the standardization and commercialization of a simple CMV cell-mediated immunity assay based on CD8+ T-cell, HLA-restricted CMV peptide stimulation, which triggers interferon-gamma production (77). Through the use of this and other cell-based non-standardized tests, it is now clear that donor and recipient CMV serostatuses alone are insufficient to assess an individual's risk for development of CMV disease (78). In chapter 4, the utility of using CMV cell-mediated immunity testing to predict progression of individual episodes of viral replication to CMV disease is explored.

The interferon-gamma produced upon CMV-specific CD8+ T-cell stimulation seems to be a practical marker for clinical use in prevention of the CMV burden in transplantation. However, this may be an oversimplification of the complexity of cellular immune responses leading to the effective control (or not) of viral replication. For example, CMV-specific T-helper cells are required for the maintenance of an antiviral suppressive state induced by the frontline action of their CD8+ T-cell counterparts (79-81). Additionally, these and other leukocyte populations involved in the CMV immune response, such as NK cells and monocytes, are functionally affected by therapeutic immunosuppression (82, 83), immune exhaustion (84), and infection itself (85). The infection both induces and benefits from a dysregulated cytokine response, shifting towards monocyte/macrophage and Th2 activation (86-89), with chemokine expression also affected (90-93). Immune genetic variability in the donor (94) and/or recipient (95, 96) further contributes towards the complex and individualized risk of CMV replication. In Chapter 5, the findings of Chapter 4 are extended with the characterization of the broader cytokine and chemokine blood responses to CMV-specific CD8+ T-cell stimulation at the onset of viral replication episodes, offering insights about their regulation by modifiable or predictable factors.

One aspect of CMV biology that remains underexplored in the transplantation context is the control of host gene expression by virus-encoded microRNAs. Shortly after the discovery of these short non-coding RNA species (97), herpesvirus microRNAs, including those from cytomegalovirus (98), began to be identified. CMV expresses microRNAs during productive infection (99, 100), and human transcript targets have been identified for only a few (101). Their expression is dependent on early kinetics viral protein synthesis (100) and has been documented to occur during lytic infection in connective, vascular, epithelial and nervous cell types (99). So

far, putative roles of microRNAs in immune evasion and in regulation of viral replication have been identified (102-105). Lymphocytes and monocytes harbor CMV (106-108); under allostimulatory conditions (109), they support expression of immediate-early transcripts (110, 111). This makes it plausible that CMV miRNAs may be expressed in the blood of transplant recipients and play important roles in immune suppression and viral dissemination. In Chapter 6, the in vivo blood expression profile of CMV miRNAs in transplant recipients with CMV disease and their correlation with virologic and clinical outcomes are characterized, and host targets of a clinically relevant viral miRNA are investigated.

In summary, this thesis is comprised of research studies that explore some current gaps in the prevention of CMV disease among transplant recipients, as well as underexplored aspects of the virus biology and its interaction with the human host. The findings presented here are contextualized and interpreted through the lens of clinical practice in order to highlight their relevance to the refinement of preventative strategies, and to encourage their acceptance and further development by the broader research community.

CHAPTER 2: The Clinical Utility of Whole Blood Versus Plasma Cytomegalovirus Viral Load Assays for Monitoring Therapeutic Response

INTRODUCTION

Therapy of cytomegalovirus (CMV) viremia or active CMV disease with ganciclovir is usually successful. However, a commonly encountered clinical question revolves around the duration of antiviral therapy so as to avoid recurrence after treatment is stopped. Based on the current guidelines for the management of CMV viremia in solid-organ transplant recipients, the standard approach is to perform weekly molecular monitoring (with a quantitative CMV viral load assay using nucleic acid testing or pp65 antigenemia assay) and continue antiviral therapy until viremia is no longer detectable (112, 113). With pp65 antigenemia or with nucleic acid testing of plasma samples, this usually results in treatment duration of between 2 and 4 weeks. The rationale for this "treat until negative" paradigm is based on high rates of recurrent viremia or recurrent clinical disease observed when the antiviral treatment is discontinued before undetectable CMV levels are achieved (71, 114, 115).

Rapid advances in molecular techniques, specifically the use of real-time polymerase chain reaction (PCR)-based assays, have resulted in the development of progressively more sensitive assays, thereby lowering the achievable detection threshold. It has been shown that more sensitive assays allow for earlier diagnosis of CMV viremia, potentially improving the efficacy of preemptive treatment strategies. Improved sensitivity of assays can also be achieved by testing different peripheral blood fractions. For example, several studies have demonstrated improved sensitivities for measurements made in whole blood versus plasma (116-118). Some recommendations have suggested that based on this improved sensitivity, whole blood detection of CMV is preferred over plasma (119-121). Similar recommendations have been made for other groups of immunocompromised hosts, such as stem-cell transplant recipients (117).

However, the rapid evolution of highly sensitive viral load assays and their common adoption in clinical practice has not been accompanied by concomitant clinical validation studies evaluating the utility of such assays. When monitoring response to CMV disease therapy, if a higher-sensitivity viral load assay is used in conjunction with a "treat-to-negative" paradigm, one might expect a significantly longer duration of therapy. Theoretically, this may translate into the added

clinical benefit of decreased clinical and virologic recurrence rates. The comparison of molecular assays for monitoring response to therapy has been difficult to perform, because in clinical practice, treatment regimens commonly vary from patient to patient even in a given center.

The purpose of this study was to determine whether more sensitive detection of residual viremia would be clinically useful for monitoring therapeutic response in patients with CMV disease. This was done by comparing two different quantitative nucleic acid assays in two different peripheral blood sample types used to monitor therapeutic response in a large prospective cohort of patients with CMV disease treated with a standard duration of antiviral therapy. Early viral kinetics and end-of-treatment viral clearance were assessed to test the hypothesis that higher sensitivity detection methods would allow better prediction of recurrence of CMV infection and disease posttreatment.

MATERIALS AND METHODS

Patient Population and Definitions – Solid-organ transplant recipients with clinical and virologic evidence of symptomatic CMV disease enrolled in a multicenter, randomized, open-label, and comparative antiviral therapy trial (VICTOR Study) were included (122). Treatment doses of IV ganciclovir (5 mg/kg IV twice daily) or oral valganciclovir (900 mg orally twice daily) were given for 21 days, followed by valganciclovir maintenance dose (900 mg orally once daily) up to day 49, having doses being adjusted for renal function. The detailed demographic and clinical characteristics of this patient cohort have been described previously (122). No significant differences in the demographic or clinical baseline characteristics and no differences in success rates at the end of treatment (day 21) or in long-term recurrence rates were observed between the treatment arms (123); therefore, for this study, both groups were studied together as a single cohort. Only the per-protocol-population was analyzed in this study (patients with detectable baseline CMV viremia more than 600 copies/mL plasma evaluated at the central laboratory).

Therapeutic Response Monitoring – All viral load assays were performed centrally, and results were not available to treating physicians. Plasma viral load was performed using a commercial plasma quantitative PCR assay (Amplicor CMV Monitor Test [Roche Molecular Diagnostics, CA]) at days 0, 3, 7, 10, 14, 17, 21, 35, 42, and 49, at months 3 and 6 follow-up visits, and on

clinical suspicion of CMV disease at any time until month 12. This assay allows for simultaneous quantitation of a 362-bp sequence within the CMV polymerase gene contained in the specimen and of a quantitation standard plasmid of the same length and a similar G:C content added in known copy numbers during sample preparation. DNA extracted from 200 µL of plasma with an automated MagnaPure System (Roche Molecular Diagnostics, CA) was eluted in 100 µL of elution buffer. An aliquot of 50 µL of eluate was used for PCR amplification in a COBAS Amplicor Analyzer (Roche Diagnostics). The limit of detection for this assay is approximately 200 copies/mL, and the limit of accurate quantitation is approximately 600 copies/mL. In addition to the plasma assay, an in-house developed real-time PCR assay was performed on whole blood samples on days 0, 3, 7, 14, and 21. A 72- to 79-bp fragment of *glycoprotein B* gene is amplified with TaqMan-MGB probes specific to each of its four genotypes. Primers, probes, reaction setup, and thermal cycler conditions were previously described in detail (124). DNA was manually extracted from 200 µL of whole blood using Qiagen DNA mini kit (Qiagen, MD) and eluted in 60 µL of nuclease-free water, 5 µL of which were added to 20 µL of master mix (25 µL reactions) for a total 45 PCR cycles. The lower limit of detection obtained for this assay is approximately 50 copies/mL, and the limit of accurate quantitation is approximately 500 copies/mL (124). Given the limited blood volume of the samples in our repository, plasma extraction for analysis with the real-time PCR assay or viral load quantitation of whole blood with Amplicor CMV Monitor Test was not possible.

Definitions – Viral eradication by day 21 was defined as viral loads below the limit of detection of each assay (<200 copies/mL plasma and <50 copies/mL whole blood). Virologic recurrence was monitored using only the plasma assay with the same thresholds used for the clinical trial (122), defined as a positive viral load within 6 months of follow-up, in patients who had initially cleared plasma viremia by day 49 (<600 copies/mL). A recurrence of symptomatic CMV disease was defined based on standard diagnostic criteria compatible with the American Society of Transplantation guidelines for use in clinical trials (125). Individual viral clearance kinetics were graphed for each patient using viral load data obtained from both assays. Early phase kinetics including logarithmic viral loads declines by days 3 and 7 and viral load half-lives were calculated for both plasma and whole blood using methods described previously (114). In short, virus load kinetics are modeled using a logarithmic decay curve, expressed by the equation

$y=y_0e^{-ax}$, where "y_0" is the initial virus load, "x" is time from start of treatment, and "a" is the decay constant. Virus load half-life is then calculated using the equation (ln2)/a.

Statistical Analysis – Categorical variables were compared using chi-square or Fisher's exact test. Continuous variables were compared using the Mann-Whitney *U* test or Wilcoxon test for paired data. Spearman's correlation coefficient was used to compare viral load data between the two assays. Binary logistic regression was used for assessment of persistent viremia time points as predictors for virologic recurrence. All statistical analysis was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL).

RESULTS

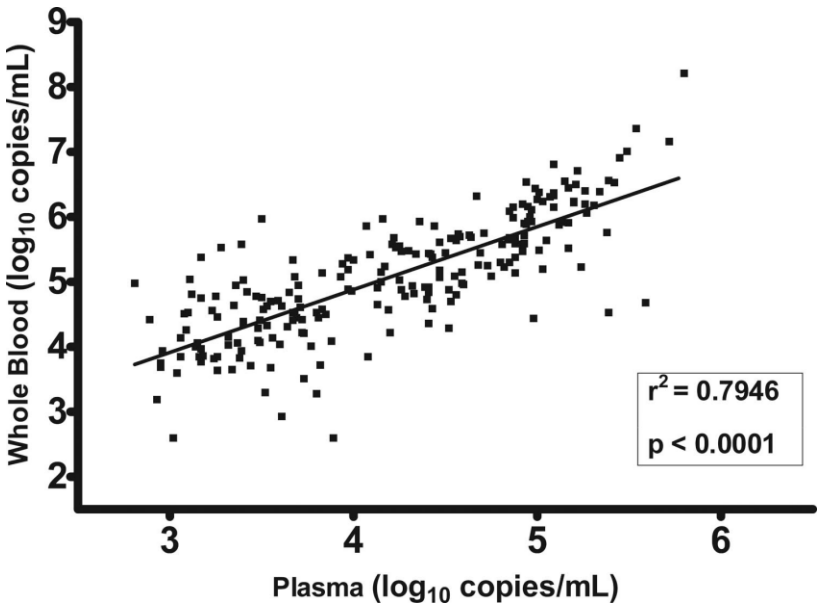
Patient Population and Outcomes – A total of 259 patients with symptomatic CMV disease and day 0 plasma viral loads more than or equal to 600 copies/mL were analyzed (per-protocol-population). Demographic and baseline clinical and immunosuppression characteristics were published previously (122, 126). Briefly, transplant types included kidney (72%), liver (7.3%), heart (6.6%), lung (6.2%), and other (7.7%). Pretransplant CMV donor/recipient serostatus was available in 71.4% of the patients and included D+/R- (n=51), D+/R+ (n=101), D-/R+ (n=18), and D-/R- (n=14). In 219 patients (84.6%), both plasma and whole blood viral load data were available on the following time points: day 0, 3, 7, and 21. This group was used as the final analysis group.

Virologic Parameters Whole Blood Versus Plasma – A comparison of virologic parameters obtained with standard plasma assay versus the real-time whole blood assay is shown in Table 2-1. The initial (day 0) median plasma viral load (17,950 copies/mL, range 645–635,000 copies/mL) was lower than the whole blood viral load (118,950 copies/mL, range 400–160,000,000 copies/mL; P<0.001). Overall, there was a good correlation between plasma and whole blood viral loads (Spearman's r2=0.79 P<0.001; Fig. 2-1). However, the absolute value for the two viral loads was different. In most patients, whole blood viral loads were approximately 1-log higher in whole blood versus plasma. However, in some patients, this difference was as great as 2-logs. Occasionally, whole blood viral loads were less than plasma loads (Fig. 2-1). The rate of logarithmic decay of viral loads between days 0 and 3 (median -1.19 vs. -0.28 log10) and days 0 and 7 (median -1.3 vs. -0.37 log10) were greater for the whole blood

Table 2-1: Comparison of viral load kinetics between whole blood real-time PCR and Plasma PCR. Viral load data on 219 patients with CMV disease treated with standard antiviral therapy are presented as median (range) and N (%). PCR, polymerase chain reaction; CMV, cytomegalovirus.

Characteristic	Whole blood	Plasma	P
Day 0 viral load (copies/mL)	118,950 (400 – 160,000,000)	17,950 (645 – 635,000)	<0.001
Log change in viral load (d 0-3)	-1.19 (-4.08 to +0.86)	-0.28 (-1.29 to +0.56)	<0.001
Log change in viral load (d 0-7)	-1.30 (-4.28 to +0.19)	-0.37 (-1.88 to +0.49)	<0.001
Undetectable by end of treatment (d 21)	65/219 (29.7%)	114/219 (52.1%)	<0.001

Figure 2-1: Day 0 viral loads in whole blood versus plasma. All viral loads are in $\log_{10}$ copies/ml.



assay when compared with the plasma assay, respectively (P<0.001). Consequently, the early median half-life of whole blood viral load was shorter than the paired plasma ones (1.7 vs. 4.72 days, respectively, P<0.001). The whole blood assay showed greater sensitivity for detection of residual viremia by the end of treatment. Undetectable whole blood viral loads (<50 copies/mL) were achieved by 29.7% (65/219) in the end of the therapeutic dose antivirals (day 21), whereas 52.1% (114/219) obtained clearance of viremia (<200 copies/mL) when measured by the plasma assay (P<0.001).

Virologic Recurrence – In the 219 patients, 180 (82.2%) achieved initial complete clearance of viremia by day 49 (plasma), and this was the subpopulation in whom virologic recurrence was assessed. Virologic recurrence rate for those who achieved undetectable plasma viremia by day 49 was 58 of 180 (32.2%). The presence of plasma viremia, but not whole blood viremia at day 21 or plasma viremia at days 28, 35, 42, or 49, was the only predictor of virologic recurrence (P=0.01). In patients with persistent plasma viremia at day 21 (n=86), the incidence of recurrence was 41.9% (Table 2-2). In patients with a negative plasma viremia at day 21, the incidence of recurrence was 23.4% (P=0.01). This translates into a positive predictive value (PPV) of 41.9% and a negative predictive value (NPV) of 76.6%. We next did an analysis to see whether the whole blood PCR was a better predictor of recurrence. Undetectable whole blood viremia by day 21 was much less likely with this assay. Persistent whole blood viremia at day 21 was not a significant predictor of recurrence (P=0.12). In patients with persistent whole blood viremia at day 21, the incidence of recurrence was 36.3%. In patients with negative whole blood viremia at day 21, the incidence of recurrence was 23.2% (P=0.12). This translates into a PPV of 36.3% and a NPV of 76.8%.

There were 49 patients who were plasma negative but whole blood positive at the end of treatment (day 21). The median whole blood viral load in these patients on day 21 was 327 copies/mL (range 50 – 4723 copies/mL). The incidence of virologic recurrence by 6 months was 23.1%, and the incidence of disease recurrence was 10.6%. This was similar to the rate of recurrence when both plasma and whole blood were negative at day 21 (virologic recurrence 23.6%; *P*=not significant).

Kinetic Analysis – Next, a more in-depth analysis of plasma versus whole blood kinetic parameters was performed to see whether early phase kinetics had improved predictive value for

Table 2-2: Viral kinetics compared with virologic recurrence. Data are presented as median (range) and N (%). Analysis restricted to subset of patients who cleared plasma viremia by d 49 (n=180) and who underwent follow-up viral load testing beyond d 49. Recurrence then assessed out to 6 months postenrolment.

Characteristic	Virologic recurrence		P
	Yes (n=58)	No (n=122)	
Whole blood day 0 viral load (copies/ml)	304,950 (850 – 8,124,500)	98,475 (400 – 22,706,350)	0.006
Plasma day 0 viral load (copies/ml)	32,825 (785-279,000)	14,700 (645-525,000)	0.031
Whole blood log- change (d 3)	-1.17 (-4.08 to +0.86)	-1.39 (-4.04 to +0.12)	0.12
Whole blood log- change (d 7)	-1.27 (-4.28 to +0.19)	-1.47 (-4.04 to +0.27)	0.015
Plasma log-change (d 3)	-0.29 (-1.26 to +0.51)	-0.24 (-1.29 to +0.36)	0.16
Plasma log-change (d 7)	-0.40 (-1.88 to +0.49)	-0.36 (-1.06 to +0.23)	0.73
Persistent whole blood viremia (d 21; n=128)	45 (36.3)	79 (64.8)	0.12
Persistent plasma viremia (d 21; n=86)	36 (41.9)	50 (58.1)	0.013

recurrence. This comparison is shown in Table 2-2. Viral loads at day 0 were statistically associated with virologic recurrence within 6 months. Early phase declines in viral load were different between whole blood compared with plasma. Only modest associations were observed for early phase declines in viral load and recurrence of CMV viremia (only statistically significant for the log-change in whole blood viral load by day 7). Both the treatment arms (ganciclovir and valganciclovir) showed similar viral clearance kinetics, as published previously (122).

Clinical Recurrence – A clinical picture compatible with CMV disease (viral syndrome or tissue-invasive disease) was verified during the day 49 to month 6 follow-up in 25 of 203 (12.3%) patients in whom adequate follow-up regarding disease recurrence was available (Table 2-3). The incidence of CMV disease recurrence in patients with positive plasma viremia at day 21 was 16 of 93 (17.2%). In patients with a negative plasma viremia at day 21, the incidence of disease recurrence was 9 of 110 (8.2%; P=0.08; PPV 17.2%; NPV 91.8%). The incidence of CMV disease recurrence at day 21 in positive whole blood patients was 21 of 139 (15.1%) compared with 4 of 64 (6.3%) in patients who had negative whole blood at day 21; (P=0.12; PPV 15.1%, NPV 93.8%). Four patients with undetectable viremia by both assays at day 21 presented with clinical recurrence during the follow-up. Three of them had CMV diagnosed within the first 3 months and one between months 3 and 6 postenrollment. All had concurrent virologic recurrence that could be confirmed by samples sent to the central laboratory (2/4) or at their center.

DISCUSSION

We report the first large comparative study of different viral load assays and blood compartments to use in monitoring therapeutic response to CMV. This is an important clinical question because current guidelines for management of CMV infection and disease suggest that the ultimate duration of antiviral therapy should be guided by viral load response (112, 113). Generally, it is recommended to continue treatment doses of antivirals until viral load is no longer detectable. The purpose of this "treat-to-negative" paradigm is to prevent CMV disease recurrence. This is based on several published studies that have demonstrated a high rate of recurrence in patients who are persistently viremic at the end of therapy. For example, in the long-term assessment of the dataset used here (VICTOR study), using a multivariable logistic regression analysis

Table 2-3: End of treatment viremia with whole blood and plasma and virologic and disease recurrence rates. Analysis restricted to subset of patients who cleared plasma viremia by d 49 (n=180) and who underwent follow-up viral load testing beyond d 49. Recurrence then assessed out to 6 months postenrolment.

End of Treatment (day 21) result	Virologic recurrence, N (%)	Disease recurrence, N (%)
Plasma negative / whole blood negative	13/55 (23.6)	4/63 (6.3)
Plasma positive / whole blood positive	36/85 (42.4)	16/92 (17.4)
Plasma negative / whole blood positive	9/39 (23.1)	5/47 (10.6)
Plasma positive / whole blood negative	0/1 (0)	0/1 (0)

including recipient CMV IgG serostatus at the time of the transplant, the only independent predictor for recurrence was failure to eradicate viremia by the end of treatment (day 21) for both clinical disease recurrence (odds ratio 3.9; P=0.012) and virologic recurrence (odds ratio 5.6; P<0.0001) (123). This has also been shown in several smaller studies as well (50, 71, 127-129). However, the rate of viral clearance at a specific time point depends, to a large part, on the sensitivity of the assay and the compartment in which viral load is measured.

We compared two different assays. The first was a plasma-based commercially available PCR assay. The second assay was a real-time PCR assay performed using whole blood samples. The latter was able to detect residual viremia in 49 extra patients, when compared with the plasma assay, for several reasons. These included the measurement of not just free viral particles, but also DNA from intracellular virus. Also, the real-time PCR technique itself allows for an overall higher sensitivity. Finally, by targeting much shorter nucleotide sequences of all four variants of glycoprotein B, specific detection is likely achieved even in fragmented DNA.

Our results suggest that when monitoring therapeutic response, the greater detection of viremia at the end of treatment may not be advantageous. We show that persistent plasma viremia but not whole blood viremia had greater statistical association with virologic recurrence, yet similar predictive values for both virologic and clinical recurrences. If one used the whole blood real-time PCR assay in this cohort to guide duration of therapy, the length of therapy would be increased for a substantial portion of patients (49 patients were plasma negative and whole blood positive at the end of day 21) to reach undetectable whole blood viral loads. This may result in increased toxicity and cost. The benefit of this would be uncertain because the plasma negative/whole blood-positive subgroup was not identified as high-recurrence risk subgroup. Other interesting observations were the different kinetics of viral clearance in whole blood versus plasma assays. The whole blood half-life was significantly shorter reflecting a more rapid initial clearance of virus. When measuring plasma viral loads, many patients had an initial plateau-phase before viral decline. This may represent free CMV DNA released from cells or tissue. Overall, early measurement of whole blood kinetics did offer some advantage in predicting recurrence, but the effect was modest (only log-decline in whole blood viral load by day 7 was significant predictor).

There are limited data in the literature assessing the utility of different assays when applied to therapeutic response monitoring (as opposed to prediction of CMV posttransplant). However, studies comparing the use of whole blood versus plasma have generally shown improved sensitivity of whole blood for the detection of CMV (116-118). In a prospective study of 82 kidney transplant patients, Garrigue et al. compared serial CMV testing of plasma versus whole blood using a real-time PCR assay. The two assay results were concordant, but whole blood assay showed greater sensitivity, such that 14.1% of samples were whole blood positive and plasma negative, and DNA was detected more frequently in whole blood (66.9%) than in plasma (53.4%) (116). In a study of 32 liver transplant patients, real-time PCR of peripheral blood leukocytes was compared with COBAS Amplicor PCR. The former was found to have greater sensitivity for detection of CMV (130). Indeed, more sensitive assays may be preferable for use in preemptive strategies, although it is also possible that overly sensitive assays may result in excess treatment if cutoffs for discontinuation of the antiviral are not used. Another important point to consider is the significant viral load variation between assays. There is currently a lack of an internationally accepted calibrator to allow a precise inter assay comparison. In a multicenter evaluation involving several North American testing laboratories, up to a 3-log variation in viral load results was verified for the same samples, not just as a consequence of the use of different assays but also as a result of the availability of different quantitation standards (131).

Our study experienced several limitations. First, this was a post hoc analysis of a clinical trial whose primary endpoint was to compare oral versus intravenous (IV) antiviral therapy. Also, the results of this analysis do not assess what the outcomes would have been if indeed treatment was carried out until viral load quantified by the different assays was negative because all patients received 21 days of treatment followed by 4 weeks of prophylaxis. This would likely have resulted in treatments sometimes shorter than 21 days, particularly in patients with viral syndrome without visceral involvement. We are unaware of studies assessing the impact of the use of secondary antiviral prophylaxis on CMV recurrence rates, and it is unknown to date what groups of patients may benefit from this strategy, even though its widespread use has been confirmed by a recent survey (54). Also, we have compared two different variables when using these assays, that is, the use of a different assay (including different DNA extraction methods) and the use of a different compartment (plasma vs. whole blood). The relative contribution of

each of these variables in the observed differences for the detection of residual viremia and on viral kinetics is unknown. A higher rate of virologic recurrence may have occurred if long-term monitoring used the whole blood assay; nonetheless, the appropriateness of its use in this setting is unknown. In addition, no conclusions about the appropriateness of the use of real-time whole blood or plasma CMV PCR assays on a preemptive therapy strategy can be drawn from this study. The strengths of this study include the large sample size, the prospective data collection, and the blinded evaluation of whole blood viral loads. In addition, all patients received uniform treatment duration and were followed up closely for both virologic and clinical recurrence of CMV.

In conclusion, we compare two different combinations of assays and blood compartments for assessment of response to ganciclovir-based anti-CMV treatment. The greater sensitivity for detection of residual viremia by the end of treatment does not seem to offer clear advantages in predicting recurrence after a standard 21 days of antiviral therapy. Further evaluation of different assays and blood compartments along with dynamic changes in treatment length and outcome analysis would be helpful in clarifying the optimal management strategy in patients with CMV disease. Viremia levels for termination of the antiviral therapy should be tailored for different therapeutic virologic monitoring tests to obtain the lowest recurrence rates possible. This may or may not translate into treating until negative, depending on the sensitivity of the therapeutic monitoring assay used. Tests with reasonable sensitivity need not necessarily be replaced by highly sensitive assays when monitoring therapeutic response.

CHAPTER 3: Clinical Utility of Molecular Surveillance for Cytomegalovirus After Antiviral Prophylaxis in High-Risk Solid Organ Transplant Recipients

INTRODUCTION

Currently, there are two common strategies used for cytomegalovirus (CMV) prevention in organ transplant recipients: prophylaxis and preemptive therapy. In a prophylaxis strategy, antivirals are commenced early posttransplant and continued for a defined period of time such as 3 months. In a preemptive strategy, patients are monitored at regular intervals (e.g., weekly for the first 12 weeks) using a molecular diagnostic assay. In patients with early evidence of viral replication, treatment is initiated to prevent the development of CMV disease. Both strategies have their advantages and disadvantages. However, in the highest risk group for CMV (donor seropositive and recipient seronegative [D+/R-]), current guidelines suggest that prophylaxis may have advantages over a preemptive strategy (112, 113).

One of the main limitations with a standard 3-month course of prophylaxis is the occurrence of late-onset CMV disease, defined as CMV disease occurring beyond 3 months posttransplant often shortly after the discontinuation of antiviral prophylaxis. The reported incidence of late-onset disease is variable but ranges from 17% to 37% in D+/R- patients receiving 3 months of prophylaxis (112). In this group, disease usually develops between 3 and 6 months posttransplant. Several strategies have been proposed to prevent late onset CMV disease. These include prolongation of antiviral prophylaxis or use of serial molecular surveillance after the discontinuation of prophylaxis. The latter strategy involves use of regular viral load or antigenemia monitoring after discontinuing antiviral prophylaxis. Patients with viral replication above a certain threshold are then treated preemptively.

This latter strategy is also sometimes referred to as a hybrid prophylaxis/preemptive therapy approach. Its purpose is specifically to provide the potential benefits of prophylaxis and yet prevent the problem of late-onset CMV disease commonly observed with prophylaxis. Clear guidelines do not exist with respect to exactly how such a strategy should be carried out (target patient population, type of test, threshold, period of surveillance, or intensity of surveillance). In addition, there are limited data on the efficacy of this approach in D+/R- patients. Our center has been using this hybrid prevention in high-risk patients. We performed once weekly viral load

testing for 8 weeks after the completion of prophylaxis. Protocol-defined preemptive therapy is then initiated based on predefined thresholds of viremia. The purpose of this study was to evaluate the efficacy of this hybrid approach for the prevention of late onset CMV disease.

MATERIALS AND METHODS

Patient Population – We conducted a retrospective chart review of all CMV D+/R- solid organ transplant recipients at our institution. Patients were identified through a solid organ transplant registry maintained by a study investigator and viral load data from the Provincial Laboratory for Public Health's database. We reviewed transplants occurring at the University of Alberta Hospital over a 4-year period. This represented a time period when a uniform prophylaxis protocol was used in conjunction with a nucleic acid testing for CMV viral load monitoring approach. We included all organ recipients who fulfilled the following criteria: (i) aged older than 18 months; (ii) CMV-seronegative recipient of organ(s) from CMV-seropositive donors; (iii) received antiviral prophylaxis; and (iv) monitored with at least one viral load measurement during the expected surveillance postprophylaxis period. The study was approved by the institutional ethics board.

Prevention Protocol – The institutional protocol was to administer universal antiviral prophylaxis to CMV D+/R- patients before the seventh day posttransplant and until 3 months posttransplant for kidney, liver, heart, and pancreas recipients (including combined organ). For D+/R- lung transplant, combined heart and lung transplant, and small bowel recipients, 6 months of antiviral prophylaxis was administered. Prophylaxis consisted of valganciclovir 900 mg/day or intravenous ganciclovir 5 mg/kg once daily followed by oral ganciclovir 3 g/day (during 2004) or valganciclovir 900 mg daily (2005 onward) upon recovery of oral intake with all doses adjusted for renal function. No molecular surveillance was performed during prophylaxis.

After prophylaxis was discontinued, weekly surveillance during the next 8 weeks was performed using a quantitative real-time plasma-based polymerase chain reaction assay. This time period was chosen based on data from a study comparing oral ganciclovir versus valganciclovir prophylaxis in 364 CMV D+/R- patients (132). In this study, after prophylaxis, a significant proportion of viremia occurred in the first 8 weeks. The lower limit of accurate quantitation was 500 copies/mL (133). Results were available to clinicians within 24 hr of sample receipt in the

laboratory. The threshold for initiation of preemptive antiviral therapy was set at 25,000 copies/mL of plasma. This was based on the quantitative relationship between our center's in-house developed assay results and a commercially available quantitative polymerase chain reaction assay used in a natural history study of CMV viral loads that best predicted CMV disease in liver transplant patients seropositive for CMV before transplant (134). In asymptomatic patients with lower levels of viral of replication, viral load was repeated once weekly. If the viral load was between 10,000 and 25,000 copies/mL, it was repeated every 3 days. In patients with greater than 25,000 copies/mL antiviral therapy consisted of either intravenous ganciclovir 10 mg/kg/day or oral valganciclovir 900 mg twice daily, adjusted for renal function, for at least 14 days or greater until viremia was undetectable. Antiviral therapy was also instituted to symptomatic patients regardless of their viral load. There was no standard approach to reduction of immunosuppression, and this was at the discretion of the treating physician. Generally, for patients on triple immunosuppression, the mycophenolate mofetil dose was reduced by 25% to 50%. Further surveillance was also performed at any time posttransplant if lymphocyte-depleting antibodies were used for treatment of rejection.

Outcomes and Analysis – Definitions of CMV disease were comparable with those in the American Society of Transplantation Infectious Diseases Guidelines (112) and American Society of Transplantation Recommendations for Screening, Monitoring and Reporting of Infectious Complications in Immunosuppression Trials in Recipients of Organ Transplantation (125). Patients were followed for 1 year from the time of transplant. Allograft rejection was defined as biopsy-proven and treated, except in lung recipients, in whom rejection could be clinically diagnosed. Descriptive statistics were used for demographics and viral load calculations. Mann-Whitney *U* test was used for comparison of continuous variables and Fisher's exact test for categorical variables. Viral kinetics analysis (doubling time) for selected cases was performed as previously described (114). Briefly, doubling time calculations require at least three viral load measurements and are performed using a mathematical model to calculate the exponential increase of the viral load before the start of antiviral therapy. In short, virus load kinetics are modeled using a logarithmic decay curve, expressed by the equation $y=y_0e^{ax}$, where "y_0" is the initial virus load, "x" is time from start of treatment, and "a" is the increase constant. Virus load doubling-time is then calculated using the equation (ln2)/a.

RESULTS

Demographics – A total of 83 CMV D+/R- patients were identified; however, 12 patients were excluded from analysis (no antiviral prophylaxis [n=2] and no postprophylaxis molecular monitoring [n=10]). The demographic characteristics and immune suppression regimens of the patient population are listed in Table 3-1. Median age at the time of transplant was 51 years (range 3–72 years). Antiviral prophylaxis lasted a median of 98 days (interquartile range 96–117 days). It consisted of intravenous ganciclovir for 1 to 2 weeks followed by oral ganciclovir (during 2004) or valganciclovir (from 2005 onward) using standard doses adjusted for renal function.

CMV Viremia and Disease – CMV viremia (>500 copies/mL) occurred in 37 of 71 (52.1%) patients during the first-year posttransplant. Of the viremic patients, 19 of 37 patients had viremia first detected during the postprophylaxis surveillance period, and 18 of 37 patients had their first detectable viremia only after surveillance (measurement performed for assessment of symptoms). Symptomatic CMV disease occurred in 29 of 71 (40.8%) patients during the first-year posttransplant. CMV disease occurred during the surveillance period in 13 of 29 (44.8%; 2 tissue-invasive and 11 viral syndrome) patients and after the surveillance period in 16 of 29 (55.2%; 1 tissue invasive and 15 viral syndrome) patients. One patient with tissue-invasive CMV disease was diagnosed at autopsy 97 days after completion of the postprophylaxis surveillance period and had no viral load investigation during the hospital admission. Median time to CMV disease was 214 days (range 93–356 days) posttransplant. All episodes of disease (except one diagnosed at autopsy) were treated with ganciclovir or valganciclovir.

Clinical Utility of Surveillance – Approximately half of the viremia episodes during the first year posttransplant were detected during the 8-week surveillance period postprophylaxis (n=19/37, 51.3%). The first positive viral load was after a median of 35 days from the beginning of the surveillance period (range 11–53 days). The median first detectable viral load during surveillance was 10,250 copies/mL (range 1620 – 2,666,500 copies/mL). The median peak viral load observed within this group was 100,550 copies/mL (range 1985 – 2,666,500 copies/mL). On the basis of routine surveillance, preemptive therapy was successfully initiated before the development of symptoms in three patients (two liver transplant and one kidney transplant). An additional three patients (all liver recipients) had asymptomatic low-level viremia, which

Table 3-1: Characteristics of 71 CMV D+/R- solid organ transplant recipients. Maintenance immunosuppression at the discontinuation of antiviral prophylaxis included Prednisone (71.8%), Cyclosporin (23.9%), Tacrolimus (63.4%), Mycophenolate mofetil (73.2%), Azathioprine (1.4%), Sirolimus (19.7%) and Other (5.6%). CMV, cytomegalovirus; D+/R-, donor seropositive and recipient seronegative; IL, interleukin.

Characteristic	
Male/Female, n	51/20
Age, median (range), in years	
< 18 years old (n=6)	13 (3 – 16)
≥ 18 years old (n=65)	52 (18 – 72)
Organ, n (%)	
Kidney	26 (36.6)
Liver	20 (28.2)
Lung	11 (15.5)
Heart	10 (14.0)
Kidney-pancreas	3 (4.2)
Heart-lung	1 (1.4)
Maintenance immunosuppression, n (%)	
Single drug	3 (4.3)
Double drug	24 (34.8)
Triple drug	43 (60.5)
Quadruple drug	1 (1.4)
Steroid-free	20 (28.2)
Induction therapy post-transplant, n (%)	
Antilymphocyte antibody	22 (31.4)
IL-2 receptor antagonist	36 (50.7)
Both	2 (2.9)
Prophylaxis, median (range), in days	98 (15 - 322)

spontaneously cleared without reaching the threshold for initiation of preemptive therapy. In the remaining 13 patients with detectable viremia during the surveillance period, patients either had symptoms (CMV disease) at the time of first detectable viremia (n=7) or became symptomatic between the first positive sample and subsequent sample but before the initiation of preemptive therapy (n=6). Virologic and sampling characteristics of each patient subgroup are summarized in Table 3-2.

A significant proportion of patients developed symptomatic CMV disease only after the end of the 8-week postprophylaxis surveillance period (n= 16/29 total disease cases). The median time to presentation of this late disease was 64 (range 9 – 180) days after the completion of the surveillance period. Viral load at disease onset was 39,830 copies/mL (range 3095 – 16,700,000 copies/mL).

Kinetic Parameters of Viral Doubling – In six patients, rapid increases in viral load during the surveillance period meant that preemptive therapy could not be started in time to prevent CMV disease. Plotting of viral loads over time before treatment initiation allowed calculation of the in vivo viral doubling time. All six patients had an exponential increase in viral load over time. An example of three of these patients is shown in Figure 3-1. The median doubling time before the start of therapy for these six patients was 1.10 days (range 0.91 – 1.46 days) reflecting rapid viral growth.

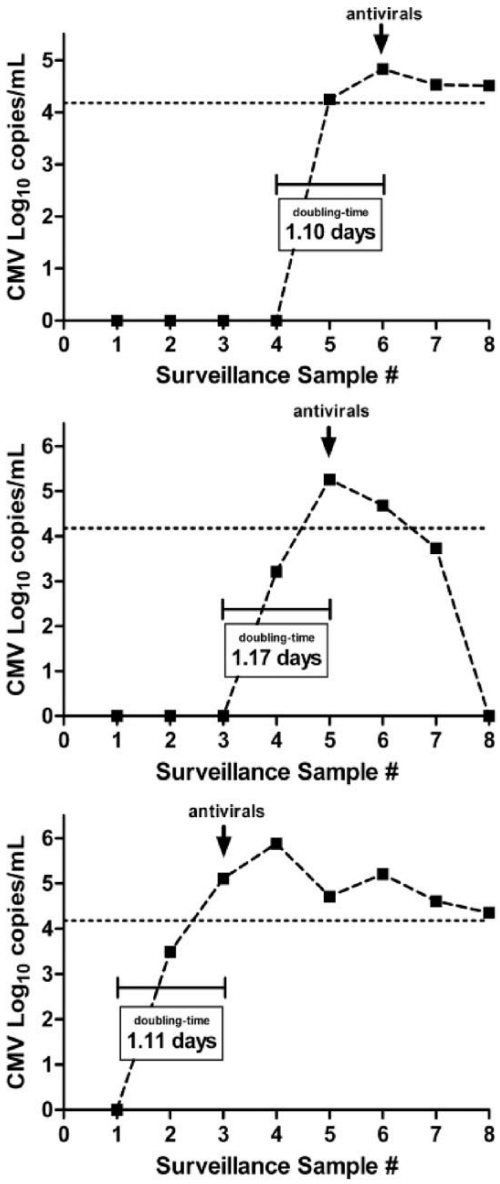
Compliance to Postprophylaxis Surveillance – We also evaluated the compliance to postprophylaxis surveillance. Overall, during the 8 weeks after the discontinuation of the antiviral prophylaxis, a median of six samples were collected at a median interval of 8 days (mean 8.64, range 3.5 – 34 days). We analyzed a best-case scenario of how many cases of disease could have been prevented if compliance with weekly monitoring for 8-week postprophylaxis was 100%. At best, an additional 3 of 29 (10.3%) cases of CMV disease may have been prevented through enhanced compliance.

Analysis by Organ Type – An analysis of the utility of postprophylaxis surveillance was carried out for each organ transplant type and is shown in Table 3-3. For example, in the 29 D+/R- kidney (or kidney/pancreas) recipients, CMV viremia occurred in 12 (41.4%) and CMV disease in 9 (31.0%). One patient benefited from preemptive antiviral therapy, whereas in most cases,

Table 3-2: Outcomes, virologic and sampling information on patients experiencing CMV viremia during the postprophylaxis surveillance period. Data are expressed as median (range). CMV, cytomegalovirus; N/A, not applicable.

19/71 viremic patients	Viral Load (copies/ml)		Samples		
Groups	**Opening**	**Peak**	**Doubling time**	**Number**	**Interval**
Spontaneous Clearance (n=3)	4,442 (1,985–5,125)	4,442 (1,985–5,125)	N/A	4 (2 –10)	6 days (6–14)
Pre-emptive therapy (n=3)	28,700 (2,335–75,000)	75,000 (12,900–75,000)	N/A	8 (7–10)	7 days (6–7)
Disease at first positive viral load (n=7)	125,950 (10,250–2,666,500)	167,500 (44,200–2,666,500)	N/A	8 (2–9)	7 days (3.5–7)
Disease occurrence between first and second positive viral loads (ie rapid doubling) (n=6)	4,585 (1,620–221,500)	200,750 (67,500–755,000)	1.10 days (0.91–1.46)	6.5 (5–9)	6 days (4–10.5)

Figure 3-1: CMV viral loads during post-prophylaxis surveillance for selected patients. The horizontal dash line represents the viremia cut-off value for pre-emptive therapy (25,000 copies/ml). Viral loads were $\log_{10}$ transformed. The arrows represent the start of antiviral therapy in symptomatic patients.



Doubling-time calculations are based on an exponential increase in viral loads modeled by the equation $y=y_0e^{ax}$ and require a minimum of 3 consecutive viral load measurements prior to the initiation of therapy. The doubling time is calculated using the formula *ln2/a*. The undetectable viral load immediately prior to the first detectable viral load is assigned a nominal value of 1 $\log_{10}$ copies/ml.

viremia and disease occurred only after the 8-week surveillance period (Table 3-3). Similar results were observed in liver transplant recipients. Of note, the highest CMV disease rate was observed in lung (including heart-lung) transplant recipients (10/12 [83.3%]), and in none of these patients was preemptive therapy successfully deployed.

DISCUSSION

Current guidelines recommend antiviral prophylaxis as the preferred strategy for CMV prevention in D+/R- transplant patients. However, after the discontinuation of prophylaxis, the occurrence of late-onset CMV disease is a major problem. The use of a hybrid strategy where preemptive monitoring is used after prophylaxis has been proposed as a way to prevent late-onset CMV disease (113); however, data assessing the utility of hybrid prevention strategies are limited. In this study, we demonstrate in a moderately large cohort of D+/R- transplant recipients that a number of limitations exist to this hybrid approach. A significant portion of disease developed only after the 8-week surveillance period. Preemptive antiviral therapy was successfully administered in only three patients, and 13 of the 71 patients still developed late-onset CMV disease during the surveillance period. Therefore, the hybrid approach was not fully effective because of a combination of factors including rapid viral doubling time, occurrence of disease after the monitoring period, and use of a high threshold for preemptive therapy. An analysis by organ subtype demonstrates that these limitations exist regardless of the type of the transplanted organ (Table 3-3).

There are limited data assessing this hybrid prophylaxis/ preemptive strategy in D+/R- transplant patients. Boillat Blanco et al. (135) assessed a similar strategy in 30 D+/R- kidney transplant patients and showed that CMV disease occurred simultaneous to the first positive viral load in five of seven patients. In an analysis of the PV16000 database, which included 364 non-lung D+/R- patients who received 3 months of antiviral prophylaxis, the utility of monitoring for predicting late-onset disease was assessed. Monitoring was performed at the time of discontinuation of prophylaxis and then every 2 to 4 weeks until month 6 and then at less frequent intervals (132). Overall, of 64 cases of late-onset disease, viral load monitoring was not able to predict CMV disease in 40 (62%) patients. One of the main criticisms of that study was that viral load monitoring was performed at infrequent intervals (every 2–4 weeks). In a study comparing a hybrid strategy (n=10) versus preemptive therapy alone (n=11) after pediatric liver

Table 3-3: Outcomes at one year post-transplant for CMV D+/R- patients. Patients are grouped by type of organ transplanted and reasons for failure of preemptive therapy are indicated. CMV, cytomegalovirus; D+/R-, donor seropositive and recipient seronegative.

Type of transplant (n=71)	CMV viremia (n=37)	CMV disease (n=29)	Benefit from Preemptive Therapy (n=3)	Reasons for failure of Preemptive Therapy
Kidney or Kidney-pancreas (n=29), n (%)	12(41.4)	9 (31.0)	1 (3.4)	Disease at first positive viral load (n=2) Disease due to rapid doubling times (0) Spontaneous clearance (0) Viremia after surveillance (n=9) Disease after surveillance (n=7)
Liver (n=20), n (%)	12 (60)	7 (35)	2 (10)	Disease at first positive viral load (n=2) Disease due to rapid doubling times (n=1) Spontaneous clearance (n=3) Viremia after surveillance (n=4) Disease after surveillance (n=4)
Lung or heart-lung (n=12), n (%)	9 (75)	10 (83.3)	0	Disease at first positive viral load (n=3) Disease due to rapid doubling times (n=3) Spontaneous clearance (0) Viremia after surveillance (n=3) Disease after surveillance (n= 4)
Heart (n=10), n (%)	4 (40)	4 (40)	0	Disease at first positive viral load (0) Disease due to rapid doubling times (n=2) Spontaneous clearance (0) Viremia after surveillance (n=2) Disease after surveillance (n= 2)

transplantation, no case of CMV disease was observed in either arm (136). In a study of 122 pediatric liver transplant recipients (43 D+/R-), a minimum of 14 days of prophylaxis was followed by surveillance. CMV disease developed in 12 patients (9.8%) during the follow-up, the majority of whom were D+/R-, had detectable CMV viremia, and had rapid development of symptoms after initial detection of viremia (137). The incidence of disease in this study was low likely because no threshold value for treatment was used, and all viremia was treated.

Alternative strategies to prevent late-onset CMV disease include simply using a preemptive strategy as opposed to a prophylaxis approach in D+/R- patients or prolonging antiviral prophylaxis beyond 3 months (138-141).

There are potential ways to improve on the efficacy of this hybrid strategy. The first would be to lower the viral load threshold at which therapy is initiated. A lower threshold would likely have prevented some other cases of CMV disease but may result in treatment of low-level viremia that would have spontaneously cleared. Indeed, optimal thresholds for use in preemptive therapy in general have not been established. The recent international CMV consensus stated that no specific threshold could be agreed upon because such thresholds were assay dependent and likely also dependent on the patient population being studied (113). To prevent cases of disease occurring after the surveillance period, an extension of the weekly surveillance period beyond 8-week postprophylaxis may be of benefit but may be difficult in patient compliance and cost. For example, to diagnose all 18 episodes of viremia that were detected after the surveillance period, an extra 26 weeks of testing would be required. In addition, based on rapid viral load doubling times observed, even more frequent monitoring (more than once weekly) may be necessary in D+/R- patients (137) consistent with previous reports assessing viral kinetics in transplant patients (142). The feasibility of intensive monitoring (i.e., sampling more than once weekly) is unclear and may be difficult because of lack of compliance and cost.

Another important issue is that the utility of this strategy may vary across different transplanted organ groups. Different transplant types may have higher or lower risk of CMV. Lung transplant patients in particular seem to be at high risk of late-onset disease (143). In our cohort, lung transplant patients did have a higher rate of viremia and disease. None of the six lung patients with viremia in the surveillance period had preemptive therapy instituted before the development

of CMV disease. In patients with the highest risk of post-prophylaxis disease, prolongation of prophylaxis may be a more viable option, whereas in specific organ types with lower rates of late-onset disease, post-prophylaxis surveillance may be more suitable. The hybrid strategy used in our study seems to be more useful in kidney and liver recipients who tended to clear viremia spontaneously and in whom preemptive strategies were sometimes successful. Further studies in individual organ groups would need to be performed to better define this.

Our study has a number of limitations. First, we only evaluated a single threshold for preemptive therapy and a single duration of surveillance. The optimal duration, frequency, and thresholds for use in a postprophylaxis preemptive strategy, therefore, cannot be well defined based on these data. However, the sample size is robust enough to provide valuable conclusions that have not been previously well shown in the literature; that is, for prevention of late-onset disease in D+/R- patients, a preemptive strategy would need longer than 8 weeks of surveillance with low thresholds for initiation of antivirals and possible twice weekly than once weekly monitoring. Another limitation of our study was the retrospective design. However, the viremia endpoints were objective, and the disease definitions based on standard ones recommended for use in clinical studies (112, 125).

In summary, the use of standard viral load surveillance after completing CMV prophylaxis in patients who were D+/R-was associated with only modest benefit, given that it did not prevent CMV disease in a number of patients. The reasons include the use of a high threshold for preemptive therapy, disease occurring beyond the surveillance period, and rapid viral load doubling times. Further studies are needed to evaluate and compare improved hybrid strategies to alternative approaches for prevention of late-onset CMV viremia and disease in D+/R- patients.

CHAPTER 4: Clinical Utility of Cytomegalovirus Cell-Mediated Immunity in Transplant Recipients With Cytomegalovirus Viremia

INTRODUCTION

Solid organ transplant recipients are at risk for cytomegalovirus (CMV) infection posttransplant. CMV reactivation may result in asymptomatic viremia or symptomatic CMV disease. Prevention strategies include preemptive therapy and antiviral prophylaxis. In preemptive strategies, patients are monitored for viremia, and those with viremia at a previously determined threshold are treated before the development of symptoms. Alternatively, many centers use antiviral prophylaxis for at-risk patients, and this significantly decreases the overall incidence of CMV disease (144). However, CMV viremia and disease is still extremely common, occurring in up to 50% of lung transplant recipients (145) and up to 38.7% D+/R- kidney recipients given a 3-month course of prophylaxis (144). Many centers therefore use a hybrid of these two strategies where prophylaxis is given and then monitoring occurs after discontinuation of prophylaxis. One of the central clinical questions with any strategy involving viral load monitoring has been what to do in patients with asymptomatic low-level viremia. Many of these patients will clear viremia spontaneously without the need for antiviral therapy and its associated costs and toxicity. However, no clear method exists for distinguishing which patients with low level viremia will have progressive increases in viral load versus spontaneous viral clearance.

The CMV-specific T-cell response seems to be a critical component of the host antiviral response during CMV reactivation posttransplant. There has been significant interest in using CMV-specific T-cell-mediated immune monitoring assays for clinical monitoring. However, such assays have not yet been adopted into standard clinical use primarily due to a lack of data evaluating these assays in well-defined clinical settings and the lack of an easy to implement commercially available assay. There are many different ways to characterize the T-cell response to CMV (summarized in Ref. (146). These include intracellular cytokine or tetramer staining with flow cytometry quantification, enzyme-linked immunosorbent spot (ELISPOT) assays, and the Quantiferon-CMV assay. The latter assay relies on enzyme-linked immunosorbent assay (ELISA) detection of interferon-γ released in whole blood upon ex vivo stimulation of CD8+ T cells with human leukocyte antigen (HLA)-restricted CMV peptides. It is commercially available in some regions (e.g., Europe and Australia) and relies on standard platforms available in most

virology and serology laboratories. A previous study by our group has shown that the lack of CMV-specific interferon response as measured with this assay was predictive of development of CMV disease in high-risk patients after discontinuation of antiviral prophylaxis (78).

In this study, we assessed the clinical utility of CMV-specific cell-mediated immunity (CMI) measurements in patients with asymptomatic low-level CMV viremia who were not receiving antiviral therapy. The Quantiferon-CMV assay was used for measurement of CMI. The specific hypothesis was that, in untreated patients with low-level CMV viremia, a detectable cell-mediated immune response will predict spontaneous viral clearance, whereas an absent CMI response would be associated with progression of viral replication and development of disease.

MATERIALS AND METHODS

Patient Enrolment – The study was approved by the institutional ethics board. Informed written consent was obtained from every patient and/or their competent representative. Adult solid organ transplant recipients with recent onset (i.e. within 1 week of first detection) low-level CMV viremia were eligible if (i) they had no symptoms of CMV disease, (ii) their viral load was below the threshold for preemptive therapy at our institution (<15,000 copies/mL), and (iii) they were not being treated for an episode of acute rejection. At our center, CMV donor seropositive/recipient seronegative (D+/R-) patients receive 3 months of prophylaxis and then undergo weekly viral load monitoring for 8 weeks. This strategy is also used for R+ patients who are lung transplant recipients or those given thymoglobulin induction. The remaining CMV seropositive (R+) patients are on a preemptive strategy with weekly viral load monitoring for the first 12 weeks posttransplant.

Cell-Mediated Immunity Testing – The CMI test was performed using the Quantiferon-CMV assay (Cellestis International, Melbourne, Australia). This assay is based on the measurement of interferon-γ released upon stimulation of whole blood with 21 class I HLA-restricted CMV peptides encompassing the most common HLA types present in the general population (77). The peptide pool includes several immunodominant CMV epitopes within the proteins pp65, IE-1, IE-2, pp50, and gB. Assays were carried out as per manufacturer's instructions. In brief, for each test, 1 mL of whole blood was collected into each of three heparinized tubes containing either coated CMV peptides (CMV tube), a positive mitogen control (MIT tube), and a negative control

with no antigen (NIL tube). After collection, tubes were shaken vigorously, and were incubated for 16 to 24 hr at 37°C. After centrifugation, supernatant was harvested and interferon-γ levels were measured (in IU/mL) by use of a standard ELISA. Results of the Quantiferon-CMV assay were not available for the management of the patients. Background levels of interferon production detected in the NIL tube are subtracted from both the values yielded by peptide (CMV) tube or mitogen (MIT) tube before result interpretation. The recommended cutoff value for CMV CMI reactivity is 0.2 IU/mL of interferon-γ, but a cutoff value of 0.1 IU/mL was also evaluated based on previous data (78). In cases where both the mitogen and the CMV tube were negative, the result was counted as negative for the purpose of analysis. The assay was performed at three time points. A baseline CMI sample was collected usually within 7 days from the first detection of viremia, and follow-up samples were drawn 7 and 14 days later.

Virologic Monitoring – After the first detectable low-level viremia, all patients underwent once weekly viral load monitoring for a minimum of 4 weeks. Viral load testing was performed using an in-house internally validated real-time polymerase chain reaction assay using DNA extracted from plasma samples (lower limit of detection 50 copies/mL) (133). The institutional threshold for initiation of preemptive therapy in asymptomatic patients was 15,000 copies/mL. In addition, any patient with symptoms consistent with CMV disease was started on antiviral therapy.

Definitions – Definitions of CMV disease were based on the American Society of Transplantation recommendations for use in clinical studies in organ transplant patients (125). Spontaneous clearance of viremia was defined as the development of a negative viral load (<50 copies/mL) in the absence of any specific antiviral treatment. Virologic and/or clinical progression was defined if the patient developed symptoms consistent with CMV disease or if the viral load rose to a level greater than 15,000 copies/mL. Patients with progression received antiviral treatment (intravenous ganciclovir or oral valganciclovir) at standard doses corrected for renal function when necessary.

Statistical Analysis – PASW Statistics 18 (SPSS, Inc., Chicago, IL) and Graph Pad Prism 4 (GraphPad Software, Inc., La Jolla, CA) were used for statistical analysis and graph generation, respectively. Fisher's exact test was used to compare categorical variables, and Mann-Whitney *U* test was used to compare continuous variables. Spearman's test was used to assess the

correlation between two continuous variables. Receiver operator characteristics curve analysis was used to evaluate the performance of CMV-specific interferon-γ on prediction of clearance of viremia. A *P* value less than 0.05 was considered statistically significant (two-tailed).

RESULTS

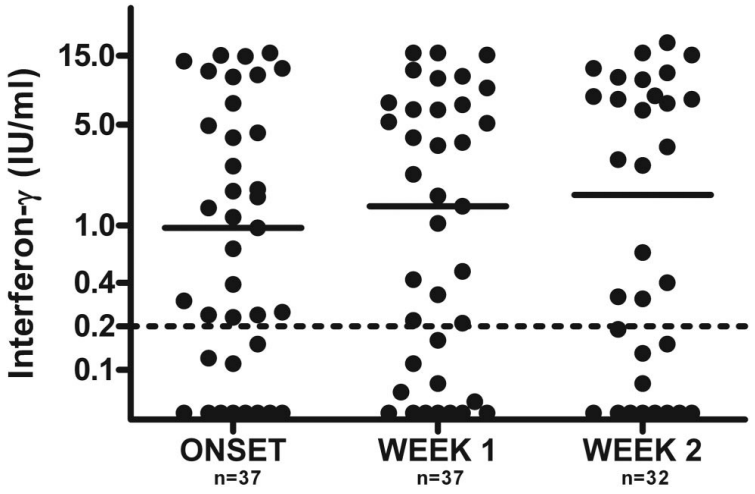
Patient Population and Outcomes – A total of 42 patients were initially enrolled. Five patients were excluded (inadequate sample collection n=1; unrelated death n=1; treated with ganciclovir for Epstein-Barr virus-related posttransplant lymphoproliferative disorder n=2; and symptomatic CMV disease at time of enrolment n=1). Characteristics of the remaining 37 evaluable patients are shown in Table 4-1. The viremia episode occurred at a median 76 days posttransplant (interquartile range [IQR] 35–203 days). All patients were asymptomatic, and the median viral load at onset was 1140 copies/mL (IQR 655–1542 copies/mL). Spontaneous clearance of viremia (without antiviral therapy or modification of maintenance immunosuppression) occurred in 29 of 37 (78.4%) patients. The remaining 8 of 37 (21.6%) either subsequently went on to develop symptomatic CMV disease (n=6; all with viral syndrome) or had a progressive increase in viremia such that preemptive therapy was initiated (n=2). Viral load at the time of antiviral treatment peaked at a median of 21,725 copies/mL (IQR 13,287–32,750 copies/mL). All of these eight patients responded clinically and virologically to intravenous ganciclovir or oral valganciclovir therapy. In patients with spontaneous clearance, a median of 21 days (IQR 10 – 42 days) elapsed between the first positive viral load and the first negative viral load. No significant differences in baseline immunosuppression or previous induction therapy were seen in the two groups (Table 4-1).

CMV-Specific CMI – The baseline CMI measurement was performed as close to onset of first detectable low-level viremia as possible (at a median of 7 days after first positive viral load; IQR 5–8). Subsequent testing was performed once weekly totalizing three measurements. Results are shown in Figure 4-1. Using a cutoff value of 0.2 IU/mL of interferon-γ, 26 of 37 (70.3%) patients had positive CMI assay at baseline measurement. By the second and third measurements, a positive CMI was present in 25 of 37 (67.6%) and 20 of 32 (62.5%) patients, respectively. Using a lower cutoff value of 0.1 IU/mL, the number of patients with a positive CMI was 29 of 37 (78.4%), 27 of 37 (73%), and 23 of 32 (67.6%), respectively, at the three time points. Of the 11 patients who were CMI negative at the initial assessment, 4 (36.4%) became

Table 4-1: Characteristics of study patients and viremia episodes. Categorical variables were compared using Chi-Square or Fisher's Exact Test and continuous variables using Mann-Whitney U Test. D=donor; R=recipient; Tac – Tacrolimus; MMF – mycophenolate mofetil; CsA – Cyclosporin.

	Spontaneous clearance (n=29)	**Antiviral treatment (n=8)**	***P***
Gender, n (%)			0.17
Male	23 (79.3)	4 (50)	
Female	6 (20.7)	4 (50)	
Age (yr), median	55	57	0.51
Transplanted organ, n (%)			0.26
Kidney	17 (58.6)	3 (37.5)	
Liver	4 (13.8)	2 (25)	
Combined	3 (10.3)	0	
Lung	3 (10.3)	3 (37.5)	
Heart	2 (6.9)	0	
CMV Serostatus, n (%)			0.51
D+/R-	3 (10.3)	0	
D+/R+	20 (69)	7 (87.5)	
D-/R+	6 (20.7)	1 (12.5)	
Induction Therapy, n (%)	22 (75.9)	5 (62.5)	0.65
Basiliximab/Daclizumab	15 (51.7)	2 (25)	
Antilymphocyte globulin	7 (24.1)	4 (37.5)	
Immunosuppression, n (%)			0.21
Tac or CsA/MMF/prednisone	21 (72.4)	7 (87.5)	
Sirolimus/MMF/prednisone	1 (3.4)	1 (12.5)	
Other	7 (24.1)	0	
Viremia episode, median			
Time post-transplant (d)	94	35	0.13
Time to spontaneous clearance (d)	21	NA	
Viral load at enrollment (copies/ml)	1,005	1,425	0.18
Peak viral load (copies/ml)	1,460	21,725	<0.001
Total Lymphocytes at enrollment (10^9/L)	1.1	0.8	0.46

Figure 4-1: CMV specific interferon-γ levels measured at the onset of viremia episode and at weekly intervals. Circles represent individual patients. Dashed line represents the cutoff of 0.2 IU/ml to consider a CMV CMI test as positive. Solid lines represent median values.



positive on at least one subsequent assessment. These 11 patients with negative CMI results were all CMV seropositive pretransplant. Neither induction or maintenance immune suppression therapy nor any organ types were associated with baseline results of CMI assay.

Clinical Prediction With CMI – The primary outcome was the ability of the baseline CMI assay result to predict spontaneous viral clearance versus progression. The results using a cutoff value of 0.2 and 0.1 IU/mL of interferon-γ are shown in Figure 4-2. In patients with a positive CMI (≥ 0.2 IU/mL), the incidence of subsequent spontaneous viral clearance was 24 of 26 (92.3%) compared with 5 of 11 (45.5%) in patients with a negative CMI at onset (*P*=0.004). Conversely, virologic and/or clinical progression occurred in 2 of 26 (7.7%) patients with a positive CMI versus 6 of 11 (54.5%) patients with a negative CMI (*P*=0.004). The results were consistent across organ groups as shown in Table 4-2. Of the two patients with a positive CMI (interferon-γ at baseline 0.39 and 1.14 IU/mL, respectively) who had disease progression, one was a CMV donor seropositive/recipient seropositive (D+/R+) kidney transplant (peak viral load=11,100 copies/mL) and the other was a D+/R+ lung transplant patient (peak viral load=18,850 copies/mL), both of whom developed viral syndrome.

Other Parameters – Interferon-γ production was also analyzed as a continuous variable. In patients who had spontaneous clearance, the baseline interferon-γ was a median of 1.73 IU/mL (IQR 0.24–10.87) vs. 0.05 IU/mL (IQR 0.01– 0.32) for patients with virologic or clinical progression (*P*=0.003). At the second time point, median interferon-γ levels were 3.75 vs. 0.07 IU/mL in the two groups, respectively; *P*=0.004. By the third time point the median levels were 3.49 vs. 0.08 IU/mL; *P*=0.038. A receiver operator characteristics curve analysis was performed for prediction of spontaneous clearance of viremia based on CMV specific interferon-γ levels. The area under the curve (AUC) was 0.843 (*P* value for difference from chance alone [AUC=0.5] was 0.003; 95% confidence interval [CI] for AUC=0.709–0.977) for the baseline sample, 0.832 (*P*=0.004; 95% CI 0.700–0.964) for the second sample, and 0.707 (*P*=0.146; 95% CI 0.526–0.888) for the third sample. We also analyzed different cutoff values for the assay (for defining a positive result) ranging from 0.1 to 0.4 IU/mL of interferon-γ (Table 4-3). Increases in the cutoff value resulted in slight improvement in positive predictive value (i.e., ability of a positive test to predict spontaneous clearance) but a significant loss in negative predictive value.

Figure 4-2: Performance of baseline (viremia onset) samples for prediction of spontaneous clearance of viremia using different cut-off values. P-values for Fisher's Exact Tests.

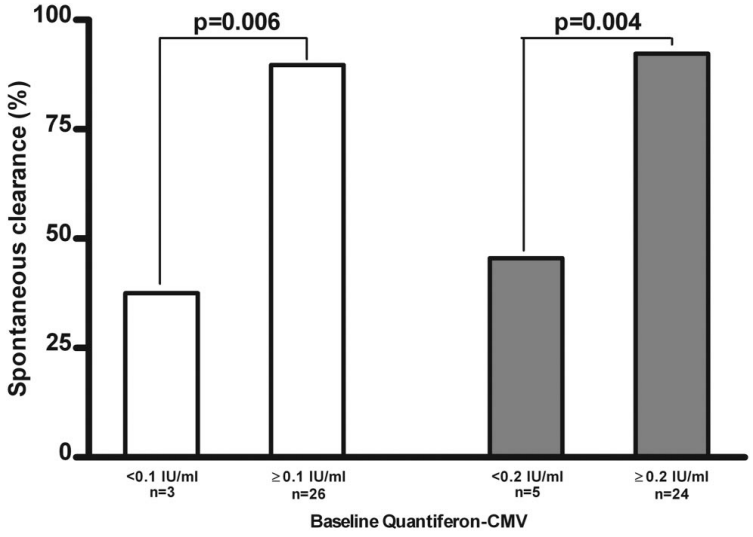


Table 4-2: Analysis of primary endpoints according to baseline CMV-CMI results (standard cut-off ≥ 2 IU/ml) and organ types. Number of patients with the outcome of interest/Number of patients with positive or negative CMV-CMI test (%). All combined organs transplant recipients achieved spontaneous clearance of viremia and had positive CMV-CMI testing. NA= not applicable; CMV, cytomegalovirus; CMI, cell-mediated immunity.

Organ Type	Spontaneous clearance among patients with positive CMV-CMI	Antiviral therapy among patients with negative CMV-CMI
Kidney (n=20)	14/15 (93.3)	2/5 (40)
Liver (n=6)	3/3 (100)	2/3 (66.7)
Combined (n=3)	3/3 (100)	NA*
Lung (n=6)	3/4 (75)	2/2 (100)
Heart (n=2)	1/1 (100)	1/1 (100)

Absolute interferon-γ values were also analyzed for their correlation with viral load measurements performed the same day. In 70 samples, both viral load and CMI assays were collected on the same day. In this subset, the CMV-specific interferon-γ production was inversely correlated with CMV viral load measured at the same time (Spearman's rho 0.318, P=0.007). For instance, the median viral load at the time of any positive test (≥0.2 IU/mL) was 225 copies/mL (IQR undetectable to 1865), whereas concurrently to a negative CMI testing (<0.2 IU/mL) the viral load was 2345 copies/mL (IQR 225–8350). Because the assay measures interferon-γ production from lymphocytes, we also compared total lymphocyte counts in the spontaneous clearance group versus those with progression, and no significant difference was seen at all time points (Table 4-1). However, absolute interferon-γ levels were positively correlated with the lymphocyte count in 85 samples where both values were concurrently available (Spearman's rho 0.386, P<0.001).

DISCUSSION

Asymptomatic low-level CMV viremia is very common in transplant patients; making the detection of viremia in such patients is the basis of preemptive prevention strategies for CMV. However, a subset of patients will spontaneously clear viremia without the need for treatment. This has meant that optimal thresholds for use in preemptive strategies have not been well defined, and the clinical dilemma of what to do in asymptomatic patients with low-level CMV viremia remains. This study demonstrates that a significant refinement of preemptive protocols could be made with the addition of CMI testing. We show that low-level viremia patients who have a detectable CMV-CMI response have a high likelihood of spontaneous clearance of viremia, whereas those with a negative CMI response have a high risk of virologic or clinical progression. In the clinical setting therefore, the use of CMI testing could help decide which patients with low-level viremia could simply be followed closely and which patients should commence antiviral treatment. This could result in more rational antiviral use, with a potential decrease in toxicity and cost. Although it could also be argued that treatment of any level of viremia may be important to prevent indirect effects of CMV, the true indirect effects of low-level CMV replication have yet to be ascertained. It should also be noted that prediction is not absolute and that some patients with detectable CMI responses and low-level viremia will still have virologic or clinical progression as noted for two patients in our study.

Table 4-3: Analysis of different cut-points of interferon-γ for defining a positive vs. a negative cell-mediated immune response to CMV. PPV= positive predictive value [ability of a positive test to predict spontaneous clearance]; NPV= negative predictive value [ability of a negative test to predict virologic and/or clinical progression]; RR=relative risk for progression (i.e. future need of antiviral therapy) with a negative vs. positive test; CI=confidence interval.

Cut-off (≥IU/ml)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	RR of progression with negative test		
					RR	95% CI	*P*
0.1	89.7	62.5	89.7	62.5	6.04	(1.82 – 20.03)	0.003
0.2	92.3	54.5	82.8	75.0	7.09	(1.69 – 29.83)	0.008
0.3	90.9	40.0	69.0	75.0	4.40	(1.02 – 18.94)	0.047
0.4	95.0	41.2	65.5	87.5	8.24	(1.12 – 60.43)	0.038

The Quantiferon-CMV assay has only been assessed in a small number of studies. Walker et al. (77) evaluated this assay in 25 heart and/or lung patients, in whom the test differentiated seronegative from seropositive transplant recipients at different times posttransplant, regardless of antiviral or immunosuppressive therapy. The association between CMV-CMI and viremia was not addressed in that study. In a previous study of 108 high-risk transplant patients, CMV CMI was assessed at the end of prophylaxis to predict late onset CMV disease (78). Patients with a positive CMI test at the end of prophylaxis had a late-onset disease rate of 5.3% vs. 22.9% in patients with a negative CMI test. Westall et al. (147) evaluated this assay longitudinally in 39 lung transplant recipients and correlated results with CMV viral loads within the bronchoalveolar lavage fluid. Although a marked decrease in the CMI response was seen at the time of viral reactivation in the lung, the assay was not particularly predictive of significant reactivation in that organ compartment. In one additional study, this assay was evaluated in a cohort of 14 viremic kidney transplant recipients (148). A lower, although not statistically significant, interferon-γ response in patients with high viral loads compared with those with low viral loads was observed. The other clinical scenario where CMI testing might be used is to guide the duration of treatment for CMV disease and to prevent recurrent CMV disease. We are not aware of any specific studies that answer this question. Finally, another area where a CMI test would be used is to guide what to do in patients with low-level viremia (i.e., to refine a preemptive algorithm). We believe that this is the first study to specifically address this question in a rigorous manner.

Several studies have assessed intracellular cytokine staining and ELISPOT for assessment of CMV CMI (72, 73, 149-158), although the exact clinical scenarios where these tests should be applied have been more difficult to interpret. Development of CMV CMI by CD4+ and CD8+ intracellular cytokine staining was associated with control of subsequent viremia episodes without development of CMV disease in kidney and liver D+/R+ patients (159). Recovery of CMV specific response as measured by ELISPOT in a cohort of small bowel/multivisceral transplant recipients early posttransplant was protective against development of moderate or severe episodes of CMV disease (160). On the other hand, viral replication was associated with significantly lower frequencies of CMV-specific CD8+ T-cells measured by flow cytometry in seropositive kidney recipients yet the assay performed poorly on predicting concurrent and future CMV replication (152). Overall, these studies help confirm the importance of CD4+ and CD8+

T-cells in control of viral replication posttransplant. These measurements are still not in routine use in clinical practice for a number of reasons. Flow cytometry allows assessment of both CD4+ and CD8+ T-cell populations, is quantitative, and allows measurement of cytokines other than interferon-γ. ELISPOT assays have been reported in several studies and also seem to be useful for measurement of the CMV-specific immune response. For both methods, complexity, cost and lack of standardization are important issues to consider. The ELISA-based Quantiferon-CMV assay is approved for in vitro diagnostics in Europe, Australia, and Korea and for investigational use only in the United States and Canada. It also uses equipment that is readily available in most laboratories. It measures primarily CD8+ T-cell responses (not CD4+) and is HLA-restricted such that patients with uncommon HLA types may not be represented in the peptide pool used for stimulation (148, 161). This could be a cause for false-negative tests.

In this study, some patients had a change in test result over subsequent measurements. A small number of patients who were initially positive became negative. This may be due to interferon-γ values near the threshold for positivity or due to subtle changes in the cell-mediated immune response possibly as a consequence of viral replication. More commonly, however, an overall interferon-γ production increase over time occurred (Fig. 4-2) presumably as an appropriate immune response to viral replication. This was especially apparent in the group that had spontaneous clearance (absolute interferon-γ increased from 1.73 to 3.75 IU/mL between baseline and follow-up measurement). Serial CMI testing could also be clinically explored, although our results indicate that a single sample collected early in the course of viremia had better prediction value when compared with follow-up testing. In addition, we show that the assessment of CMV-CMI in solid organ transplant during a viremia episode does not simply reflect the pretransplant serostatus of the patient, suggesting it rather reflects the functional status of CMV-specific CD8+ T cells at the time of assessment.

Our study had a number of limitations. First, the CMI assay was not truly obtained on the same day as the onset of viremia, due to the time to obtain the initial viral load results (usually 48 hr), and return of the patient for obtaining informed consent and collecting the CMI assay. Therefore, our “baseline” CMI assessment actually reflects a few days after onset of low-level viremia in most instances. However, this situation likely reflects how the assay would be used in clinical practice, where asymptomatic viremic patients could be asked to return for assessment of their

CMI response. Another limitation of our study was the small sample size. This was partly due to the stringent criteria for enrolment. These criteria were necessary so that a clear clinical question could be answered from the study, which we believe we have done. Our study population was also primarily R+ patients. Viral kinetics may differ between D+/R- patients and R+ patients. However, R+ patients are the primary group for which a preemptive strategy is recommended as a reasonable option to prophylaxis (112, 113). Finally, our study analyzed a heterogeneous organ type group, with predominance of kidney recipients. The overall results are consistent across organs. We believe that the next step would be to perform a larger multicenter validation trial.

In summary, we show that in organ transplant patients with asymptomatic low-level CMV viremia, the measurement of CMV-specific CMI using the Quantiferon-CMV assay can help predict which patients will spontaneously clear virus versus which patients will have progression. This could be used in the clinical setting as an adjunctive tool in a preemptive protocol to allow more rational and targeted antiviral use. Further studies could compare the efficacy and cost effectiveness of preemptive strategies using virologic monitoring alone versus preemptive strategies using a combination of virologic and immunologic monitoring to help guide antivirals.

CHAPTER 5: CCL8 and the Immune Control of Cytomegalovirus In Organ Transplant Recipients

INTRODUCTION

Monitoring virus-specific, cell-mediated immune responses is a promising way to predict CMV reactivation, and may ultimately be useful in the prevention and management of CMV infection among transplant recipients (162). In particular, CMV-specific cytotoxic T-cells seem to be an important biomarker for predicting virologic and clinical outcomes. For example, assessments of CMV-specific IFN-γ responses pre-transplant (163) or upon completion of antiviral prophylaxis (78, 164) have been shown to predict CMV disease. Recently, we showed that the CMV-specific IFN-γ response measured at the onset of CMV viremia in solid organ transplant recipients had the potential to predict spontaneous clearance vs. progressive viral replication (165). Cell-mediated immunity tests can not only help define riskgroups for CMV disease, but also determine subgroups of patients capable of achieving suppression of viral replication exclusively by immunological means – an elusive goal at the present stage of CMV vaccine development (166). However, cytotoxicity exerted by CMV-specific T-cells is only one component of the highly complex multipronged cellular and humoral response leading to immune control of the virus. An understanding of other factors and players involved in immunologic control of CMV beyond the accepted roles of cytotoxic T-cells may facilitate the development of novel and improved preventative strategies.

The objective of this study was to perform an in-depth analysis of CMV cell-mediated immune responses by comprehensively profiling cytokine and chemokine expression upon *ex vivo* stimulation of whole blood from transplant recipients shortly after the onset of CMV viremia. We hypothesized that immune control of viral replication would be associated with distinct cytokine and chemokine responses, which were expected to offer new insights about the contributions of other peripheral blood cell subpopulations to the immunologic control of infection. As our findings implicated the participation of a chemokine in the immune control of CMV viremia, we then investigated its cellular origin and regulation by immunosuppressive agents. Among a second cohort of transplant recipients, we explored the use of genetic variability in this chemokine promoter for prediction of CMV viremia.

MATERIALS AND METHODS

Patients, Samples and Outcomes – Two distinct solid organ transplant recipient cohorts were analyzed using samples obtained from two previously-published studies (164, 165). For the first group (n=37) (165), blood samples collected at the onset of asymptomatic CMV viremia (i.e., the first samples obtained upon patient enrollment) were utilized for cytokine and chemokine profiling. Patients were followed clinically and with weekly CMV PCR. The outcomes assessed in this cohort were either spontaneous viral clearance or progressive viremia/symptomatic CMV disease requiring intravenous ganciclovir or oral valganciclovir. The second group (n=67) was comprised of CMV donor seropositive/recipient seronegative (D+/R-) transplant recipients who were previously enrolled in non-interventional clinical trials at two Canadian centers (164). All patients received 3-6 months of antiviral prophylaxis, and blood samples collected upon discontinuation of treatment were utilized for single nucleotide polymorphism genotyping. The outcomes assessed in this cohort were the development of CMV viremia and CMV disease up to 1 year post-transplant. Definitions of CMV viremia and CMV disease were derived from the respective original studies. In addition, self-declared healthy adult volunteers (n=8) donated blood samples which were used to investigate the blood leukocyte origins of cytokines and chemokines. The original studies and the required amendments were reviewed and approved by the local Ethics Research Board. All patients and healthy volunteers provided written informed consent.

Plasma Cytokine and Chemokine Measurements - In the first cohort (n=37 individual patient samples at onset of CMV viremia), whole blood was incubated overnight in the presence or absence of 22 CMV peptides utilizing the commercially available QuantiFERON-CMV assay (QTF; Qiagen Inc.) according to the manufacturer's instructions. Quantitation of 65 cytokines and chemokines (EGF, Eotaxin/CCL11, FGF-2, Flt-3 ligand, Fractalkine/CX3CL1, G-CSF/CSF3, GM-CSF/CSF2, GRO/CXCL1, IFN-α2, IFN-γ, IL-10, IL-12(p40)/IL12B, IL-12(p70)/IL12A, IL-13, IL-15, IL-17/IL17A, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10/CXCL10, MCP-1/CCL2, MCP-3/CCL3, MDC/CCL22, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-AA, PDGF-AB/BB, RANTES/CCL5, TGF-α, TNF-α, TNF-β, VEGF, sCD40L, sIL-2Rα, MCP-2/CCL8, MCP-4/CCL13, ENA-78/CXCL5, SDF-1α+β/CXCL12, BCA-1/CXCL13, I-309/CCL1, MIP-1δ/MIP-5/CCL15, TARC/CCL17, 6Ckine/CCL21,

EOTAXIN-2/CCL24, EOTAXIN-3/CCL26, CTACK/CCL27, IL-23, LIF, THPO, TRAIL/TNFSF10, SCF/KITLG, TSLP, IL-20, IL-21, IL-28A, IL-16, IL-33/NF-HEV) was performed on the resultant stimulated and unstimulated plasma using Milliplex Map Human Cytokine/Chemokine kits (Millipore) according to the manufacturer's protocol. The assays were performed using the Luminex™ 100 system (Luminex) by Eve Technologies Corp. (Calgary, AB, Canada). Results beyond the extremes of the standard curves were attributed to either the lowest or the highest standard concentration of the respective analyte.

Cytokine/Chemokine-Based Classification of Viremia Outcomes - In order to reduce the complexity of the data to the optimal number of cytokines and chemokines capable of accurately classifying patients into a particular outcome (spontaneous viral clearance vs. progressive viremia), the Gene Expression Model Selector (GEMS) (167) system was used. The software defines the optimum algorithms and parameters to support vector machine-based classification through comparative performance assessment generated by nested cross-validation procedures. A standard SVM with Gaussian kernel was employed for classification, utilizing z-normalized cytokine/chemokine ratios (versus unstimulated controls); the optimal method devised for the data was a signal-to-noise ratio in a one-versus-rest fashion.

Single Nucleotide Polymorphism (SNP) Genotyping - Patients in cohort 2 (n=67 D+/R- patients followed for CMV infection after antiviral prophylaxis) were genotyped for a CCL8 SNP. A custom TaqMan SNP qPCR assay for SNP rs3138035 was designed by Life Technologies (assay ID **AHAA9YL)** based on the CCL8 promoter reference sequence obtained from NCBI. Synthetic oligonucleotides (Integrated DNA Technologies) were utilized to validate the qPCR assay. Plasma genomic DNA automated extraction was performed using the DNA Investigator Kit (Qiagen). The qPCR was performed using a StepOne Real-Time PCR instrument and results were analyzed using the StepOne Software v2.2 (Life Technologies).

Cellular Origin of CCL8 and IFN-γ – Peripheral blood mononuclear cells (PBMCs) were obtained from CMV-seropositive (n=4) and CMV-seronegative (n=4) samples by Ficoll gradient centrifugation of whole blood and cryopreserved until further use. After thawing, cells were washed and resuspended in RPMI 5% human serum at a density of 5 x 10^6 cells/mL and stimulated overnight at 37ºC in QuantiFERON NIL or CMV antigen tubes. Cell viability was

confirmed using amine-reactive dye (LIVE/DEAD stain, Life Technologies). Fc receptors were blocked (eBioscience) prior to surface staining with antibodies (eBioscience, Biolegend or BD Biosciences) belonging to panel 1 (CD3-PerCP-Cy5.5, CD4-PE-Cy7, CD8-APC-H7 and CD19-APC) or panel 2 (CD3-PerCP-Cy5.5, CD14-PE, CD19-APC and CD56-APC-Cy7). Antigen choices were based on the standardized immunophenotyping proposed for the Human Immunology Project (168). Sorted leukocyte populations included monocytes (CD3-/CD19-/CD14+), NK cells (CD3-/CD19-/CD14-/CD56+), B-cells (CD3-/CD19+), CD4+ T-cells (CD3+/CD8-/CD4+), and CD8+ T-cells (CD3+/CD4-/CD8+). Cells were maintained on ice throughout the staining process and formaldehyde fixation was not performed in order to better preserve their RNA content. Cell sorting and purity checks were performed in FACSAria III (BD Biosciences). Cells were sorted directly into lysis buffer and RNA was extracted using an RNeasy Plus micro kit (Qiagen). Reverse transcription was performed using High Capacity cDNA Reverse Transcription kits followed by qPCR using TaqMan assays (CCL8-Hs04187715_m1, IFN-γ-Hs00989291_m1; RPL13A-Hs04194366_g1 and RN18S1-Hs03928985_g1; all Life Technologies). Each PCR reaction contained the cDNA equivalent of 10 ng of total RNA, as quantified by spectrometry (NanoDrop). Relative quantitation against unstimulated cells (i.e., NIL tube) was performed by the delta-delta-Ct method using RLP13a (169) as the endogenous control for B-, T- and NK cells; and 18S rRNA for monocytes. These were selected based on the smallest level of variation found between CMV and NIL samples of CMV seropositive healthy volunteers (data not shown).

Effect of Immunosuppression on Monocyte CCL8 Expression – THP-1 cells cultured in RPMI 10% FCS were plated at a density of 200,000 cells/well in 96-well plates and pre-treated for 4 hours at 37ºC with serial dilutions of the immune suppressive agents tacrolimus, mycophenolate mofetil and rapamycin (all Enzo Life Sciences). The dose range for each drug was defined to ensure comparable metabolic activity between treated and untreated cells, as per MTT assay (data not shown; Roche). IFN-γ was added (final concentration 1 μg/mL; Peprotech) and cells were incubated for an additional 6 hours before RNA extraction. Cells were lysed in plate and automated RNA extraction was performed using an RNeasy Micro kit (Qiagen).

Statistical Analysis – Two-tailed testing was applied to all analyses, with $p < 0.05$ considered to be statistically significant. To facilitate comparison with previously published data, during the

receiver operating characteristic curve analysis we subtracted background values found in the unstimulated blood (i.e., NIL tube) from the values measured in the CMV peptide-stimulated blood, analogous to the QuantiFERON-CMV manufacturer recommendations for IFN-γ. All analyses were performed using PASW Statistics 18.0.0 (IBM) or Prism 6 (GraphPad Software).

RESULTS

CMV peptide stimulation broadly alters the blood expression of cytokines and chemokines – To analyze the blood secretome in the context of an MHC class I-restricted CMV peptide-triggered interferon-γ response, 65 cytokines and chemokines were measured in paired non-stimulated and CMV peptide-stimulated plasma samples of 37 organ transplant recipients at the onset of CMV viremia. Quantitation within the assay limits was achieved in the majority of the specimens for 60 of the 65 cytokines/chemokines (Appendices Table S5-1). Of the 5 remaining cytokines/chemokines, IL-3, IL-9, IL-21, IL-28A and CCL26 were detected in less than 50% of paired patient samples. Appendices Table S5-2 summarizes the effects of peptide stimulation on the levels of cytokines and chemokines. CMV peptides triggered significant up-regulation in expression of several proteins in addition to IFN-γ, while promoting down-regulation of a selected set of cytokines and chemokines.

Responses to CMV peptide stimulation differ according to viremia outcomes – For this cohort of CMV-viremic transplant recipients, those who had spontaneous clearance of viremia showed a significantly higher *ex vivo* production of IFN-γ compared to those who had progressive viremia (165). We next compared the levels of cytokines/chemokines between patients with these two clinical outcomes. First, we analyzed background levels of cytokines and chemokines present in the non-stimulated blood samples. Univariate analysis revealed that only the level of chemokine IL-8 level significantly varied between the two groups of patients, with patients achieving immune control of viremia showing the highest baseline levels of this chemokine (median 8744 vs. 5334 pg/mL; p=0.042) (AUC 0.737 95% CI 0.552 – 0.922; p=0.042). We then performed a univariate analysis to determine the cytokine/chemokine expression levels following CMV antigen stimulation, relative to their background levels, in patients with spontaneous clearance vs. viral progression (Table 5-1 and Appendices Table S5-3). Patients achieving spontaneous clearance of viremia showed a distinct secretome with increased expression of 15 and decreased

expression of 9 cytokines/chemokines upon CMV peptide stimulation. Among those with progressive viremia, reduced expression of both CCL4 and CXCL12 was a distinctive feature.

Generating a cytokine/chemokine-based classifier of viremia outcomes – We postulated that identifying the specific cytokine and chemokine contributions that lead to the most precise classification of virologic outcomes during monitored CMV replication episodes would improve our understanding of the hierarchy of inflammatory mediators involved in the immunologic control of replication. We utilized the Gene Expression Model Selector (GEMS) (167) to devise highly accurate classification models based on cytokine/chemokine level changes promoted by CMV peptide stimulation (i.e., CMV:NIL ratio). The measured effects of CMV peptide stimulation yielded a classifier with an accuracy of 80% in a 10-fold cross-validation performed on the same dataset. The cytokines and chemokines that most contributed to the classification model are ranked in Table 5-2. CCL8, IFN-γ and CXCL10 demonstrated the highest increase in expression upon CMV peptide stimulation and closely correlated (CCL8 and CXCL10: Spearman's rho 0.642, $p<0.001$; IFN-γ and CCL8: Spearman's rho 0.912, $p<0.001$; IFN-γ and CXCL10: Spearman's rho 0.571, $p<0.001$).

CCL8 and CXCL10 as isolate predictors of viremia outcomes – The discriminatory power of CMV peptide-elicited IFN-γ production to predict spontaneous clearance of viremia has been defined previously for this sample set (AUC 0.843 95% CI 0.709 – 0.977; p=0.003) (165). For comparison purposes, the levels of other cytokines and chemokines following CMV peptide stimulation of whole blood were also individually analyzed. Receiver operating characteristic curves confirmed the discrimination capacity of CCL8 (AUC 0.849, 95% CI 0.721 – 0.978; p=0.003) and CXCL10 (AUC 0.841, 95% CI 0.707 – 0.974; p=0.004). No other cytokine/chemokine demonstrated a statistically significant discriminatory capacity following CMV peptide stimulation (Figure 5-1).

Monocytes are the source of CCL8 – To better contextualise cytokine and chemokine production in the antiviral response evoked by CMV peptide stimulation, we next sought to identify the cell types primarily responsible for producing the top-ranked cytokine (IFN-γ) and chemokine (CCL8) in the generated classifier. For that, PBMCs from QTF-CMV positive and negative healthy individuals were incubated overnight in QTF CMV or NIL tubes, and sorted by

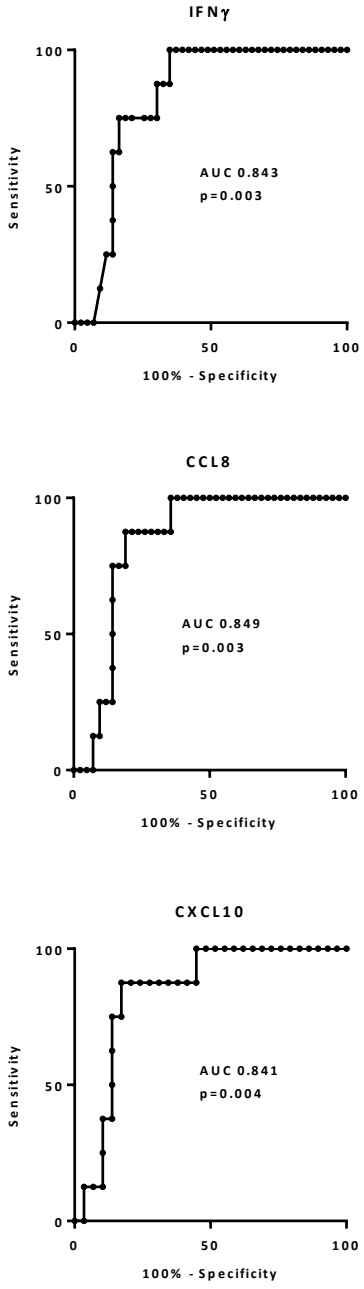
Table 5-1: Outcome-specific cytokine and chemokine responses among 37 CMV-viremic transplant recipients. A two-tailed paired Wilcoxon signed-rank test was utilized to describe the outcome-specific behavior of individual cytokines and chemokines upon MHC-I-restricted CMV peptide stimulation in samples from CMV-viremic patients with progressive (n=8) or spontaneous clearance of viremia (n=29). Ratios of levels in Quantiferon CMV and NIL tubes and interquartile ranges are shown for statistically distinct cytokine/chemokine behaviors between the two groups. A P-value < 0.05 was considered statistically significant.

	Progressive Viremia (n=8)		**Spontaneous Clearance (n=29)**	
Protein	**CMV/NIL median (IQR)**	**p-value**	**CMV/NIL median (IQR)**	**p-value**
CCL8	1.20 (1.00 - 1.51)	.161	5.62 (2.44 - 30.30)	.000
CXCL10	0.99 (0.91 - 1.04)	.674	2.01 (1.14 - 6.61)	.000
IFNγ	0.84 (0.76 - 0.97)	.401	1.91 (0.83 - 9.34)	.002
TNFβ	1.26 (0.94 - 1.85)	.401	1.78 (1.05 - 4.28)	.004
EGF	1.08 (1.01 - 1.49)	.063	1.72 (1.30 - 2.34)	.000
IL-1ra	1.18 (1.06 - 1.99)	.263	1.66 (1.06 - 2.99)	.001
CCL5	1.12 (0.67 - 1.36)	.889	1.56 (1.06 - 2.43)	.001
IL-4	0.96 (0.92 - 1.19)	.866	1.37 (0.95 - 1.99)	.008
IL-2	1.17 (0.95 - 1.42)	.674	1.34 (0.97 - 2.35)	.035
sIL-2Rα	0.91 (0.75 - 1.75)	.889	1.33 (0.99 - 1.60)	.003
CX3CL1	1.51 (1.12 - 1.65)	.674	1.32 (0.99 - 1.70)	.018
TRAIL	1.11 (0.90 - 1.54)	.208	1.18 (0.96 - 1.47)	.017
FGF2	1.26 (0.76 - 1.47)	.674	1.17 (1.00 - 1.41)	.005
CCL13	1.07 (0.84 - 1.64)	.401	1.14 (0.92 - 1.50)	.025
KITLG	1.11 (1.00 - 1.37)	.263	1.10 (1.01 - 1.28)	.003
IL-6	0.68 (0.16 - 1.21)	.093	0.43 (0.24 - 0.98)	.000
GCSF	0.47 (0.17 - 0.87)	.050	0.50 (0.22 - 1.45)	.033
CCL7	0.61 (0.42 - 0.85)	.069	0.61 (0.41 - 1.01)	.001
CXCL1	0.65 (0.60 - 1.04)	.161	0.71 (0.50 - 0.89)	.000
CCL22	0.70 (0.51 - 1.11)	.123	0.71 (0.61 - 0.94)	.019
IL-16	0.74 (0.62 - 0.90)	.093	0.73 (0.61 - 1.01)	.015
IFNα2	0.93 (0.74 - 1.02)	.093	0.76 (0.57 - 0.92)	.048
IL-28A	1.00 (1.00 - 1.28)	.593	0.86 (0.31 - 1.00)	.026
THPO	0.85 (0.67 - 1.23)	.401	0.87 (0.72 - 1.05)	.023
CCL4	0.33 (0.23 - 0.69)	.012	0.65 (0.48 - 1.17)	.230
CXCL12	0.81 (0.65 - 0.90)	.028	1.00 (0.70 - 1.19)	.677

Table 5-2: Relative importance of cytokines and chemokines when classifying patients according to CMV viremia outcomes. The 10 top-ranked cytokine and chemokine responses in whole blood after CMV MHC-I-restricted peptide stimulation contributing to an average classification accuracy of 80% are summarized, along with respective median fold-changes seen in patients with spontaneous viral clearance or progressive viremia requiring antiviral therapy. Positive and negative fold-changes correspond to increased or decreased levels upon CMV peptide stimulation, relative to background levels. Shading ($p<0.05$) and underlining ($p<0.001$) indicate statistically significant fold-changes based on a two-tailed paired Wilcoxon signed-rank test.

Rank	Protein	Progressive Viremia (n=8) Fold-change	Spontaneous Clearance (n=29) Fold-change
1	CCL8	+1.20	+5.62
2	IFN-γ	-1.19	+1.91
3	CCL5	+1.12	+1.56
4	CXCL10	-1.01	+2.01
5	CCL4	-3.03	-1.54
6	CCL2	-1.14	+1.04
7	PDGF-AB/BB	-1.02	+1.12
8	IL-4	-1.04	+1.37
9	IL-8	-1.41	-2.08
10	CCL3	-5.00	-3.70

Figure 5-1: Performance of IFN-γ, CCL8, and CXCL10 in the prediction of immune clearance of CMV viremia. Protein levels of cytokines/chemokines elicited by whole blood stimulation with CMV MHC-I-restricted peptides were tested as predictors of spontaneous immune clearance of ongoing CMV viremia among 37 organ recipients. Receiver Operating Curve analyses were performed using protein concentrations measured in background-subtracted, peptide-stimulated samples. AUC: area-under-the-curve.



flow cytometry (Appendices Figures S5-1 and S5-2), yielding cell phenotypes of high purity (>95%) suitable for studying mRNA gene expression. IFN-γ mRNA transcription was evident in CD4+ and CD8+ T-cells of CMV seropositive individuals. Higher IFN-γ levels were also detected in NK cells from these individuals (Figure 5-2). Amongst the leukocyte populations analyzed, monocytes were the only cell type in which CCL8 mRNA could be detected, with CMV-seropositive individuals showing median expression levels 2-$\log_{10}$ higher than CMV-seronegative individuals in the CMV-stimulated sample (Figure 5-2).

CCL8 promoter polymorphism is associated with risk of CMV viremia following donor–derived primary CMV infection – A potentially relevant factor affecting cytokine/chemokine levels is the genetic variability among hosts. In particular, polymorphisms in the gene promoter region may impact the epigenetic regulation of gene expression. Using a TaqMan-based SNP assay, we determined the CCL8 promoter SNP rs3138035 genotypes of 67 D+/R- transplant recipients. Patients from distinct rs3138035 genotype groups differed only in terms of utilization rate and type of induction immunosuppression therapy (Appendices Table S5-4), and the risk of CMV viremia in this cohort seemed not to be affected by these variables (Appendices Figure S5-4). Individuals who were homozygous for the CCL8 promoter minor allele (TT) were significantly more likely to experience viremia after discontinuation of primary antiviral prophylaxis (Log-rank Mantel-Cox test for difference between CC/CT/TT curves p=0.0178; Figure 5-3). These individuals had a risk of viremia that was 3.62 times greater than carriers of at least one major C allele (logrank hazard ratio; 95% CI 2.077 – 51.88). There was no association between CCL8 promoter genotypes and the development of symptomatic CMV disease in the follow up (Log-rank Mantel-Cox test p=0.7114).

Immunosuppression adversely affects the production of CCL8 – Immunosuppressive drugs are known modulators of cytokine/chemokine expression in response to CMV antigens (170). We hypothesized that the expression of CCL8 is also affected by therapeutic immunosuppression. Known inducers of CCL8 expression include IFN-γ, IL-1β (171, 172) and TNF-α (173). Since the expression of both IL-1β and TNF-α was reduced by CMV peptide stimulation (Appendices Table S5-3) independent of viremia outcomes, we assessed the effects of immunosuppression on the IFN-γ-induced CCL8 expression in monocytes. Undifferentiated monocytic THP-1 cells pre-

Figure 5-2: Expression of CCL8 and IFN-γ transcripts in response to CMV MHC-I-restricted peptides. Gene transcript expression was measured in peripheral blood mononuclear cells of CMV-seropositive (n=4) and CMV-seronegative (n=4) healthy volunteers. The relative expression levels of CCL8 (top) and IFN-γ (bottom) between cells stimulated with MHC-I-restricted CMV peptides (i.e., CMV tube) and each individual's corresponding unstimulated cells (i.e., NIL tube) are shown. CCL8 transcripts were exclusively detected in monocytes (t test p=0.112), whereas IFN-γ was present in T CD4 (t test p=0.065), T CD8 (t test p=0.775) and NK cells (t test 0.096).

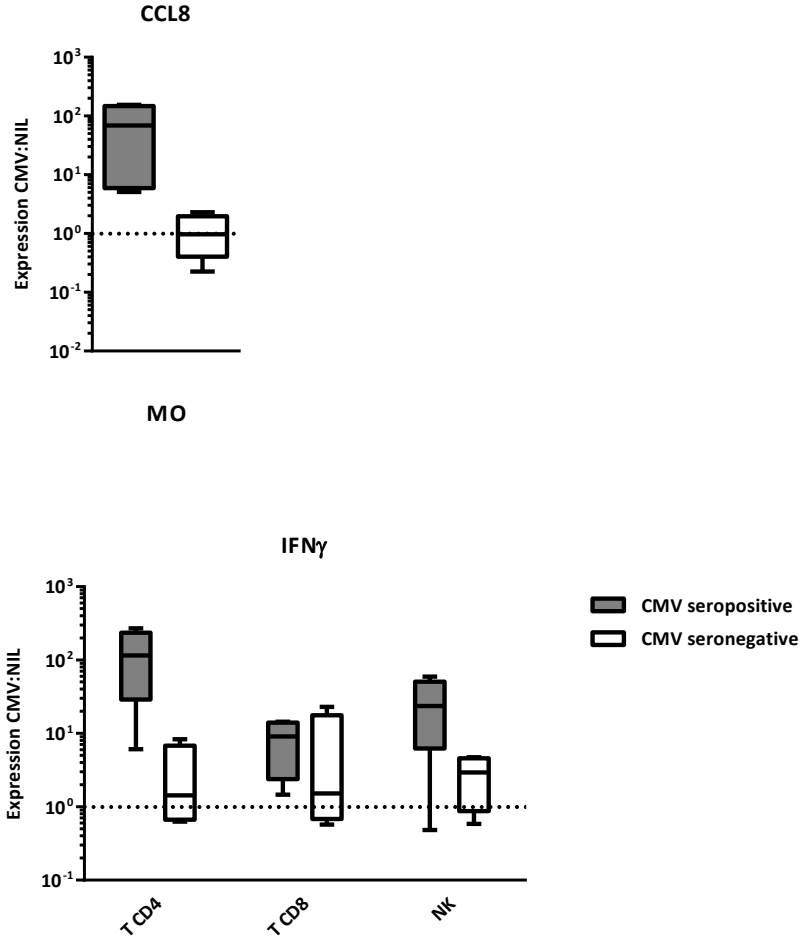
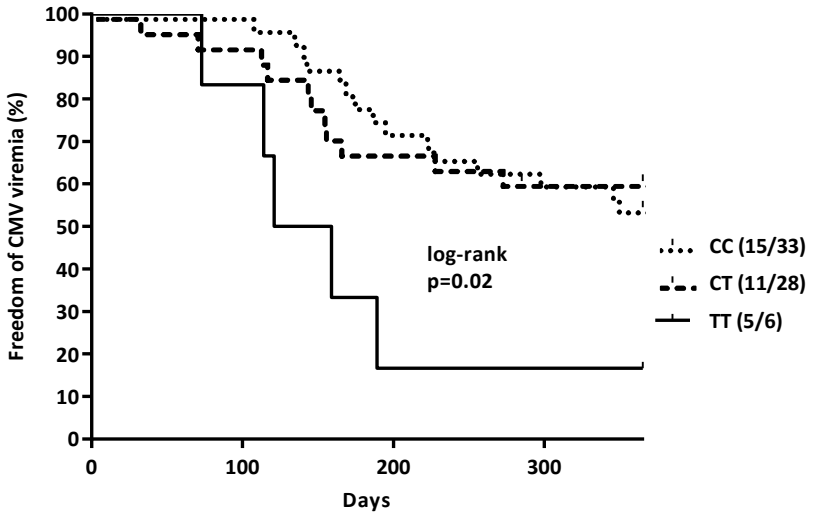


Figure 5-3: Incidence of CMV viremia in D+/R- patients according to CCL8 promoter SNP rs3138035 genotype. A Log-rank (Mantel-Cox) test was utilized to compare the Kaplan-Meier curves representing the freedom from CMV viremia during the first year post-transplant. It indicated a differential risk for D+/R- patients according to their rs3138035 genotypes, with those homozygous for the minor allele (TT) at increased risk of CMV viremia.



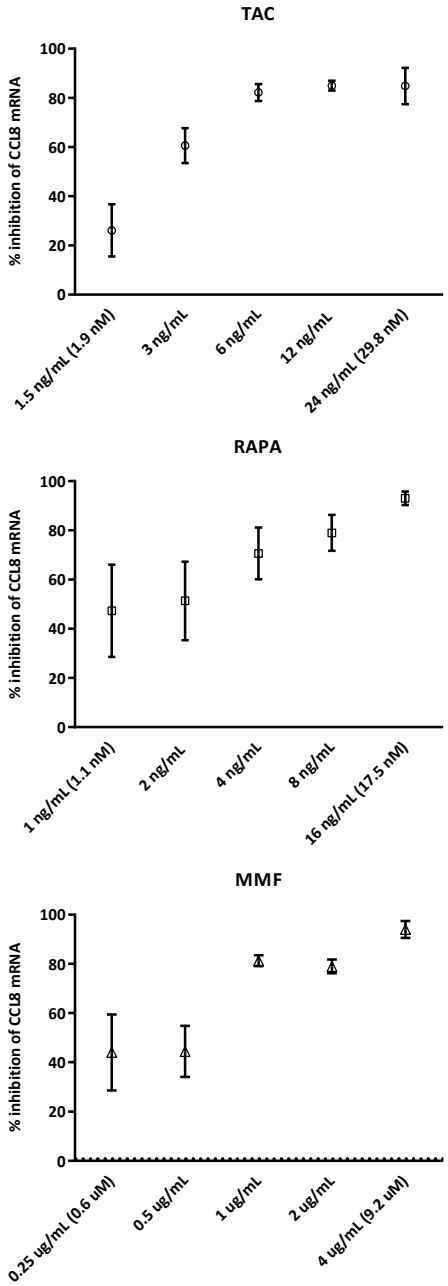
treated with and subjected to increasing concentrations of rapamycin, tacrolimus or mycophenolate were stimulated with a fixed IFN-γ dose. We observed a dose-dependent inhibitory effect on CCL8 mRNA expression (Figure 5-4) that was especially pronounced for tacrolimus, implicating these drugs in the regulation of monocyte chemokine responses.

DISCUSSION

The immune response against CMV is both complex and diverse. In the current study, we further characterize it by comprehensively profiling the expression of cytokines and chemokines following *ex vivo* CMV peptide stimulation. Beyond the established importance of T-cell responses as a biomarker for CMV outcomes in transplantation, we evaluate the relevance of other host immune cells and chemokines in the efficient immunologic control of CMV. In particular, we demonstrate that the monocytic chemokine CCL8 produced in response to *ex vivo* CMV peptide stimulation is associated with spontaneous viral clearance in patients with CMV viremia. We additionally show that a mutation in the promoter region of CCL8 is associated with an increased risk of CMV replication in high-risk D+/R- transplant patients. Finally, we demonstrate that monocytes are the main source of CCL8 production in response to CMV stimulation and that CCL8 expression is modulated by commonly used immunosuppressive drugs. Whilst very important as a predictor of CMV outcomes, the prototypic cytotoxic CD8+ T -cell response is only one part of a larger dynamic network of other relevant and interdependent immune cells, in which chemokines play an often underestimated role.

Aside from their role in leukocyte trafficking between bone marrow, bloodstream, secondary lymphoid organs, and tissues, chemokines are also key elements involved in T-helper cell polarization (174) and T -cell differentiation (175). CCL2, a chemokine with high homology to CCL8, aside from its role in promoting the influx of leukocytes in infected tissues (176, 177), is critical to both generation and survival of memory CD8 T-cells (176). CCL8 elicits chemotaxis of activated T-cells primarily through CCR5 (178), a receptor present in CMV-specific CD4+ T-cell subsets (179), including central memory and effector/memory phenotypes (180). Amongst CD8+ T-cells, CCR5 expression is highest in naïve cells (181), likely favoring cross-priming by monocytes (182), and is up-regulated in clonally proliferating antigen-experienced

Figure 5-4: Effect of immunosuppressive drugs on IFN-γ-induced expression of the CCL8 transcript. CCL8 transcript expression was measured in undifferentiated monocytic THP-1 cells pre-incubated with serial dilutions of the immunosuppressive drugs tacrolimus (TAC), mycophenolate mofetil (MMF) or rapamycin (RAPA) and stimulated with a fixed dose of recombinant human IFN-γ. Relative expression levels were obtained by comparison to IFN-γ-stimulated cells not treated with any of the drugs. Mean ± SEM are representative of four independent experiments.



CD8+ T-cells in response to their CMV cognate ligands (183). Monocyte expression of CCL8 in response to IFN-γ in a milieu comprised of other collaborating chemokines and cytokines would be aimed at priming naïve CD8 T-cells and differentiating central memory into effector/memory CD8+ T-cells, thereby promoting an integrated and efficient antiviral response. CMV, however, is very proficient at evading the immune response of the host, and the multitude of virally-mediated mechanisms targeting chemokines further corroborates their central role in achieving virologic control. For example, during lytic infection the viral IE-2 protein blocks the expression of chemokines CCL8, CCL5, CCL3 and IL-8 by CMV-infected fibroblasts (93). Conversely, CMV hijacks the host chemokine system of latently-infected CD34+ progenitor cells to attract CCR5+ CD4+ T-cells via increased expression of CCL8, only to suppress their effector function with two other components of its secretome, TGF-β and IL-10 (184). The immune control of viral replication would therefore be contingent on overcoming the viral maneuvers that otherwise subvert the host chemokine system.

Another layer of regulation of CCL8 expression is determined by therapeutic immunosuppression, at both transcriptional and post-transcriptional levels. CCL8 expression depends on transcription factor phosporylation by the mitogen-activated protein kinases ERK 1/2 and p38 (185), which occurs following IFN-γ stimulation in monocytic THP-1 cells (186). This can be inhibited by tacrolimus (187) and mycophenolic acid (188). Post-transcriptional regulation of IFN-γ-induced transcript stability is also dependent on such enzymes (189, 190), with evidence indicating a faster decay of mRNA transcripts in association with calcineurin inhibitors (191) and rapamycin (192). We observed a dose-dependent decrease in CCL8 gene expression in THP-1 cells pre-treated with tacrolimus, mycophenolate mofetil or rapamycin, which is most likely consequent to the interaction between these two mechanisms, and indicates that the effects of therapeutic immune suppression go beyond their intended T- and B-cell targets, also affecting chemokine expression by antigen-presenting cells.

Connecting the *ex vivo* and *in vivo* importance of CCL8 in CMV infection outcomes is the association between the promoter SNP rs3138035 and susceptibility to CMV viremia in D+/R- patients. This single-nucleotide polymorphism (C/T) also has been shown to be associated with clinical outcomes in non-small cell lung cancer (193). The minor allele (T) frequency is lowest in Africans (8%) and Asians (9%), intermediate in Americans (22%) and highest in Europeans

(40%) (1000 Genomes Project phase 1 (194). This SNP is flanked upstream by the transcriptional insulator CTCF, which is itself up-regulated by CMV infection and plays a repressor role in CMV major immediate promoter gene expression (195). CTCF is responsive to IFN-γ (196), and may be destabilized by downstream promoter sequences (197). It is therefore likely that the rs3138035 TT genotype exhibits distinct responses to both CTCF-mediated CMV repression and IFN-γ-triggered CCL8 induction in monocytes, culminating in reduced CCL8 expression levels. This may explain the CCL8 SNP association with CMV viremia seen in our patients. Additional data suggestive of the relevance of CCL8 to CMV outcomes are the polymorphisms associated with its preferred receptor, CCR5. In recipients of allogeneic stem cell transplants, CCR5 promoter SNPs rs2734648 and rs1800023 were associated with an increased risk of CMV disease (198). Further, rs1800023 was associated with high CMV viral loads in tracheal aspirates of non-immunosuppressed critically ill patients with CMV reactivation (199).

One important potential implication of our data is the utilization of CCL8 as an immunoadjuvant in CMV vaccines, with the goal of concurrently triggering robust adaptive cellular and humoral responses. CMV vaccine candidates reaching phase 2 clinical trials have achieved some success in stimulating either cellular or humoral responses, but not both simultaneously (166). Chemokines have been explored as a new class of vaccine adjuvant, and have already been tested in animal models for hepatitis C (200), malaria (201) and HIV (202, 203). This strategy has already been specifically validated for a CCR5-targeting chemokine-antigen fusion DNA vaccine in mice, which resulted in enhanced CD4+ T-cell, CD8+ T-cell, and B-cell responses (204). Theoretically, intradermal co-delivery of immunoadjuvant CCL8 and CMV antigen-coding DNA plasmids should result in priming of naïve and induction of memory Th1 CD4+ T-cells by CD1a+ dendritic cells (205), simultaneously yielding Langerhans cells capable of attracting naïve CD8+ T-cells to secondary lymph nodes for priming (206, 207). To this end, priming efficiency for CMV-specific CD8+ T-cell clones may potentially increase when dendritic cells expressing both CCL8 and CMV antigens (208) recruit CCR5+ helper polyclonal CD8+ T-cells. Memory CD8+ T-cells could also arise from such interactions with dendritic cells, contingent on help from CD4+ T-cells (209). CCR5 is also expressed by naïve B-cells (210, 211), thereby allowing for their interaction with CD14+ dermal dendritic cells expressing CMV antigens and

CCL8 for B-cell priming (212). Immunization of CMV-seropositive individuals may be especially challenging, given the multiple viral immune evasion mechanisms expressed in infected cells. CCL8 is spared by the scavenging activity of virally-encoded chemokine receptors in CMV-infected cells (213), while guiding naïve CD8+ CCR5+ T-cell priming by dendritic cells in lieu of virus-infected macrophages in draining lymph nodes (207).

In summary, our findings suggest that the chemokine CCL8 is important to controlling CMV replication in transplant recipients. This knowledge could potentially be translated into personalized monitoring and prophylactic strategies for patients at increased risk for CMV replication, and contribute to the improvement of candidate CMV vaccines.

CHAPTER 6: Hcmv-miR-UL22A-5p: a Biomarker in Transplantation with Broad Impact on Host Gene Expression and Potential Immunological Implications

INTRODUCTION

Cytomegalovirus (CMV) infection remains a common problem after organ transplantation despite considerable progress achieved with preventive strategies. Viral replication and disease occurring after completion of antiviral prophylaxis (214, 215) or therapy (216) still pose significant challenges in the care of this patient population. Current strategies to predict the risk of CMV infection rely on pre-transplant donor and recipient serostatuses and post-transplant viral load monitoring. With the goal of closing the existing gaps in CMV prevention, alternate biomarkers are being increasingly explored. Notably, host antiviral cell-mediated immune responses have been clinically explored in several contexts, including pre-transplant CMV risk assessment (163, 217), individualization of the duration of antiviral prophylaxis (147), and CMV disease risk stratification in the pre-emptive setting (165).

In its interaction with the host, viral factors are important determinants in the pathogenesis of CMV infection. For example, the viral genome encodes a number of immune evasion proteins that subvert the host immune response (218). In addition, factors such as viral strain variation (219), and the development of antiviral resistance may influence the development of CMV disease following transplantation. An improved understanding of how viral factors may contribute to the pathogenesis of CMV infection may uncover new biomarkers and therapeutic targets. Recently, multiple microRNAs (miRNAs) of viral origin have been described (98-100, 220). MiRNAs are small, non-coding RNA species involved in the post-transcriptional regulation of gene expression. CMV miRNAs have been implicated in the regulation of viral replication (102-104, 221-223), immune modulation (224, 225) and immune evasion (105, 226, 227). *In vivo* evidence of the link between CMV miRNAs and disease processes is just now emerging, with the description of hcmv-miR-UL112-3p as a biomarker of essential hypertension (228). The clinical relevance of viral miRNAs in the setting of CMV reactivation following transplantation has not been previously described.

In the present study, we assess the *in vivo* expression of CMV miRNAs in solid organ transplant recipients, and examine their potential as predictors of clinical and virological endpoints. Based

on these findings, we further investigate the regulation of host gene expression by the virus through its miRNA using a combination of experimental and bioinformatic approaches. Our findings suggest an *in vivo* functional role of a viral miRNA on the modulation of a major host transcriptional factor, with potential implications for viral pathogenesis.

MATERIALS AND METHODS

Patient Population **–** We first assessed CMV miRNA expression in transplant recipients with CMV viremia. The study population consisted of solid organ recipients with symptomatic CMV disease participating in a multicenter randomized controlled trial (229). Patients were diagnosed with CMV disease and treated with either ganciclovir or valganciclovir for 21 days, followed by 28 days of valganciclovir maintenance therapy. All patients with detectable CMV viral loads (≥ 600 copies/mL of plasma) at the time of enrollment were considered eligible. Whole blood samples obtained at the start of antiviral therapy were utilized (n=245). All patients provided informed consent for participation in the primary study (ClinicalTrials.gov identifier NCT00431353). The miRNA sub-study was approved by the Scientific Advisory Board of the Oslo University Hospital and by the Oslo Regional Ethics Committee (S-04011) (Oslo, Norway).

MiRNA Real-Time Quantitative PCR **–** Whole blood samples stored at -80ºC were thawed and placed in RNAlater solution overnight prior to total RNA extraction using the RiboPure-Blood kit (both from Life Technologies). Early Access Custom TaqMan Small RNA assays were designed by Applied Biosystems based on sequences from miRBase v11.0. Assays were optimized such that low or no background levels (Ct values > 40) were observed when testing samples of CMV-seronegative individuals. The assay was performed as per the manufacturer's protocol. Total RNA (10 ng per sample/assay) was reverse-transcribed using miRNA-specific stem-loop reverse-transcription primers (Life Technologies) (230). Quantitation was achieved through the use of standard curves created by sequential dilution of synthetic single-stranded RNA oligos (Integrated DNA Technologies). Results were reported as copies per µg of total RNA.

MiRNA Mimic Transient Transfection **–** Confluent MRC-5 lung fibroblasts (ATCC CCL-171) were released from the plate with Trypsin-EDTA and washed in EMEM containing 10% FBS (GIBCO). Cells re-suspended in complete medium were seeded on a pre-incubated mixture of

hcmv-miR-UL22A-5p miScript mimic or AllStars Negative Control siRNA (both Qiagen) and lipofectamine RNAiMAX (Life Technologies) in serum-free medium. AllStars Hs Cell Death Control siRNA (Qiagen) was utilized as a transfection control. The final concentration of all double-stranded RNA oligonucleotides was 50 nM.

RNA-Induced Silencing Complex Immunoprecipitation – Immunoprecipitation of the Argonaute-2 (AGO2) protein was performed using the Magna RIP kit with the RIPAb+ AGO2 monoclonal antibody (Millipore). Previously (48 h) transfected cells were washed, scraped and pelleted in cold PBS before lysis in RIP buffer containing protease and RNase inhibitors (Millipore). Half of each lysate was immunoprecipitated overnight at 4°C, while the remainder was stored at -20ºC. RNA extraction was performed manually with miRNeasy mini kit (Qiagen). RNA integrity and mRNA content were assessed with Agilent Bioanalyzer assays (Agilent). A Gene Expression 4x72K Hs18.0 array (Roche NimbleGen) containing 24,000 different 60-mer probes, each in triplicate, was utilized. Microarray processing was performed at the Alberta Transplant Applied Genomics Centre (University of Alberta, Edmonton, AB, Canada). Briefly, 200 ng of total RNA or 25 ng of immunoprecipitated RNA were used for preparation of cDNA libraries, subject to unbiased linear pre-amplification over 17 PCR cycles (WTA2 kit, Sigma Aldrich, St. Louis, MO, USA), prior to Cy3-labeling (One-Color DNA Labeling kit, Roche Nimblegen). These were hybridized onto the microarray slides and scanned on a MS200 Microarray scanner (Roche Nimblegen). Data extraction was performed using Nimblescan software (Roche Nimblegen), and arrays were summarized by the Robust Multi-array Analysis algorithm. For a given gene, the ratio between gene probe intensities in the immunoprecipitated and the non-immunoprecipitated portions of a sample was calculated first, and the ratio of the ratios obtained for samples transfected with hcmv-miR-UL22A-5p or with a scrambled sequence oligo determined the relative association of that gene with AGO2.

Proteomics – Previously (48 h) transfected cells were washed, scraped and pelleted in cold PBS before lysis in a RIP buffer (Millipore) containing a protease inhibitor cocktail. Lysates were centrifuged and supernatants were utilized for quantitative proteomics, performed at the University of Victoria Genome BC Proteomics Centre (Victoria, BC, Canada). Protein concentrations were determined using BCA protein assay (Sigma). Samples (100 µg each) were precipitated overnight in acetone at 4°C followed by resolubilization in 0.5M TEAB, 0.2% SDS.

Proteins were reduced with TCEP and alkylated with MMTS, followed by digestion with trypsin (Promega) and labeling with the appropriate isobaric tag label (hcmv-miR-UL22A-5p label 114; miR-NC label 116). Labeled peptides were then combined and separated by strong cation exchange HPLC. Fractions were reduced in volume by speed-vac and analyzed by LC-MS/MS. The length of the reverse gradient used was 2 hours per HPLC strong cation exchange fraction. Samples were analyzed by reversed-phase nanoflow (300 nL/min) HPLC with nano-electrospray ionization using a quadrupole time-of-flight mass spectrometer (QStar pulsar I, Applied Biosystems) operated in positive ion mode. A drill-down strategy similar to (231) was utilized to increase the depth of identification of peptides and proteins. Exclusion lists were created from the first analytical round of data, and applied as a filter for re-analysis of the remaining sample. All data were analyzed using Protein Pilot Software 4.0.8085 with Paragon Algorithm 4.0.0.0, 148083. Raw files were searched against the Homo sapiens UniprotKB database released in May2011.

Luciferase Reporter Assay – Cloning was performed by GeneCopoeia (Rockville, MD) using vectors encoding secreted alkaline phosphatase (SEAP) controlled by the CMV MIE promoter for normalization purposes. Segments of BMPR2 (NM_001204.6) 3'UTR flanking conserved binding sites were cloned downstream of Gaussia luciferase CDS under the control of the SV40 promoter (vector pEZX-MT05). MRC-5 cells were reverse-transfected with miRNA mimics as described above, followed by transfection of reporter plasmid 24 hours later, using Endofectin-Plus (Genecopoeia). Culture supernatant was harvested at the times indicated and stored at -20ºC until analyzed. Luciferase activity was measured using the Secrete Pair Gaussia Luciferase Assay kit (Genecopoeia) and normalized with alkaline phosphatase using the Phospha-Light System (Life Technologies).

Bioinformatics – TargetScan Human Custom release 5.2 (232) was used to identify conserved canonical binding sites for the hcmv-miR-UL22A-5p seed region (nt 2-8). MRNA-miRNA interactions were predicted using RNAhybrid (233) with sequences obtained from the UCSC Table Browser (234). Upstream regulator analysis based on curated experimental data was performed using IPA (Ingenuity® Systems). Pathway enrichment analysis was performed using DAVID Bioinformatics Resources v6.7 with the human genome as the background (235).

Western Blot – Cell lysis was performed in plate with RIPA buffer (1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, PMSF, sodium orthovanadate and protease inhibitor in TBS). Whole cell lysates were sonicated and quantified by Micro BCA Protein assay (Pierce Thermo Scientific). Prior to C-MYC detection, 50 µg of lysate were resolved by discontinuous (4.0/8.0%) SDS-PAGE, transferred onto nitrocellulose membrane, blocked in 5% non-fat milk and incubated overnight at 4°C with C-MYC antibody clone 9E10 (sc-40, Santa Cruz Biotechnology) 1:500. C-MYC was detected using an HPR-conjugated secondary antibody 1:10,000 (sc-2055, Santa Cruz Biotechnology) and film exposure.

Statistical Analyses – Fisher's Exact Test, the Mann-Whitney U Test, and Spearman's rho (all two-tailed) were used to analyze categorical data, continuous data, and correlations, respectively. the Forward Stepwise (Conditional) method was used to perform the multivariate logistic regression with probabilities for stepwise entry and removal of 0.05 and 0.10, respectively, and a classification cut-off of 0.5 with a maximum of 20 iterations, including the constant in the model.

RESULTS

Viral miRNAs are detectable in the blood of patients with CMV disease – The *in vivo* expression of CMV miRNAs during clinical infection post-transplant has not been previously assessed. We tested blood samples of a large cohort (n=245) of organ transplant patients with CMV disease. Nine CMV miRNAs were analyzed in samples collected at the onset of antiviral therapy (day 0). CMV miRNAs were readily detectable upon diagnosis of CMV disease among the majority of patients (Table 6-1). Overall, 91.8% of the samples contained at least one viral miRNA, with hcmv-miR-UL112-3p and hcmv-miR-UL22A-5p detected in over 70% of the patients. Significant variation in the number of miRNA copies was observed, over a 3-$\log_{10}$ range.

Direct, yet not universal correlations between viral miRNA levels and DNAemia – The CMV miRNAs US25-1(102), US25-2-5p, US25-2-3p (221) and UL112-3p (102) all have been proposed to inhibit viral replication *in vitro*. Therefore, we analyzed whether quantitative miRNA levels correlated with concurrent DNA viral load. Viremia levels in these patients varied (median 18,900, range 645 – 750,000 copies/mL). Inverse correlations between DNA

Table 6-1: CMV miRNA detection in blood samples at the onset of CMV disease. Detection and quantitation of CMV miRNAs against a synthetic oligonucleotide standard curve is represented (n=245). Results are expressed in $\log_{10}$ copies/microgram of total RNA.

miRNAs	**US5-1**	**US-5-2-3p**	**UL-22A-5p**	**US25-1-5p**	**US-25-2-3p**	**US-25-2-5p**	**US-33-3p**	**UL36-5p**	**UL112-3p**
% detected	18.4	19.2	73.5	45.7	30.2	43.7	27.8	41.6	73.9
Median*	3.81	3.76	4.46	4.35	4.05	3.87	3.91	4.18	4.31
Min*	3.49	3.37	3.45	3.46	3.61	3.21	3.48	3.61	3.63
Max*	4.88	5.33	6.98	6.76	5.98	5.54	5.76	5.92	5.39

**** in log_{10} copies/microgram of total RNA***

and miRNA levels would therefore corroborate the cell culture findings. Unexpectedly, we found that the majority of viral miRNAs had positive statistically significant correlations ($p<0.001$ for all comparisons) with CMV DNA levels (miRNAs: US5-2-3p, Spearman's rho 0.261; UL22A-5p, rho 0.629; US25-1-5p, rho 0.622; US25-2-3p, rho 0.621; US25-2-5p, rho 0.615; US33-3p, rho 0.441; and UL36-5p, rho 0.515). Levels of miRNAs US5-1 (Spearman's rho 0.022, $p=0.732$) and UL112-3p (rho 0.125, $p=0.051$) showed no significant correlation with DNA viral load.

Hcmv-miRNAs and viral kinetics – Next, we examined the association of viral miRNAs with the kinetics of viral replication in response to therapy. All patients were monitored with viral load testing at regular intervals for evidence of clinical recurrence. Detection of viral miRNAs at baseline, with the exception of miRNAs US5-1 and UL112-3p, was significantly associated with failure to clear viremia by the end of therapy (day 21) (Table 6-2). These associations, however, were not significant once adjusted for baseline viral load. Importantly, none of the viral miRNAs was associated with more rapid clearance of viremia. In fact the opposite could be observed for hcmv-miR-UL36-5p; levels at baseline were positively correlated with viral DNA half-life during the first week of antiviral treatment (Spearman's rho 0.228, $p=0.001$). By the completion of antiviral therapy on day 49, 174 patients achieved complete clearance of viremia (<600 copies/mL of plasma), comprising the population in which recurrence of viremia was studied. Follow-up for the next 6 months revealed recurrent viremia in 62/174 (35.6%) patients. We then analyzed whether baseline miRNA detection was associated with virologic recurrence. Recurrent viremia was significantly associated with detection of miRNAs UL22A-5p, US25-2-5p and UL36-5p at baseline (Table 6-3). Once adjusted for concurrent CMV DNA levels, only detection of hcmv-miR-UL22A-5p at the beginning of antiviral therapy was independently predictive of subsequent viral replication after its discontinuation (odds ratio 3.024, 95% CI 1.35 – 6.8, $p=0.007$).

Table 6-2: CMV miRNA detection at diagnosis of CMV disease and persistent viremia (≥ 600 copies/mL) by day 21 of antiviral treatment. A two-tailed Fisher's Exact test was utilized to examine the predictability of persistent viremia after 3 weeks of antiviral therapy, based on detection of CMV miRNAs at initiation of treatment (n=232). P-values < 0.05 were considered statistically significant.

	viremic (%) Day 21		
miRNA	**miRNA Detected**	**miRNA Not detected**	**p-value**
US5-1	18/44 (40.9)	73/188 (38.8)	0.864
US5-2-3p	24/45 (53.3)	67/187 (35.8)	0.041
UL22A-5p	78/169 (46.2)	13/63 (20.6)	<0.001
US25-1-5p	61/104 (58.7)	30/128 (23.4)	<0.001
US25-2-3p	45/65 (69.2)	46/167 (27.5)	<0.001
US25-2-5p	60/101 (59.4)	31/131 (23.7)	<0.001
US33-3p	36/63 (57.1)	55/169 (32.5)	0.001
UL36-5p	56/95 (58.9)	35/137 (25.5)	<0.001
UL112-3p	65/169 (38.5)	26/63 (41.3)	0.763

Table 6-3: CMV miRNA detection at diagnosis of CMV disease and virologic recurrence within 6 months. Two-tailed Fisher's Exact or Mann-Whitney U tests were utilized to describe the predictability of virologic recurrence within 6 months of discontinuation of antiviral therapy in patients with undetectable viremia at day 49, according to detection of CMV miRNAs at the beginning of therapy (n=174). A multivariate logistic regression model included variables with p-values < 0.05 in univariate analyses. P-values < 0.05 were considered statistically significant. OR: odds ratio; CI: confidence interval.

	virologic recurrence (%) within 6 months			Multivariate		
Day 0 miRNA	**Detected**	**Not detected**	**p-value**	**OR**	**95% CI**	**p-value**
US5-1	15/33 (45.5)	47/141 (33.3)	0.227			
US5-2-3p	16/34 (47.1)	46/140 (32.9)	0.162			
UL22A-5p	53/127 (41.7)	9/47 (19.1)	0.007	3.022	1.35 – 6.8	0.007
US25-1-5p	32/73 (43.8)	30/101 (29.7)	0.077			
US25-2-3p	21/46 (45.7)	41/128 (32)	0.109			
US25-2-5p	32/70 (45.7)	30/104 (28.8)	0.025			0.177
US33-3p	19/45 (42.2)	43/129 (33.3)	0.285			
UL36-5p	34/72 (47.2)	28/102 (27.5)	0.010			0.091
UL112-3p	48/128 (37.5)	14/46 (30.4)	0.474			
	Yes	**No**	**p-value**			
Baseline Viral Load (in copies/mL)	27,575 (6,462 – 107,375)	12,675 (2,550 – 53,375)	0.010			0.316

RISC-associated mRNAs contain binding sites for hcmv-miR-UL22A-5p with regulatory potential – Given the strong association with recurrent viremia, we assessed potential targets of hcmv-miR-UL22A-5p. Mature miRNAs typically target mRNA 3'UTRs sequences complementary to their "seed region" (nt 2-8; i.e., canonical interaction), thereby inhibiting protein translation. This process involves the participation of the protein Argonaute-2 (AGO2) in the RNA-induced silencing complex (RISC). One tool for experimental determination of miRNA-mRNA associations is the profile of mRNAs associated with RISC upon expression of a given miRNA. We immunoprecipitated AGO2 of cells transfected with an hcmv-miR-UL22A-5p mimic or with a negative control oligonucleotide, and compared the relative association of mRNA transcripts with RISC to their overall abundance in the cells, using microarrays. Compared to the negative control, hcmv-miR-UL22A-5p had a 2-fold or higher association of mRNA transcripts with AGO2 for 7,418/24,000 microarray probes, corresponding to 6,764 unique genes (Figure 6-1A). Non-conserved, non-canonical interactions within 3'UTR, the coding sequence, or 5'UTR could be predicted for the vast majority of transcripts. Conserved putative binding sites allowing for canonical mRNA to hcmv-miR-UL22A-5p interactions were present in the 3'UTR of only 33 genes. We confirmed the regulatory potential of hcmv-miR-UL22A-5p in luciferase reporter assays, using two distinct sites present on the 3'UTR of the bone morphogenetic protein receptor type II (BMPR2) (Figure 6-1B), the mRNA transcript amongst those containing conserved binding sites with the highest AGO2 enrichment (9.75-fold higher upon hcmv-miR-UL22A-5p transfection). Levels of luciferase activity were significantly down-regulated for a site containing an 8mer match encompassing the miRNA seed region ($p<0.001$) (Figures 6-1C and 6-1D).

Hcmv-miR-UL22A-5p regulates proteins involved in antigen presentation and the transcription factor C-MYC – Intrigued by the multitude of enriched mRNA targets in association with the RISC machinery and by the paucity of conserved 3'UTR sites for canonical mRNA-miRNA interactions, we proceeded to investigate the effects of hcmv-miR-UL22A-5p at the protein level (see *Materials and Methods*). In total, 971 proteins were identified with a confidence threshold of 95%. Significant changes in protein levels were observed for 25 proteins, with a decrease in 13 and, unexpectedly, an increase in 12 proteins upon transfection of hcmv-miR-UL22A-5p (Table 6-4).

Figure 6-1A: Distribution of gene probe association with Argonaute-2 upon hcmv-miR-UL22A-5p transfection, expressed relatively to that determined by transfection of a scrambled sequence negative control oligonucleotide (i.e., fold-change). Positive enrichment values denote preferential association with RISC upon hcmv-miR-UL22A-5p transfection. AGO2: argonaute-2; NC: negative control.

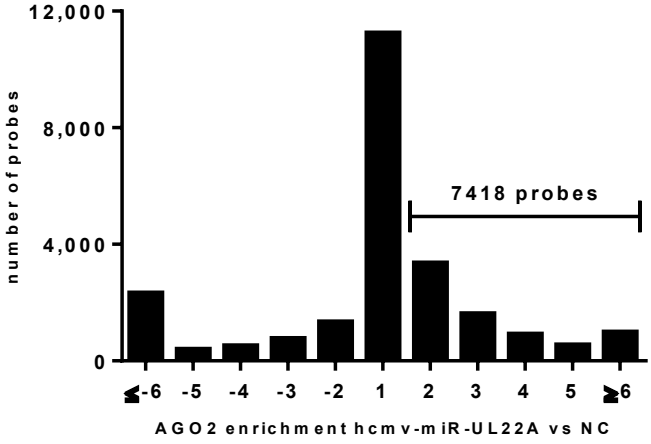


Figure 6-1B: Conservation of canonical binding sites (highlighted) for the seed-region of hcmv-miR-UL22A-5p present in the 3'UTR of the BMPR2 mRNA. Hsa: Homo sapiens; Ptr: Pan troglodytes (chimpanzee); Mml: Macaca mulata (rhesus); Mmu: Mus musculus (mouse) Rno: Rattus norvegicus (rat).

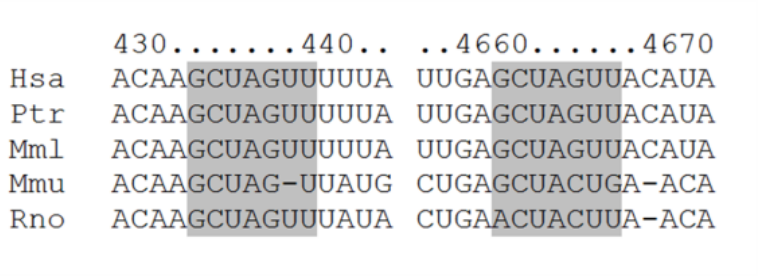


Figure 6-1C: Predicted miRNA-mRNA interactions between hcmv-miR-UL22A-5p and BMPR2 conserved canonical binding sites (highlighted). MUT: mutated.

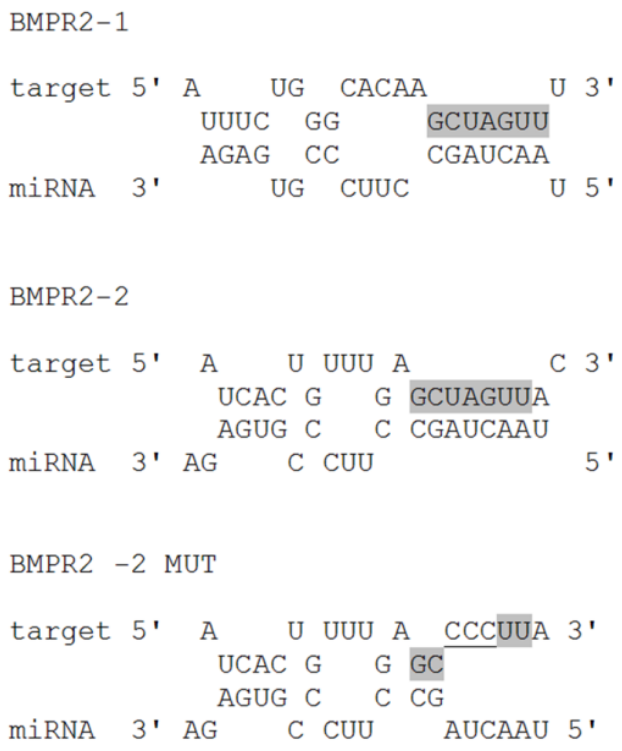
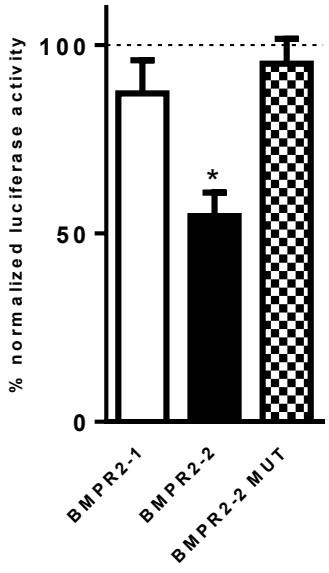


Figure 6-1D: Luciferase reporter assays for the sites of BMPR2. Activity of luciferase in cells transfected with hcmv-miR-UL22A-5p normalized to that of cells transfected with negative control oligonucleotide. * p-value < 0.001 for comparison with negative control.



Interestingly, pathway enrichment analysis suggested the involvement of the affected proteins HSP90AB1, PDIA3, HSPA5, CANX and HSPA8 in antigen processing and presentation (KEGG pathway hsa04612; FDR-corrected p=0.047). Computational miRNA target prediction suggested that only a single protein – alpha enolase (ENO1) – has a canonical binding site on its 3'UTR, potentially explaining its down-regulation. We then hypothesized that transcriptional regulators also may have been targeted. An upstream regulator analysis revealed the experimentally validated association between the transcription factor C-MYC and 13/25 significantly changed proteins (Table 6-5). Accordingly, transfection of hcmv-miR-UL22A-5p clearly modified the expression pattern of the C-MYC protein in lung fibroblasts, with the truncated transcription-repressive isoform c-myc-s predominating at the cost of reduced expression of the transactivating isoforms c-myc-1/2 (Figure 6-2A). Multiple putative non-canonical binding sites could be predicted, with the most thermodynamically favorable mRNA-miRNA interactions situated on the 5'UTR and on the coding sequence unique to the large isoforms c-myc-1/2, upstream of the translation initiation codon of c-myc-s (Figure 6-2B).

DISCUSSION

The assessment of host and pathogen miRNA responses is an exciting area of investigation, both for improving our understanding of viral pathogenesis, and for developing biomarkers and therapeutic targets (236, 237). This is especially true in CMV infection after transplantation, where the complex interaction between viral and host factors contribute to pathogenesis, and where current preventive and treatment strategies have been only partially successful. In this study, we provided novel evidence of *in vivo* viral miRNA expression in transplant recipients with CMV disease, probing it in the context of viral kinetics. We found no evidence for inhibitory effects on viral replication, but did observe a novel association between a viral miRNA and recurrence of CMV viremia after initial treatment, suggesting a potential immunoregulatory role. We provided further *in vitro* data to support broad regulatory potential over the host gene expression. These data corroborate the existence of other yet unexplored roles for CMV miRNAs in viral pathogenesis and as biomarkers for clinical and virological outcomes following transplantation.

Table 6-4: Statistically significant protein level changes upon hcmv-miR-UL22A-5p transfection. FC: fold-change, relative to levels in cells transfected with negative control oligonucleotide.

UniProtKB Accession	Gene	Protein Name	FC	p-value
Q5T7J1	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	-8.47	0.026
A8K4W6	PGK1	Phosphoglycerate kinase 1	-4.17	0.022
A8K486	PPIA	Peptidyl-prolyl cis-trans isomerase	-3.70	0.016
P11142	HSPA8	Heat shock cognate 71 kDa protein	-2.70	0.035
P06733	ENO1	Alpha-enolase	-2.36	0.002
B4E2U0	PGD	6-phosphogluconate dehydrogenase, decarboxylating	-2.13	0.045
Q53YD7	EEF1G	EEF1G protein	-2.11	0.017
B4DG39	GPI	Glucose-6-phosphate isomerase	-2.01	0.039
P08238	HSP90AB1	Heat shock protein HSP 90-beta	-2.00	0.023
P13639	EEF2	Elongation factor 2	-1.79	0.022
P08758	ANXA5	Annexin A5	-1.79	0.027
B7ZAT2	CCT2	T-complex protein 1 subunit beta	-1.74	0.033
Q5U077	LDHB	L-lactate dehydrogenase	-1.39	0.048
P30101	PDIA3	Protein disulfide-isomerase A3	1.45	0.020
P11021	HSPA5	78 kDa glucose-regulated protein	1.53	0.022
P02452	COL1A1	Collagen alpha-1(I) chain	1.57	0.027
B4DJ30		cDNA FLJ61290, highly similar to Neutral alpha-glucosidase AB	1.69	0.048
P08123	COL1A2	Collagen alpha-2(I) chain	1.72	0.014
P07237	P4HB	Protein disulfide-isomerase	1.79	0.007
Q8TB01	CKAP4	Similar to cytoskeleton-associated protein 4	2.09	<0.001
P50454	SERPINH1	Serpin H1	2.21	<0.001
B4DGP8	CANX	Calnexin	2.44	0.043
A6NG51	SPTAN1	Spectrin alpha chain, non-erythrocytic 1	2.78	0.042
P05161	ISG15	Ubiquitin-like protein ISG15	3.31	0.014
Q60FE2	MYH9	Myosin-9	4.29	<0.001

Table 6-5: Transcription factor analysis for proteins significantly affected by hcmv-miR-UL22A-5p transfection.

Transcriptional Regulator	p-value of overlap	Target molecules in the dataset
MYC	2.76E-11	ANXA5,CANX,COL1A1,COL1A2,EEF2,ENO1,GPI,IFIT1,LDHB, PGK1,PPIA,SERPINH1
HSF1	2.39E-08	CCT2,HSP90AB1,HSPA8,PGK1,SERPINH1,SPTAN1
HTT	3.66E-08	CANX,EEF2,GPI,HSP90AB1,HSPA5,HSPA8,LDHB,SERPINH1, SPTAN1
MYCN	2.93E-07	CKAP4,COL1A1,EEF1G,EEF2,HSP90AB1,MYH9
CREB3L1	6.76E-07	COL1A1,COL1A2,HSPA5
SP3	2.38E-06	COL1A1,COL1A2,HSPA5,PGK1,SERPINH1
CEBPA	2.77E-06	CKAP4,COL1A1,COL1A2,HSPA5,ISG15,PGD
TP53	7.69E-06	COL1A1,COL1A2,GPI,HSP90AB1,HSPA8,ISG15,MYH9,P4HB, SERPINH1
JUNB	7.74E-06	COL1A1,COL1A2,EEF2,SPTAN1
HIF1A	2.54E-05	ENO1,GPI,HSPA5,PGK1,PPIA

Figure 6-2A: Expression of C-MYC protein isoforms in human fibroblasts transfected with hcmv-miR-UL22A-5p (left lane) or negative control oligonucleotide (right lane). Beta-tubulin was utilized as loading control.

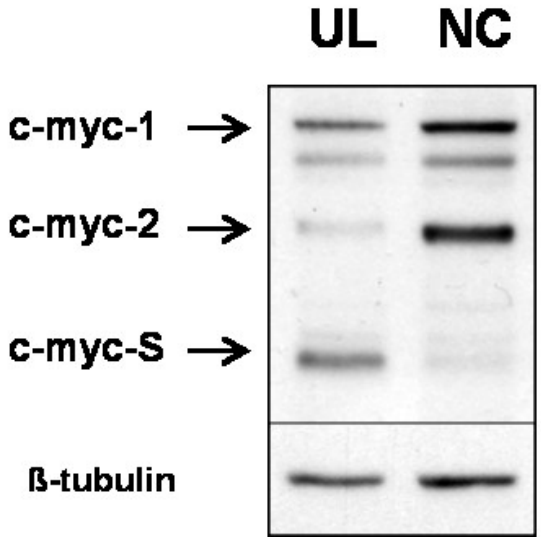
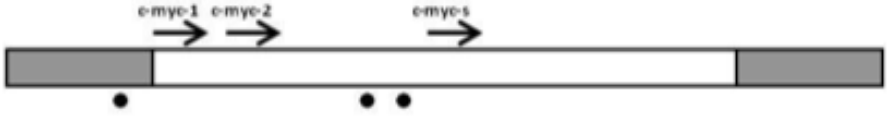


Figure 6-2B: Predicted C-MYC binding sites for hcmv-miR-UL22A. Top – C-MYC transcript representation annotated with initiation codons for protein isoforms (arrows) and predicted hcmv-miR-UL22A-5p binding sites (dots). Bottom – Predicted miRNA-mRNA interactions between hcmv-miR-UL22A-5p and the most thermodynamically favorable C-MYC binding sites. MiRNA seed-region is highlighted. 5'UTR: 5-prime untranslated region; CDS: coding sequence.



MYC 5'UTR

```
target 5' C    CCGAC            U    3'
           UCUC     GCGGGGAGGCUA
           AGAG     UGCCCUUCCGAU
miRNA  3'                      CAAU 5
```

MYC CDS-1

```
target 5' U    CCUU      GACAACGAC       G  3'
           UCUC    CGGGGA          GGC GGU
           AGAG    GCCCUU          CCG UCA
miRNA  3'      U                     A   AU 5'
```

MYC CDS-2

```
target 5' U   C     CC  CCA     A   3'
           UCU CACGG  GA   GCUGG
           AGA GUGCC  CU   CGAUC
miRNA  3'               UC      AAU 5'
```

Consistent with *in vitro* data assessing miRNA expression in lytic CMV infection models, several viral miRNAs were readily detectable in patients with CMV disease. Hcmv-miR-UL22A-5p was the second-most consistently detected CMV miRNA amongst the miRNAs tested. It is expressed during lytic infection (when it is one of the most abundant miRNAs of viral origin) (220, 238), but not during latency (239). It is unclear why miRNAs were not detected in certain patients. Possibly, viral miRNAs are not uniformly expressed in all clinical strains, or during all stages of clinical infection. Mutations generating variability in viral miRNA sequences (224) have not yet been described for CMV miRNAs, with multiple lab-adapted human cytomegalovirus strains showing high (100%) sequence conservation for hcmv-miR-UL22A-5p (99). Sequence variability in clinical strains may be unlikely, and yet could be a factor limiting detection of certain miRNAs using highly specific TaqMan probes (240).

In vitro, several CMV miRNAs have been found to down-regulate viral replication in fibroblasts miR-US25-1 (102), miR-US25-2-3p (221), miR-US33 (241) and miR-UL112 (103). If a biologically relevant *in vivo* consequence of viral miRNA expression was inhibition of viral replication, an effect on viral kinetics in patients with CMV infection could then be observed. Our data did not support an inhibitory effect of any measured viral miRNA. Instead, for the majority of viral miRNAs, detection and quantitation were directly associated with concurrent DNA viral load. In addition, no viral miRNA was associated with faster or an increased likelihood of viral clearance. We also found that higher levels of hcmv-miR-UL36-5p anticipated a longer half-life (i.e., slower decay) of CMV viremia early in the course of antiviral therapy. Interestingly, this miRNA has been shown to possess proviral properties when evaluated in an *in vitro* culture model (222). Overall, our findings suggest that *in vivo*, these miRNAs may not have an inhibitory effect on viral replication, or alternately, that this effect may be largely outweighed by opposing forces of other host and viral factors.

Viral miRNAs also may play a potential role in immune evasion and immune modulation. for example, it has been suggested that hcmv-miR-UL112-3p regulates the antiviral interferon-responsive gene IRF-1 (228) and the stress-induced ligand MICB (105). Albeit the correlation of *in vitro* findings with clinically relevant immunologic evasion is largely unknown, it can be inferred by analysing viral kinetics in relation to putative viral immune evasion products. This was the first study to attempt this for CMV miRNAs, and we demonstrated among a large cohort

of patients that hcmv-miR-UL22A-5p detection at the start of antiviral therapy is independently associated with recurrence of viremia, even after adjustment for total viral load. This is particularly intriguing, since no apparent beneficial or detrimental effects of this miRNA on CMV replication have been demonstrated in fibroblast cell lines (241, 242). Therefore, we attempted to discern how hcmv-miR-UL22A-5p might be related to immune evasion. Utilizing a proteomics approach, we found that a number of the proteins affected by hcmv-miR-UL22A-5p belong to the family of heat-shock proteins, known to activate antigen-presenting cells and to promote antigen presentation (243). It is possible that hcmv-miR-UL22A-5p may interfere with activation and expansion of virus-specific T-cells, thereby impairing the immune containment of infection. This process may have even persisted during the ganciclovir-based therapy received by these patients, given that the expression of CMV miRNAs is not affected by foscarnet, another viral DNA polymerase inhibitor (100). Further evaluation would be needed to confirm this potential role.

An additional implication of our findings lies in CMV's ability to utilize monocytes and macrophages as vehicles for its own dissemination to tissue compartments (244). Primary CMV infection of monocytes has been shown to induce a pro-inflammatory M1 macrophage phenotype (87), which is proposed to drive infected monocytes from blood into tissue. This is seen despite transactivation of the C-MYC promoter by the viral immediate-early proteins IE1 and IE2 (87, 245), which would otherwise drive the alternative polarization of these cells into the anti-inflammatory M2 phenotype. (246). Based on our data, we propose that hcmv-miR-UL22A-5p inhibits the translation of the transactivating isoforms of C-MYC, c-myc-1 and c-myc-2, while favoring the expression of its repressive isoform c-myc-s. This would modulate the IE1/IE2-initiated MYC-dependent alternative polarization, and contribute towards the establishment of a favorable M1 macrophage phenotype for viral dissemination. Although herein demonstrated at the protein level, the exact target within C-MYC has yet to be determined. Recently, an additional pathway for alternative polarization to the M2 macrophage phenotype involving BMPR2 as the receptor for the bone morphogenetic protein-7 (BMP-7) was characterized (247). The regulatory interaction between hcmv-miR-UL22A-5p and BMPR2 described here provides an additional mechanism facilitating viral dissemination through maintenance of the inflammatory M1 macrophage phenotype.

In summary, we have provided the first evidence that CMV miRNAs are detectable in transplant recipients experiencing symptomatic viral replication. We have performed a unique *in vivo* correlation of miRNA expression with viral kinetic parameters, and have provided clinical evidence for potential immune evasion effects associated with viral miRNA expression. Finally, we have shown evidence for their significant role regulating host gene expression, which warrants further exploration in order to understand the pathogenesis of CMV and identify biomarkers for clinical disease post-transplantation.

CHAPTER 7: Conclusion

The research studies that comprise this thesis contribute to an exciting and evolving field in which translational approaches to CMV management post-transplant are being evaluated more carefully in order to improve outcomes and decrease the burden associated with this infection in specific clinical settings. The contributions of the present body of work include (i) highlighting the limitations of current prediction and prevention strategies, (ii) evaluating standard molecular diagnostics, and (iii) evaluating novel host and pathogen immunodiagnostic strategies.

Antiviral treatment of CMV replication episodes, both symptomatic (i.e., CMV disease) and asymptomatic (i.e., CMV viremia), has traditionally followed the "treat until negative" paradigm. For cases in which a CMV protein (e.g., the pp65 antigenemia assay) or its nucleic acid are monitored in the blood, this strategy translates into administering an appropriate antiviral drug to the patient at least until the viral protein or DNA are no longer detectable, usually upon repeated testing. The purpose of this therapeutic approach is to decrease the rates of virologic and clinical recurrence of infection after the antiviral agent is discontinued. While this approach may have been clinically successful with the first generation of commercially available CMV antigenemia and competitive PCR monitoring assays, newer technologies such as real-time PCR have resulted in significantly enhanced detection thresholds. While in some instances increased test sensitivity is highly desirable (e.g., detection of cancerous cells), in others it may lead to over-diagnosis and/or over-treatment. In Chapter 2, the findings of our study corroborate the latter scenario, suggesting that increased sensitivity for the assessment of therapeutic response may not translate into reduced recurrence rates. Although limited by its non-interventional nature, our study provides a valuable clinical cautionary note of the potential to excessively prolong antiviral therapy when ultra-sensitive assays are used to determine response.

Several centers employ in-house PCR assays for economic and academic reasons. Multiple combinations of PCR platforms (e.g., plate vs. capillary, DNA-binding dyes vs. fluorescent reporter probes), test matrices (e.g., serum vs. plasma vs. whole blood vs. leukocytes) and quantitation standards (e.g., in-house plasmids vs. titered virus) such as the ones reported in Chapter 2 result in assays with very distinct performances (131). Therefore, a proper comparison of preventative and therapeutic strategies against CMV infection involving the use of molecular

monitoring as well as reproducibility of successful approaches is greatly limited. In 2010, the World Health Organization released a universal standard for quantification of CMV; to date, clinical laboratories are still transitioning to reporting quantitative CMV DNA results in International Units per mL. As new data based on WHO standard calibrated assays emerge, researchers are beginning to revisit the clinical correlates of viral clearance in therapeutic response (248) and viral replication kinetics in pre-emptive antiviral treatment (249). Guidelines for prevention and management of CMV infection in transplantation are likely to be affected, since reproducible data may soon be available that will inform new evidence-based CMV viremia thresholds for the start and discontinuation of antiviral therapy.

Nonetheless, molecular monitoring of CMV has inherent limitations that are unlikely to be overcome by the use of a common reference quantitation standard. In Chapter 3, the challenges of preventing CMV viremia and disease solely by using quantitative PCR as a predictive monitoring tool are illustrated. Antiviral drug prophylaxis has become the standard of care for most patients at risk for CMV infection post-transplant (250), given its significant impact in reducing the incidence of CMV disease and CMV-associated mortality (251) and possibly also in protecting against the indirect effects of infection on the allograft (252). Its benefits may be increased by extending its duration beyond the typical first 100 days post-transplant, although at the cost of increased toxicity (144). The incidence of CMV infection and disease in transplant recipients remains significant despite antiviral prophylaxis, with 30% of D+/R- kidney recipients in one trial still experiencing infection and/or disease in the first year post-transplant after discontinuation of prophylaxis (253). Pre-emptive antiviral treatment is an acceptable alternative for prevention of CMV disease, being associated with a significantly lower incidence of leukopenia compared to antiviral prophylaxis (254).

Our center adopts a hybrid approach for prevention of CMV disease in CMV-naïve transplant recipients of CMV-infected organs; this includes initial antiviral drug prophylaxis followed by molecular surveillance of viral replication. Failure to prevent CMV disease with weekly monitoring of CMV DNAemia was a finding of our study which has been corroborated by others (135). Perhaps the most important inference from our data is the individual nature of the risk of CMV replication, which is not adequately predicted by grouping patients according to pre-transplant donor and recipient CMV serostatuses. Amongst "high-risk" CMV D+/R-

patients given antiviral prophylaxis, many who developed viremia underwent spontaneous clearance, while those at imminent risk of CMV disease could not be identified in a timely manner. Refinements to the CMV serostatus-based risk stratification standards that permit a more rational allocation of prophylaxis and molecular monitoring resources are clearly needed.

For D+/R- patients, preventative strategies can be potentially tailored according to the development of CMV-specific T-cell responses. QuantiFERON-CMV performed at the end of antiviral prophylaxis further qualified the risk of CMV disease within the first year post-transplant (164). Patients with a positive test are at very low risk of CMV disease and potentially may be managed with standard pre-emptive treatment, whereas those with a negative or indeterminant test may benefit from prolonged prophylaxis or pre-emptive treatment initiated at lower cut-offs; CMV-seropositive organ recipients receiving CMV-seropositive organs may also be at risk for CMV disease. Given the relatively high incidence of CMV viremia/disease among D+/R+ patients (255) (who are typically managed with pre-emptive therapy), some authors have suggested extending antiviral prophylaxis to this group (256). For CMV-seropositive organ recipients, individualized preventative strategies may be devised pre-transplant, with the assessment of virus-specific T-cell responses further segregating the post-transplant risk of viral replication and disease (69, 163, 217). CMV-specific T-cell testing may also be informative when asymptomatic viral replication is detected in CMV R+ patients by determining a need for antiviral therapy. The data in Chapter 4 support this concept, with the QuantiFERON CMV test predicting immune containment of viral replication among a population of patients composed predominantly of CMV-seropositive individuals. A few individuals in our study subsequently developed symptoms despite positive IFN-gamma responses. This might be explained by the host's genetic makeup, since an association between a host genotype with high levels of IFN-γ production and increased risk of CMV disease has been reported among lung transplant recipients (257). Indeed, additional opportunities to individualize CMV risk stratification in transplantation include characterizing the genetic determinants of a host's immune responses against the virus. Our group has found a specific risk attributable to IL-28B polymorphisms (258), whereas data in Chapter 5 reveal a novel association between a polymorphism in the promoter of CCL8 and increased incidence of CMV replication.

CMV-specific T-cell generation and expansion seems to be tightly connected to cells from the monocytic lineage, as suggested by the associations between monocyte-derived CCL8 and the CMV outcomes measured in the populations reported in Chapter 5. Differentiating from monocytes upon their exit from the blood and entry into the peripheral tissues, macrophages may contribute to the expansion of CMV-specific T-cells through antigen presentation (259). Monocytic cells, however, are also able to support CMV reactivation *in vivo,* as exemplified by myeloid dendritic cells (260). Given the *in vivo* expression of CMV miRNA UL22A upon lytic infection and its putative role in the regulation of the major host transcription factor MYC, as collectively suggested by the data in Chapter 6, it would be very interesting to investigate if this miRNA targeting of host transcripts in monocytic cells negatively affects CMV cell-mediated immunity.

Another fascinating opportunity for CMV miRNA research lies in exploiting the viral miRNAome to promote viral reactivation from latency in the presence of antiviral drugs such as ganciclovir or foscarnet, which are active only against actively replicating virus. Such an approach could potentially eliminate infected cells, analogous to the cytolytic activation strategies being developed to combat EBV-positive lymphomas (261-264). Hcmv-miR-UL36 has been shown to target the latency determinant viral gene UL138 (222). Exogenous overexpression of miR-UL36 may help trigger reactivation from latency, making the virus susceptible to the action of currently available antiviral drugs targeting the viral DNA polymerase. This could potentially translate into a treatment to eliminate CMV -infected cells from allografts during *ex vivo* perfusion. Coupled with inhibitors of viral miRNAs involved in immune evasion, this strategy may allow for enhanced immunologic clearance of CMV. Hcmv-miR-US4-1, for example, targets ERAP1, impairing CMV peptide presentation on MHC-I to CD8+ T-cells (226). Inhibition of hcmv-miR-UL112, which contributes to immune evasion by targeting the NK cell ligand MICB (227) and IL-32 (265), may also contribute to increased immune elimination of infected cells, with the additional effect of countering the targeting of antiviral proteins BclAF1 (266) and IRF1 (228).

After decades of very meaningful progress that has enabled the expansion of transplantation as a first-line therapy for organ failure, the field is moving towards tailoring CMV prevention and management strategies to individual needs. Categorization based on donor and recipient CMV

serostatuses and PCR-based molecular monitoring offers a framework for management. Moving forward, personalization of CMV preventative and therapeutic strategies by incorporating virus-specific, cell-mediated immunity and genetic risk markers may further limit the burden caused by this infection.

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Table S1-1: Definitions of CMV disease in solid organ transplant recipients. Reproduced under license (number 3491521143469) from Humar A, Michaels M. American Society of Transplantation recommendations for screening, monitoring and reporting of infectious complications in immunosuppression trials in recipients of organ transplantation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2006;6(2):262-74.

Table 2: Definitions of CMV disease in solid organ transplant recipients

Disease type	Probable	Definite
CMV syndrome	One or more of the following: 1. Fever >38°C for at least 2 days 2. New or increased malaise 3. Leukopenia 4. ≥5% atypical lymphocytes 5. Thrombocytopenia 6. Elevation of hepatic transaminases (ALT or AST) to 2 × upper limit of normal (applicable to nonliver transplant recipients) plus evidence of CMV in blood by viral culture, antigenemia or a DNA/RNA-based assay	Clinical and laboratory findings as in 'probable' case and no other cause of symptoms/signs identified
Pneumonia[1]	Signs and/or symptoms of pulmonary disease in the absence of other documented cause plus evidence of CMV in blood and/or[3] bronchoalveolar lavage (BAL) fluid by viral culture, antigenemia or a DNA/RNA-based assay	Signs and/or symptoms of pulmonary disease plus detection of CMV in lung tissue by culture, immunohistochemical analysis or *in situ* hybridization[4] with or without evidence of CMV in blood or BAL fluid by viral culture, antigenemia (BAL) or a DNA/RNA-based assay
Gastrointestinal disease	Symptoms of upper or lower gastrointestinal disease plus macroscopic mucosal lesions on endoscopy plus evidence of CMV in blood or biopsy tissue by viral culture, antigenemia or an RNA/DNA-based assay	Symptoms or signs of upper or lower gastrointestinal disease plus detection of CMV in gastrointestinal tissue by culture, immunohistochemical analysis or *in situ* hybridization[4]
Hepatitis	Elevation of bilirubin and/or hepatic enzymes in the absence of other documented cause of hepatitis[2] plus evidence of CMV in blood by anti-genemia or a DNR/RNA-based assay	Elevation of bilirubin and/or hepatic enzymes plus detection of CMV in liver tissue by culture, immunohistochemical analysis or *in situ* hybridization[4]
CNS disease	CNS symptoms in the absence of other documented cause plus evidence for CMV in CSF samples by viral culture or DNA-based assay	CNS symptoms plus detection of CMV in CNS tissue by culture, immuno-histochemical analysis or *in situ* hybridization[4]
Retinitis	Not applicable	Lesions typical of CMV retinitis must be confirmed by an ophthalmologist
Other tissue invasive disease (nephritis, cystitis, myocarditis, pancreatitis, etc.)	Evidence of organ dysfunction in the absence of other documented cause[2] plus evidence of CMV in blood by viral culture, antigenemia or DNA/RNA-based assay	Symptoms/signs of organ dysfunction plus detection of CMV in affected tissue by culture, immunohistochemical analysis or *in situ* hybridization[4]

[1]Superinfection or coinfection with other pathogens may occur and should be noted when present.
[2]If affected organ is the allograft, acute rejection must be excluded as a cause for the clinical symptoms.
[3]The detection of CMV in both BAL and peripheral blood strengthens the evidence for probable CMV pneumonitis.
[4]Although, immunohistochemistry and *in situ* hybridization techniques are more sensitive for the detection of CMV-infected cells than morphologic examination, the presence of typical cytomegalovirus inclusions should be considered evidence of definite disease.

Table S5-1: Quantitation yields of cytokines and chemokines within the linear range of detection in QuantiFERON CMV and NIL samples from 37 CMV-viremic transplant recipients. The number of NIL and CMV samples in which a given cytokine/chemokine was detected within the linear quantitation range of the assay is summarized. Total number of patients n=37.

Protein	Gene Symbol	Gene Name	n quantitated	
			NIL	CMV
6Ckine	CCL21	chemokine (C-C motif) ligand 21	32	29
BCA-1	CXCL13	chemokine (C-X-C motif) ligand 13	37	37
CD40LG	CD40LG	CD40 ligand	37	37
CTACK	CCL27	chemokine (C-C motif) ligand 27	37	37
EGF	EGF	epidermal growth factor	36	36
ENA-78	CXCL5	chemokine (C-X-C motif) ligand 5	37	37
Eotaxin	CCL11	chemokine (C-C motif) ligand 11	37	37
Eotaxin-2	CCL24	chemokine (C-C motif) ligand 24	37	37
Eotaxin-3	CCL26	chemokine (C-C motif) ligand 26	9	9
FGF-2	FGF2	fibroblast growth factor 2 (basic)	37	37
Flt-3 ligand	FLT3LG	fms-related tyrosine kinase 3 ligand	37	37
Fractalkine	CX3CL1	chemokine (C-X3-C motif) ligand 1	37	37
G-CSF	CSF3	colony stimulating factor 3 (granulocyte)	37	37
GM-CSF	CSF2	colony stimulating factor 2 (granulocyte-macrophage)	37	37
GRO	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	37	37
I-309	CCL1	chemokine (C-C motif) ligand 1	37	37
IFNalpha2	IFNA2	interferon, alpha 2	36	37
IFNγ	IFNG	interferon, gamma	37	37
IL-10	IL10	interleukin 10	36	37
IL-12(40p)	IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	37	37
IL-12(70p)	IL12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	36	35
IL-13	IL13	interleukin 13	37	24
IL-15	IL15	interleukin 15	37	37
IL-16	IL16	interleukin 16	37	37
IL17	IL17A	interleukin 17A	36	37
IL-1alpha	IL1A	interleukin 1, alpha	30	37
IL-1beta	IL1B	interleukin 1, beta	37	37
IL-1ra	IL1R1	interleukin 1 receptor, type I	37	37
IL-2	IL2	interleukin 2	37	37
IL-20	IL20	interleukin 20	20	26
IL-21	IL21	interleukin 21	3	8
IL-23	IL23A	interleukin 23, alpha subunit p19	30	33
IL-28A	IL28A	interleukin 28A (interferon, lambda 2)	18	24
IL-3	IL3	interleukin 3 (colony-stimulating factor, multiple)	0	2
IL-33	IL33	interleukin 33	35	36
IL-4	IL4	interleukin 4	35	36
IL-5	IL5	interleukin 5 (colony-stimulating factor, eosinophil)	26	37
IL-6	IL6	interleukin 6 (interferon, beta 2)	37	37
IL-7	IL7	interleukin 7	36	37
IL-8	IL8	interleukin 8	37	37
IL-9	IL9	interleukin 9	17	9
IP-10	CXCL10	chemokine (C-X-C motif) ligand 10	37	36
LIF	LIF	leukemia inhibitory factor	31	30
MCP-1	CCL2	chemokine (C-C motif) ligand 2	37	37
MCP-2	CCL8	chemokine (C-C motif) ligand 8	36	37
MCP-3	CCL7	chemokine (C-C motif) ligand 7	37	37
MCP-4	CCL13	chemokine (C-C motif) ligand 13	37	37
MDC	CCL22	chemokine (C-C motif) ligand 22	37	37
MIP-1alpha	CCL3	chemokine (C-C motif) ligand 3	26	36
MIP-1beta	CCL4	chemokine (C-C motif) ligand 4	36	37
MIP-1delta	CCL15	chemokine (C-C motif) ligand 15	37	37
PDGF-AA	PDGFA	platelet-derived growth factor alpha polypeptide	37	37
PDGF-AB/BB	PDGFB	platelet-derived growth factor beta polypeptide	37	37
RANTES	CCL5	chemokine (C-C motif) ligand 5	37	37
SCF	KITLG	KIT ligand	37	37
SDF-1alpha+beta	CXCL12	chemokine (C-X-C motif) ligand 12	32	33
sIL-2Ralpha	IL2RA	interleukin 2 receptor, alpha	37	37
TARC	CCL17	chemokine (C-C motif) ligand 17	37	37
TGFalpha	TGFA	transforming growth factor, alpha	37	37
TNFalpha	TNF	tumor necrosis factor	37	37
TNFbeta	LTA	lymphotoxin alpha (TNF superfamily, member 1)	33	37
TPO	THPO	Thrombopoietin	37	37
TRAIL	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	37	37
TSLIP	TSLP	thymic stromal lymphopoietin	30	32
VEGF	VEGFA	vascular endothelial growth factor A	37	36

Table S5-2: Overall cytokine and chemokine responses of 37 CMV-viremic transplant recipients after whole blood incubation in QuantiFERON CMV and NIL tubes. A two-tailed paired Wilcoxon signed-rank test was utilized to describe the overall behavior of cytokines and chemokines upon CMV epitope stimulation in the samples of 37 CMV-viremic transplant recipients. Values are given in pg/mL. $P < 0.05$ was considered statistically significant.

	Decreased Expression Upon CMV Peptide Stimulation						Increased Expression Upon CMV Peptide Stimulation				
	NIL		CMV				NIL		CMV		
	Median	IQR	Median	IQR	p-value		Median	IQR	Median	IQR	p-value
IL-8	8460.94	6394.45-9131.62	3971.77	2541.51-4396.51	.000	CCL8	27.26	20.54-54.67	161.73	41.30-375.21	.000
CCL3	3406.64	1791.26-10000	778.68	335.58-2682.63	.000	EGF	33.20	19.14-62.77	57.83	32.40-111.42	.000
VEGF	87.74	74.09-123.63	59.26	39.97-80.33	.000	CXCL10	1734.08	936.53-3938.04	4179.61	2268.67-8584.38	.000
IL-6	1084.58	326.77-2092.07	412.83	93.47-873.97	.000	IL-1ra	43.40	24.76-71.64	64.20	39.37-100.44	.001
CSF2	69.34	49.77-148.15	23.41	15.07-69.48	.000	KITLG	19.86	12.97-34.36	23.11	15.78-37.25	.001
CXCL1	1988.19	1453.54-3205.97	1512.23	1061.34-2174.99	.000	CCL5	1270.06	811.32-2111.62	2075.16	1183.09-3111.19	.002
CCL7	5267.09	2114.39-9443.41	3034.62	1973.63-4244.45	.000	TNF-β	4.12	1.41-8.08	5.44	3.60-11.48	.002
IL-1β	885.56	202.01-1992.1	384.30	111.63-874.48	.001	IFN-γ	21.21	15.42-36.39	41.93	17.41-295.75	.006
TNF-α	129.17	37.06-414.9	46.76	22.93-163.87	.001	TRAIL	35.26	21.94-43.77	45.21	32.13-59.53	.008
IL-16	243.69	132.60-444.95	178.68	120.35-307.11	.004	IL-4	6.42	4.01-15.55	10.39	4.67-19.65	.010
CCL22	432.13	196.97-677.59	309.04	159.45-681.28	.004	FGF-2	42.08	30.63-53.01	46.66	34.44-65.73	.014
CSF3	109.31	39.11-338.77	40.64	15.09-149.38	.006	sIL-2R-α	22.05	10.70-37.14	27.19	16.84-46.53	.014
IFN-α2	27.77	21.17-40.69	20.27	13.42-29.85	.009	CCL13	30.56	13.03-44.55	33.26	21.81-45.47	.020
TPO	519.44	343.44-871.03	466.68	313.19-862.17	.021	CX3CL1	54.43	41.91-111.23	70.60	51.56-114.83	.021
CCL4	1165.38	569.78-2499.77	712.04	389.99-1433.77	.028	IL-2	6.30	2.73-16.31	8.48	5.47-16.56	.029

Table S5-3: Outcome-specific statistically significant cytokine and chemokine responses of 37 CMV-viremic transplant recipients after whole blood incubation in QuantiFERON CMV and NIL tubes. A two-tailed paired Wilcoxon signed-rank test was utilized to describe the outcome-specific behavior of individual cytokines and chemokines upon MHC-I-restricted CMV peptide stimulation in the samples of CMV-viremic patients who subsequently received antiviral therapy (n=8) and in patients with spontaneous clearance of viremia (i.e., "No antiviral"; n=29). Concentrations are given in pg/mL. $P < 0.05$ was considered significant.

	Antiviral (n=8)				No antiviral (n=29)			
	NIL	CMV	CMV/NIL	p-value	NIL	CMV	CMV/NIL	p-value
	median pg/ml (IQR)		median (IQR)		median pg/ml (IQR)		median (IQR)	
IL-8	5333.68 (2585.91 - 8640.82)	2648.01 (2178.59 - 4253.82)	0.71 (0.51 - 0.94)	.012	8744.02 (7226.04 - 9192.81)	4044.27 (2606.41 - 4411.38)	0.48 (0.44 - 0.60)	.000
CCL3	4884.83 (1266.59 - 10000.00)	863.13 (287.54 - 1878.65)	0.20 (0.14 - 0.37)	.012	3272.16 (1791.26 - 10000.00)	778.68 (374.26 - 2864.60)	0.27 (0.15 - 0.72)	.000
VEGF	90.48 (67.73 - 120.54)	51.29 (39.78 - 83.58)	0.70 (0.40 - 0.84)	.012	87.74 (77.64 - 129.43)	59.73 (43.92 - 80.33)	0.61 (0.42 - 0.77)	.000
GMCSF	114.87 (40.12 - 138.44)	23.09 (13.25 - 79.55)	0.49 (0.27 - 0.67)	.012	67.98 (49.77 - 150.77)	23.41 (15.07 - 60.28)	0.34 (0.27 - 0.67)	.002
IL-1β	763.17 (198.88 - 1831.89)	392.26 (132.83 - 1086.88)	0.84 (0.35 - 0.93)	.036	885.56 (203.87 - 2055.22)	384.30 (109.01 - 874.48)	0.53 (0.32 - 1.16)	.007
TNFα	184.64 (17.20 - 879.95)	26.94 (15.28 - 170.29)	0.56 (0.25 - 0.89)	.036	129.17 (42.79 - 379.21)	46.76 (31.56 - 163.87)	0.49 (0.28 - 1.05)	.010
CCL8	31.74 (21.15 - 54.86)	39.72 (21.57 - 86.28)	1.20 (1.00 - 1.51)	.161	26.45 (19.63 - 53.11)	224.51 (95.29 - 757.02)	5.62 (2.44 - 30.30)	.000
CXCL10	2701.94 (2099.87 - 3474.70)	2393.54 (1983.53 - 3340.87)	0.99 (0.91 - 1.04)	.674	1257.89 (872.66 - 4511.96)	5222.32 (3261.72 - 9728.95)	2.01 (1.14 - 6.61)	.000
IFNγ	25.74 (9.31 - 46.60)	25.15 (5.84 - 41.50)	0.84 (0.76 - 0.97)	.401	21.01 (15.42 - 33.12)	57.75 (20.98 - 634.35)	1.91 (0.83 - 9.34)	.002
TNFβ	5.43 (2.01 - 7.85)	5.27 (4.09 - 7.31)	1.26 (0.94 - 1.85)	.401	3.89 (1.22 - 10.70)	6.52 (3.18 - 12.41)	1.78 (1.05 - 4.28)	.004
EGF	34.57 (7.81 - 64.13)	34.98 (7.98 - 102.48)	1.08 (1.01 - 1.49)	.063	33.20 (20.63 - 60.98)	65.13 (37.14 - 125.05)	1.72 (1.30 - 2.34)	.000
IL-1ra	36.58 (10.82 - 71.38)	48.67 (21.53 - 69.92)	1.18 (1.06 - 1.99)	.263	43.40 (26.33 - 71.64)	65.21 (41.26 - 142.63)	1.66 (1.06 - 2.99)	.001
CCL5	1641.42 (918.77 - 2232.22)	1895.21 (744.38 - 3080.07)	1.12 (0.67 - 1.36)	.889	1270.06 (802.54 - 1928.61)	2075.16 (1222.87 - 3111.19)	1.56 (1.06 - 2.43)	.001
IL-4	3.09 (0.77 - 10.34)	3.86 (0.75 - 15.31)	0.96 (0.92 - 1.19)	.866	6.93 (5.21 - 17.80)	10.90 (6.98 - 21.31)	1.37 (0.95 - 1.99)	.008
IL-2	5.96 (2.29 - 13.35)	5.68 (2.77 - 15.44)	1.17 (0.95 - 1.42)	.674	6.46 (3.02 - 17.58)	8.90 (5.91 - 18.05)	1.34 (0.97 - 2.35)	.035
sIL-2Rα	21.99 (18.97 - 35.06)	21.64 (18.14 - 52.10)	0.91 (0.75 - 1.75)	.889	23.18 (10.07 - 37.85)	28.02 (15.22 - 46.53)	1.33 (0.99 - 1.60)	.003
CX3CL1	56.89 (26.43 - 102.33)	82.22 (45.45 - 102.92)	1.51 (1.12 - 1.65)	.674	54.43 (41.91 - 115.24)	68.61 (51.56 - 128.91)	1.32 (0.99 - 1.70)	.018
TRAIL	38.17 (24.79 - 39.73)	42.68 (34.40 - 58.64)	1.11 (0.90 - 1.54)	.208	33.12 (20.51 - 46.40)	45.21 (29.39 - 59.77)	1.18 (0.96 - 1.47)	.017
FGF2	41.15 (28.78 - 55.49)	41.80 (29.31 - 60.62)	1.26 (0.76 - 1.47)	.674	43.80 (31.01 - 53.01)	46.66 (35.20 - 71.07)	1.17 (1.00 - 1.41)	.005
CCL13	19.96 (7.78 - 34.82)	27.00 (6.37 - 37.15)	1.07 (0.84 - 1.64)	.401	31.48 (13.68 - 50.43)	34.12 (26.05 - 63.22)	1.14 (0.92 - 1.50)	.025
KITLG	18.06 (11.63 - 20.86)	17.25 (15.58 - 27.78)	1.11 (1.00 - 1.37)	.263	20.74 (13.59 - 40.20)	23.97 (16.51 - 45.70)	1.10 (1.01 - 1.28)	.003
IL-6	973.30 (240.45 - 2032.69)	421.04 (87.40 - 760.57)	0.68 (0.16 - 1.21)	.093	1321.23 (326.77 - 2092.07)	412.83 (93.47 - 890.40)	0.43 (0.24 - 0.98)	.000
GCSF	48.78 (20.59 - 426.35)	22.44 (12.73 - 60.57)	0.47 (0.17 - 0.87)	.050	112.10 (55.46 - 338.77)	43.98 (15.09 - 238.50)	0.50 (0.22 - 1.45)	.033
CCL7	3145.05 (510.31 - 8851.56)	2894.70 (361.16 - 3750.31)	0.61 (0.42 - 0.85)	.069	5410.73 (2187.07 - 9935.54)	3282.35 (2020.10 - 5035.57)	0.61 (0.41 - 1.01)	.001
CXCL1	1521.66 (1244.67 - 2408.39)	1407.49 (745.32 - 2091.08)	0.65 (0.60 - 1.04)	.161	2385.86 (1691.56 - 3316.54)	1665.04 (1080.37 - 2325.09)	0.71 (0.50 - 0.89)	.000
CCL22	271.91 (74.28 - 503.12)	194.65 (67.92 - 268.46)	0.70 (0.51 - 1.11)	.123	448.21 (237.85 - 892.90)	323.35 (176.75 - 877.99)	0.71 (0.61 - 0.94)	.019
IL-16	191.16 (99.23 - 663.65)	147.79 (87.87 - 225.22)	0.74 (0.62 - 0.90)	.093	247.77 (159.31 - 444.95)	205.41 (128.16 - 320.48)	0.73 (0.61 - 1.01)	.015
IFNα2	25.20 (19.47 - 28.72)	20.47 (14.50 - 25.75)	0.93 (0.74 - 1.02)	.093	28.61 (21.17 - 41.82)	20.27 (13.08 - 30.90)	0.76 (0.57 - 0.92)	.048
IL-28A	9.77 (5.03 - 9.77)	9.77 (5.31 - 9.77)	1.00 (1.00 - 1.28)	.593	9.77 (9.77 - 23.60)	9.77 (2.41 - 17.93)	0.86 (0.31 - 1.00)	.026
THPO	651.09 (379.47 - 838.82)	478.62 (329.20 - 1054.54)	0.85 (0.67 - 1.23)	.401	466.68 (343.44 - 937.05)	411.64 (309.60 - 751.42)	0.87 (0.72 - 1.05)	.023
CCL4	1064.62 (328.18 - 4955.99)	526.88 (119.62 - 897.75)	0.33 (0.23 - 0.69)	.012	1248.44 (695.58 - 2499.77)	799.29 (453.31 - 1829.69)	0.65 (0.48 - 1.17)	.230
CXCL12	922.66 (630.76 - 1369.24)	655.77 (305.85 - 954.53)	0.81 (0.65 - 0.90)	.028	804.62 (416.01 - 1208.46)	732.11 (380.01 - 998.98)	1.00 (0.70 - 1.19)	.677

Table S5-4: Outcome-specific non-statistically significant cytokine and chemokine responses of 37 CMV-viremic transplant recipients after whole blood incubation in QuantiFERON CMV and NIL tubes. A two-tailed paired Wilcoxon signed-rank test was utilized to describe the outcome-specific behavior of individual cytokines and chemokines upon MHC-I-restricted CMV peptide stimulation in the samples of CMV-viremic patients who subsequently received antiviral therapy (n=8) and in patients with spontaneous clearance of viremia (i.e., "No antiviral"; n=29). Concentrations are given in pg/mL. $P < 0.05$ was considered significant.

	Antiviral (n=8)				No antiviral (n=29)			
	NIL	CMV	CMV/NIL	p-value	NIL	CMV	CMV/NIL	p-value
	median pg/ml (IQR)		median (IQR)		median pg/ml (IQR)		median (IQR)	
IL-20	53.32 (48.83 - 73.13)	73.81 (48.83 - 130.64)	1.11 (1.00 - 1.35)	.075	48.83 (48.83 - 96.12)	69.37 (48.83 - 113.76)	1.00 (0.83 - 1.42)	.741
TGF-α	24.30 (12.94 - 27.56)	15.75 (12.29 - 20.50)	0.77 (0.59 - 1.05)	.123	22.83 (13.61 - 26.17)	18.39 (9.87 - 25.90)	0.88 (0.66 - 1.13)	.275
CCL2	1633.09 (1376.31 - 1908.13)	1560.40 (962.28 - 1953.22)	0.88 (0.81 - 0.99)	.123	1845.97 (1581.13 - 2052.68)	1993.98 (1554.79 - 2088.49)	1.04 (0.91 - 1.15)	.456
IL-5	0.50 (0.22 - 0.70)	0.35 (0.25 - 0.51)	0.71 (0.55 - 1.00)	.123	0.64 (0.45 - 0.95)	0.48 (0.34 - 1.21)	0.77 (0.64 - 1.52)	.665
CCL21	30.12 (19.53 - 63.37)	19.53 (13.34 - 32.79)	0.90 (0.47 - 1.12)	.128	48.43 (27.14 - 77.58)	34.17 (19.53 - 70.26)	0.90 (0.67 - 1.12)	.162
IL-7	7.75 (1.48 - 16.28)	3.93 (1.46 - 13.45)	0.78 (0.62 - 1.16)	.161	5.08 (30.2 - 7.85)	3.53 (2.49 - 7.14)	0.84 (0.62 - 1.31)	.275
IL-21	19.53 (19.53 - 19.53)	19.53 (6.51 - 19.53)	1.00 (0.84 - 1.00)	.180	19.53 (19.53 - 19.53)	19.53 (19.53 - 19.53)	1.00 (1.00 - 1.00)	.237
IL-12(p40)	53.30 (26.52 - 59.32)	38.90 (21.00 - 53.74)	0.90 (0.64 - 1.06)	.208	38.22 (23.91 - 60.36)	44.87 (28.35 - 61.05)	1.08 (0.83 - 1.45)	.315
IL-1α	30.66 (4.97 - 102.78)	24.16 (13.65 - 26.73)	0.89 (0.36 - 5.44)	.208	33.21 (6.82 - 83.36)	25.40 (18.29 - 55.93)	1.27 (0.75 - 2.11)	.387
LIF	19.53 (14.08 - 30.47)	19.53 (15.68 - 23.10)	0.84 (0.65 - 1.00)	.225	23.35 (12.04 - 38.21)	24.29 (17.06 - 36.50)	1.00 (0.75 - 1.30)	.840
CD40LG	241.71 (23.65 - 1469.48)	149.10 (43.79 - 429.84)	1.07 (0.61 - 2.24)	.263	75.35 (20.51 - 286.28)	73.07 (38.40 - 178.17)	1.10 (0.74 - 1.80)	.770
CCL1	1.12 (0.79 - 2.66)	1.75 (0.81 - 3.02)	1.16 (1.00 - 1.25)	.263	1.81 (0.92 - 4.59)	1.90 (1.15 - 4.38)	1.10 (0.88 - 1.23)	.846
TSLP	4.41 (2.44 - 9.30)	9.13 (4.21 - 9.77)	1.82 (0.83 - 3.62)	.326	5.56 (3.40 - 14.95)	4.17 (2.45 - 13.60)	0.87 (0.48 - 1.47)	.239
IL-10	21.65 (4.55 - 212.82)	16.77 (4.31 - 39.52)	0.86 (0.26 - 1.36)	.327	23.99 (10.81 - 79.31)	16.71 (8.75 - 69.98)	0.92 (0.46 - 1.55)	.567
IL-17	2.46 (0.92 - 3.83)	1.86 (1.34 - 4.63)	1.20 (0.77 - 2.05)	.327	2.61 (1.63 - 4.92)	3.35 (1.73 - 5.74)	1.13 (0.77 - 1.46)	.846
IL-9	0.72 (0.64 - 1.13)	0.64 (0.64 - 0.64)	0.99 (0.71 - 1.00)	.345	0.64 (0.64 - 0.74)	0.64 (0.64 - 0.64)	1.00 (1.00 - 1.18)	.421
CCL15	2378.21 (1610.33 - 3362.72)	2315.27 (1227.11 - 2775.59)	0.98 (0.65 - 1.04)	.401	2646.31 (1653.88 - 4182.80)	2519.57 (1666.30 - 3206.34)	0.89 (0.77 - 1.08)	.098
IL-13	1.42 (0.56 - 3.47)	0.64 (0.64 - 2.96)	0.95 (0.67 - 1.18)	.483	2.11 (0.97 - 7.05)	0.64 (0.47 - 14.07)	0.83 (0.28 - 1.57)	.673
CCL24	1346.09 (826.67 - 2125.28)	1665.88 (709.44 - 2935.51)	1.03 (0.77 - 1.72)	.484	1475.04 (624.20 - 2680.74)	1240.82 (667.14 - 3117.52)	1.06 (0.86 - 1.38)	.230
CXCL13	41.97 (22.71 - 86.32)	38.71 (20.20 - 100.17)	1.05 (0.86 - 1.51)	.484	28.75 (14.69 - 53.97)	34.80 (19.59 - 47.39)	0.99 (0.84 - 1.37)	.456
IL-12(p70)	4.78 (1.24 - 6.00)	4.69 (1.16 - 6.50)	1.02 (0.90 - 1.22)	.499	4.01 (2.66 - 8.22)	6.06 (3.84 - 8.22)	1.22 (0.85 - 1.97)	.094
PDGF-AB/BB	12192.80 (6940.24 - 14585.83)	12329.48 (5683.54 - 13821.34)	0.98 (0.90 - 1.06)	.575	11958.65 (7461.63 - 12924.87)	13944.95 (7462.97 - 15279.74)	1.12 (0.89 - 1.37)	.078
CCL11	435.74 (226.31 - 640.48)	578.58 (205.28 - 773.51)	1.00 (0.81 - 1.36)	.575	395.51 (251.16 - 606.25)	375.29 (239.82 - 587.88)	0.95 (0.80 - 1.13)	.133
CCL17	15.57 (11.99 - 33.66)	13.29 (11.31 - 39.82)	0.97 (0.82 - 1.06)	.575	30.07 (18.71 - 48.81)	27.32 (20.00 - 51.77)	0.99 (0.82 - 1.19)	.991
CCL26	48.83 (20.80 - 48.83)	48.83 (21.19 - 48.83)	1.00 (1.00 - 1.01)	.593	48.83 (48.83 - 48.83)	48.83 (48.83 - 48.83)	1.00 (1.00 - 1.00)	.612
PDGF-AA	778.93 (611.99 - 1145.10)	741.99 (534.71 - 1739.14)	0.85 (0.77 - 1.17)	.674	916.36 (629.00 - 1420.50)	936.91 (566.88 - 1327.94)	0.93 (0.73 - 1.15)	.198
FLT3LG	28.43 (19.58 - 53.93)	34.03 (21.77 - 56.71)	0.83 (0.76 - 1.21)	.674	31.25 (15.86 - 43.67)	25.77 (13.81 - 52.55)	0.97 (0.86 - 1.09)	.596
IL-15	11.26 (5.51 - 16.38)	10.14 (8.78 - 19.39)	0.96 (0.82 - 1.16)	.889	9.90 (5.88 - 20.14)	10.12 (7.77 - 19.92)	1.11 (0.88 - 1.45)	.325
IL-33	8.10 (3.29 - 22.44)	10.46 (2.03 - 21.46)	0.90 (0.55 - 1.50)	.889	9.36 (4.35 - 77.30)	9.64 (5.48 - 64.39)	1.13 (0.61 - 1.41)	.611
IL-3	0.64 (0.64 - 0.64)	0.64 (0.64 - 0.64)	1.00 (1.00 - 1.00)	1.000	0.64 (0.64 - 0.64)	0.64 (0.64 - 0.64)	1.00 (1.00 - 1.00)	.655
CXCL5	3456.61 (790.24 - 5143.23)	2428.61 (946.44 - 4018.59)	1.13 (0.70 - 1.93)	1.000	3888.70 (2675.99 - 5186.21)	2806.34 (1299.48 - 8123.89)	0.93 (0.45 - 1.70)	.754
CCL27	437.21 (385.12 - 547.16)	439.95 (324.82 - 623.48)	0.98 (0.82 - 1.17)	1.000	487.25 (378.42 - 544.18)	463.45 (358.51 - 601.14)	0.98 (0.86 - 1.19)	.905

Table S5-5: Characteristics of D+/R- patients according to CCL8 promoter SNP rs3138035 genotype. Two-tailed one-way ANOVA or independent sample Kruskal-Wallis tests were used to compare normally and non-normally distributed continuous data, respectively. A Two-tailed Chi-square test was used to compare categorical data. $P < 0.05$ was considered statistically significant.

	CC (n=33)	CT (n=28)	TT (n=6)	p-value
Age, y, mean (SD)	49.97 (13.8)	46.93 (14.71)	43.33 (17.42)	0.109
Sex, M/F, No.	23/10	22/6	5/1	0.640
Type of transplant				0.410
Kidney	19 (57.6)	11 (39.3)	1 (16.7)	
Kidney-pancreas	4 (12.1)	2 (7.1)	2 (33.3)	
Liver	5 (15.2)	8 (28.6)	2 (33.3)	
Lung	3 (9.1)	3 (10.7)	1 (16.7)	
Heart	0	3 (10.7)	0	
Other	2 (6)	1 (3.6)	0	
Antiviral prophylaxis				
IV GCV	5 (15.2)	5 (17.9)	0	0.591
VGC	32 (97)	27 (96.4)	6 (100)	0.897
OGCV	1 (3)	1 (3.6)	0	0.897
Duration, d, median (IQR)	98 (90-131)	98 (98-143)	139 (90-232)	0.507
Induction therapy				0.008
None	1 (3)	1 (3.6)	2 (33.3)	
Basiliximab	16 (48.5)	20 (71.4)	1 (16.7)	
Thymoglobulin	16 (48.5)	7 (25)	3 (50)	
Maintenance				
Steroids	29 (87.9)	23 (82.1)	5 (83.3)	0.815
Tacrolimus	28 (84.8)	25 (89.3)	5 (83.3)	0.854
Cyclosporin	3 (9.1)	3 (10.7)	0	0.706
MMF/MPA	28 (84.8)	25 (89.3)	5 (83.3)	0.854
Azathioprine	1 (3)	0	0	0.593
mTOR inhibitors	3 (9.1)	1 (3.6)	1 (16.7)	0.478
Other	1 (3)	0	0	0.593
QTF at end of prophylaxis				0.489
Positive	3 (10)	3 (13.6)	2 (33.3)	
Negative	21 (70)	14 (63.6)	2 (33.3)	
Indeterminate	6 (20)	5 (22.7)	2 (33.3)	

Figure S5-1: Hierarchical gating strategy for fluorescence-activated cell sorting of lymphocytes. Cells with forward (FSC-A) and side scatter (SSC-A) characteristics compatible with lymphocyte populations were initially selected (top left). Single (top right and middle left) live (middle right) cells were then separated according to expression of the markers CD19 (B -cells) (bottom left), CD3+/CD4+ (T -helper) and CD3+/CD8+ (T -cytotoxic) (bottom right).

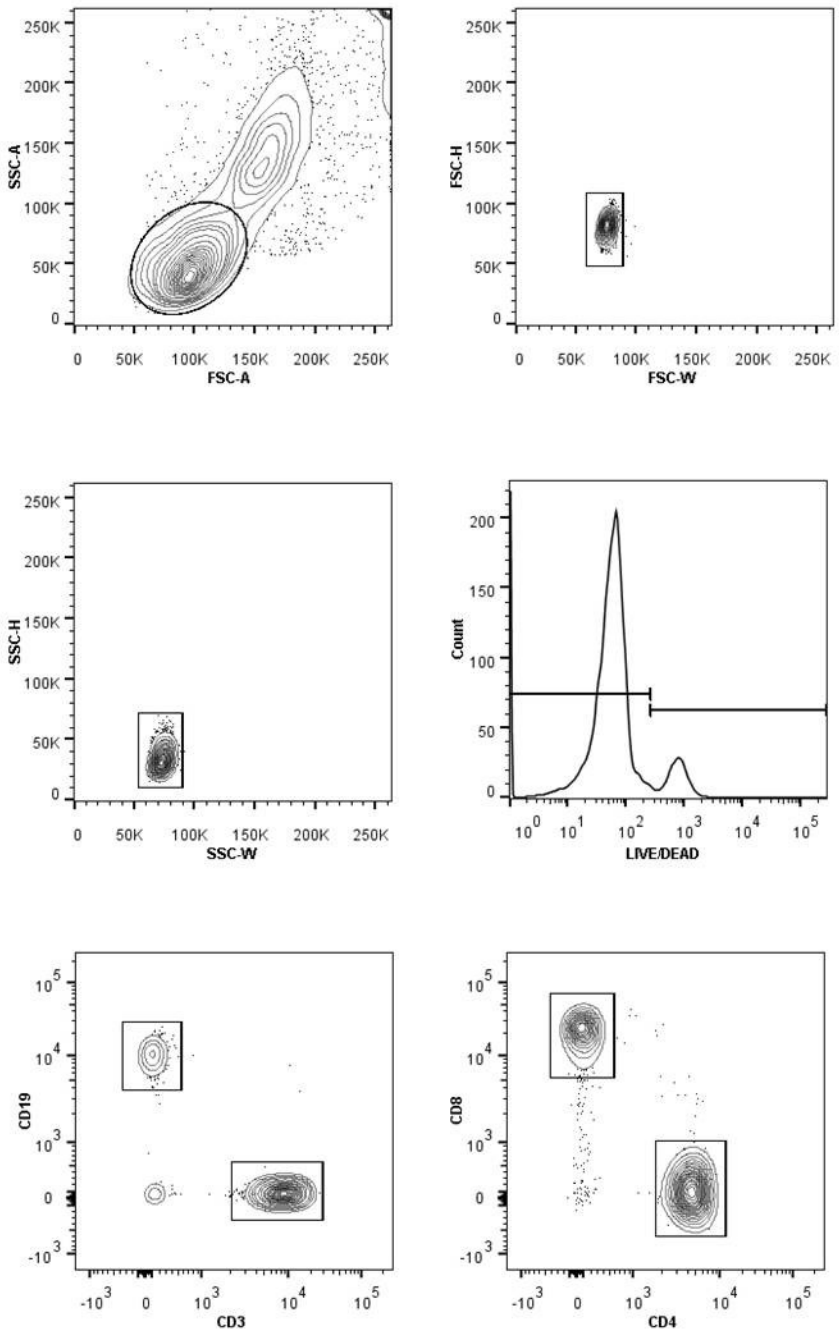

Figure S5-2: Hierarchical gating strategy for fluorescence-activated cell sorting of monocytes and NK cells. A non-restrictive gate containing all PBMC subpopulations (top left) was initially selected. Only live cells (top right; leftmost peak with the lowest amine-reactive dye staining) proceeded to a B- and T -cell exclusion step (i.e., selection of CD3-/CD19- cells) (bottom left) prior to sorting according to expression of the markers CD56+/CD14- (NK cells) and CD14+ (monocytic cells).

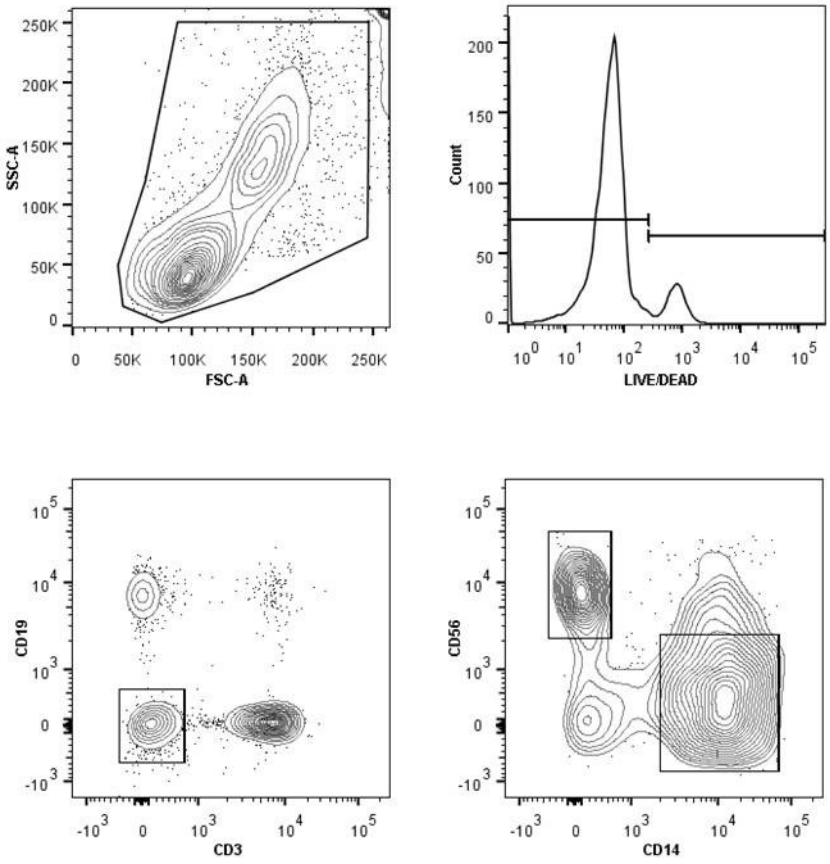

Figure S5-3: Incidence of CMV viremia in D+/R- patients according to induction of immune suppression. A Log-rank (Mantel-Cox) test was utilized to compare the Kaplan-Meier curves representing freedom from CMV viremia during the first year post-transplant. It indicates a similar risk of CMV viremia, regardless of the induction immune suppression regimen utilized.