Improving the Assignment of Cytomegalovirus Infection Status in Adults and Children Awaiting Solid Organ Transplant

by

Catherine Burton

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School of Public Health
University of Alberta

Abstract

Cytomegalovirus (CMV), a herpes-virus, is widespread in the human population as a lifelong and largely asymptomatic infection in immunocompetent people, but it is a major cause of morbidity in solid organ transplant (SOT) recipients. Knowledge of pre-transplant CMV infection status, generally determined using serology to detect IgG antibodies to CMV, is critical in stratifying the risk of CMV disease post-transplant and affects decisions regarding the use of anti-viral prophylaxis and enhanced surveillance including additional laboratory testing for CMV disease post-transplant. Unfortunately, serology-based determination of CMV infection status has limitations in situations where passive antibodies may lead to falsely positive CMV serology, such as in young infants who may have maternal antibody and in individuals who have received a recent transfusion of plasma-containing blood products. While guidelines suggest that CMV serology may be unreliable in infants less than 12 or 18 months of age, the time to clearance of passive maternal CMV IgG is not well established.

Our first objective with this program of research was to gain insight into the age of clearance of passive maternal CMV IgG in infants to aid in identification of the group of infants in whom CMV serology may be unreliable due to passive maternal antibody. This was accomplished with a retrospective review of pre-transplant CMV serology and viral detection studies for all infants <18 months of age awaiting solid organ transplant at the Stollery Children's Hospital over a 20 year period. The results suggested that maternal CMV IgG was cleared before 12 months of age, thus supporting that 12 months, as opposed to 18 months, may be a more appropriate cut-off for considering CMV serology potentially unreliable due to passive maternal antibody.

Our second objective was to evaluate the use of novel assays, not affected by potential passive antibody, to more accurately assign CMV infection status in adults and children awaiting SOT, especially in those whose true CMV infection status may be obscured by potential passive antibody. In our first study, we evaluated our experience with the use of CMV culture and nucleic acid amplification tests (NAAT) to detect CMV shedding, a marker of true CMV infection, in young infants with potential passive antibody awaiting SOT. In our second study, we prospectively evaluated the use of CMV NAAT as well as the detection of CMV-specific CD4+ T cells by flow-cytometry, and the CD27-CD28- CD4+T cell phenotype as adjuncts to CMV serology in the assignment of CMV infection status in adults and children awaiting solid organ transplant. Our results clearly highlight that CMV NAAT, from urine and saliva or throat samples, is a useful adjunct to CMV serology in CMV seropositive infants with potential passive antibody as it confirms true positive CMV infection status in a significant number of infants. Detection of CMV-specific CD4+ T cells showed promise as an adjunct to CMV serology in pre-transplant CMV risk stratification in children >12 months of age and in adults but lacked sensitivity in identifying true positive CMV infection status in young infants (<12 months). T cell phenotype analysis for CD27-CD28- CD4+T cells is unlikely to be a valuable tool in establishing true CMV infection status in children but may have a role in clarifying true pre-transplant CMV infection status in adults with unreliable CMV serology.

Taken together our studies support that CMV NAAT, of urine and saliva or throat swab, should be implemented for routine pre-transplant screening of CMV seropositive infants less than 12 months of age and could be considered in older children who have been transfused and have no pre-transfusion sample available. Detection of CMV-specific CD4+ T cells showed promise as an adjunct to CMV serology in determining true CMV infection status in adults and

children >12 months of age with potential passive antibody but further evaluation, with larger numbers of pre-SOT subjects, is needed. However, detection of CMV-specific CD4+ T cells should not be used in isolation to determine CMV infection status in CMV seropositive infants < 12 months with potential passive antibody.

Preface

This thesis is an original work by Catherine Burton. The research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board: "Assignment of Epstein-Barr virus and cytomegalovirus infection status in children and adults awaiting solid organ transplantation: evaluation of viral shedding and cell-mediated immunity assays as an alternative to serology" (Pro0042807, Oct 15, 2013), "Cytomegalovirus transfusion transmission in solid organ transplant patients in the era of universal leukoreduction" (Pro00035419, Nov 22, 2013), "Oral ganciclovir for CMV prevention in pediatric solid organ transplant recipients" (Pro00041030, Nov 10, 2012), and "Estimating CMV Serostatus misclassification based on serology assay results" (Pro00043588, April 10, 2014).

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List of Abbreviations

AST - American Society of Transplantation

CD (in CD4, CD8, CD69, CD27, CD28) – Cluster of differentiation

CMI – Cell mediated immunity

CMV – Cytomegalovirus

CMV IgG –Immunoglobulin G antibody to Cytomegalovirus

CMV-TC – Cytomegalovirus-specific T cells

D/R – Donor/Recipient

ELISA – Enzyme-linked immunosorbent assay

ELISpot – Enzyme-linked immunospot assay

FACS – Fluorescence-activated cell sorting

ICS – intracellular cytokine staining

ICU – Intensive care unit

IFN-γ – Interferon gamma

IVIG – Intravenous Immunoglobulin

NAAT- Nucleic Acid Amplification Test

OD – optical density

PCR – Polymerase Chain Reaction

PPA – Potential passive antibody

SOT – Solid Organ Transplant

Chapter 1

Introduction

1.1 Statement of the Problem

Organ transplantation is a life-saving treatment for people with organ failure but patients must take immunosuppressant medications after transplant to prevent their immune system from rejecting the organ. Unfortunately, immunosuppressant medications make organ transplant recipients more susceptible to infection. Cytomegalovirus (CMV) is widespread in the human population, as an asymptomatic or self-limited infection in immunocompetent individuals, but it is a major cause of morbidity in solid organ transplant (SOT) recipients.[1,2] CMV can cause systemic and tissue-invasive disease in SOT recipients, frequently affecting the allograft, and CMV infection is also associated with an increased risk of other opportunistic infections and acute and chronic graft injury in SOT recipients.[1,2,3,4] Several factors, including organ transplanted and the net state of immunosuppression, affect the risk of CMV disease but the most important risk factor for CMV disease is CMV infection status mismatch between organ donor and recipient with a CMV positive donor and a CMV negative recipient.[1,2]

Determination of pre-transplant CMV infection status is critical in stratifying the risk of CMV disease and it affects post-transplant management decisions regarding the use of antiviral prophylaxis and enhanced surveillance including additional laboratory testing for CMV infection.[1,2] The antiviral drugs used for universal prophylaxis often have their own toxicity and, although enhanced surveillance can lead to early detection of CMV infection and lessen morbidity and possible mortality, the laboratory testing is costly and is an added burden for the transplant recipient. [1,2,4]

Pre-transplant CMV infection status is generally determined using CMV serology to detect IgG antibodies to CMV. Unfortunately, serology-based identification of CMV infection status has limitations in situations where passive antibodies to the virus may exist, such as in children less than 12 or 18 months of age who may have maternal antibody, or in individuals who have received a recent transfusion of plasma-containing blood products or intravenous immunoglobulin.[1,2] Potentially false positive CMV serology results, in populations where passive CMV antibodies may be present, complicates the risk stratification process for CMV disease. Alternative methods to accurately assign CMV infection status in the solid organ transplant population are needed.

1.2 Cytomegalovirus in the Solid Organ Transplant Population

1.2.1 Epidemiology

Human Cytomegalovirus is a large double-stranded DNA virus, of the family *Herpesviridae*.[5] CMV is widespread in the human population, with seroprevalence ranging from 40-60% in developed countries to close to 100% in developing countries.[1,5-8] CMV is never cleared from previously exposed individuals; it establishes latency in the host and can be reactivated in some clinical settings.[5] Transmission from person to person commonly occurs via contact with infected secretions (including saliva, urine, respiratory and genital secretions); CMV can also be efficiently transmitted via organ or hematopoietic stem cell transplantation.[5] Without antiviral prophylaxis most CMV negative patients who receive an organ from a CMV positive person would develop CMV infection, with a significant proportion developing clinical disease.[9]

1.2.2 Clinical Manifestations and Consequences of CMV Infection

In immunocompetent hosts, acute CMV infection is frequently asymptomatic, especially if acquired early in childhood, or results in a self-limited illness.[5] In contrast, in

immunocompromised individuals, including SOT recipients, CMV infection is a cause of significant morbidity. CMV infection is defined as the detection of virus, viral nucleic acid, or proteins, by CMV culture, CMV NAAT or CMV antigen testing respectively, in any body fluid or tissue, and it may occur with or without any clinical signs or symptoms.[10] When CMV infection is accompanied by clinical signs and symptoms it is termed CMV disease which is further classified as CMV syndrome or tissue-invasive CMV disease. CMV syndrome is characterized by fever, fatigue or malaise, leukopenia, thrombocytopenia and elevation of hepatic aminotransferases.[10] Tissue-invasive disease can affect almost any organ or tissue in the body in a transplant recipient, causing: colitis, pneumonitis, hepatitis, nephritis, myocarditis,retinitis and other disease states.[1,5,8,10] CMV has a predilection for the transplanted organ and CMV disease can result in loss of function of the transplanted organ.[1,8]

CMV is an immunomodulatory virus, thought to have both immunosuppressive and inflammatory properties, and has many adverse indirect effects, not thought to be related to direct viral invasion of tissues. CMV infection in SOT recipients is associated with increased rates of bacterial, other viral and fungal infections, post-transplant lymphoproliferative disease, and graft dysfunction.[1,3,4]

1.2.3 Risk Factors

The risk of CMV disease in SOT recipients is affected by several factors, including the organ transplanted, host factors, and the net state of immunosuppression, but a critically important risk factor is CMV infection status mismatch between organ donor and recipient with a CMV positive donor and a CMV negative recipient (CMV D+/R-).[1,9] In this situation a recipient, with no immunity against CMV, is exposed to CMV transmitted from the organ at a time of intense immunosuppression when it is difficult to mount an effective immune response against the virus.[9] Without anti-viral prophylaxis, more than 50% of CMV negative recipients receiving

organs from CMV positive donors develop CMV disease.[11,12] Recipients who are CMV seropositive are also at risk of reactivating CMV and developing CMV disease post-transplant while CMV seronegative recipients of a CMV seronegative organ (D-/R-) are at the lowest risk of CMV disease.[9]

1.2.4 Pre-Transplant Risk Stratification of Post-Transplant CMV-Related Complications

CMV Serology

CMV serology, using an assay to detect anti-CMV Immunoglobulin G (CMV IgG) antibodies in the blood, is routinely performed in all SOT donors and recipients prior to transplant to determine CMV infection status. Although many different CMV serology assays are available, enzyme-linked immunosorbent assays (ELISAs) are commonly used for detection of CMV IgG antibodies. In the ELISA, enzyme-linked antibodies, specific to CMV IgG, bind to CMV IgG in the patient blood sample, then a reagent is added and the bound enzyme converts this reagent to a detectable signal, commonly producing a color change. The intensity of the color-generating reaction is measured and results in an optical density (OD) value, which, although considered semi-quantitative, reflects the CMV IgG antibody titer in the patient sample, although the correlation may not be linear.[13] Despite the semi-quantitative nature of the assay result, the reported results are often simply positive or negative based on a pre-established OD cut-off.

A negative CMV serology result on pre-transplant screening should represent a true negative CMV infection status; CMV serology is repeated at the time of transplant to determine if CMV infection has occurred since initial testing. Unfortunately, a positive CMV serology result does not always represent true positive CMV infection status in situations where passive CMV IgG may exist. CMV IgG can be passively transferred via infusion of plasma-containing

blood products and intravenous immunoglobulin (IVIG) preparations; CMV IgG also passes across the placenta from mother to infant in utero. This passive CMV IgG is ultimately cleared from the circulation over a period of months but the time to clearance of passive maternal CMV IgG is not well established. It is very difficult to determine the time to clearance of passive maternal CMV IgG because of the relatively high rate of CMV infection in infants of CMV seropositive mothers, related to transmission through breast milk and from close contact with infected secretions.[14,15] We would expect that, with advancing infant age, infants with passive maternal CMV IgG would have lower CMV IgG titers than infants with true positive CMV IgG from CMV infection.[15] It is unclear whether, in infants with potential passive antibody, the OD value, a surrogate of CMV IgG antibody titer, would be useful in distinguishing true infection from passive maternal antibody.

Risk-Stratification for Post-Transplant CMV-Related Complications

All SOT recipients are considered "at-risk" for CMV disease if the donor and/or recipient CMV serostatus is positive, with D+/R- having the greatest risk (See Table 1.1).[1,2] CMV seronegative recipients of CMV seronegative organs (D-/R-) are at low risk of CMV-related complications post-transplant although they are still at risk of community-acquired CMV infection. While organ transplanted and immunosuppressive regimen are important considerations, the post-transplant CMV prevention strategy is largely based on the donor/recipient CMV infection status, inferred from CMV serostatus, thus highlighting the importance of accurate determination of CMV infection status.

Current guidelines suggest that CMV serology may be unreliable for infants less than 12 or 18 months of age.[1,2] In CMV seropositive infants, it is prudent to repeat CMV serology at the time of transplant to determine if there has been or clearance of passive antibody, in previously CMV seropositive infants, but transplant frequently occurs during the time that passive antibody

may still be present. Given the potential for passive maternal antibodies in infants less than 12-18 months of age, current guidelines recommend assuming the highest risk donor/recipient CMV scenario for CMV seropositive infants, which is to consider a CMV seropositive infant CMV negative if the donor is CMV seropositive (assume D+/R- scenario) but to consider a CMV seropositive infant CMV positive if the donor is CMV negative (assume CMV D-/R+ scenario).[1,2] The same assumption of highest risk scenario is applied in adults or older children in whom serology may be unreliable because of recent transfusion. While this strategy ensures that no potentially high-risk situation is missed, it leads to unnecessary anti-viral use and additional monitoring, which is associated with potential toxicity and additional costs.

1.2.5 CMV Prevention Strategies in SOT Recipients

The main strategies for prevention of CMV in at-risk, SOT recipients are universal prophylaxis with antivirals and pre-emptive therapy.[1,2,4] Universal prophylaxis is the administration of anti-viral drugs to potentially at-risk SOT recipients for some pre-determined period of time post-transplant. Pre-emptive therapy involves regular laboratory monitoring for CMV replication in blood, with CMV NAAT or CMV antigenemia assay, with administration of antivirals if CMV replication reaches a certain threshold. Prophylactic anti-viral use for 3-12 months post-transplant has been shown to significantly reduce the risk of CMV disease and enhanced monitoring can lead to earlier detection of infection and can lessen the associated morbidity.[12,16] The American Society of Transplantation guidelines on CMV in SOT recommend universal antiviral prophylaxis for 3-12 months, depending on organ transplanted, whenever there is CMV mismatch (D+/R-), and recommend either universal prophylaxis or pre-emptive therapy when the recipient is CMV seropositive (regardless of donor serostatus) except in lung and small bowel recipients where universal prophylaxis is still the recommended prevention strategy (Table 1.1).[1] CMV seronegative recipients of CMV seronegative organs are considered

to be at low risk of CMV-related complications post-transplant and thus no prevention strategy is applied in this group.

1.2.6 Challenges of CMV Risk-Stratification and Prevention in Populations with Unreliable CMV Serology

In situations where there is potential passive antibody and uncertainty exists over whether recipient CMV seropositivity is truly positive or falsely positive from passive antibody, it is difficult to determine the actual risk of CMV-related complications and apply the appropriate preventive strategy. If the recipient's CMV seropositive result is considered a true positive and the donor is CMV seronegative the recipient would be considered at moderate risk and receive either universal prophylaxis or pre-emptive therapy, which would be unnecessary if the recipient was truly CMV seronegative with passive antibody. If a truly CMV seronegative recipient with passive antibody is considered CMV seropositive and receives an organ from a CMV positive donor, this would be treated as a moderate risk scenario where pre-emptive therapy would be appropriate when in reality it is the highest risk scenario where universal prophylaxis is warranted. While the first scenario overestimates the risk of CMV and results in unnecessary prophylaxis or pre-emptive therapy, the second scenario, where the risk of CMV is underestimated, is considered more dangerous, hence the guidelines suggesting adoption of the highest risk scenario when there is potential for passive antibody (see Table 1.1 which outlines risk levels and treatment options).[1,2]

The anti-viral drugs most commonly recommended for prevention of CMV are oral valganciclovir and intravenous ganciclovir.[1,2] Leukopenia is common with these drugs and can be very challenging to manage in SOT patients on multiple other medications.[1] The significant costs, including medication and testing costs, associated with both the universal prophylaxis and pre-emptive therapy approaches are justifiable for prevention of CMV disease in those at high-

risk but would likely not be justified if used in low-risk populations, thus further highlighting the importance of clearly identifying those at high-risk of CMV disease post-SOT.[4] The assumption of the highest-risk scenario in CMV seropositive infants with unreliable serology undoubtedly leads to overuse of anti-virals and laboratory testing for CMV and thus excess costs and potential drug toxicities.

1.3 Potential Strategies to Improve Risk Stratification in Populations with Unreliable Serology

1.3.1 Detection of Viral Shedding

Young children infected with CMV often have detectable virus, in urine or oral secretions, for months to years after primary infection and CMV is never cleared from previously exposed individuals, and can be reactivated. Thus, identification of virus shed in saliva, throat swab and urine samples or identification of latent virus in the peripheral blood may be an alternative method to confirm true positive CMV infection status in CMV seropositive people with potential passive antibody.[17-20] Current guidelines suggest that culture or nucleic acid amplification tests (NAAT) of urine, saliva and throat swabs may be useful to identify CMV DNA, a marker of true infection, in CMV seropositive infants with unreliable serology, but the utility of these tests in assigning pre-transplant CMV infection status has not been systematically evaluated.[1,2]

While CMV shedding is common in young children, most studies of CMV shedding among children do not report corresponding CMV serology so cannot be used to determine the proportion of CMV seropositive children shedding CMV.[17] Most of the published data regarding CMV shedding in infants comes from studies of infants with congenital CMV infection; much less is know about the natural history of shedding in infants who acquire CMV post-natally. A study of CMV shedding in healthy CMV seropositive young children reported CMV shedding in

23% (3/13) of infants 0-3 months, all (8/8) infants 4-12 months and 64% (9/14) of infants 13-24 months, suggesting that passive maternal antibody accounts for the majority of the CMV seropositivity in infants <4 months of age, and that the prevalence of viral shedding among truly CMV infected CMV seropositive children decreases with increasing age > 12 months.[18] A companion study showed that CMV shedding in young children was intermittent but was highly correlated with initial shedding status.[19] A study of Ugandan infants with post-natal CMV infection found quite consistently detectable CMV DNA in saliva for over a year after primary infection although the CMV shedding patterns in some of the infants suggested that reinfection may have occurred, which, due to differences in CMV seroprevalence and living conditions, would be much more common in a developing country than in North America; thus, these results may not be generalizable to North American infants.[20] While CMV shedding is less common in adults than children, it is more common in immunocompromised adults than in heathy adults so detection of CMV shedding may be more common in the population of adults, with chronic or critical illness, awaiting SOT and, if positive, would help to confirm true positive CMV infection status in adults with potentially unreliable positive CMV serology.[17,21]

1.3.2 Detection of CMV-Specific T cells

Assessment of the specific cellular immune responses to CMV is another potential method to determine true CMV infection status. Unlike serology, assays of cell mediated immunity (CMI) are not confounded by the presence of passively acquired immunoglobulin .[22-24] CMV-specific T cells will only be present if the individual has mounted a response to CMV infection. Three of the more common CMI assays, enzyme-linked immunospot assays (ELISpot), enzyme-linked immunosorbent assays (ELISA) and the intracellular cytokine staining technique (ICS) apply the principle of detecting cytokines, commonly interferon gamma (IFN-γ), produced by activated virus-specific T cells after stimulation of whole blood or peripheral blood

mononuclear cells with the viral antigens or peptides of interest. The cytokines are detected differently in each assay, with ELISpot and the ICS technique both having the advantage of being able to quantify the number of virus-specific T cells producing the cytokine while ELISA assays only measure the overall amount of cytokine secreted. The ICS technique, where the cytokine(s) of interest is/are stained in the activated cell and then detected with flow cytometry, has the additional advantage of being able to characterize the phenotype and functionality of the virus-specific T cells through the use of co-staining for cell-surface molecules.[24] The cell surface marker CD69 is commonly used as a marker of T cell activation.[25,26]

Although both CD4+ and CD8+ T cells are important in controlling acute and chronic viral infections, there has been particular interest in the study of CD4+ CMV-specific T cells in the SOT population as it appears that they play a critical role in protection against CMV disease, and dominate the cellular immune response to persistent CMV infection.[26-28] Detection of CD69+IFN-γ producing CMV-specific CD4+T cells after stimulation with CMV-antigen lysate, using intracellular cytokine staining (ICS) and flow-cytometry, has been investigated in healthy adults, adults with chronic renal failure and in adult renal transplant recipients, as well as in healthy children > 18 months of age, and was found to assign CMV infection status with excellent sensitivity and specificity (100% sensitivity and 98% specificity in adults, 100% sensitivity and 100% specificity in children >18 months), but it has not been investigated in chronically or critically ill adults or children awaiting SOT.[23,25] The assay also showed promising results in healthy infants <18 months of age with potential passive antibody, although CMV seropositive infants without detectable CMV-specific T cells were presumed to have false positive CMV serology despite not including viral detection methods for comparison in the study.[25]

1.3.3 T cell Phenotyping

T cells can be characterized and divided into subsets based on their expression of cell surface markers, the T cell phenotype.[29] T cell phenotype can be rapidly determined from a small volume of blood using flow cytometry to detect fluorescent-labeled antibodies to the cell surface markers of interest.

A body of evidence supports that, in adults, the majority of CMV-specific T cells are of an end-stage differentiated phenotype, characterized by loss of expression of the co-stimulatory proteins CD27 and CD28.[30-33] Naïve T cells express CD27 and CD28 but after repeated cycles of replication, likely driven by frequent CMV reactivation, they lose this expression and become CD27 and CD28 negative (CD27-CD28-). While CD27-CD28- CD8+ T cells are present in CMV seropositive and seronegative adults, CD27-CD28- CD4+T cells seem to be quite specific to CMV seropositive adults.[30] When investigated in a cohort of adults, who were either healthy, on hemodialysis or post-renal transplant, the frequency of CD27-CD28- CD4+T cells was a reliable marker of CMV serostatus and was strongly correlated with the frequency of CMV-specific CD69+ IFN-γ producing CD4+ T cells.[34] To our knowledge, little literature exists regarding the CD27-CD28- CD4+ T cell phenotype in children, who are much more likely to have had recent primary CMV infection than adults, and it is unknown whether this phenotype is associated with CMV seropositivity in children.[35]

Further study of the correlation between the CD27-CD28- CD4+ T cell phenotype, CMV serology, and the frequency of CMV-specific CD69+ IFN-γ producing CD4+ T cells in adults and children awaiting SOT is needed to determine if this relatively rapid and simple assay could improve pre-transplant CMV risk-stratification especially in populations with potentially unreliable CMV serology.

1.4 Summary

CMV is a major cause of morbidity in SOT recipients, but much of this morbidity is preventable with post-transplant CMV prevention strategies. Accurate pre-transplant CMV risk stratification, based on donor and recipient CMV-infection status, is critical to selecting the ideal post-transplant CMV prevention strategy but the use of CMV serology to determine CMV infection status has limitations in infants with potential passive maternal antibody and in individuals with recent transfusion of plasma-containing blood products or IVIG, where passive CMV IgG may exist and lead to false positive CMV serology. Alternative methods to accurately assign CMV infection status in this vulnerable population are needed.

1.5 Objectives

1) Gain insight into the age of clearance of passive maternal CMV IgG in infants and thus more clearly identify the group of infants in whom CMV serology may be unreliable.
2) Evaluate the use of three adjunct assays: CMV NAAT, flow cytometry-based detection of stimulation-induced CMV-specific CD4+ T cells, and CD27-CD28-CD4+T cell phenotype, to improve the assignment of CMV infection status in adults and children awaiting SOT across all allograft types, particularly in those whose true infection status may be obscured by passive antibodies.

The first and part of the second objective were addressed by a retrospective review of pre-transplant CMV serology as well as CMV throat and urine viral culture and CMV NAAT results for all infants <18 months of age awaiting solid organ transplant at the Stollery Children's Hospital over a 20 year period (Chapter 2). The second objective was addressed with a prospective study of the use of CMV NAAT, flow-cytometry-based detection of CMV-specific CD4+ T cells, and the $CD27^{-}CD28^{-}$ CD4+ T cell phenotype as adjuncts to CMV serology in the

assignment of CMV infection status in adults and children awaiting solid organ transplant (Chapter 3).

Table 1.1 Risk of CMV-Related Complications and Recommended Prevention Strategies by Donor and Recipient CMV Infection Status

Donor CMV Infection Status	Positive	Positive	Negative	Negative
Recipient CMV Infection Status	Negative	Positive	Positive	Negative
Risk for CMV Complications	High	Moderate	Moderate	Low
Prevention Strategy	Universal Prophylaxis	Universal Prophylaxis or Pre-emptive therapy	Universal Prophylaxis or Pre-emptive therapy	None

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Chapter 2

Assignment of Cytomegalovirus Infection Status in Infants Awaiting Solid Organ Transplant: Viral Detection Methods as Adjuncts to Serology

2.1 Introduction

Determination of pre-transplant Cytomegalovirus (CMV) infection status is critical in stratifying the risk of CMV disease and affects decisions regarding the use of anti-viral prophylaxis and monitoring for CMV infection post-transplant. Without antiviral prophylaxis most CMV negative patients who receive an organ from a CMV positive person develop CMV infection, with a significant proportion developing clinical disease.[1] Prophylactic anti-viral use for 3-12 months post-transplant has been shown to substantially reduce the risk of CMV disease and enhanced monitoring can lead to earlier detection of infection and can lessen the associated morbidity.[1,2,3] Unfortunately, serology-based identification of CMV infection status has limitations and may be falsely positive in situations where passive antibodies exist, such as in infants who may have maternal antibody and in individuals who have received a recent transfusion of plasma-containing blood products or intravenous immunoglobulin. The time to clearance of maternal antibodies to CMV (CMV IgG) is not well established but current guidelines suggest that CMV serology may be unreliable for infants less than 12 or 18 months of age. [2,3]

When serology is unreliable, culture or nucleic acid amplification tests (NAAT) of urine or throat swabs may be useful to identify CMV shedding, a marker of true infection, in CMV seropositive infants, but the utility of these tests in assigning pre-transplant CMV infection status

has not been evaluated.[2,3] If CMV culture or NAAT are negative or are not performed, guidelines recommend assuming the highest risk donor/recipient CMV scenario for CMV seropositive infants, which is to consider a CMV seropositive infant CMV negative if the donor is CMV seropositive.[2,3] As this strategy may lead to unnecessary anti-viral use and additional monitoring, which is associated with potential toxicity and additional costs, it is important to clearly identify the group of infants in whom serology may be unreliable and to evaluate the role of CMV viral detection assays as an adjunct to serology in assignment of pre-transplant CMV infection status.

Our goal was to more clearly define the group of infants in whom serology may be unreliable, and to evaluate our experience with the use of CMV culture and NAAT to detect viral shedding among CMV seropositive infants awaiting SOT. We reviewed pre-transplant CMV serology as well as CMV throat and urine viral culture and NAAT results for infants <18 months of age awaiting solid organ transplant at our institution looking for differences in CMV seropositivity proportions and CMV IgG optical density (OD) values, a surrogate of CMV IgG titer, by age group. In CMV seropositive infants, we also examined relationships between CMV viral shedding, age and serology OD values to determine if serology OD values and/or detection of viral shedding, used alone or together, may improve recipient CMV serostatus classification, assisting in differentiating CMV currently or previously infected infants from uninfected infants with passive maternal or transfusion-acquired antibody.

2.2 Methods

Institutional Protocols:

CMV Serology was routinely performed in all children being assessed for solid organ transplant since 1994. Since January 2000, our local protocol has also recommended submitting

a throat swab and urine sample for CMV viral detection on all CMV seropositive infants <18 months of age awaiting solid organ transplant. CMV was detected using shell-vial culture between 2000-2012; in January 2013 CMV culture was replaced with CMV DNA detection by NAAT.

Detection of CMV-Specific Antibodies (Serology) and CMV DNA

CMV-specific IgG antibodies (serology) were detected by enzyme immunoassay (Siemens Enzygnost Anti-CMV/IgG, Siemens Healthcare Diagnostics Products GmbH, Marburg/Germany). Serology results used were as reported by the lab and were based on lab guidelines for optical density cut-offs of <0.1 AU/mL for negative, >0.2 AU/mL for positive and between 0.1 and 0.2 AU/mL for indeterminate CMV serology. Though-considered semi-quantitative, the OD value is a surrogate of CMV IgG antibody titer.

Throat swabs, collected in viral transport media, and urine specimens, collected in sterile containers, were processed for CMV shell vial culture according to routine procedures (Merifluor CMV, Meridian Bioscience Inc, Cincinnati, OH, USA). Nucleic acid amplification testing (NAAT) for CMV from throat swab, collected in universal transport media (UTM-RT, COPAN diagnostics INC, USA) and urine samples, collected in sterile containers, was performed using the RealStar® CMV PCR Kit 1.0 (Altona Diagnostics, Hamburg, Germany).

Data Collection:

Infants

Data on the age, and CMV serology results from all infants <18 months awaiting solid organ transplant at our institution between April 1994 and September 2014 was obtained from a transplant database. CMV IgG OD values, for all infants with positive CMV serology, were

extracted from the Provincial Laboratory for Public Health laboratory information system. Results of CMV culture or NAAT testing of throat swab or urine samples, for CMV seropositive patients transplanted after January 2000, were also extracted from this system. Transfusion data was obtained from the Stollery Children's Hospital blood bank database. We considered infants to have had relevant recent transfusion if they had received transfusions of red cells, plasma, platelets, cryoprecipitate or whole blood or if they had received intravenous immune globulin within the 3 months prior to the date of their CMV serology test. The CMV prevalence in Canadian blood donors is 42% (personal communication, Canadian Blood Services).

Normative Adult OD Values

To establish a reference range of OD values in CMV seropositive adults, we reviewed CMV serology and OD values for 1016 sequential Canadian Blood Services (Edmonton) blood donors. Samples were collected in 2015 and tested in our research lab with the same CMV serology assay (Siemens Enzygnost Anti-CMV/IgG) as the infants in our study.

Data Analysis:

For analysis, infants were classified into 3 age groups based on age at the time of sampling for serology: 0 to <6 months, 6 to <12 months and 12 to 18 months. For the analysis of pre-transplant CMV serology by age group a single CMV serology result was used for each infant; if there were multiple pre-transplant CMV serology results, the pre-transplant serology result closest to the time of transplant was used. For analysis of age at sero-reversion, we documented the age when infants with a previous positive CMV serology were first found to have a negative CMV serology result.

For the analysis of CMV seropositive infants, all infants who had a positive CMV serology at some point pre-transplant were included, even if the CMV serology immediately pre-transplant was no longer positive. If there were multiple positive pre-transplant CMV serology

results, the serology result closest to the time of CMV viral detection test (urine or throat culture or NAAT) was used. If there were multiple viral detection tests performed, the result closest to the time of transplant was used. If there was no viral detection test performed, the positive serology result closest to the time of transplant was chosen. Infants were classified as CMV culture positive or NAAT positive if at least one of their samples (throat or urine) was positive.

In addition to analyzing OD values in CMV seropositive adult blood donors, comparisons were made between OD values in CMV seropositive women of childbearing age (18-45 years) and OD values in CMV seropositive infants in each of the 3 age groups, to examine the impact of potential passive maternal antibody.

Continuous and ordinal variables are presented as medians with 25^{th},75^{th} percentiles. To compare medians, we used the Kruskal-Wallis test, and the Mann-Whitney U test for pairwise comparisons with Bonferroni correction for multiple pairwise comparisons. Categorical variables are presented as counts with percentages and were analyzed using the Chi-squared test or Fischer's Exact test. All tests were two-sided and a p-value of < 0.05 was considered statistically significant. When multiple pairwise comparisons were made, p values were multiplied by the number of comparisons and reported as an adjusted p value.[4] Data analysis was performed with STATA 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

Ethics approval for this study was obtained from the University of Alberta Health Research Ethics Board. This study was supported by a Canadian Blood Services Research and Development Grant (00539).

2.3 Results

Pre-Transplant CMV Serology for Infants <18 months of Age

There were 152 infants, less than 18 months of age, transplanted at our institution between January 1994 and September 2014; Table 2.1 summarizes pre-transplant CMV serostatus and age, by allograft type. Pre-transplant CMV serology results by age group for the 152 transplanted infants are presented in figure 2.1. The proportion of CMV seropositive infants decreased from 52% (25/48) in the 0-6 month group to 43% (32/75) in the 6-12 month group and 28% (8/29) in the 12-18 month group but there were no statistically significant differences in the proportion of CMV positive infants between the three groups (p=0.109).

CMV IgG Optical Density in CMV Seropositive Infants

A total of 73 of the 152 infants (48.0%) had a positive CMV serology result at some point prior to transplant. At the time of transplant 65 remained CMV seropositive, 6 had sero-reverted to negative and 2 had indeterminate CMV serology. CMV IgG OD values were available for 72/73. The distribution of CMV IgG OD by age group is shown in figure 2.2. Median OD increased from 0.78 (0.36,1.12) in the 0-6 month group to 1.33 (0.81,1.16) in the 6-12 month group and 1.62 (1.36,2.41) in the 12-18 month group. Median OD was significantly higher in both the 6-12 and 12-18 month groups compared to the 0-6 month group (adjusted p=0.007, adjusted p=0.004) but there was no significant difference in median OD between the 6-12 and 12-18 month groups (adjusted p=0.25). Compared to the 0-6 month group there is a wider spread of CMV OD values in the 6-12 month group with a suggestion of clustering in a higher OD group, likely reflecting true CMV infection, and a lower OD group, likely reflecting waning maternal antibody. By 12-18 months the lower OD cluster is no longer present and all CMV OD values are greater than 1. Complete transfusion data was available for 67/72 infants (93.1%): 32/67 (47.8%) had received transfusions within 3 months of their positive CMV serology result

so may have had transfusion-related passive CMV IgG. When the transfused infants are excluded there is more clustering into higher and lower OD groups in the 6-12 month age group.

For comparison, we reviewed the CMV serology OD values for 1016 sequential Canadian Blood Services donors, age 18 to 76 years; 39% were CMV seropositive with a median OD value among seropositives of 1.58 (1.17,2.14). There were 280 women of childbearing age (18-45 yrs.) in this cohort: 41% were CMV seropositive with median OD value among seropositives of 1.49 (1.13,1.98). The median OD of seropositive women of childbearing age was significantly higher than in seropositive infants age 0-6 months (adjusted $p<0.001$) but there were no significant differences in median OD between women of childbearing age and either the 6-12 or 12-18 month groups (adjusted $p=0.073$, adjusted $p=1.0$).

Six infants sero-reverted while awaiting transplant. The age of documented sero-reversion ranged from 0.2 to 1.12 years and all of the infants had an OD value <1 on their positive sample(s) (OD range 0.206 to 0.93). Four of these infants had received recent transfusions prior to their initial positive serology and transfusion history was unavailable in one child so it is unclear if the sero-reversion was due to disappearance of passive maternal or passive transfusion-acquired antibodies or both. The child with no history of blood transfusion had documented sero-reversion at 0.36 years.

CMV Viral Detection Assays in CMV Seropositive Infants

Of the 59 infants <18 months of age transplanted between January 2000 and September 2014, 83.0% (49/59) had at least one sample (throat or urine) sent for CMV viral detection and 18/49 (36.7%) had detectable virus/viral DNA in at least on sample. CMV viral detection results by age group, for the 49 infants with samples sent, are summarized in table 2.2. The median time between sample collection for CMV serology and CMV viral detection was 2 (0,6) days.

Transfusion data was available for all 59 infants. Recent transfusion was common in this population and was more frequent in infants with negative compared with positive CMV viral detection studies (54.8% (17/31) vs. 27.8% (5/18)) but this difference did not reach statistical significance (p=0.082).

CMV culture was performed in 45 seropositive infants (from urine in 44 and throat in 23). There were no statistically significant differences in the proportion with a positive CMV culture between the age groups (p=0.20). Of the 22 infants who had both a urine and throat sample sent for CMV culture, the results were concordant in 20 cases (17 negative and 3 positive). In 2 cases, a 2.9 and a 4.8 month old, the urine CMV culture was positive and the throat culture was negative.

CMV NAAT was only performed in 7 infants and was positive in 4 (57.1%): 4 had throat samples (3 positive), 2 had urine samples (1 positive) and 1 had urine and throat samples (both negative). Only 3 children had both CMV culture and CMV NAAT performed and results were concordant in all 3 cases (2 negative and 1 positive).

CMV IgG Optical Density and CMV Culture in CMV Seropositive Infants

Figure 2.3 illustrates the distribution of CMV IgG OD values by age in CMV-seropositive infants with positive and negative CMV cultures. Among CMV seropositive infants with positive CMV culture, there is an increasing trend in OD with increasing age. Among CMV seropositive infants with negative CMV cultures there is also an overall increasing trend in OD with age. These trends remain when transfused infants are excluded (data not shown).

Median OD by CMV culture status and age group are presented in figure 2.4. There are no statistically significant differences in median OD between infants with positive and negative CMV culture overall (p=0.13) or within age groups (0-6 months p=0.43, 6-12 months p=0.22).

While there were no significant differences in median OD between the 3 age groups, among CMV-seropositive culture negative infants (p=0.09), median OD value was higher in the 6-12 month group compared with the 0-6 month group which is contrary to what we would expect based on waning passive maternal CMV antibody. The 2 seropositive infants >12 months of age had high OD values but negative CMV culture, and neither had a history of recent transfusion.

CMV IgG Optical Density and CMV NAAT in CMV Seropositive Infants

Figure 2.5 illustrates the distribution of CMV IgG OD values by age in the 7 CMV-seropositive infants with samples tested for CMV NAAT. The 3 CMV seropositive infants with negative CMV NAAT were < 12 months of age and all 3 had a history of recent transfusion; 2 of the infants, 4.7 and 11.5 months of age, had relatively low OD values (<0.5 AU/mL) while an 11 month old infant had a high OD value (>1 AU/mL) but had received IVIG the day prior to serologic testing. Both CMV seropositive infants >12 months of age had a high OD value (>1 AU/mL) and had positive CMV NAAT.

2.4 Discussion

In our cohort the proportion of CMV seropositive infants decreased from 52% in the 0-6 month group to 43% in the 6-12 month group and 28% the 12-18 month group, consistent with waning passive maternal antibody. Studies of CMV seroprevalence in infants in Turkey and China, where almost all pregnant women are seropositive, described the highest seroprevalence in infants 0-6 months of age and the lowest in infants 6-12 months, consistent with waning passive maternal antibody, then increasing seroprevalence after 12 months of age, reflecting true CMV infection.[5,6] The seroprevalence of CMV in infants less than 1 year of age in countries

more similar to Canada, with lower prevalence of CMV, is largely unknown. Seroprevalence studies in the United States and in Germany, where the overall prevalence of CMV is approximately 50%, report estimated prevalence of CMV IgG positivity in 1-2 year old children of 12.3% and 21.5%, which is more similar to our cohort.[7,8,9] We might have expected our cohort of infants awaiting solid organ transplant to have a lower rate of CMV infection than healthy infants as they spend a significant amount of time in hospital pre-transplant thus may be at lower risk of community-acquired CMV infection, and many of them are not able to breast feed so they likely have decreased exposure to CMV. In support of this, several studies have documented lower prevalence of CMV shedding among young children with chronic medical conditions compared to healthy children.[10] The fact the seroprevalence of CMV in our cohort of infants 12-18 months of age was actually slightly higher than the estimates for 1 year old infants in the U.S. and Germany likely just reflects the small sample size of infants over 12 months in our study.

The current American Society of Transplantation guidelines suggest that passively acquired maternal CMV IgG should be considered in pediatric SOT donors and recipients <18 months of age and the International Consensus Guidelines on the management of CMV in SOT suggest a cut-off of 12 months.[2,3] More clearly establishing the time to clearance of maternal CMV antibody would allow identification of the group of infants in whom serology may be unreliable, who would benefit from additional investigations and/or modified CMV risk-stratification, but there are very few studies that have addressed this question. The relatively high rate of CMV infection in infants from CMV seropositive mothers, related to transmission through breast milk and from close contact with other infected secretions, makes it difficult to accurately establish the time to clearance of passive maternal CMV IgG. The CMV OD data from our cohort suggests that passive maternal CMV IgG clears before 12 months, as the CMV

seropositive infants in the 12-18 month group all had higher OD values (>1 AU/mL) which would be expected with true CMV infection as opposed to with waning passive maternal antibody, and had median CMV OD that was very similar to our comparison group of seropositive adult blood donors. A study of 121 CMV seropositive very low birth weight pre-term Korean infants documented a mean age of seroreversion of 5.5 months, with 97.5% of infants becoming seronegative by 10 months of age, but these results may not be generalizable to full term infants who would be expected to have a higher CMV IgG titer at birth.[11] A Chinese study investigating the kinetics of CMV IgG levels in serial samples in 40 infants born to CMV seropositive mothers found that 8 infants cleared maternal CMV IgG between 3.5 and 8 months of age, and the other 32 infants had decreasing levels of CMV IgG over the first 3.5 months and then had significantly higher levels at 8 months, consistent with true infection prior to 8 months of age.[5] Studies looking at the time to clearance of maternal IgG antibodies to other viruses that are less commonly acquired in the first year of life, including measles, mumps, rubella and varicella, report rapid decreases in virus-specific IgG antibody levels in the first 3-6 months of life and little or no detectable antibody by 12 months.[12,13] Based on our study and the limited additional available evidence it appears that the 18 month cut-off to consider passive maternal antibody, when establishing pre-transplant CMV infection status, is likely too long and a 12 month cut-off is more appropriate.

Current guidelines suggest that, in infants whose serology may be unreliable due to passive maternal antibody, CMV culture or NAAT of urine and throat swabs may be helpful in identifying truly infected infants, but there is very limited data to support this recommendation.[2,3] Most studies of CMV shedding among children do not report corresponding CMV serology so cannot be used to determine the proportion of CMV seropositive children shedding CMV, and

many involve congenitally infected infants or older infants and young children (age 6 months to 6 years).[10] In our study the proportion of CMV seropositive infants shedding CMV in throat or urine, by culture or NAAT, increased from 30% in the 0-6 month age group to 47% in the 6-12 month age group and 50% in the 12-18 month age group. The majority of our data was from CMV culture. A recent cross-sectional US study assessed CMV shedding in saliva by NAAT, in healthy CMV seropositive children aged 0-4 years found that 23% (3/13) of infants 0-3 months were shedding CMV, all infants aged 4-12 months (8/8) were shedding CMV, and 64% (9/14) of infants aged 13-24 months were shedding CMV. [14] This suggests that passive maternal antibody accounts for the majority of the CMV seropositivity in infants <4 months of age and that at a very young age (<12 months) truly infected infants have detectable viral shedding but the prevalence of viral shedding among truly CMV infected CMV seropositive children decreases with increasing age. A companion study enrolled some of the CMV seropositive children from this study for a repeated measures study of CMV shedding patterns over a 3 month period.[15] They found that CMV shedding was intermittent but was highly correlated with initial shedding status: seropositive children who were shedding at the initial visit had CMV DNA detected in 84% of follow-up saliva specimens while those who were not shedding at the initial visit only had CMV DNA detected in 16% of follow-up saliva specimens. Importantly, due to the way the saliva and urine samples were collected and processed to allow home collection in these studies, the limits of NAAT detection were considerably higher for the saliva (1600 copies/mL) and urine (16000 copies/mL) than they would be for samples collected in a hospital setting where the approximated limits of detection would be closer to 500 copies/mL. A recent study of infants with congenital CMV, who are presumed to have more prolonged CMV shedding than children with post-natally acquired CMV, found that over 20% of asymptomatic congenitally CMV-

infected infants had cleared CMV DNA from the urine by 12 months of age.[16] A positive CMV culture or NAAT test is clearly useful in establishing true CMV infection and, even in very young infants <6 months of age, up to 30% of CMV seropositive infants in our cohort were shedding CMV. Given the well-recognized improved sensitivity of CMV NAAT over CMV culture, CMV NAAT may be a more promising adjunct in the assignment of CMV infection status in infants pre-transplant, and sampling at multiples sites, urine and throat, as well as repeated sampling may improve the sensitivity of the detection of CMV viral shedding.[17] The relatively high prevalence of true CMV infection in these young pre-transplant infants also highlights the importance of re-screening CMV seronegative infants before transplant, with CMV serology and CMV culture or NAAT, to detect primary CMV infection. A negative CMV viral detection result in a CMV seropositive infant should not be definitive evidence of a true negative CMV status, especially at >12 months of age, as CMV shedding can be intermittent and it appears that older infants are less likely to have detectable shedding than younger infants.

In CMV seropositive infants with positive CMV culture in our cohort, there was an increasing trend in OD with increasing age, which may reflect the fact that infants are being infected early and that OD is low in acute infection and increases over the next 2-4 months.[18,19] Surprisingly, among CMV seropositive infants with negative CMV culture there was also an increasing trend in OD with age which raises concern that CMV culture may not be sensitive enough to pick up true CMV infection, especially at older ages. As we only had 7 seropositive infants with CMV NAAT studies in our cohort we were unable to determine if reporting CMV OD values would offer any additional benefit, when combined with CMV NAAT studies, in assigning CMV infection status. Further study of CMV serology OD in relationship to CMV NAAT, with larger numbers, may clarify whether there is any additional benefit to reporting the

OD values, perhaps to trigger repeat sampling for CMV NAAT if CMV OD is high and CMV NAAT is negative.

The major limitation of our study is that, for most subjects, we had only a single serology result so we could not document CMV sero-reversion and establish a true negative CMV infection status in infants suspected to have false positive CMV serology from passive maternal antibody, thus we could not determine the negative predictive value of CMV culture or NAAT nor could we definitively define the age of clearance of maternal CMV IgG. We did not have access to maternal CMV serology results so we made the assumption that young CMV seropositive infants who had not been transfused could have had passive maternal antibody. Unfortunately, we had a very small number of infants in the 12- 18 month age group which limited our ability to draw strong conclusions about the time to clearance of maternal CMV IgG and the sensitivity of CMV viral shedding assays in these older infants.

Despite these limitations, our study clearly highlights that CMV viral detection studies are a useful adjunct to CMV serology in CMV seropositive infants awaiting solid organ transplant and supports that 12 months, as opposed to 18 months, may be a more appropriate cut-off for considering potential passive maternal antibody. Though our experience with CMV NAAT was limited, our preliminary results, along with the well-recognized improved sensitivity of CMV NAAT compared to CMV culture, are encouraging and more research is warranted in evaluating the role of CMV NAAT for establishing CMV infection status in infants awaiting transplant.

Table 2.1 Age and CMV Serostatus by Allograft Type for Infants Awaiting Transplant

	Allograft Type				
	Liver n=89	Heart n=57	Multivisceral n=5	Kidney n=1	Total n=152
CMV Seropositive* n (%)	43 (48.3%)	21 (36.8%)	1 (20.0%)	0	65 (42.8%)
Age (yrs.) at CMV serology, Median (25,75$^{\%ile}$)	0.74 (0.56, 0.99)	0.45 (0.22,0.69)	0.77 (0.45,1.05)	1.41	0.64 (0.45,0.93)

*If there were multiple pre-transplant CMV serology results available, the serology result closest to the time of transplant was used

Table 2.2 CMV Viral Detection in CMV Seropositive Infants Awaiting Transplant

	Age Group			
	0-6 months	6-12 months	12-18 months	Total
CMV Viral Detection: Any*	n=30	n=15	n=4	n=49
Positive n(%)	9 (30.0)	7 (46.7)	2 (50.0%)	18 (36.7)
CMV Viral Detection: Culture	n=29	n=14	n=2	n=45
Positive n(%)	8 (27.6%)	7 (50%)	0	15 (33.3)
CMV Viral Detection: NAAT	n=3	n=2	n=2	n=7
Positive n(%)	2 (66.7%)	0	2 (100%)	4 (57.1)

*Considered positive if CMV culture and/or NAAT from throat and/or urine sample was positive

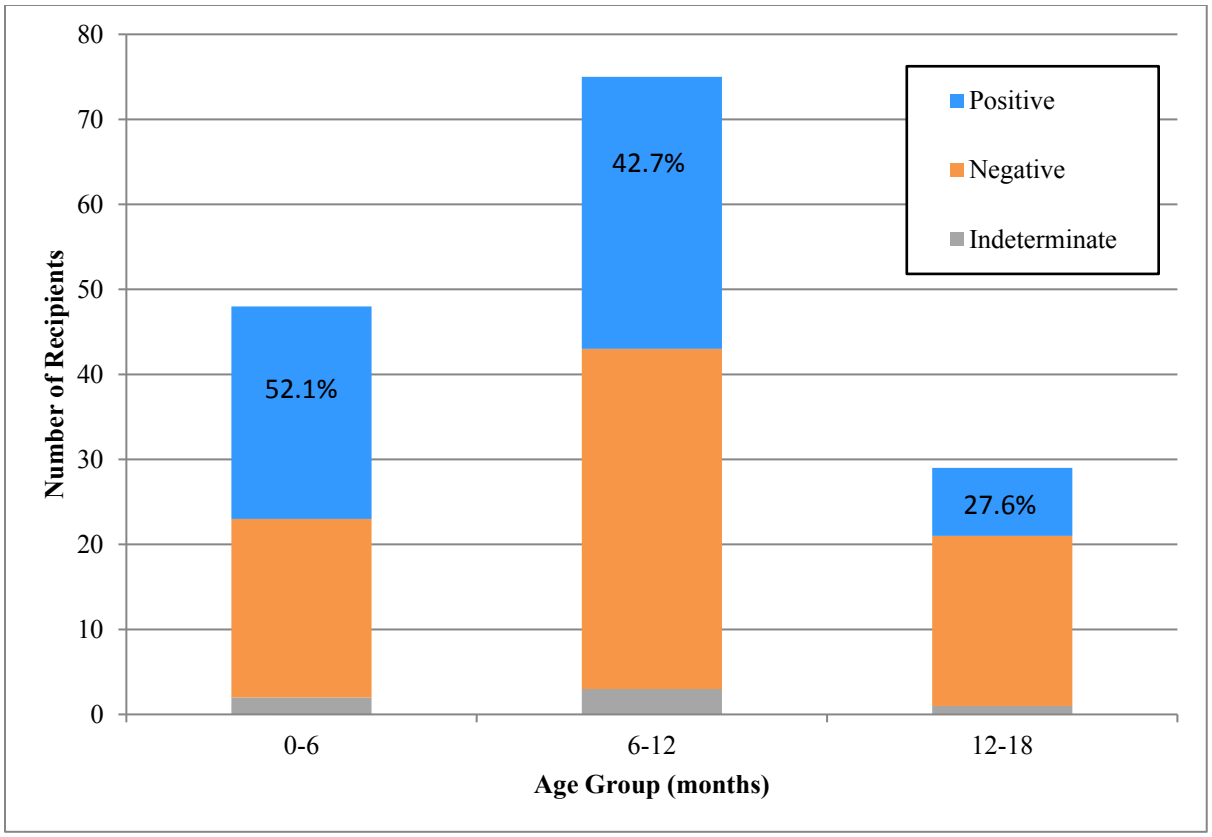

Figure 2.1 Infant CMV Serology by Age Group

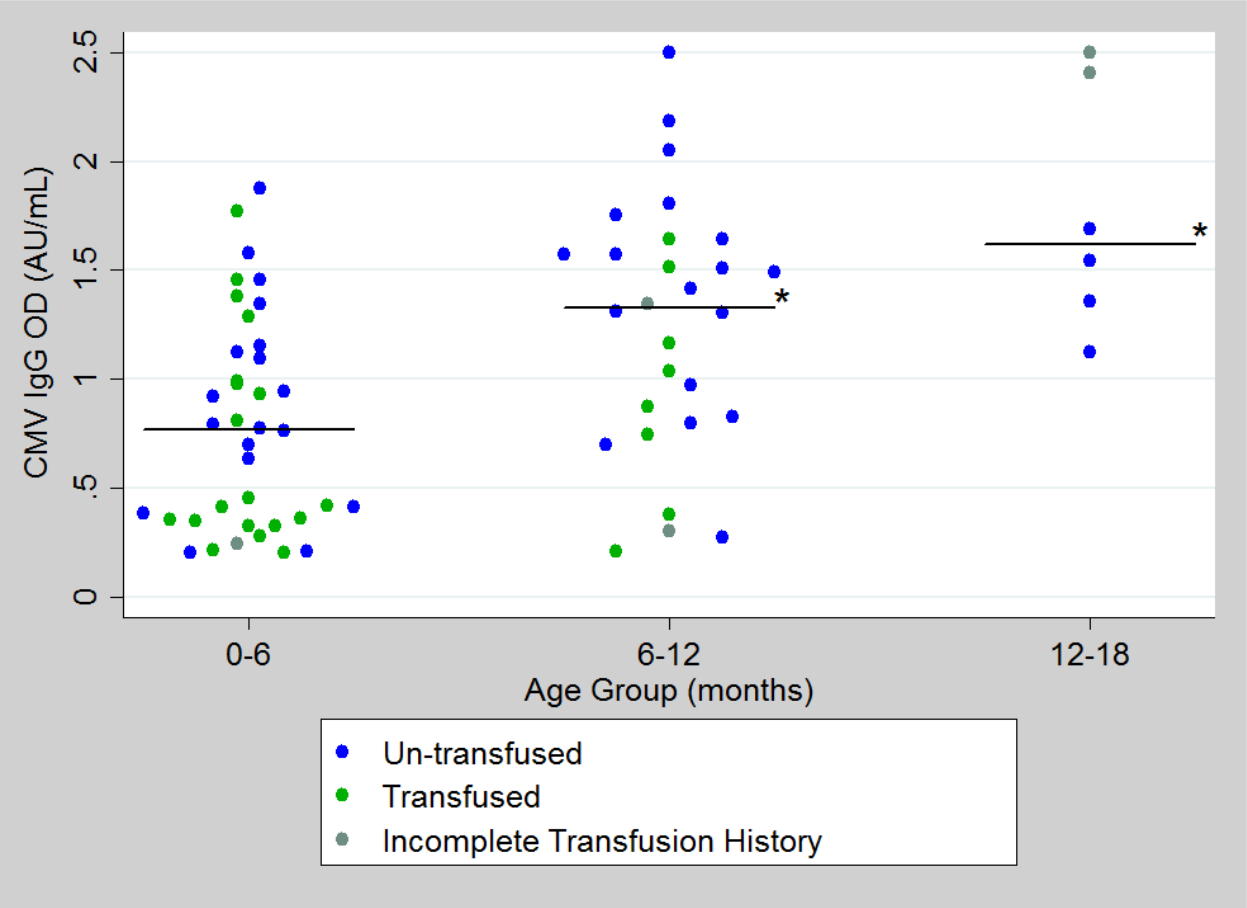

Figure 2.2 CMV IgG Optical Density (OD) by Age Group in CMV Seropositive Infants Awaiting Transplant

Median OD was similar between the 6-12 and 12-18 month age groups ($p=0.25$).

* Indicates significant difference when compared to 0-6 month group ($p<0.05$)

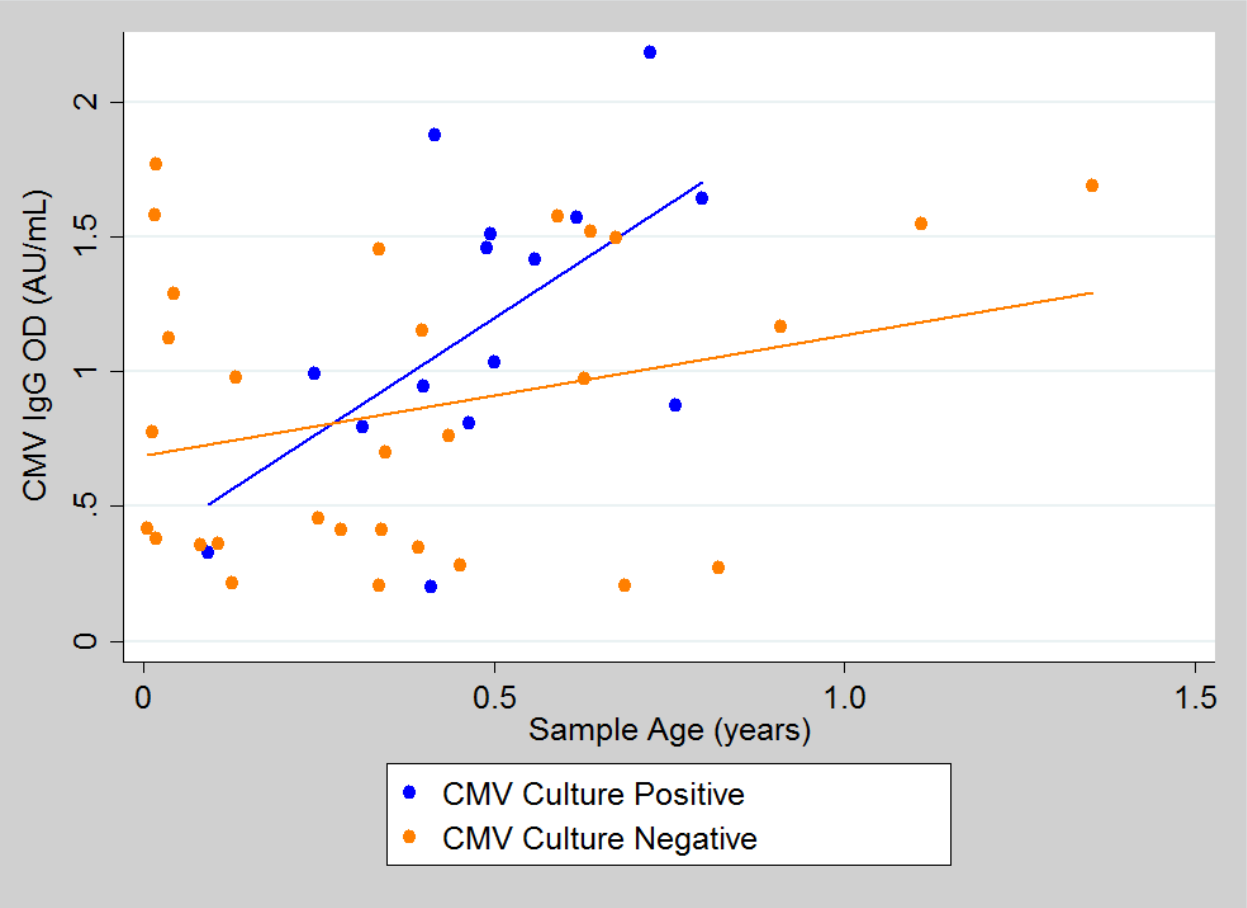

Figure 2.3 CMV IgG Optical Density (OD) in CMV Seropositive Infants with Positive or Negative CMV Culture, by Age

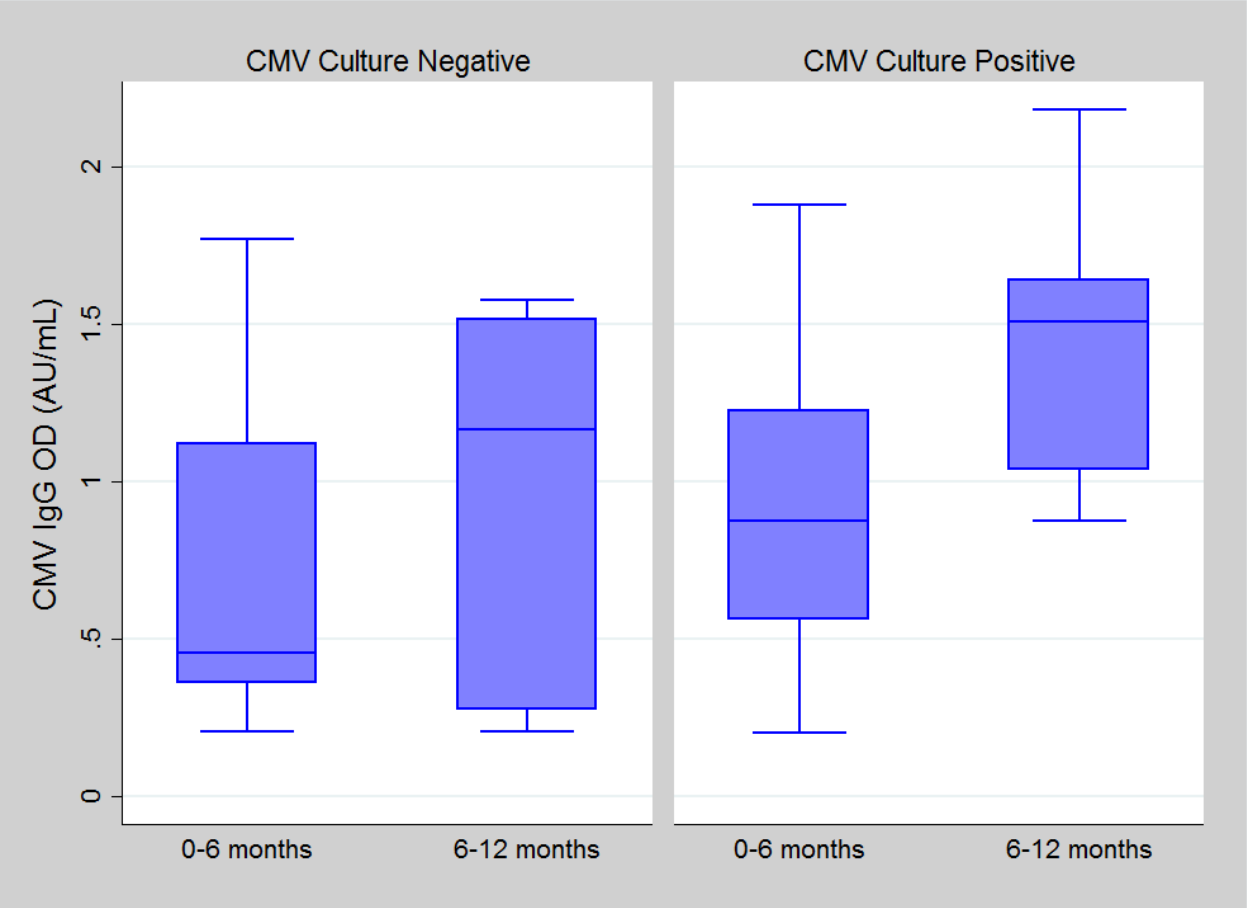

Figure 2.4 Median CMV IgG Optical Density (OD) by Age Group in Infants with Positive or Negative CMV Culture

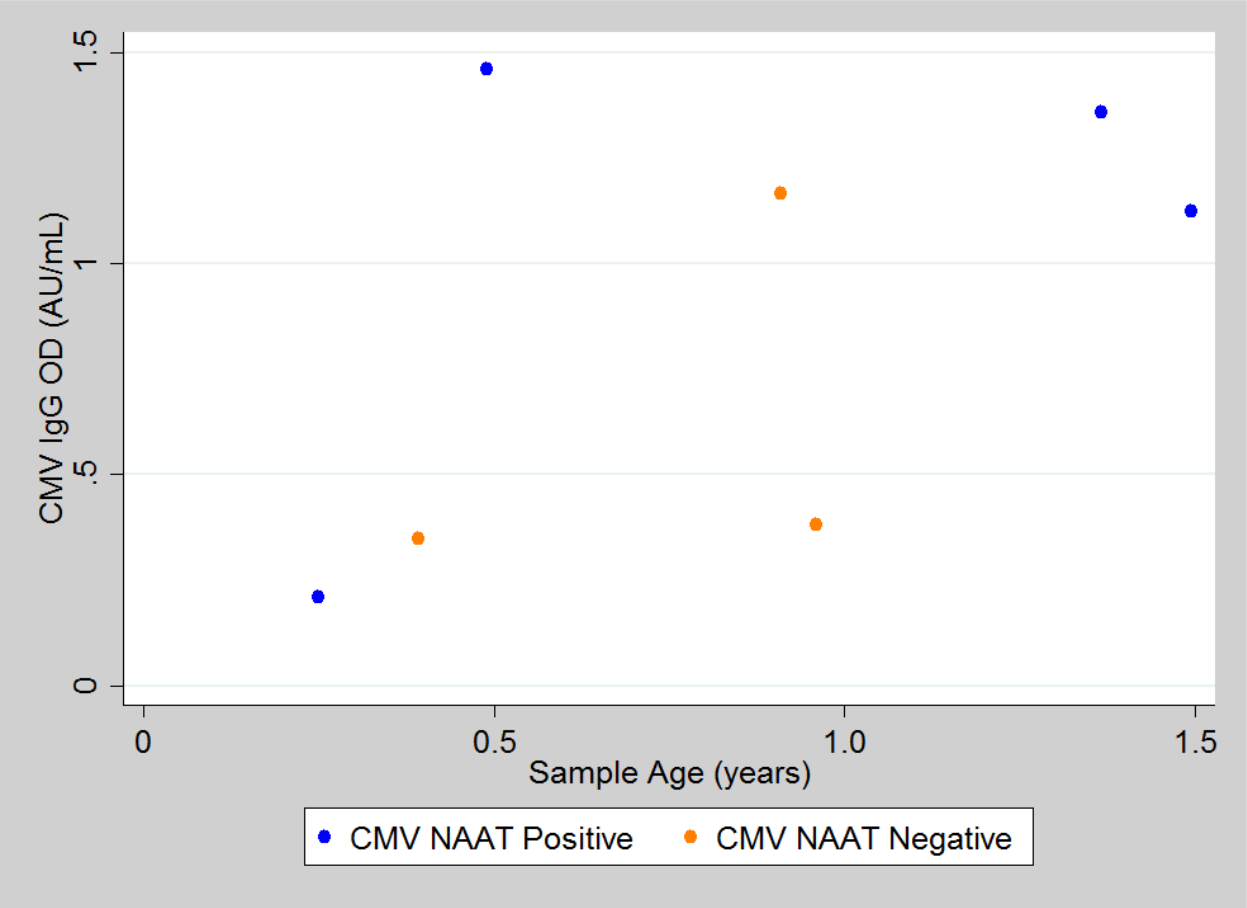

Figure 2.5 CMV IgG Optical Density (OD) in CMV Seropositive Infants with Positive and Negative CMV Nucleic Acid Amplification Test (NAAT) Results

All of the infants with negative CMV NAAT were < 12 months of age and had recent transfusion.

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Chapter 3

Viral Shedding, CMV-Specific T Cells and CD27-CD28-CD4+ T Cell Phenotype as Adjuncts to Serology for Improving Assignment of Cytomegalovirus (CMV) Infection Status in Adults and Children Awaiting Solid Organ Transplantation

3.1 Introduction

Determination of previous cytomegalovirus (CMV) infection, using CMV IgG serology screening, in organ donors and recipients, is routine and critical in stratifying the risk of CMV disease, a major cause of morbidity in solid organ transplant (SOT) recipients.[1] However, false positive serology results can occur due to passive antibody, in individuals with recent transfusion of plasma-containing blood products or intravenous immunoglobulin (IVIG) and following transplacental transfer of maternal antibodies in infants of CMV seropositive mothers.

The time to clearance of maternal antibodies to CMV (CMV IgG) is not well established but current guidelines suggest that CMV serology may be unreliable in infants less than 12 and possibly up to18 months of age. Assuming the highest risk donor/recipient CMV scenario for these infants is recommended: CMV seropositive infant recipients are treated as seronegative if they are receiving an organ from a CMV seropositive donor and are treated as seropositive if they are receiving an organ from a CMV seronegative donor.[1,2] This strategy results in unnecessary use of anti-virals, with resulting adverse effects and additional costs, and/or monitoring post-transplant. Many of these infants are not infected although CMV transmission by breast milk or other intimate contact is not uncommon in the first year of life.

Young children infected with CMV often have persistent or intermittent shedding of virus, in urine or oral secretions, for months to years. Direct detection of virus shed in urine or oropharyngeal secretions, or identification of latent virus in peripheral blood, with nuclear acid amplification testing (NAAT), is proof of true CMV infection in infants with potential passive antibody (PPA), but the utility of CMV NAAT in assigning pre-transplant CMV infection status has not been systematically evaluated. [3,4,5] Detection of CD4+ CMV-Specific T cells (CMV-TC) is not confounded by the presence of PPA and has shown promising results as an alternative to serology in assignment of CMV infection status in adults and healthy children, but has not been evaluated in ill adults and children awaiting SOT.[6,7,8] The majority of CMV-specific T cells have an end-stage differentiated phenotype, characterized by loss of the expression of the co-stimulatory receptors CD27 and CD28 on CD4+ and CD8+ T cells, after repeated cycles of antigenic stimulation.[9-12] The presence of $CD27^{-}CD28^{-}$ CD4+ T cells appears to be quite specific to CMV seropositive adults and the frequency of these cells has been investigated as a marker of CMV infection status in adults, including patients on hemodialysis, but has not otherwise been evaluated in adults awaiting SOT or in children.[13]

With this study we aimed to evaluate the use of three adjunct assays: CMV Nucleic Acid Amplification Testing (NAAT), flow cytometry-based detection of stimulation-induced CMV-specific CD4+ T cells (CMV-TC assay), and CD27-CD28-CD4+T cell phenotype to improve the assignment of CMV infection status in adults and children awaiting SOT across all allograft types, particularly in those whose true infection status may be obscured by PPA.

3.2 Methods

Patient Selection

Following approval from the Heath Research Ethics Board of the University of Alberta (Pro00042807), we enrolled adult and pediatric inpatients and outpatients awaiting SOT (heart, lung, liver, kidney, or liver+small bowel) at the University of Alberta/ Stollery Children's hospitals (Edmonton, Alberta, Canada) between July 2014 and February 2016. One control was selected for each pre-SOT subject. Adult controls were healthy volunteers matched by gender and age (+/- 5 years) and pediatric controls were immune-healthy children undergoing cardiac catheterization matched by age (+/- 2 years). Informed consent (assent) was obtained from all participants. Exclusion criteria included: previous SOT, use of immunosuppressive medications in the past 3 months, acute febrile illness and, for controls, known congenital immunodeficiency.

Data on age and sex, history of cardiac surgery with thymectomy (children only) use of immunosuppressant mediations and recent transfusion history was obtained from health records and/or personal/parental report. Additional data collected from pre-SOT subjects included: reason for transplant, organ transplanted, waitlist status at enrollment, hospitalization status, medication history and additional organ specific information including the presence of ventricular assist device (VAD) in cardiac transplant candidates, current dialysis in renal transplant candidates, and MELD or PELD score in liver transplant candidates.

Specimen Collection:

Collection of a throat swab, saliva sample, urine sample and blood sample was attempted on all subjects and controls. Throat samples were obtained with sterile polyester tipped swabs. Urine samples were clean catch samples in sterile containers, or were collected in pediatric urine collector bags. In adults and children old enough to gargle, saliva samples were collected by gargling sterile water then spitting the gargled liquid into a sterile collection tube. For infants and younger children, saliva specimens were collected using the VersiSAL®1 Saliva Collection

Device (Oasis Diagnostics® Vancouver, WA, USA). EDTA blood samples for CMV NAAT and CMV serology were stored at 4°C and processed within 48 hours. Heparinized blood samples for the CMV-TC assay and CD27-CD28- CD4+T cell phenotype were stored at room temperature and processed within 24-48hours of collection.

Specimen Processing and Testing:

Detection of CMV DNA by NAAT

CMV DNA was detected and quantified directly in all samples using our in-house developed real-time polymerase chain reaction (PCR) assay, as previously described.[14] The conversion factor for the assay is 5.65 genome copies/mL=1 IU/mL. Samples were considered definitively positive if CMV viral load was >500 copies/mL (2.7 log10 copies/mL). Samples with <500 copies/mL were considered positive only if they remained positive on repeat testing.

Detection CMV-Specific IgG antibodies (Serology)

CMV-specific IgG antibodies were detected by enzyme immunoassay (Siemens Enzygnost Anti-CMV/IgG, Siemens Healthcare Diagnostics Products GmbH, Marburg/Germany) or chemiluminescent microparticle immunoassay (Architect™ CMV IgG, Abbott Diagnostics, Abbott Park,IL).

Detection of CMV-Specific CD4+T cells (CMV-TC assay)

CD4+ T cells specifically activated by CMV lysate were identified via fluorescence-activated cell sorting (FACS) using intracellular cytokine staining (ICS) for IFN-γ and activation marker CD69 expression after 6-hour stimulation with CMV-antigen lysate as previously described by M. Sester et al.[6-8,15] Analysis was performed on a BD FACSCanto™. CMV-specific

CD4+ T cell frequencies were calculated by subtracting the percent CD69+IFN-γ+ CD4+T cells in the control culture from the percent obtained after stimulation with CMV-antigen lysate.

Samples were excluded from analysis if cell viability by Propidium Iodide (PI) staining was <80%. Results were considered valid if the positive control, and/or CMV antigen, elicited ≥ 0.05% CD4+CD69IFN-γ+ T cells, and if there was at least a 20% increase in CD4+CD69+IFN-γ+ T cells obtained after CMV-specific stimulation compared to control stimulation and background reactivity, otherwise results were considered indeterminate.

Phenotypic Analysis of CD4+ T cells for expression of CD27 and CD28 (CD27-CD28-CD4+ T cell phenotype)

Peripheral blood mononuclear cells (PBMC) isolated from whole blood using density gradient centrifugation were FACS-stained for CD4-V450, CD28-PerCP-Cy5.5, CD28-PerCP-Cy5.5 and CD27-APC-EF780 fixed in 1% paraformaldehyde in FACS buffer until reading on a BD FACSCanto™. Samples were excluded from analysis if PI viability was <80%.

Data Analysis

CMV seropositive individuals were considered to have PPA if they were ≤ 18 months of age and/or had received transfusion(s) of red cells, plasma, platelets, cryoprecipitate or whole blood or IVIG within the 2 months prior to blood sample collection. In order to validate the CMV-TC and CD27-CD28- CD4+ T cell phenotype assays in subjects and controls with potential passive antibody we used positive CMV NAAT results to supplement positive CMV serology results as confirmation of true positive CMV infection status in CMV seropositive individuals with PPA (figure 3.1).

All analyses were stratified according to adult or pediatric samples and CMV serostatus. In adults, analyses were stratified according to subject or control status. In children, initial analyses were stratified according to subject or control status but, to further investigate the use of the CMV-TC and CD27-CD28-CD4+ T cell phenotype assays in children with PPA, further analysis was performed combining all children as there were both pediatric subjects and controls with PPA. Similarly, to investigate the potential effect of thymectomy on the performance of the CMV-TC and CD27-CD28-CD4+ T cell phenotype assays, analysis was performed combining all children as both pediatric subjects and controls may have had previous thymectomy.

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

Categorical variables are presented as counts with percentages and were analyzed using the Chi-squared or Fischer's exact test. Continuous variables are presented as medians with 25th and 75th percentiles or means with standard deviations. The Kruskal-Wallis and Man-Whitney U test were used to compare the distributions of continuous variables between groups, with Bonferroni correction for multiple pairwise comparisons. Spearman's correlation coefficient was used to compare frequencies of CMV-TC and CD27-CD28- CD4+T cells. Receiver operator characteristics (ROC) analyses were performed for CMV-TC and CD27-CD28- CD4+T cell phenotype data to determine cut-offs to distinguish CMV seropositive from seronegative individuals in adults and children using the Liu method, of maximizing the product of the sensitivity and specificity. Using these cut-offs, CMV-TC and CD27-CD28-CD4+ T cell

phenotype were classified as positive (at or above the cut-off) or negative (below the cut-off) and agreement between CMV-TC and CD27-CD28- CD4+T cell phenotype was assessed by Kappa statistic.

All tests were two-sided and a p-value of <0.05 was considered statistically significant. When multiple pairwise comparisons were made, p values were multiplied by the number of comparisons and reported as an adjusted p value.[16] Data analysis was performed with STATA 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

3.3 Results

We enrolled 150 participants: 50 adult and 25 pediatric subjects awaiting SOT and 50 adult and 25 pediatric controls (Table 3.1). Within the adult and pediatric populations, subjects and controls were similar with respect to median age, gender, and proportion CMV seropositive. Among children, the proportion with thymectomy was significantly higher among controls than subjects (p=0.005).

Detection of CMV DNA by NAAT

Adults

Saliva, blood and urine samples were collected for CMV NAAT for all 100 adults and throat swabs were collected for 99/100. Only 2/48 (4.2%) CMV seropositive adults, both subjects awaiting lung and liver transplants, had a positive CMV NAAT result. (Table 3.2)

Children

All 50 children had blood samples and throat swabs collected, 49/50 had urine samples (25/25 subjects, 24/25 controls) and 41/50 had saliva samples (21/25 subjects, 20/25 controls). Saliva collection was unsuccessful in 6/20 infants < 18 months (3 subjects, 3 controls) and in 3/8

children ages 1.5-3 years (1 subject, 2 controls) due to insufficient saliva to saturate the collection device or child refusal of saliva collection device.

CMV NAAT was positive, in at least 1 specimen, in 8/17(47.1%) CMV seropositive children (5/7 subjects, 3/10 controls). Positive CMV NAAT confirmed true positive CMV infection status in 6/11(54.6%) CMV seropositive children with PPA (Table 3.2). The 5 CMV seropositive children with PPA and negative CMV NAAT ranged from 0.07 to 0.96 years of age: 4 were subjects and had all been transfused and 1 was a control, age 0.2 years, who had not been transfused. There was no significant difference in median age between CMV seropositive children with positive and negative CMV NAAT (0.87 yrs.(0.6,4.5) vs. 0.96 yrs. (0.2,8.2) p=0.85). CMV NAAT was only positive in 1/5 CMV seropositive children >5 years of age: a critically ill 15 year old awaiting lung transplant.

No CMV seronegative adult or child had a positive CMV NAAT result.

CMV-Specific CD4+T cell Assay (CMV-TC Assay) and CD27-28- CD4+T cell Phenotype

Results for CMV-TC assay and CD27-CD28-CD4+T cell phenotype were available for 145/150 and 142/150 participants respectively. Four samples (1 adult control, 2 pediatric controls, 1 adult subject) were excluded from both assays due to poor cell viability. Four samples were excluded for technical reasons, 1 from both assays (pediatric subject) and 3 from immunophenotyping (1 each pediatric subject and control and 1 adult subject).

CMV-TC Assay

The CMV-TC assay yielded valid results in 142/145 individuals; results from 1 adult (age 27 yrs.) and 2 pediatric subjects (0.96 and 4 yrs.) were indeterminate, due to high background reactivity, and were excluded from further analysis. All adults and children had a good response

to the SEB positive control. Frequency of CMV-TC in seropositive adults and children are presented in figure 3.2 and ROC analyses are summarized in Table 3.3.

Adults

A cut-off of 0.075% CMV-TC distinguished CMV seropositive from CMV seronegative adult subjects with reliable serology with a sensitivity of 80% and specificity of 93%. Sensitivity decreased to 76% when subjects with unreliable serology were included.

Children

When results from pediatric subjects and controls were combined, CMV TC frequencies were significantly higher in CMV seropositive children with reliable serology compared to CMV seronegative children (adjusted p=0.003); CMV-TC frequencies were similar between CMV seropositive children with reliable and unreliable serology (adjusted p=0.083) and between CMV seronegative children and CMV seropositive children with unreliable serology (adjusted p=1.0). Frequency of CMV-TC was similar in CMV seropositive children with reliable serology with or without previous thymectomy (p=0.85). Among CMV seropositive individuals with reliable serology, frequencies of CMV-TC were significantly lower in children than in adults (p=0.0019).

Given the lower frequencies of CMV-TC in children, we performed a ROC using CMV-TC assay results from children with reliable serology, and determined that a lower cut-off of 0.035% CMV-TC distinguished CMV seropositive from seronegative children with improved sensitivity compared to the 0.075% cut-off determined from adults (80% vs. 60%) while maintaining high specificity (94% versus 97%) (Table 3.3). Figure 3.3 highlights that, even using the lower 0.035% CMV-TC cut-off, there were 2 CMV seropositive infants with positive CMV

NAAT who did not have detectable CMV-TC. Two CMV seronegative controls, ages 3 and 11 years, had detectable CMV-TC.

CD27-28- CD4+T cell Phenotype

Figure 3.4 shows the distribution of CD27-CD28-CD4+T cell frequencies in adults and children by CMV serostatus. ROC analyses are outlined in table 3.4.

Adults

A cut-off of 0.46% CD27-CD28- CD4+ T cells distinguished CMV seropositive from CMV seronegative adult subjects with reliable serology with a sensitivity of 87% and specificity of 74%. Sensitivity decreased to 71% when subjects with unreliable serology were included.

Children

When results from pediatric subjects and controls were combined, CD27-CD28-CD4+ T cell frequencies were significantly higher in CMV seropositive children with reliable serology compared to CMV seronegative children (adjusted p=0.009) (Figure 3.5A). CD27-CD28-CD4+ T cell frequencies were similar between CMV seropositive children with reliable and unreliable serology (adjusted p=0.11) and between CMV seronegative children and CMV seropositive children with unreliable serology (adjusted p=1.0). CD27-CD28-CD4+ T cell frequencies in children with and without thymectomy are shown in figure 3.5B. There was no significant difference in median age between the children with and with and without a history of thymectomy (2.6yrs.(1.6,4.5) vs. 6.0yrs.(0.8,11.3), p=0.7).

Among CMV seronegatives, children had significantly higher CD27-CD28-CD4+T cell frequencies compared adults (p=0.034) but when children with previous thymectomy were excluded the difference was no longer statistically significant (p=0.23). There was no significant difference in CD27-CD28-CD4+T cell frequencies between seropositive children and adults with

reliable serology (p=0.85) and this did not change when children with thymectomy were excluded (p=0.26).

Given the differences in CD27-CD28- CD4+ T cell frequency between adults and children and between children with and without thymectomy we performed ROC analyses using the CD27-CD28- CD4+T cell frequency results from children with reliable serology and, from children with reliable serology without a history of thymectomy (Table 3.4). Figure 3.6 illustrates that issues with sensitivity and specificity exist across all ages regardless of which of the 3 potential cut-off values of CD27-CD28-CD4+T cell frequency is used to try to distinguish CMV seropositive from CMV seronegative children.

Correlation between CMV TC Assay and CD27-CD28- CD4+T cell Phenotype

There was a moderate positive correlation between frequencies of CD27-CD28- CD4+T cells and CMV-TC in adult controls (Spearman's rho 0.68, p<0.001) and in adult subjects (Spearman's rho 0.52, p<0.001), a weak positive correlation in pediatric controls (Spearman's rho 0.47, p=0.03) and no significant correlation in pediatric subjects (Spearman's rho 0.21, p=0.36). When children with previous thymectomy were excluded there was a high positive correlation between the 2 assays in pediatric controls (Spearman's rho 0.79, p=0.004) but there was still no significant correlation in pediatric subjects (Spearman's rho 0.22, p=0.34).

Using the cut-offs established from ROC analysis in Adult Controls (0.075% CMV-TC and 0.46% CD27-CD28- CD4+ T cells) to classify CMV infection status, there was moderate agreement between the 2 assays in adults (kappa 0.69, p<0.001). Using the cut-offs established from ROC analysis in children with reliable serology (0.035% CMV-TC and 1.96% CD27-CD28- CD4+T cells) to classify CMV infection status, there was very poor agreement between the 2 assays in children (kappa 0.26, p=0.045). Excluding children with thymectomy and using

the cut-offs established from ROC analysis in children with reliable serology without thymectomy (0.035% CMV-TC and 0.70% CD27-CD28- CD4+T cells), there was still poor agreement between the 2 assays (kappa 0.34, p=0.022).

Table 3.5 shows CD27-CD28-CD4+T cell phenotype among individuals with reliable serology who had discordant serology and CMV-TC results.

CMV NAAT, CMV-TC and CD27-CD28-CD4+ T cell Phenotype in Seropositive Adults and Children with Potential Passive Antibody

Figure 3.7 illustrates the results of CMV NAAT, CMV-TC assay and CD27-CD28-CD4+ T cell phenotype in adults and children with PPA.

3.4 Discussion

The current study confirmed that while detection of CMV DNA is uncommon in CMV seropositive adults it is common CMV seropositive children (47%). Moreover, CMV NAAT is clearly a useful adjunct to CMV serology in infants as it confirmed true positive CMV infection status in 5/10 CMV seropositive infants < 18 months of age. Although it is well documented that infants with primary CMV infection have prolonged viral shedding, studies in healthy infants have shown that shedding of CMV can be intermittent and that the prevalence of shedding is lower in infants 12-24 months of age compared with those 4-12 months of age thus, while a positive CMV NAAT result confirms seropositivity, a negative CMV NAAT does not exclude previous CMV infection in CMV seropositive infants, especially in those 12-18 months.[3,4,5]

The current study suggests that, in combination with urine sampling, saliva sampling may be more useful than throat swab for clarifying CMV status. The CMV viral loads from positive saliva samples were generally high, a finding also reported in previous studies, and CMV NAAT from saliva was positive in 2 cases where throat swabs were negative.[4,5,17] Although our success

rate with saliva collection in children was only 82%, collection was generally quick (1 to 2 minutes) and well tolerated in those in whom collection was successful.

The CMV-TC assay we used was adapted from one previously evaluated in healthy adults, and those with chronic renal failure or post-renal transplant. CMV serostatus, as determined by a CMV IgG enzyme immunoassay, was predicted with 100% sensitivity and 98% specificity, using a cutoff of 0.05% CMV-TC.[7] Using a slightly higher detection limit, of 0.075% CMV-TC, our assay had comparable excellent sensitivity and specificity in assigning CMV infection status in healthy adults (92%, 100%). The difference in cut-off value may arise from different gating strategies: while we strictly adhered to quadrants determined in negative controls, previous studies have modified the quadrant separations to capture all events visually attributed to a population. In adults awaiting SOT, of all organ types, including patients hospitalized and listed at a high-status, we found that the CMV-TC assay discriminated between CMV seropositive and seronegative adults awaiting SOT well (sensitivity 80%, specificity 93%). The lower sensitivity, compared to controls, is likely due to the high severity and chronicity of illness in our pre-SOT subjects which may impair their cell-mediated immune responses. Although validation with larger patient numbers will be required, our study suggests that this CMV-TC assay may be a useful adjunct to serology in adults awaiting SOT, across multiple organ types, who have PPA.

In our study, there were 3 CMV seropositive adult subjects, without recent transfusion, who did not have detectable CMV-TC, and, perhaps more surprisingly, 2 CMV seronegative adult subjects who had detectable CMV-TC. Similar discordant results have been found in other studies and it has been suggested that there may be patients who are seronegative despite

previous CMV infection: the presence of CMV-specific T cells may better predict the transplant candidate's ability to control CMV replication post-transplant.[7,18-20]

The CMV-TC assay we used was adapted from one previously evaluated in healthy children.[8] Using a detection limit of 0.05%, these investigators found perfect agreement between CMV serology and detection of CMV-TC in children > 18 months of age. In line with previous studies we found that, among CMV seropositive individuals with reliable serology, children had significantly lower CMV-TC frequencies than adults, probably reflective of a general immaturity of the adaptive immune response in early childhood[21-25] Using a lower detection limit of 0.035%, we observed a similar correlation between CMV-TC and serology children with reliable serology [excellent specificity (94%) and good sensitivity (80%)]. In Ritter's study of 13 CMV seropositive children < 18 months of age, 8 had detectable CMV-TC; the other 5 were presumed to have passive maternal antibody but viral shedding was not studied.[8] We were unable to detect CMV-TC in 2 CMV seropositive infants < 12 months of age who had positive CMV NAAT results. Therefore, unfortunately, it appears that the absence of detectable CMV-TC cannot be used to assume that a seropositive infant has only passive antibodies. As in the adult cohort, we found that 2/31 CMV seronegative children had detectable CMV-TC. Studies of young children with congenital or post-natally acquired CMV infection have documented an impaired cellular immune response to primary CMV infection, with increasing CMV-TC responses with age.[21-26] The variability in CD4+ T cell response to primary CMV infection in infants and young children, who are much more likely than adults to have recent primary CMV infection, limits the use of CMV-TC analysis, at least on its own, as alternative to serology in infants with PPA. However, the combination of CMV NAAT and CMV-TC may provide more useful information than either test alone in identifying infants with poor immune control of CMV, such as those with positive

NAAT but no detectable CMV-TC, who may be at particularly high risk of CMV-related complications post-transplant.

CD27/CD28 double negativity is an established phenotype of CMV-TC.[9-13] Compared to CMV-TC assays, T cell phenotyping is more rapid, simple, and requires a lower blood volume. Previous evaluation of the frequency of CD27-CD28- CD4+T cells in healthy adults, dialysis patients and post-renal transplant patients, found that a frequency of ≥ 0.44% CD27-CD28- CD4+T cells discriminated between CMV seropositive and seronegative individuals with 93% sensitivity and 97% specificity and that the frequency of CD27-CD28- CD4+T cells and stimulation-induced CMV-TC were strongly positively correlated (rho=0.73).[13] In our cohort of adult subjects awaiting SOT we found that a similar frequency of ≥ 0.46% CD27-CD28- CD4+ T cells identified CMV seropositive adult subjects with reliable serology with good sensitivity (87%) and reasonable specificity (74%). There was moderate positive correlation between the frequency of CD27-CD28- CD4+T cells and CMV-TC in adults with higher correlation in controls (rho=0.68) than in subjects (rho=0.52). Although not significantly different, seropositive adult subjects had higher CD27-CD28-CD4+ T cell frequencies and lower CMV-TC frequencies than controls, perhaps related to premature immune aging and waning cellular immunity in patients with chronic or critical illness, which may explain the poorer correlation between the 2 assays in subjects awaiting SOT. While the CD27-CD28-CD4+T cell phenotype shows promise in helping to resolve unreliable positive CMV serology in adults with PPA, its use may be limited by its disappointing specificity.

To our knowledge the CD27-CD28- CD4+T cell phenotype has not previously been evaluated as a marker of CMV infection status in children, although a study of Gambian infants suggested that a lower percentage of CMV-TC had lost expression of CD27 in CMV seropositive

infants compared to CMV seropositive adults.[23] Given the differences in immune responses in young children and the fact that CMV infection is much more likely to have been recent in children compared with adults, it is not surprising that we found that use of a specific frequency of CD27-CD28- CD4+T cells lacked sensitivity in defining CMV status in children. As these cells are of an end-differentiated phenotype it was, however, somewhat surprising that among seronegatives, children had higher frequencies of CD27-CD28- CD4+T cells than did adults while seropositive adults and children had similar CD27-CD28- CD4+T cell frequencies. This may be at least partly attributed to the fact that many of the children in our study had a previous thymectomy. Prior studies have documented a decreased proportion of naïve CD4+ T cells and an increased proportion of highly differentiated memory T cells in children and young adults who had thymectomy in infancy.[27-29] Interestingly one group found that these immune alterations were especially strong in CMV seropositive individuals with a strong CMV-TC response.[29] Although the inclusion of children with thymectomy likely affected the T cell phenotype analysis in children in our study, even after excluding these children, the frequency of CD27-CD28-CD4+T cells was not a reliable marker of CMV seropositivity in children.

Limitations of the study are that it was performed in a single institution in a developed country where the seroprevalence of CMV in children >12 months of age is likely in the range of 12-32% and the sample size was small.[30-32] Completely healthy pediatric controls would have been ideal but it is difficult to obtain consent for blood draws from children.

Our study highlights that saliva and urine samples for CMV NAAT should be obtained from all CMV seropositive infants <18 months of age awaiting SOT as, when positive, they confirm true CMV infection in infants with PPA. The CMV-TC assay, detecting CD69+IFN-γ producing CMV-specific CD4+T cells by flow-cytometry, lacked sensitivity in identifying true

positive CMV infection status in infants < 12 months of age but may still be useful in infants when used in combination with CMV NAAT to identify children at higher risk for symptomatic donor acquired CMV infection. T cell phenotype analysis for CD27-CD28- CD4+T cells is unlikely to be a valuable tool in establishing true CMV infection status in children but clarifies aspects of prematuration of the immune system in the context of thymectomy. Although their performance was inferior in adults awaiting SOT compared with healthy controls, both the CMV-TC assay and CD27-CD28- CD4+T cell phenotype show promise as adjuncts to CMV serology in pre-transplant CMV risk stratification in adults.

Table 3.1 Characteristics of Subjects and Controls

Characteristics	Adult		Pediatric	
	Subjects n=50	Controls n=50	Subjects n=25	Controls n=25
Age in yrs. median (25^{th},75^{th})	49.1 (32.4,59.9)	48.6 (35.0, 60.4)	1.86 (0.54, 9.65)	2.59 (1.0, 9.1)
Infants <= 18 mos. of Age, n (%)	NA	NA	12(48.0)	8 (32.0)
Thymectomy[†], n (%)	NA	NA	3 (12.0)	13 (52.0)
Sex Male, n (%)	32 (64.0)	32 (64.0)	13 (52.0)	13(52.0)
CMV Seropositive, n (%)	21 (42.0)	27 (54.0)	10 (40.0)	7 (28.0)
Potential Passive Antibody*, n	7	0	6	5
Organ and Wait-list status[‡]				
Kidney, n (%)	15 (30.0)[§]		6 (24.0)[§]	
Liver, n (%)	15 (30.0)[††]		11 (44.0)**	
status 0	3		3	
status 1	5		3	
status 2	7		2	
status 3	0		1	
status 3F	0		1	
status 4	0		1	
Lung, n (%)	11 (22.0)		2 (8.0)	
status 1	3		0	
status 2	8		2	
Heart, n (%)	9 (18.0)		6 (24.0)	
status 0	1		0	
status 1	1		1	
status 2	2		1	
status 3	5[#]		2	
status 3.5	0		1	
status 4	0		1[§§]	
Hospitalized, n (%)	17 (34.0)		12 (48.0)	
ICU, n (%)	2 (4.0)		6 (24.0)	

[†] Partial or complete thymectomy due to previous cardiac surgery

[*] Potential Passive Antibody due to recent transfusion and/or age <18 months

[‡] Waitlist status in Canada: higher numbers reflect more urgent status. For heart and liver, status ≥3 considered high status, for lung status 2 reflects rapidly deteriorating condition.

[§] 8/15 (53.3%) adults and 3/6 (50.0%) pediatric pre-renal Transplant candidates on dialysis

[††] Average MELD 22.9 (SD 6.12)

** Average PELD 29.1 (SD 8.6)

[#] 4/5 on ventricular assist device

[§§] on ventricular assist device

Table 3.2 Positive CMV NAAT by Specimen Type

Subject or Control	Age (Years)	PPA Yes/No (Source)†	Whole Blood (log10 copies/mL)	Urine (log10 copies/mL)	Throat Swab (log10 copies/mL)	Saliva (log10 copies/mL)
Control	0.25	Yes (Maternal)	1.81	5.27	5.66	9.31
Control	0.56	Yes (Maternal)	2.85	5.78	5.66	NA*
Control	0.64	Yes (Maternal)	Neg	4.54	6.22	8.59
Control	0.70	Yes (Maternal)	Neg	NA*	2.21	NA*
Subject	1.05	Yes (Maternal)	Neg	4.54	5.54	7.88
Subject	3.99	No	Neg	Neg	Neg	4.14
Control	4.92	No	Neg	2.54	2.28	4.58
Subject	15.60	Yes (Transfusion)	Neg	4.64	Neg	4.05
Subject	23.10	No	Neg	2.67	Neg	Neg
Subject	59.90	Yes (Transfusion)	Neg	2.37	Neg	Neg

†Potential passive antibody (PPA) sources: Maternal (due to age <18 months) or Transfusion (within 2 months prior to positive CMV serology)

* unable to collect

Table 3.3 ROC Analyses: Frequency of CMV-TC to Distinguish CMV Seropositive from CMV Seronegative Individuals

Population Used to build ROC	Cut-off value (% CMV-TC)	Population applied to	Sensitivity at cut-off	Specificity at cut-off	AUC
Adult controls †	0.075%	Adult Controls n=49	92%	100%	0.96
Adult controls †	0.075%	Adult Subjects-Reliable Serology n=42	80%	93%	
Adult controls †	0.075%	All Adult Subjects n=48	76%	93%	
Adult controls †	0.075%	Children*-Reliable Serology n=41	60%	97%	
Adult controls †	0.075%	All Children* n=45	43%	97%	
Children*- Reliable Serology‡	0.035%	Children*-Reliable Serology n=41	80%	94%	0.87
Children*- Reliable Serology‡	0.035%	All Children* n=45	57%	94%	
Children*- Reliable Serology, No Thymectomy§	0.035%	Children*-Reliable Serology without Thymectomy n=29	86%	95%	0.91
Children*- Reliable Serology, No Thymectomy§	0.035%	All children* without Thymectomy n=32	60%	95%	

†26 CMV seropositive, 23 CMV seronegative adult controls used to build ROC

*Pediatric Subjects and Controls

† 10 CMV seropositive and 31 CMV seronegative children (subjects and controls)

§ 7 CMV seropositive and 22 CMV seronegative children (subjects and controls) without thymectomy

Table 3.4 ROC Analyses: Frequency of CD27-CD28- CD4+ T cells to Distinguish CMV Seropositive from CMV Seronegative Individuals

Population Used to build ROC	Cut-off value (% CD27-CD28- CD4+ T cells)	Population applied to	Sensitivity at cut-off	Specificity at cut-off	AUC
Adult controls †	0.46%	Adult Controls n=49	88%	83%	0.86
Adult controls †	0.46%	Adult Subjects-Reliable Serology n=42	87%	74%	
Adult controls †	0.46%	All Adult Subjects n=48	81%	74%	
Adult controls †	0.46%	Children*-Reliable Serology n=40	82%	52%	
Adult controls †	0.46%	All Children* n=45	75%	52%	
Children*- Reliable Serology‡	1.96%	Children*-Reliable Serology n=40	64%	90%	0.77
Children*- Reliable Serology‡	1.96%	All Children* n=45	44%	90%	
Children*- Reliable Serology, No Thymectomy§	0.70%	Children*-Reliable Serology without Thymectomy n=28	71%	71%	0.71
Children*- Reliable Serology, No Thymectomy§	0.70%	All children* without Thymectomy n=32	55%	71%	

†26 CMV seropositive, 23 CMV seronegative adult controls used to build ROC

*Pediatric Subjects and Controls

‡ 11 CMV seropositive and 29 CMV seronegative children (subjects and controls)

§ 7 CMV seropositive and 21 CMV seronegative children (subjects and controls) without thymectomy

Table 3.5 CD27-CD27-CD4+ T cell phenotype in Adults (A) and Children (B) with Reliable CMV Serology but Discordant Serology and CMV-TC Results

A)

		CMV Seropositive	CMV Seronegative
		CMV-TC Negative	CMV TC-Positive
CD27-CD28-CD4+ Tcell Phenotype †	Positive	3*	2‡
	Negative	2§	0

†based on 0.46% cut-off

* 2 subjects and 1 control

‡ 1 subject and 1 control

§ 2 subjects

B)

		CMV Seropositive	CMV Seronegative
		CMV-TC Negative	CMV TC-Positive
CD27-CD28-CD4+ Tcell Phenotype†	Positive	2*	0
	Negative	0	2†

†based on 1.96% cut-off

* controls

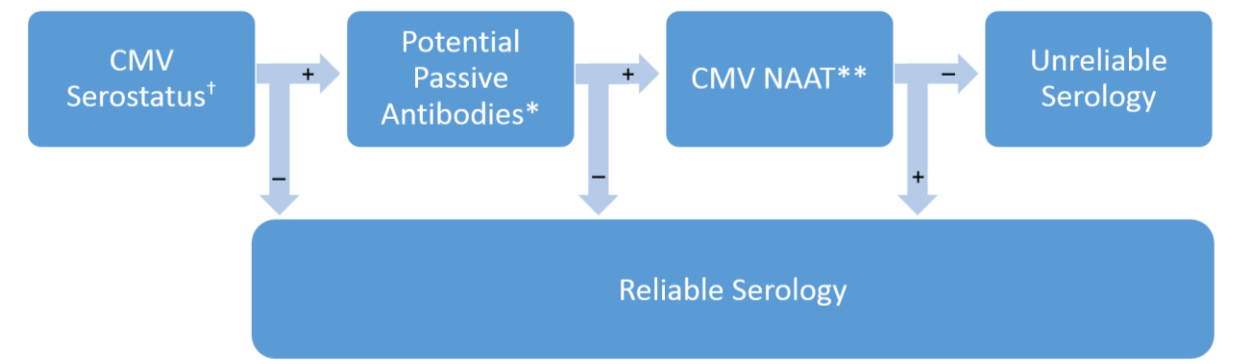


Figure 3.1 Classification of CMV serology as reliable or unreliable
†All individuals had CMV NAAT performed, regardless of CMV serostatus
*Potential Passive Antibodies present (+) if age <18 mos. and/or recent transfusion
**CMV NAAT positive (+) if NAAT result from at least 1 specimen was positive

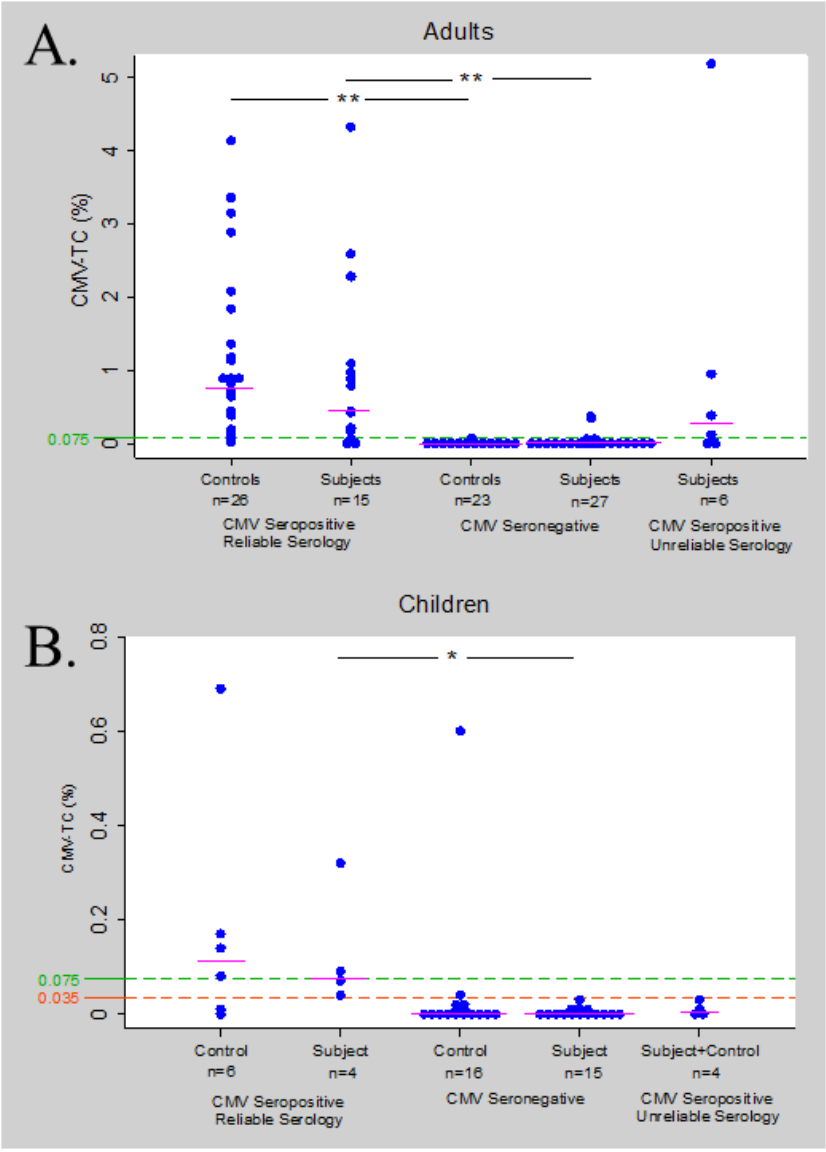


Figure 3.2 Comparison of CMV-TC T cell frequencies between CMV seropositive and seronegative subjects and controls in adults (A) and children (B)

(A) Adults: CMV-TC frequencies are similar between CMV seropositive controls and subjects with reliable serology (p=1.0) and between CMV seronegative controls and subjects (p=1.0). CMV-TC frequencies in CMV seropositive subjects with unreliable serology did not differ significantly from those in CMV seropositive subjects with reliable serology (p=1.0) or from CMV seronegative subjects (p=0.22). **p<0.0001. Green line (0.075%) represents the detection limit for CMV-TC in adults.

(B) Children: CMV-TC frequencies are similar between CMV seropositive controls and subjects with reliable serology (p=1.0) and between CMV seronegative controls and subjects (p=1.0). There was no significant difference in CMV-TC frequencies between CMV seropositive controls with reliable serology and CMV seronegative controls (p=0.088). *p=0.0032. Green (0.075%) and orange (0.035%) lines represent the detection limits for CMV-TC determined in the adult and pediatric populations respectively. Note the difference in Y axis scale between the graphs for adults (0 to 5%) and children (0 to 0.8%).

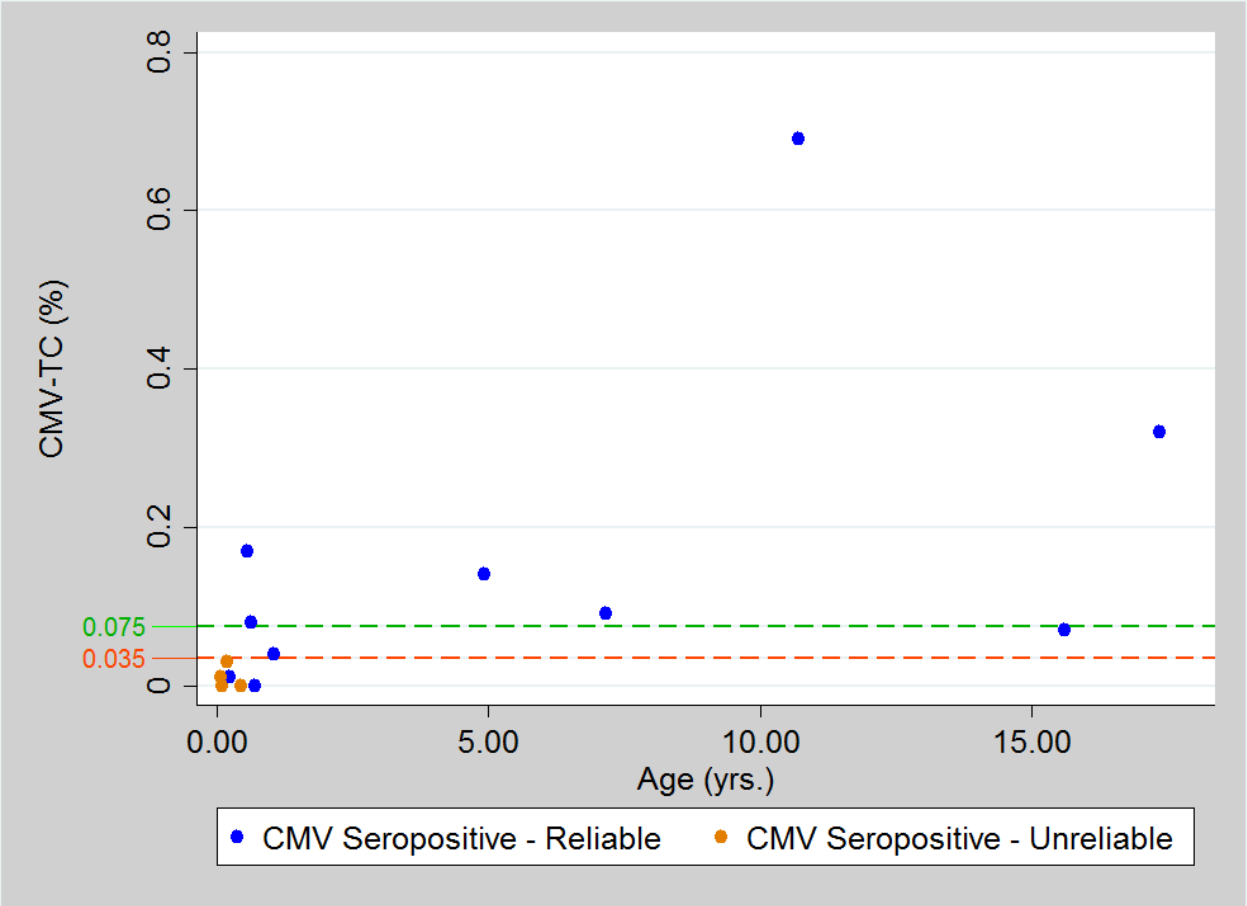


Figure 3.3 CMV-TC by age in CMV seropositive children with reliable and unreliable serology
Of 5 CMV seropositive infants <18 months with reliable serology (positive CMV NAAT), only 2 and 3 had CMV-TC above the 0.075% and 0.035% cut-offs, respectively. A 15 year-old in the ICU on extracorporeal membrane oxygenation awaiting lung transplant had reliable serology (transfused but had positive CMV NAAT) and had CMV-TC below the 0.075% but above the 0.035% cut-off. None of the 4 CMV seropositive infants <18 mos with unreliable serology (negative CMV NAAT) had detectable CMV-TC regardless of cut-off. Green (0.075%) and orange (0.035%) lines represent the detection limits for CMV-TC determined in the adult and pediatric populations respectively.

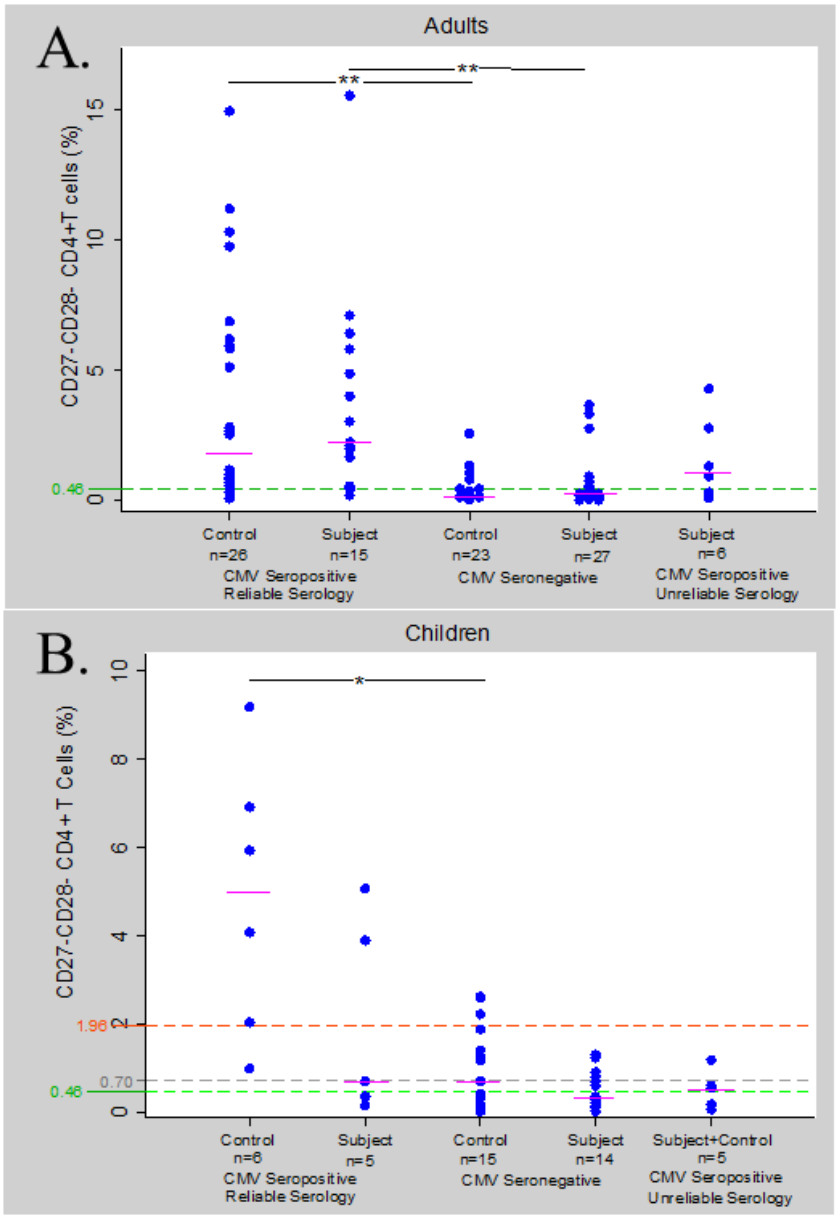
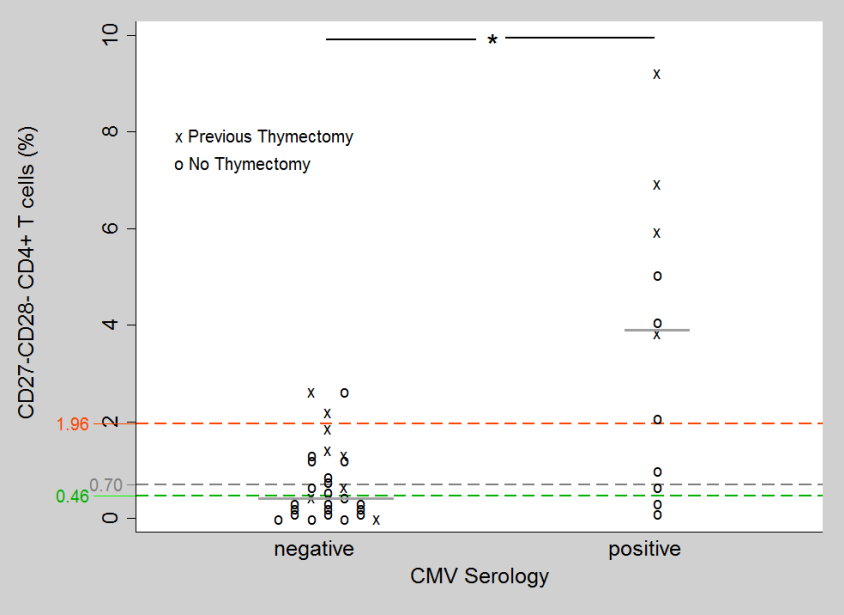


Figure 3.4 Comparison of CD27-CD28- CD4+ T cell frequencies between CMV seropositive and seronegative subjects and controls in Adults (A) and children (B)

(A) Adults: CD27-CD28- CD4+ T cell frequencies are similar between CMV seropositive controls and subjects with reliable serology (p=1.0) and between CMV seronegative controls and subjects (p=1.0). CD27-CD28- CD4+ T cell frequencies in CMV seropositive subjects with unreliable serology did not differ significantly from those in CMV seropositive subjects with reliable serology (p=0.97) or from CMV seronegative subjects (p=0.33). **p<0.001. Green line (0.46%) represents the cut-off determined in adults to distinguish CMV seropositive from seronegative individuals.

(B) Children: CD27-CD28- CD4+ T cell frequencies are similar between CMV seropositive controls and subjects with reliable serology (p=0.27) and between CMV seronegative controls and subjects (p=0.79). There was no significant difference in CD27-CD28- CD4+ T cell frequencies between CMV seropositive subjects with reliable serology and CMV seronegative subjects (p=0.66). *p=0.026. The orange, grey and green lines represent the cut-offs determined to distinguish CMV seropositive from seronegative individuals in adults (0.46%), children without thymectomy (0.70%) and all children (1.96%)

A.



B.

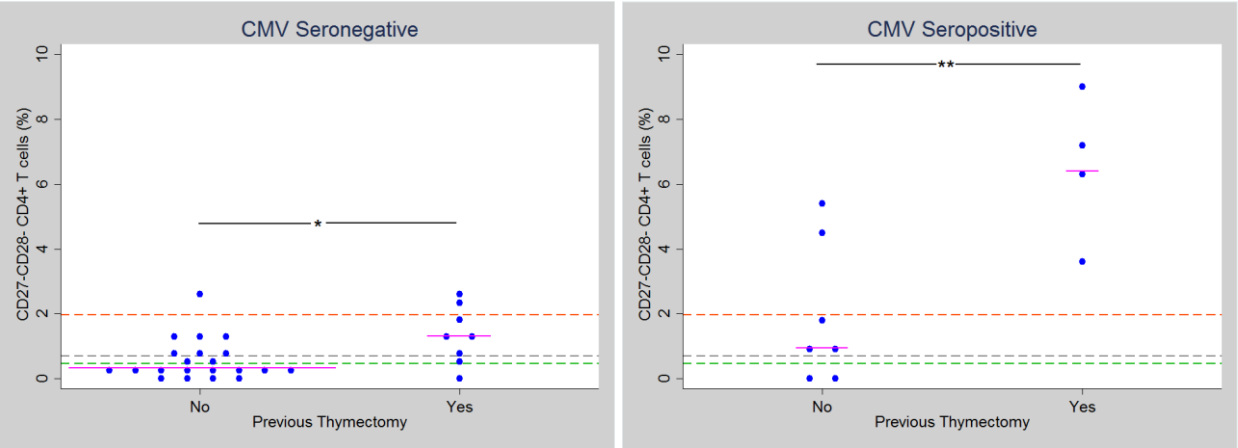


Figure 3.5 CD27-CD28- CD4+ T cell frequencies CMV seropositive and seronegative children with reliable serology with and without thymectomy

(A) CD27-CD28- CD4+ T cell frequencies are significantly higher among CMV seropositive than CMV seronegative children but several CMV seronegative children, with and without thymectomy have frequencies above the 0.46% and 0.70% cut-offs. *p=0.009. X denotes a history of thymectomy.

(B) Among CMV seronegative and CMV seropositive children with reliable serology CD27-CD28- CD4+ T cell frequencies are significantly higher in children with a history of thymectomy. *p=0.028 **p=0.023.

The orange, grey and green lines represent the cut-offs determined to distinguish CMV seropositive from seronegative individuals in adults (0.46%), children without thymectomy (0.70%) and all children (1.96%).

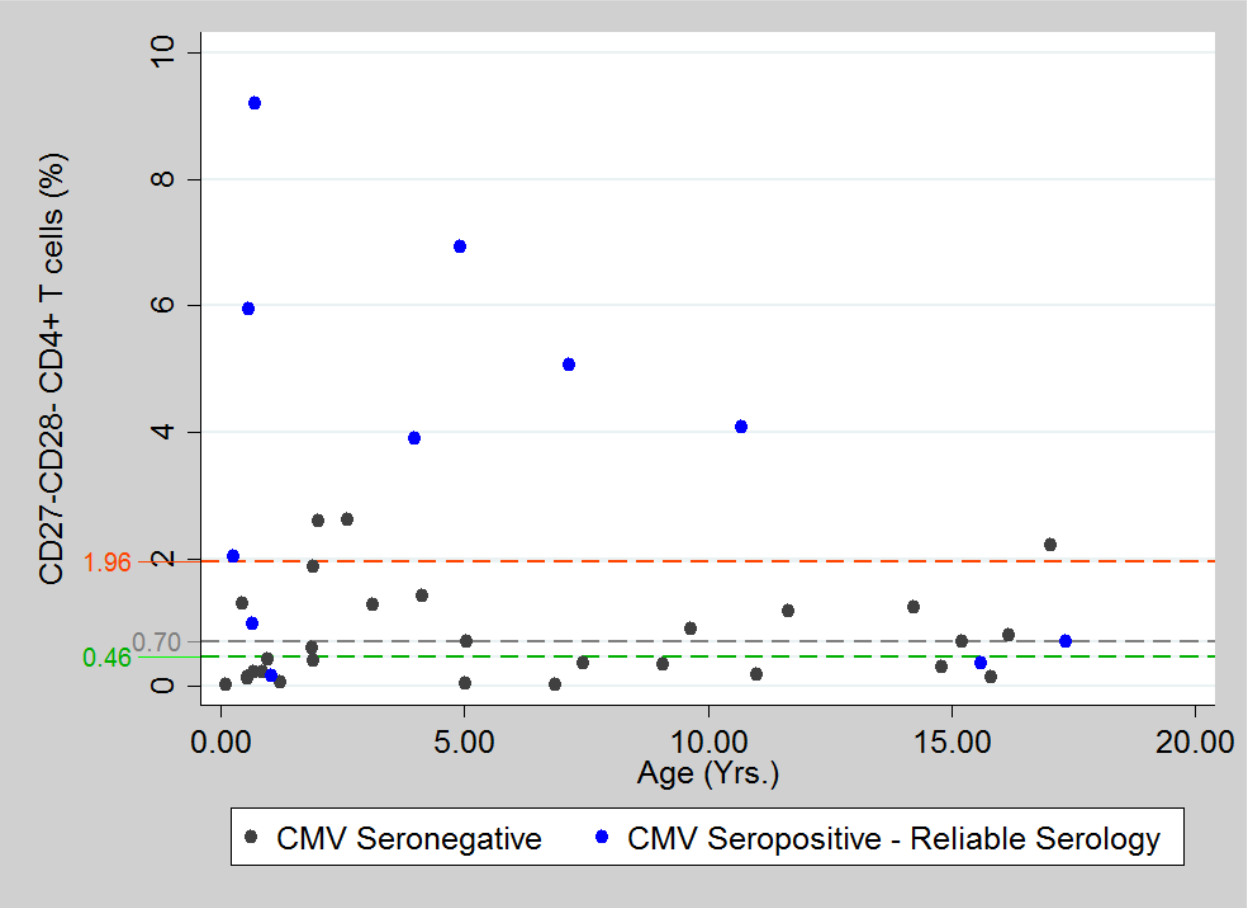


Figure 3.6 CD27-CD28-CD4+ T cell frequency by age in CMV seronegative and CMV seropositive children with reliable serology

Children of all ages were misclassified using any of the 3 potential cut-offs of CD27-CD28-CD4+ T cells attempting to distinguish CMV seropostive from CMV seronegative children. The orange, grey and green lines represent the cut-offs determined to distinguish CMV seropositive from seronegative individuals in adults (0.46%), children without thymectomy (0.70%) and all children (1.96%).

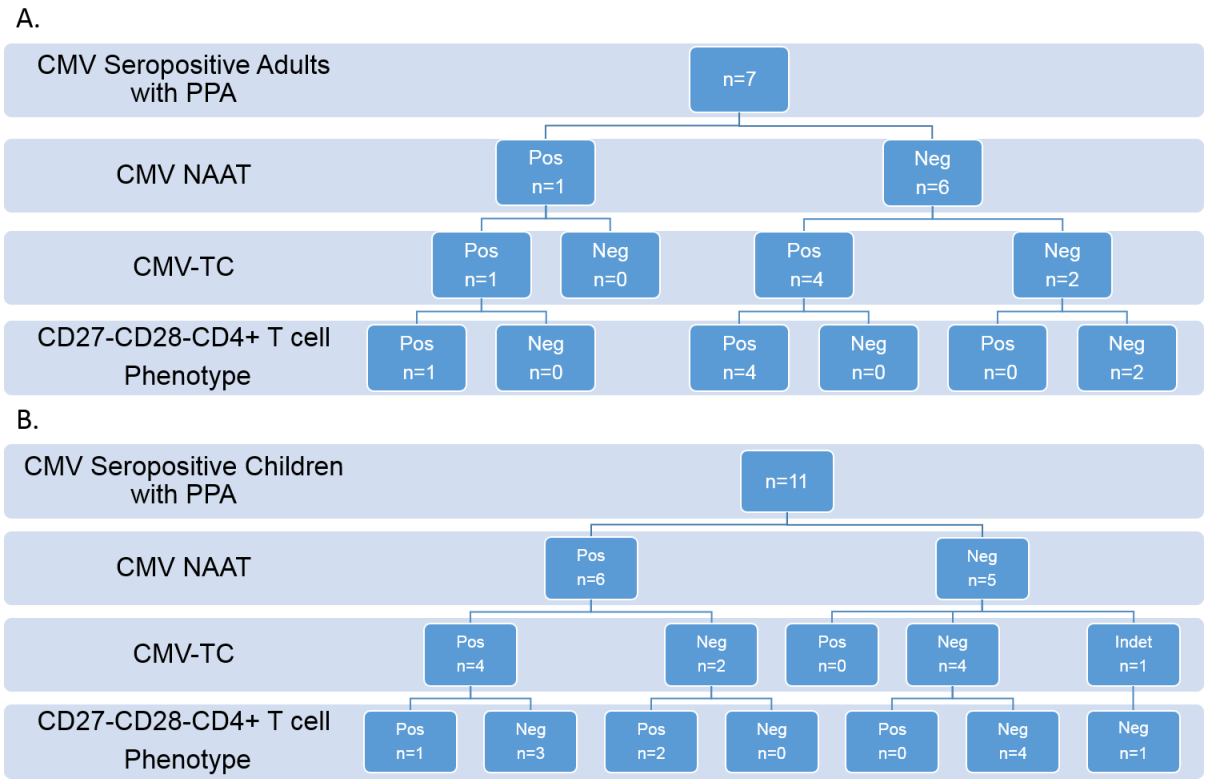


Figure 3.7 CMV-NAAT, CMV-TC and CD27-CD28-CD4+ T cell phenotype in CMV Seropositive Adults and Children with Potential Passive Antibody (PPA)

(A) Adults: There was 100% agreement between the CMV-TC assay and CD27-CD28- CD4+T cell phenotype in the 7 adults with potential PA, (classified 5 as positive and 2 as negative). CMV-TC considered positive if >= 0.075%. CD27-CD28-CD4+ T cell phenotype considered positive if >= 0.46%.

(B) Children: Two children, controls aged 3 and 8 months, with positive CMV NAAT had no detectable CMV-TC. There was poor agreement between CMV-TC assay and CD27-28-CD4+ T cell phenotype in children with PPA. CMV NAAT considered positive if at least one of throat, saliva, urine or blood was positive. CMV-TC considered positive if >= 0.035%. CD27-CD28-CD4+ T cell phenotype considered positive if >= 1.96%.

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Chapter 4

Summary

4.1 Summary of Research and Implications for Future Research

CMV is the most common infection after solid organ transplant and it has a major impact on transplant outcomes.[1,2] Significant advances have been made in the prevention of CMV disease, with universal antiviral prophylaxis or pre-emptive therapy.[3,4] The choice of the most appropriate CMV prevention strategy relies on accurate determination of CMV infection status in organ donors and recipients as the donor/recipient CMV infection status is a key predictor of the risk of CMV disease post-transplant.[1,2] Unfortunately, serology-based identification of CMV infection status has limitations in situations where passive antibodies may exist, such as in young infants who may have maternal antibody, and in individuals who have received a recent transfusion of plasma-containing blood products. Alternative methods to accurately assign CMV infection status in the solid organ transplant population are needed.

The goals of this program of research were to more clearly identify the age of infants in whom CMV serology may be falsely positive due to passive maternal antibodies, and to evaluate the use of alternative assays, not confounded by the presence of passively acquired antibodies, to determine CMV infection status in adults and children awaiting solid organ transplant.

Determining the time to clearance of passive maternal CMV IgG is challenging as time, with documentation of CMV sero-reversion, is the only gold standard. Even with a longitudinal study design with repeated blood collection in CMV seropositive infants of seropositive mothers, the high rate of CMV infection in infants of CMV seropositive mothers, related to transmission through breast milk and close contact with other infected secretions, makes determination of time to clearance of maternal CMV IgG challenging.[5,6] Our retrospective review of CMV serology in

72 CMV seropositive infants less than 18 months of age awaiting SOT suggests that passive maternal CMV IgG has cleared before 12 months of age. These findings are supported by other studies of CMV sero-reversion and the kinetics of CMV IgG in infants of CMV seropositive mothers, and by studies of time of clearance of maternal IgG antibodies to other viruses.[6-9] This evidence supports that 12 months, as opposed to 18 months, is a more appropriate cut-off for considering potential passive maternal antibody in young children awaiting SOT; CMV serology should be considered to be reliable in children over 12 months of age, who have not had recent transfusion.

Our retrospective and prospective studies both support that detection of CMV viral shedding, with CMV culture or CMV NAAT, is an important adjunct to CMV serology in CMV seropositive infants with potential passive maternal antibody as it confirms true CMV infection status in many infants, thus improving the accuracy of CMV risk-stratification in these infants. Our prospective evaluation of the detection of CMV-TC to assign CMV infection status showed promising results in children over 12 months of age but, the CMV-TC assay lacked sensitivity in identifying true positive CMV infection status in infants less than 12 months. In line with a previous study evaluating the detection of CMV-TC in children less than 18 months of age, we were able to detect CMV-TC in some CMV seropositive children <18 months, but the additional use of CMV NAAT in our study allowed us to show that the absence of detectable CMV-TC cannot be used to assume that a CMV seropositive infant has only passive antibodies.[10] A negative CMV-TC assay result should not be used to rule out a true positive CMV infection status in CMV seropositive infants less than 12 months of age. However, we believe that further study of the use of the CMV-TC assay in combination with CMV NAAT is warranted as the combination of assays may be useful to identify children who are truly infected but have poor

immune control of CMV, who may be at higher risk of CMV-related complications post-transplant.[11] Longitudinal studies to further characterize the development of CMV-specific T cell responses and their relationship to viral shedding in young children with post-natally acquired CMV infection would help us to better interpret and use both the CMV NAAT and CMV-TC assays in the context of pre-transplant assessment and prediction of post-transplant CMV disease.

While our evaluation of CD27-CD28- CD4+ T cell phenotype suggests that the frequency of CD27-CD28- CD4+ T cells is not a reliable marker of CMV infection status in children, our findings of high frequencies of this phenotype post-thymectomy may be of value in future investigation into pre-maturation of the immune system associated with childhood thymectomy.[12-14]

Our prospective study suggests that CMV NAAT has a limited role as an adjunct to CMV serology in clarifying true CMV infection status in adults with potential passive antibody as CMV shedding is uncommon in adults. The CMV-TC assay showed promise as an adjunct to CMV serology in assigning CMV infection status in adult subjects with potential passive antibody, although validation with larger patient numbers will be required. It may also be useful in determining true CMV infection status in transfused deceased organ donors, in whom no pre-transfusion specimen is available, although its use may be limited by a higher percentage of indeterminate results in deceased donors as compared to living individuals.[15] Sensitivity and specificity were lower in adult subjects than in controls but the discordant results, between CMV serology and CMV-TC assay, may have important clinical implications. A previous study found that among CMV seropositive recipients of organs from CMV seropositive donors, recipients without detectable CMV-specific CD8+ T cell immunity had a significantly increased incidence

of CMV DNAemia post-transplant compared with recipients with detectable T cell immunity.[11] Another study, in pre-renal transplant patients, found that CMV-specific T cells were detectable in some CMV seronegative individuals and those individuals had lower rates of CMV infection post-transplant.[16] Further studies of pre-transplant assessment of CMV-specific cellular immunity and post-transplant CMV-related outcomes are needed to determine whether analysis of CMV-specific T cell responses may be more useful than CMV serology alone in pre-transplant risk-stratification for post-transplant CMV-related complications. While the CD27-CD28- CD4+ T cell phenotype assay is faster and easier to perform and had similar sensitivity to our CMV-TC assay in discriminating seropositive from seronegative adult subjects awaiting SOT, the specificity of the assay was disappointing and may limit the use of this assay as an adjunct to serology in assigning CMV infection status in adults with potential passive antibody.

4.2 Implications for Future guidelines

A major translational goal of this program of research was to contribute to the development of revised and more extensive clinical practice guidelines for screening, monitoring and management of CMV in pediatric SOT patients. Current International Consensus Guidelines on the Management of Cytomegalovirus Infection in Solid Organ Transplantation suggest that infants less than 12 months of age may have false positive CMV serology due to passive maternal antibodies, while the American Society of Transplantation (AST) guidelines suggest a cut-off of 18 months.[1,2] Our retrospective study adds to the limited additional available evidence and supports that, when establishing CMV infection status, 12 months is likely a more appropriate cut-off than 18 months for considering false positive CMV serology due to passive maternal antibody.[6-9] Decreasing the age from 18 to 12 months would mean that far fewer infants

would require additional investigations to try to accurately establish CMV infection status and there would be fewer infants in whom the highest risk scenario of donor/recipient serostatus would have to be assumed, thus reducing unnecessary use of additional testing and anti-virals.

This program of research was designed to address one of the areas for future research identified in the 2013 International Consensus Guidelines on the Management of Cytomegalovirus Infection in Solid Organ Transplantation which was to "assess the utility of cell-mediated immunity assays and NAAT using a variety of sample types for the interpretation of passive immunity due to transfusion of blood products and maternal antibodies".[2] Current International guidelines suggest that CMV culture or NAAT of urine or throat swabs may be helpful to identify truly infected CMV seropositive infants with potential passive antibody, while AST guidelines suggest that CMV culture of urine should be performed in these infants but that the role of CMV NAAT in CMV risk assessment has not been fully investigated.[1,2] The results of our studies support that CMV culture and CMV NAAT, when positive, are very useful adjuncts to serology to confirm true positive CMV infection status in CMV seropositive infants with potential passive antibody. Sampling at multiple sites, urine and saliva ideally, may identify more truly infected infants than sampling of urine alone. Our results, in combination with the well-recognized improved sensitivity of CMV NAAT over CMV culture, support that CMV NAAT, of urine and saliva or throat swab, should be implemented for routine pre-transplant screening of CMV seropositive infants less than 12 months of age and could be considered in older infants and children who have been transfused and have no pre-transfusion sample available.[17] Our evaluation of the use of a CMV cell-mediated immunity assay (our CMV-TC assay), which used flow-cytometry to identify CMV-specific CD4+ cells by CD69 expression and intracellular IFN-γ quantification after stimulation with CMV-antigen lysate, supports that

this assay performs quite well even in ill adults and children >12 months, across all organ types, awaiting SOT. Further evaluation of this assay with larger patient numbers, is needed to determine if and how this assay should be incorporated into pre-transplant CMV risk-stratification guidelines. Our finding that the CMV-TC assay lacks sensitivity in identifying true positive CMV infection status in infants < 12 months of age, has important implications for future guidelines as it suggests that the absence of detectable CMV-TC should not be used to rule out true CMV infection in infants. Future studies are needed to investigate whether the combined use of CMV NAAT and a CMV-TC assay may improve pre-transplant CMV risk-stratification, by identifying children with poor immune control of CMV, those with positive CMV NAAT and no detectable CMV-TC, who may be at particularly high risk of CMV-related complications post-transplant.

4.3 Conclusion

Accurate CMV-risk stratification is essential in optimizing CMV-prevention strategies, decreasing unnecessary use of anti-virals and additional post-transplant monitoring for CMV, and ultimately improving outcomes and quality of life in SOT recipients. Through this program of research, we have contributed to improved CMV-risk stratification in many ways. We have better identified the group of infants in whom CMV serology may be unreliable due to passive maternal antibody, we have shown that CMV NAAT is an important adjunct to serology in CMV seropositive infants with potential passive antibody, and that the detection of CMV-specific CD4+ T cells is a promising adjunct to CMV serology in adults and children with potential passive antibody awaiting SOT, but that the lack of detectable CMV-specific T cells should not be used to rule-out true CMV infection in infants < 12 months of age. We believe that these

findings will lead to changes in guidelines and in practice that will serve to improve pre-transplant CMV risk-stratification, and ultimately improve outcomes in SOT recipients.

4.4 References

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