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Cytomegalovirus Latency Promotes Cardiac Lymphoid Neogenesis and Accelerated Allograft Rejection in CMV Naïve Recipients

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Abstract

Human Cytomegalovirus (HCMV) infection is associated with the acceleration of transplant vascular sclerosis (TVS) and chronic allograft rejection (CR). HCMV-negative recipients of latently HCMV infected donor grafts are at highest risk for developing CMV-disease. Using a rat heart transplant CR model, we have previously shown that acute rat CMV (RCMV) infection following transplantation significantly accelerates both TVS and CR. Here, we report that RCMV-naïve recipients of heart allografts from latently RCMV-infected donors undergo acceleration of CR with similar kinetics as acutely infected recipients. In contrast to acutely infected recipients, treatment of recipients of latently infected donor hearts with ganciclovir did not prevent CR or TVS. We observed the formation of tertiary lymphoid structures (TLOs) containing macrophages and T-cells in latently infected hearts prior to transplantation but not in uninfected rats. Moreover, pathway analysis of gene expression data from allografts from latently infected donors, indicated an early and sustained production of TLO-associated genes compared to allografts from uninfected donors. We conclude that RCMV-induced TLO formation and alteration of donor tissue T-cell profiles prior to transplantation in part mediate the ganciclovir-insensitive rejection of latently infected donor allografts transplanted into naïve recipients by providing a scaffold for immune activation.

Keywords

Cytomegalovirus; Chronic Rejection; Transplant Vascular Sclerosis; Latency

Introduction

Human cytomegalovirus (HCMV) infections are life long and the virus persists in a latent state during which time gene expression is limited and infectious virus is not produced. Critical to the ability of HCMV to maintain life-long infection is the capacity to establish cellular sites of viral persistence while avoiding recognition and elimination by the host immune system. Clinical studies have directly associated HCMV infection with the

acceleration of solid organ allograft chronic rejection (CR) due to increased transplant vascular sclerosis (TVS). Although all transplant recipients are at risk for developing CMV disease, seronegative recipients of allografts from seropositive donors are at the highest risk for CMV infection and consequently for graft loss due to accelerated TVS [1-3]. When an allograft from a CMV latently infected donor is transplanted into a naïve (uninfected) recipient, the immediate environment is one of inflammation due to ischemia/reperfusion and the obligatory immune suppression therapy given at the time of transplantation to prevent acute allograft rejection. This environment promotes CMV reactivation, which is considered to be the major source of viral infection. Direct evidence for the role of the transplanted organ as a source of HCMV is supported by finding of HCMV genetic material in the vasculature of transplanted organs [4]. In cardiac transplant recipients, HCMV infection either *de novo*, or by reactivation in the recipient, nearly doubles the 5-year rate of graft failure due to accelerated TVS [5]. Importantly, kidney transplant graft survival was decreased in asymptomatic HCMV infected recipients during the first 100 days post transplantation compared to patients with no HCMV infection, suggesting that the presence of HCMV alone, whether asymptomatic or manifesting overt infection, has a negative impact on graft survival [6, 7]. Supporting the role of HCMV in the development of CR is the observation that treatment of heart transplant recipients with ganciclovir, a potent inhibitor of viral replication, delayed the development time to allograft rejection in comparison to an untreated HCMV seropositive group [8, 9].

Ganciclovir is costly and has significant side effects such as pancytopenia and nephrotoxicity and ganciclovir resistance is common; hence an understanding of the mechanisms associated with HCMV-associated TVS is important. It has been difficult to determine these mechanisms due to the multifactorial etiology of TVS, in addition, HCMV is ubiquitous throughout the population making it nearly impossible to understand how *de novo* infection vs. reactivation affects TVS. Using rat transplantation models of CR, we have demonstrated that acute infection of the allograft recipient with RCMV dramatically decreases the mean time to develop TVS and rejection [10-16]. Although these observations demonstrate a link between CMV and TVS, the viral characteristics, including source and replication status associated with the acceleration of disease need further clarification. In this report, we examine how the source (donor vs. recipient) and replication status (acute or latent) of RCMV infection influence the progression to the development of TVS and CR. We also evaluate the impact of ganciclovir therapy on accelerated TVS. The findings from this study have the potential to direct further treatment strategies in humans to minimize the risk of CMV associated CR.

Materials and Methods

Heart transplantation

F344 rat donor hearts were transplanted heterotopically into Lewis recipients [17, 18]. All animals were housed in the Portland Veterans Administration Medical Center animal facility, which is AAALAC accredited and complies with the USDA and HHS requirements for animal care. To determine whether status or source of virus infection affected graft survival either the donor (D) or recipient (R) were infected with 1×10^5 plaque-forming units (PFU) of the Maastricht strain of RCMV [18-20] intraperitoneally (i.p.) at 1 day post-op (POD) (acute R infection), 7 days pre-op (acute D infection) or 6 months pre-op (latent R and D infection). Uninfected allograft R served as controls. Animals were examined daily for overall health and CR was determined by monitoring heartbeat grade, and upon graft failure the hearts were harvested [17]. Paraffin embedded tissue sections were stained with elastic von Gieson. The extent of neointimal formation (TVS) was determined by the neointimal index ($NI = (\text{intima area} / \text{lumen} + \text{intima area}) \times 100$) [18, 21]. To evaluate the impact of anti-viral therapy on accelerated TVS, the recipients in the ARI group and LDI

group were treated with ganciclovir (Roche) (20mg/kg/day i.p. for 45 days following transplantation). The severity of TVS in the uninfected compared to the RCMV-infected graft recipients (+/- ganciclovir) were analyzed using Student's *t* test. *P* values <0.05 were considered statistically significant.

RCMV-specific enzyme-linked immunoabsorbent assay (ELISA)

ELISA plates were coated with RCMV lysates (5µg/ml) overnight, washed and blocked with 2% milk in 0.05% Tween-PBS. Triplicate serial dilutions of serum samples were incubated for 2hrs at room temperature followed by 3 washes with 0.05% Tween-PBS. The plates were incubated with horseradish peroxidase-conjugated anti-rat IgM or IgG for 1hr, followed by addition of chromogen OPD substrate to allow detection and quantification of bound antibody molecules. Average antibody titers are calculated from absorbance data (n=3).

TaqMan PCR detection of RCMV

Total genomic DNA was extracted from rat native and graft hearts and submandibular glands using DNazol. A total of 0.5µg of DNA was analyzed by TaqMan PCR using a probe/primer set recognizing RCMV DNA polymerase sequence [18, 22]. The sensitivity of detection for this assay was <100 copies.

Illumina Microarray Analysis

Allograft gene expression profiles from recipients of latently infected allografts and uninfected controls was analyzed using Illumina RatRef-12 arrays containing >22,000 probes selected from the NCBI RefSeq database. RNA samples from non-transplanted hearts were the baseline controls. Presence and differential expression of a transcript was determined using Genome Studio by Illumina. Heat maps displaying fold change expression data were generated using the Open Source Software R. Pathway analysis was performed using MetaCore by GeneGO. Normalized expression levels for each gene were compared to uninfected allografts and native hearts using Student's *t*-test. The *p* values of <0.05 were considered significant.

Quantitative RT-PCR

Gene expression was quantified using real-time RT-PCR. Primer sets include: CCL19-Fwd: gaccaagcctcccgatctg, Rev: catgctcacactcagttcaca; CCL21b-Fwd: cacaggcaaagaggagctaga, Rev: gaagcagacaagggtgtgacaa; CXCL13-Fwd: tcctaagaagcagacgcattac, Rev: gtcgaagcattattccctcatg. Rat fibroblasts and smooth muscle cells were infected with RCMV and RNA was harvested using Trizol at 8, 16, 24 and 36hpi. cDNA was generated using Superscript III (Invitrogen) and analyzed by real-time PCR. Gene amplicons served as quantification standards (sensitivity 100 copies/gene). Results were analyzed using ABI StepOne Real-Time PCR system and normalized to L32 [18, 23].

Immunohistochemistry

Antibodies to rat CD8α (BD Biosciences) and ED1 (Serotec) were incubated with the heart tissue sections for 45min at room temperature in a Dako Immunostainer (39). Subsequently, slides were incubated with mouse-specific peroxidase-conjugated secondary antibodies and developed using a MAB kit (Dako). Slides were counterstained with hematoxylin and visualized by microscopy.

Flow Cytometry

Heart, spleen, and blood samples were harvested from control and infected rats at 21 or 180dpi. Following exsanguination, the rats were perfused with PBS. The hearts were digested

in CollagenaseD (200 unit/ml) at 37°C for 40min. The spleen and digested heart tissue were macerated through a wire mesh and strained through a 70µm nylon cell strainer. Lymphocytes were separated from heart and spleen tissue suspensions and diluted whole blood using Ficoll-Paque Plus (GE Healthcare). After centrifugation, the white blood cell layer was resuspended in RPMI 1640 media (Mediatech). The number of lymphocytes was determined using a Hemavet HV950 Multispecies Hematologic Analyzer (Drew Scientific). For each sample, 2×10^6 lymphocytes were stained for 20min with fluorescently labeled anti-rat antibodies to CD4 (APC-Cy7, Biolegend), CD8 (PerCP-Cy5.5, BD Biosciences), and CD62L (PE, BD Biosciences). Cell cycle status was determined by measuring Ki-67 expression (BD Biosciences). The cells were analyzed using an LSRII Flow Cytometer and the data was analyzed with FlowJo software (Tree Star).

Results

RCMV Infection Source and Status Influences the Development of TVS/CR

In human transplant recipients the source of virus infection (donor vs. recipient) and the status of viral infection (active vs. latent) can be used to stratify the risk of developing CMV disease and allograft rejection. In this study, we use a rat heart transplant CR model to characterize the importance of source and status of RCMV as it relates to time to chronic allograft rejection and severity of TVS by performing transplants in acutely (A) or latently (L) RCMV-infected donors (D) or recipients (R). For these experiments, F344 rat hearts were transplanted into Lewis recipients (see Table 1). The D or R rats were acutely (R=day 1post-op; D=7 days pre-op) or latently (6 months pre-op for both D and R) infected with RCMV. Upon graft failure, the hearts were harvested and evaluated histologically for TVS (NI, neointimal index). Acutely infected R (ARI) rejected their naïve donor grafts significantly earlier when compared to naïve recipients of grafts from acutely infected D (POD 43 vs. 53, $p=0.01$, $n=6$ and 4 respectively), whereas uninfected controls, receiving uninfected hearts, rejected at day 90 (Figure 1 and Table 1). Similarly, at the time of rejection, the ARI also demonstrated increased TVS formation in their grafts compared to those from ADI (NI=74 vs. 50, $p=0.01$). Importantly, similar to the human scenario, recipients of latently infected D (LDI) ($n=8$) rejected their grafts significantly earlier than did the latently infected R of CMV naïve grafts ($n=7$) (POD 54 vs. 63, $p=0.01$) with more severe TVS at the earlier rejection time (NI=63 vs. 51, $p=0.01$) (Figure 1 and Table 1). Viral DNA was detected in the graft tissues and/or salivary glands at rejection in all groups except R of grafts from LDI (Table 2). Despite the absence of detectable virus at endpoint, RCMV-specific antibodies were present in all allograft recipients including those receiving hearts from latently infected donors (Table 2). These data demonstrate that the status of CMV infection (acute vs. latent) and the source (donor vs. recipient infection) affect the degree of CMV-acceleration of TVS and CR.

Ganciclovir Reduces Acute but not Latent RCMV-accelerated TVS/CR

To assess the role of viral replication in acute and latent RCMV-accelerated rejection, we treated allograft recipients with ganciclovir ($n=6$), a drug that inhibits late viral gene expression. Ganciclovir reduced RCMV-accelerated CR of allografts in ARI by increasing time to rejection from POD 43 to POD 75 ($p<0.001$) and reducing TVS from NI=80 to NI=65 ($p<0.001$) (Table 3). This finding indicates that viral replication/late gene expression is critical in the RCMV-accelerated allograft disease processes in ARI. However, ganciclovir treatment of the recipients of allografts from latently infected donors failed to impact the development of graft TVS when compared to that of the graft in the untreated group (NI=65 vs. 63; $p=NS$, time to rejection was 52 vs. 54; $p=NS$ (Table 3).

This finding indicates that a fundamental difference exists in the mechanisms that define CMV-accelerated rejection between the ARI and LDI groups. To determine whether this difference is in the sequence of events and/or factors that mediate rejection we analyzed the kinetics of TVS formation in ARI and LDI. Assessment of these grafts revealed that RCMV infection in both LDI and ARI groups significantly increased the severity of TVS in heart allografts at POD28, 35 and 45 compared to uninfected controls (n=4 each). In addition, the rate of NI formation between POD21 and rejection for both groups is significantly different when compared to uninfected controls (i.e. line slope: LDI=1.226, $R^2=0.9725$; ARI=1.699, $R^2=0.9932$; and Uninfected=0.3477, $R^2=0.8695$) (Figure 2). To determine whether RCMV reactivated in the LDI group we quantified the levels of viral genomic DNA in the recipient salivary glands at POD 7-28. Viral DNA was first detected in recipient salivary glands at POD21 (Figure 3). We used RCMV-specific ELISA to detect IgM and IgG levels in the LDI group (donors and recipients). The recipients of allografts from latently infected donors had predominately an IgM response at POD21, whereas the donor group (180dpi) had predominately an IgG antibody response (Figure 4) indicating that RCMV recently reactivated in the recipients of hearts from LDI animals.

RCMV Induces TLO-Genes Expression

We have previously shown that genes involved in inflammation, chemotaxis and wound healing are up-regulated in ARI allografts compared to uninfected controls [18, 23, 25]. In the current study, we used DNA microarrays to analyze gene expression in recipient allografts from LDI. The fold change values of the allograft (n=3 for each of the infected and uninfected transplant groups) were compared to uninfected non-transplanted hearts (n=4). In general, allografts from the LDI group had increased expression of a number of genes involved in inflammation and wound healing including a number of cytokines (IL1 β , IL6, IL16, IL18, LtB), chemokines (CCLs 2, 3, 4, 5, 7 & 19, CXCLs 1, 2, 9, 10, 11, 12 & 13), cell adhesion molecules (integrins α 1, α 4, α 11, α L, & β 2, ICAM-1 and VCAM-1), inflammatory signaling molecules (Hck, Csk, Lck, Apobec 1, IRFs 5 & 7, TLRs 2 & 6, and Jaks 2 & 3) as well as genes that encode proteins with extracellular matrix remodeling activities (MMPs 3, 7, 12, & 14; TIMP-1, cathepsins E, S & W) and growth factor activities (angiogenin, angiopoietin, connective tissue growth factor, endothelin, osteopontin, thrombospondin-2, TGF) (Figure 5). Up-regulation of these genes has the potential to promote allograft rejection by increasing alloreactivity and promoting unchecked wound repair and fibrosis. Interestingly, a few genes were down-regulated in comparison to normal heart tissues and allografts from uninfected recipients. These included a family of fibroblast growth factors (FGF 1, 12, 13 and 16) as well as the TGF- β receptor; however, the implications of down-regulating these genes is yet to be realized, and warrants further investigation. Pathway analysis of the microarray data from latently infected allografts following transplantation indicated an early (day 7) and sustained production of TLO-associated genes compared to uninfected allografts. These genes included the homeostatic chemokines CCL19, CCL21, and CXCL13, lymphocyte adhesion molecules ICAM-1, MAdCAM, and VCAM-1 and Lymphotoxin B (Table 4), suggesting that cardiac allografts from latently infected donors undergo active lymphoid neogenesis, and more specifically the generation of tertiary lymphoid organs (TLOs).

TLO formation is initiated by lymphoid tissue inducer cells (LTi) that function to up-regulate cell adhesion molecule, cytokine and chemokine expression in stromal cells [26-36]. The production of the homeostatic chemokines CXCL13, CCL21b and CCL19 are crucial for the formation of TLOs [32, 37]. Typically, a number of different cell types can act as stromal cells including fibroblasts, endothelial cells, immune cells, and pericytes that can differentiate into smooth muscle cells (SMC). We next determined whether RCMV infection directly promotes the activation of a TLO phenotype in rat fibroblasts and SMC by

measuring homeostatic chemokine gene synthesis. While low levels of CXCL13 and CCL19 are present in uninfected SMC, RCMV infection dramatically increased expression of CCL19, CCL21b and CXCL13 (Figure 6). In fibroblasts, RCMV infection also induced CCL21b and CXCL13 but did not increase expression of CCL19. Expression of these TLO-associated chemokines is critical for the initial stages of lymphoid neogenesis leading to the recruitment of lymphocytes and additional LTi's [38].

Latent RCMV Infected Pre-Transplant Hearts Contain Increased Levels of Macrophages And T-cells

Given that latent RCMV infection induces the expression of TLO associated genes, we performed immunohistochemical staining for T-cells and macrophages on sections from latently infected (180 dpi) non-transplanted hearts as well as uninfected control hearts. We detected increased numbers of resident ED1+ macrophages and CD8 α + T-cells within the hearts from each of the latently infected animals but not in uninfected controls (n=3) (Figure 7). In addition, within the hearts from the three latently infected animals, both macrophages and CD8 α positive T-cells were observed in large clusters. In contrast, clusters of macrophages and/or T-cells were absent in hearts from uninfected animals.

We then characterized the T-cell subsets from PBMC, splenocytes, and heart-derived lymphocytes from RCMV-infected donor hearts at 21 and 180dpi using flow cytometry, and compared them to cells harvested from uninfected control rats (n=4 for each group). We found the frequency of CD4 T-cells is increased in hearts from latently infected donors compared to both uninfected controls and tissues from rats infected for 21 days (27.8% vs. 20.4% and 20.4%; respectively) (Figure 8). However, the percentage of CD4 T-cells in the blood and spleen from latently infected rats is significantly reduced compared to both uninfected controls and at 21dpi (blood: 39.1% vs. 49.5% and 51.3%, respectively; $p < 0.001$ and spleen: 23.9% vs. 31.5% and 35.6%, respectively; $p < 0.001$) (Table 5). Frequency of CD8 T-cells is similarly increased in the infected hearts compared to uninfected controls with the percentage escalating from 19.5% at 21dpi to 37.1% at 180dpi (Figure 8). In contrast to CD4 T-cells, the percentage of CD8+ T-cells also increases in both blood (20.4% vs. 20.9% and 26%, respectively) and spleen (13.3% vs. 15.5% and 24.6%, respectively) of latently infected animals.

We also found that the frequency of CD4 and CD8 effector memory cells increased in a time-dependent fashion, with the latently infected donors exhibiting significantly higher levels in spleen (Table 5) and heart (Figure 8) compared to those from rats at 21dpi ($p < 0.001$). In fact, 93.6% of CD4+ T cells and 88.7% of CD8+ T-cells are of the effector memory phenotype in the heart tissues from rats at 180dpi (Figure 8). Interestingly, both the CD4+ and CD8+ T-cells isolated from the infected heart tissues were actively proliferating prior to the time of harvest as evidenced by the presence of Ki67, a marker of recent entry into the cell cycle (Figure 8). These findings demonstrate that the RCMV latently infected donor hearts contain a substantial number of activated T-cells prior to transplantation.

Discussion

The donor and recipient HCMV serologic status may be the most important factor in determining the development of severe HCMV disease and consequently accelerated allograft CR. In the current report, we examined the effects of RCMV infection status and source on the acceleration of TVS and CR using a rat heart transplant model. We show the source of infection (donor vs. recipient) and the infection status (acute vs. latent) prior to transplantation had a major impact on allograft survival. The ARI group rejected with the most rapid kinetics (POD43) and most severe TVS (NI=74). While both the ADI and LDI groups rejected at nearly the same time (POD53 vs. 54, respectively), the allograft TVS

associated with the LDI was significantly more severe than the ADI group (NI=63 vs. 50, respectively). As expected from the experience with human solid organ transplants, the latently infected recipient group (LRI) rejected with the slowest kinetics (POD62) with a degree of TVS similar to uninfected controls at POD90 (NI=50 vs. 51, respectively). The overall effect of status and source of virus on RCMV-accelerated rat heart allograft rejection parallels the human transplantation scenario [5].

The best evidence supporting a role for HCMV in the development of TVS/CR is that treatment with ganciclovir delays the time to allograft rejection [8, 9]. In our study, inhibiting active viral replication by treating the ARI group with ganciclovir substantially increased the time to rejection from POD42 to 75. This was in contrast to treatment of the LDI group, which was not affected by ganciclovir and developed allograft vasculopathy similar to the untreated group. This novel finding suggests the mechanisms of rejection and requirements for viral replication differ between the two groups. In the ARI group, virus replication is crucial for the infection of the allograft heart, which potentiates the host immune response to both virus and graft leading to an early rejection process. Whereas in the LDI model, persistently infected cells already exist within the allograft and viral immediate early and/or early genes are, for the most part, responsible for inducing CR. In addition, the donor immune response to the latent virus may prime the allograft for rejection by recruiting immune cells into the allograft prior to transplantation. We found that hearts from RCMV latently infected rats have an abundance of macrophages and T cells organized in TLOs. Uninfected rats were devoid of these structures and they did not harbor activated cardiac T-lymphocytes. TLOs are aggregates of immune cells that form under conditions of chronic inflammation in tissues that do not normally contain lymphoid organs. Similar to peripheral lymphoid structures, TLOs are scaffolds for immune activation. TLOs form during many different chronic inflammatory diseases including arthritis, atherosclerosis, cancer, and following various viral infections [26-36]. TLOs initially form through the recruitment of LTi's that stimulate the surrounding stromal cells and up-regulate expression of cell adhesion molecules and homeostatic chemokines, which effectively attract lymphocytes. A genome-wide microarray analysis of heart allografts from infected (LDI) and uninfected transplant groups revealed up-regulation of genes involved in inflammation and tissue remodeling processes. These genes included a number of chemokines such as CCL2, 3, 4, 5, 7 and 19 as well as CXCL 9, 10, and 13, known to recruit inflammatory cells. Similarly, adhesion molecule expression was more highly induced in the allografts from latently infected donors compared to uninfected allografts including integrins, selectins as well as ICAM-1 and V-CAM-1. These adhesion molecules promote the extravasation and tissue localization of the chemoattracted immune cells. While a number of cytokines were up-regulated in the allografts from the LDI group compared to uninfected controls the most highly expressed included IL-1 β , IL-6, IL-16, IL-18 and lymphotoxin β . These cytokines are mediators of cell-mediated immunity and could thus enhance the allo-response in the latently infected donors. Importantly, we found that by POD7, heart allografts from latently infected donors demonstrated a significant increase in TLO chemokine, adhesion molecule and LT β expression compared to allografts from uninfected donor rats. It is thought that expression of the homeostatic chemokines, alone, can initiate TLO formation by attracting lymphocytes and LTi's, this is particularly true of CXCL13 [38]. RCMV infection of SMC and fibroblasts, which are potential cardiac stromal cells, increases expression of the homeostatic chemokines including CXCL13. However, RCMV infection did not affect LT α , LT β , or LT β R expression (data not shown). Interestingly, mouse aortic SMC have been shown to differentiate into LTi's upon treatment with both TNF and an agonistic anti-LT β R mAb, and the production of these cells is associated with the development of atherosclerosis in mice [46]. We and others have demonstrated that MCMV accelerates atherosclerosis; however, the effects of CMV on TLO formation in this model were never specifically addressed. Along these lines, Vleigen et al. using similar models, found that MCMV

promotes the accumulation of T cells and macrophages in the atherosclerotic vasculature [47-51]. It is quite likely that persistent CMV infections associated with the vasculature or heart tissues can promote TLO formation by activating genes involved in lymphoid neogenesis, which ultimately enhances inflammatory processes that are specifically associated with chronic disease.

TLOs have also been associated with allograft rejection [44, 45] by supporting naïve T cell activation and the generation of effector and memory T-cells [34]. In deed, we detected the presence of T-cell and macrophage foci in latently infected hearts but not in the hearts from uninfected rats. This finding implies that latent RCMV infections, while asymptomatic, are persistent in the heart tissues and that these tissues harbor active anti-viral reactive immune cells. Approximately, 90% of the CD4+ and CD8+ T cells present in the hearts of latently infected rats were of the effector phenotype, whereas, those found within hearts of control animals or those infected for 21 days were not. Both CD4+ and CD8+ T-cells present in latently infected hearts were Ki67+ suggesting they were actively replicating. Interestingly, CMV infections are known to promote long-term alterations of T-cell profiles including T-cell memory inflation and the accumulation of effector memory CD8 T-cells [40-43]. These data suggest that the presence of effector CD4+ and CD8+ T-cells promote graft rejection via enhancing the local alloreactive immune response even in the absence of replicating virus (ganciclovir treated LDI group).

In summary, we show that, similar to the clinical transplant scenario, naïve recipients of heart allografts from latently infected donors undergo CR acceleration with similar kinetics to acutely infected recipients. Interestingly, treatment of recipients of latently infected donor hearts with ganciclovir did not prevent TVS and CR, which differs from its effect on rejection in ARI. We show that TLOs form in hearts of latently infected rats and transplantation of these hearts into naïve recipients significantly accelerates the time to rejection. Our data suggest that latent CMV infection induces TLO formation in the graft prior to procurement and this subsequently leads to enhancement of immune activation following transplantation. Further studies defining the mechanisms of this complex interplay between virus and TLO formation are necessary to develop appropriate therapeutic strategies to prevent and treat this important clinical transplantation problem.

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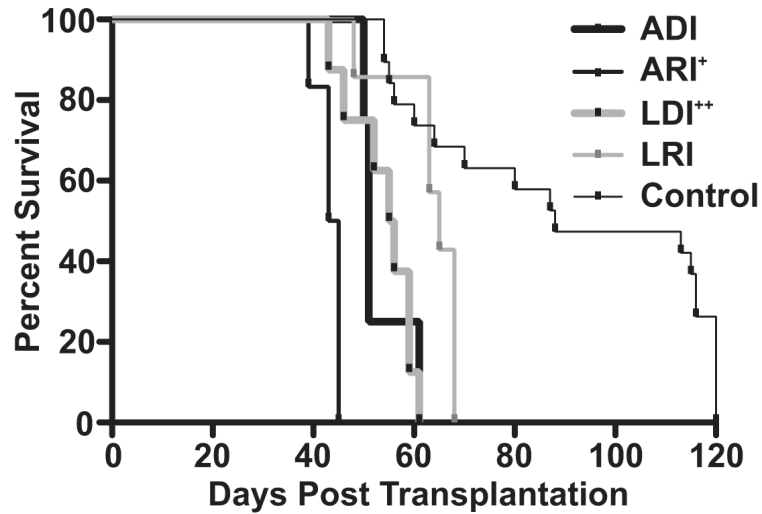


Figure 1. RCMV infection source (donor vs. recipient) and status (acute and latent) are important for virus acceleration of heart allograft chronic rejection

Shown is a survival curve for heterotopic heart grafts (F344) transplanted into Lewis recipients and monitored over a 120-day period for clinical rejection. Groups included: 1. ADI (POD 51, donor was infected 7 days prior to transplantation, n=4); 2. ARI (POD 43, recipient was infected POD 1, n=6); 3. LDI (POD 55, donor was infected 180 days prior to transplantation, n=8); 4. LRI (POD 63, recipient was infected 180 prior to transplantation, n=7); and 5. Control (uninfected donor and recipient). + = ARI vs. ADI $p < 0.01$, ++ = LDI vs. LRI $p < 0.01$, ADI/ARI/LDI/LRI accelerate graft rejection vs. control $p < 0.001$.

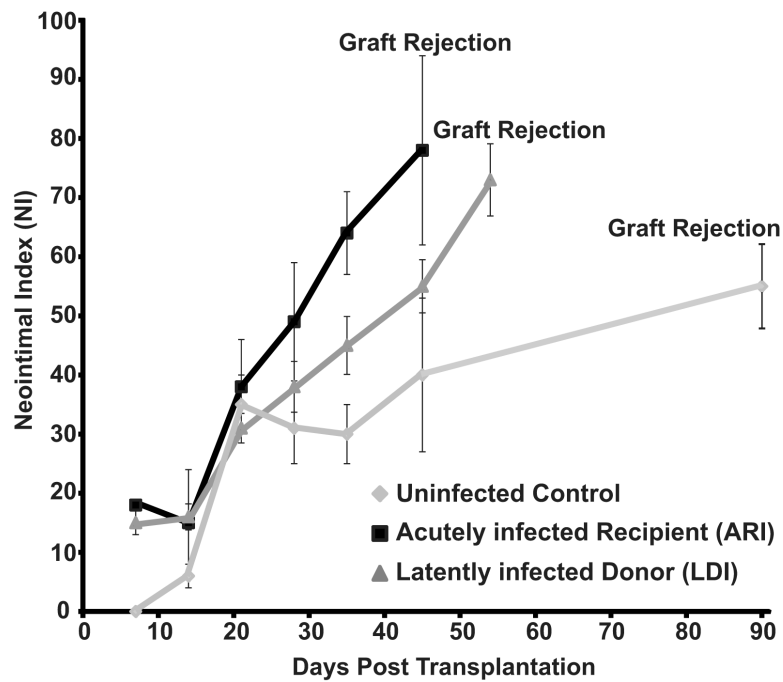


Figure 2. RCMV infection accelerates the time to develop TVS in graft hearts

Comparison of the time to allograft rejection and TVS (mean NI) of graft heart vessels of uninfected controls, RCMV-acutely infected recipients (ARI) and recipients of RCMV-latently infected donor hearts (LDI); $n=6$ for each. The time to rejection for the ARI and LDI was significantly decreased compared to that of the uninfected controls ($p<0.05$). Similarly, the increase NI parallels that of the time to rejection ($p>0.05$).

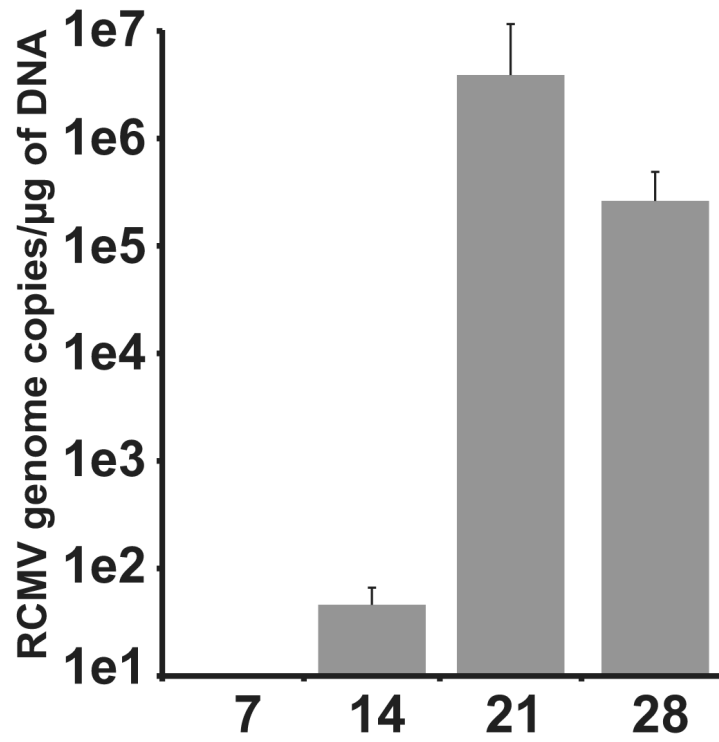


Figure 3. RCMV detection in salivary glands from recipients of latently infected donor hearts
RCMV genome copy was determined by real time PCR at 7, 14, 21, and 28 days post transplantation in recipients of latently infected donor hearts (n=4 at each time point).

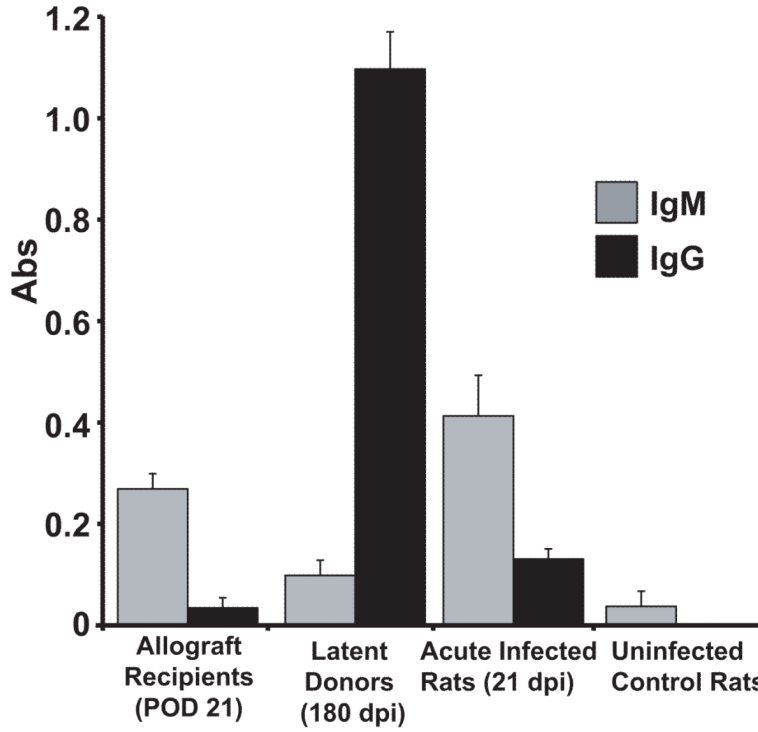


Figure 4. Recipients of RCMV-latently infected donor hearts have predominately an IgM RCMV-specific Antibody Response
RCMV-specific ELISA plates were coated with lysates from RCMV infected fibroblasts. Plasma samples from allograft recipients of hearts from latently infected rats (LDI) at POD21, latently infected heart donor rats, acutely infected rats (21dpi) and uninfected controls (n=4 for each). Plasma was diluted 1:50. The secondary anti-rat IgM or IgG antibody conjugated to HRP was used for detection. Recipients of RCMV infected donor hearts have predominantly an IgM antibody response, which is statistically significant compared the IgG response (p<0.05).

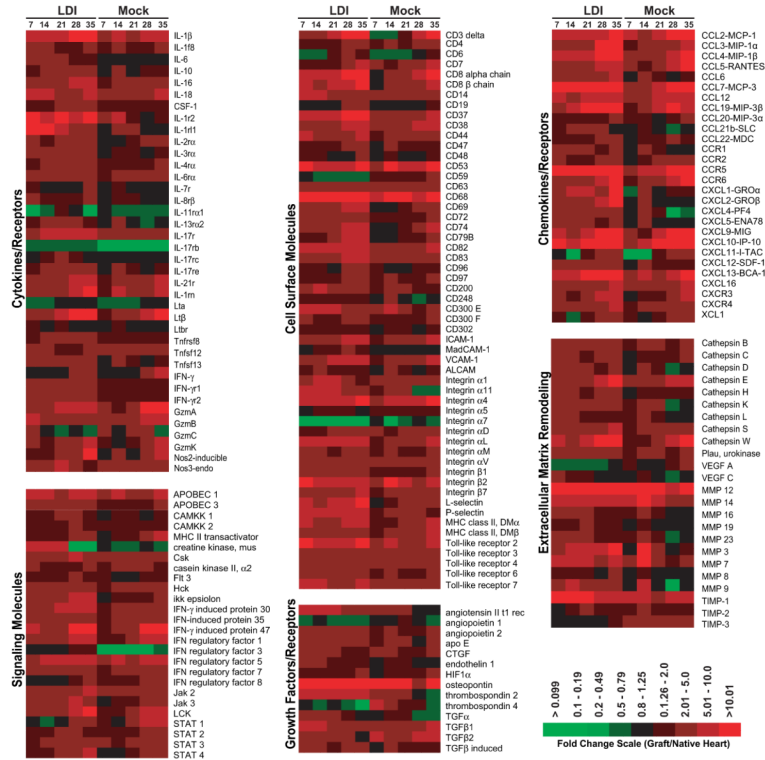


Figure 5. Gene expression profiling identifies differences between allografts from recipients of latently infected donors and uninfected controls

Microarrays were prepared from total graft and native hearts from recipients of latently infected grafts (LDI) and uninfected controls (Mock) harvested at 7, 14, 21, 28, and 35–post transplantation. Shown is a heat map representing the fold change values of the allograft (n=3) compared to uninfected non-transplanted heart (n=4). The data shown is limited to the identified genes displaying significant dysregulation (up or down) when compared to control hearts (LDI vs. mock infected p<0.05 for at least one of the time points). The data were subdivided into categories based upon predicted biological function using the computer program GeneGo by Metagene.

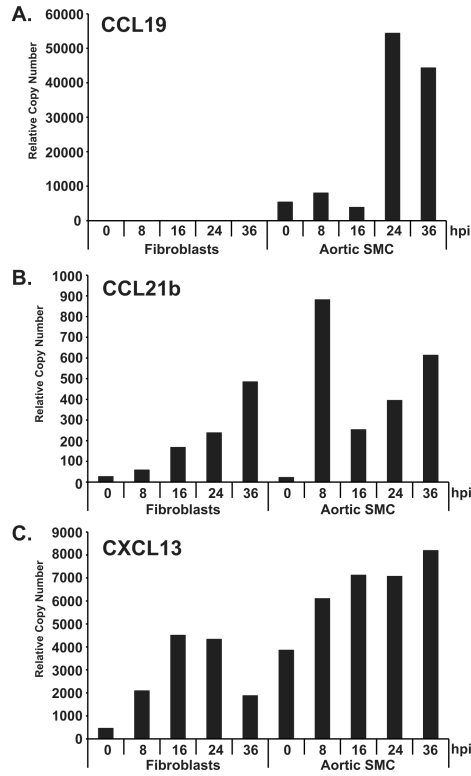


Figure 6. RCMV Infection Induces SMC and Fibroblast Expression of TLO Associated Chemokines

Quantitative RT-PCR was used to quantify CCL19 (A), CCL21b (B), and CXCL13 (C) chemokine expression in samples of RCMV infected primary fibroblasts and aortic SMC. RNA samples were analyzed in triplicate and normalized to expression of L32 (cellular ribosomal protein), and the relative copy number was determined using amplicon standards to each gene.

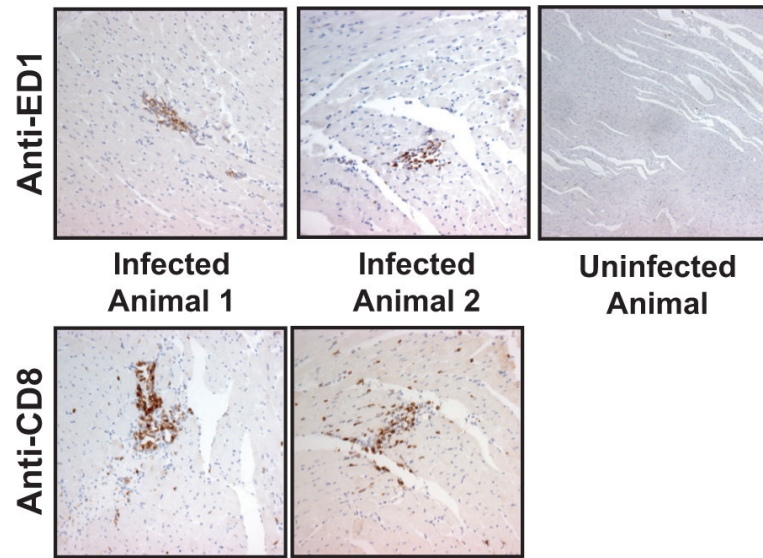


Figure 7. Hearts from RCMV latently infected rats contain macrophages and T cells
Paraffin embedded heart sections from latently infected rats or controls were probed using antibodies directed against ED1 (Mac) and CD8 (T-cell). (mag=40x).

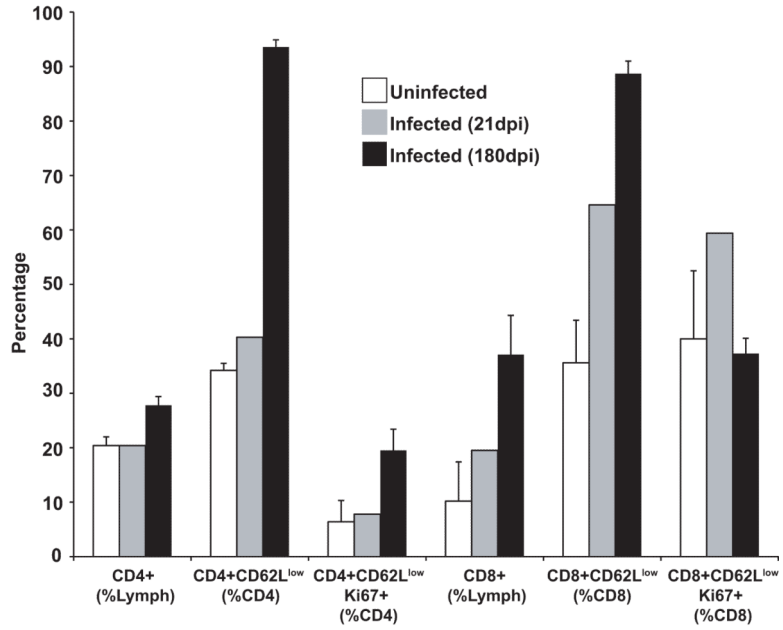
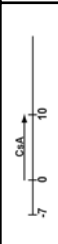



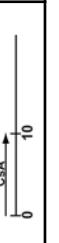


Figure 8. Latently infected rat hearts contain activated CD4 and CD8 T cells

Peripheral blood was harvested from uninfected rats or rats infected with RCMV at either 21 or 180 dpi (n=4 for each). Total lymphocytes were isolated from the rat hearts and these cells were stained for the cell surface markers CD4, CD8, and CD62 as well as for the intracellular marker of cellular proliferation, Ki67 and then analyzed by flow cytometry. Hearts from latently infected rats contain a significantly increased level of activated CD4+ and CD8+ effector T-cells compared to hearts from uninfected controls (p<0.05).

Table 1
 Status and source of RCMV infection is important for the acceleration of TVS in heart allografts.

Experimental Design	Transplantation Timeline	n=	Donor Infected (day)	Recipient Infected (day)	Rejection POD	Histology	N.I. (±SD)
ADI Acute Donor Infection / pre tx day 7 / Naive Recipient		4	-7	-	53	CR*	50±10
ARI Acute Recipient Infection / post tx day 1 / Naive Donor		6	-	+1	43 ⁺	CR	74±7 ⁺⁺
LDI Latent Donor Infection / Pre tx day 180 / Naive Recipient		8	-180	-	54 [#]	CR	63±7 ^{##}
LRI Latent Recipient Infection / pre tx day 180 / Naive Donor		7	-	-180	62	CR	51±7
Control		8	-	-	90	CR	51±7

* CR = Chronic Rejection,

⁺ = p<0.01 vs. ADI,

⁺⁺ = p<0.01 vs. ADI,

[#] = p<0.01 vs. LRI,

^{##} = p<0.01 vs. LRI

Table 2

RCMV Detection and serology of allograft recipients at the time of rejection.

	Recipient Serology	RCMV DNA Copies/ μ g		
		Native Heart	Graft Heart	Salivary Glands
ACUTE				
ADI (n=4)	+	n.d.	n.d.	1×10^5
ARI (n=6)	+	3×10^3	3×10^2	3×10^7
LATENT				
LDI (n=8)	+	n.d.	n.d.	n.d.
LRI (n=7)	+	n.d.	n.d.	3×10^5

N.D. = not detected, assay detection limit is 100 copies of the RCMV genome.

Ganciclovir (20mg/kg/day for 30 days) slows RCMV-accelerated TVS in allografts from acutely infected recipients (ARI) but not in allografts from latently infected donors (LDI).

Table 3

Group	n=	Infection Day	POD	Histological Presentation	Neoinimal Index (TVS)+/-SD
LDI	8	-180	54	Chronic Rejection	63+/-7
LDI+Ganciclovir	6	-180	52	Chronic Rejection	65+/-3
ARI	6	1	43	Chronic Rejection	80+/-5
ARI+Ganciclovir	6	1	75*	Chronic Rejection	65+/-4**

* vs. POD ARI p<0.001,

** vs. ARI NI p<0.001

Table 4
Early and sustained induction of TLO associated genes in allografts from latently infected donors

Shown are fold change values compared to uninfected non-transplanted hearts (n=3 for each time point).

	Latently Infected Donor (LDI)						Uninfected Control					
	7	14	21	28	35	7	14	21	28	35		
POD	7	14	21	28	35	7	14	21	28	35		
CCL19	4.5*	6.6	14.8	17.4	11.8	1.6	2.7	7.8	10.2	7.8		
CCL21	1.4	2.1	3.1*	2.2*	1.0	1.2	1.4	1.1	0.7	0.8		
CXCL13	9.0*	10.0	11.9	13.2	13.8	3.2	7.1	4.9	9.8	11.8		
ICAM-1	3.9*	3.7	4.5	5.9	5.2	1.3	3.1	2.7	3.1	5.6		
MADCAM1	1.1*	2.0	1.3	3.0	1.7	0.8	1.2	0.8	0.9	0.9		
VCAM-1	3.6*	4.9	6.0	7.7	7.7	1.7	4.4	3.2	5.0	6.8		
Lymphotoxin A	0.7	0.6	1.0	1.2	0.9	0.6	0.5	0.7	0.9	0.9		
Lymphotoxin B	4.5*	3.8*	6.4	10.7*	11.4	2.0	2.2	3.1	6.4	12.2		

* {p<0.05 infected vs. uninfected}

Table 5

Phenotyping of T cells from spleen and blood of RCMV infected rats at 21 and 180 dpi. n=6

		Uninfected Control	Infected 21 dpi	Infected 180 dpi
CD4+ (% lymphocytes)	Blood	51.3 ± 3.6	49.5 ± 1.5	39.1 ± 3.5
	Spleen	35.6 ± 2.1	31.5 ± 2.2	23.9 ± 3.3
CD4+CD62L ^{low} (% CD4)	Blood	7.2 ± 1.5	18.7 ± 1.7	17.3 ± 3.1
	Spleen	16.4 ± 5.2	27.8 ± 5	40.9 ± 5.5
CD4+CD62L ^{low} Ki67+ (% CD4)	Blood	1.8 ± 0.4	2.6 ± 0.2	3.7 ± 1.1
	Spleen	2.8 ± 0.6	5.1 ± 1.3	7.4 ± 1.4
CD8 (% lymphocytes)	Blood	20.4 ± 1.1	20.9 ± 1.6	26.0 ± 3.8
	Spleen	13.3 ± 1.9	15.5 ± 1.9	24.6 ± 3.2
CD8+CD62L ^{low} (% CD8)	Blood	3.3 ± 1.8	16.1 ± 2.3	11.7 ± 5.5
	Spleen	11.3 ± 1.9	19.7 ± 4.7	18.1 ± 3.3
CD8+CD62L ^{low} Ki67+ (% CD8)	Blood	37.1 ± 8.2	46.7 ± 4.8	40.2 ± 12.0
	Spleen	25.9 ± 2.9	35.3 ± 6.5	39.0 ± 7.2