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ORIGINAL ARTICLE

# Cytomegalovirus-enhanced development of transplant arteriosclerosis in the rat; effect of timing of infection and recipient responsiveness

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## Summary

Cytomegalovirus (CMV) is put forward as a risk factor for transplant arteriosclerosis (TA). In this article, we studied CMV-enhanced development of TA in rats in different donor/recipient combinations in relation to the timing of infection. Recipient rats transplanted with an aortic allograft (BN to Lew) were infected with rat CMV (RCMV) at different time-points relative to transplantation. The virus-induced effects on TA development were also determined in other strain combinations (PVG to AO and DA to WF). Finally, transmission of RCMV from aortic grafts and its effect on TA was studied. RCMV infection enhanced TA development only in Lew recipients and only after infection early post-transplantation (days 1–5). Virus transmission to the recipient only occurred from 5 and 10 days infected aortic donor-grafts, however without affecting TA development. These data indicate that the acute alloresponse and acute CMV infection need to occur simultaneously to enhance TA. This effect, however, appears to be strain combination dependent and therefore cannot be generalized.

## Introduction

Although cardiac transplantation has almost become a routine procedure in clinical practice, it has not achieved its goals as a long-term treatment for patients with end-stage heart failure. The most common cause of death and retransplantation following heart transplantation is development of cardiac allograft vasculopathy or transplant arteriosclerosis (TA) in the intragraft arteries. TA affects up to 50% of patients 5 years after transplantation as diagnosed by angiography [1,2]. Pathogenesis of TA seems to be multifactorial but precise mechanisms involved in the development of this remodeling process still remain obscure. Risk factors appear to include cold ischemia time and reperfusion injury, major histocompatibility complex (MHC) disparity between donor and recipient and number of rejection episodes [3]. There is also accumulating data suggesting that cytomegalovirus (CMV) infection plays a role in the pathophysiology of TA [4]. Grattan *et al.* for the first time showed an association between CMV infection and the development of TA by demonstrating a significantly increased incidence of severe TA in CMV-infected cardiac transplant recipients compared with uninfected controls [5]. These data have been recently confirmed in a prospective study showing that CMV infection in cardiac transplant patients increases the risk for TA [6]. Also, results from therapeutic trials of antiviral drugs favor for a role of CMV infection in the development of TA. Ganciclovir prophylaxis has been shown to ameliorate TA in human cardiac allografts [7]. However, these beneficial effects of ganciclovir prophylaxis have not been observed in the group at highest risk,

namely the seronegative recipient of an allograft from a seropositive donor (D+/R−) [8]. As appropriate treatment for subjects in the latter group to prevent CMV-enhanced TA development is still lacking, research focusing on understanding the underlying mechanism of CMV-enhanced TA development is warranted.

In addition, in experimental transplant models, the association between CMV infection and TA has been studied. Especially in the aortic transplant model in rats, which is a well established and reproducible model for cardiac allograft vasculopathy [9], we and others showed that rat CMV (RCMV), administered 1 day after transplantation, enhances both the perivascular inflammatory response [10,11] and neointima formation [10,12–14].

The increase in perivascular inflammation observed after RCMV infection peaks around 1 month after transplantation, whereas the effects on neointima formation can be detected 2–3 months after transplantation. Based on this observation, the mechanism by which CMV may predispose to TA may include a general enhancement of alloreactivity resulting in an increased perivascular inflammatory response after viral infection and eventual neointima formation. In line with this, Orloff *et al.* have shown that tolerization of the recipient for donor alloantigens, thus minimizing the rejection response upon transplantation, also prevents the RCMV-induced acceleration of TA in cardiac allografts [15].

In this study we analyzed the effect of timing of CMV infection of the recipient (relative to transplantation) on the development of enhanced TA after aortic allografting in rats. In addition, transfer of virus by an aortic allograft and its possible effect on subsequent TA development was studied. Finally, the generality of our results on CMV-enhanced TA after aortic allografting was determined by performing aortic transplantation and CMV infection in different rat strain combinations.

The results obtained indicate that although RCMV does enhance the development of TA if infection occurs 1 day (early) after transplantation, this effect cannot be generalized. Possibly the interplay between the host immune system, the developing alloreactive response and the antiviral response determine the final effect of CMV on the development of TA. As these responses may differ in different rat stains (and likewise in different human individuals), the final outcome may be different and this may reflect the variation as is observed in the human transplant population with respect to the development of TA.

## Materials and methods

### Animals

Specified pathogen free male Piebald Virol Glaxo (PVG, RT-$1^c$), Albino Oxford (AO, RT-$1^u$), Dark Agouti (DA, RT-$1^a$), Wistar Furth (WF, RT-$1^u$) were obtained from Harlan (Zeist, The Netherlands). Lewis (Lew, RT-$1^l$) and Brown Norway (BN, RT-$1^n$) rats used for RCMV infection experiments were bred at the central animal facility of the Maastricht University. Rats were 8–10 weeks of age, maintained under clean conventional conditions, and were fed standard rat chow and acidified water *ad libitum*. All animals received humane care in compliance with the Principles of Laboratory Animal Care (NIH Publication No. 86–23, revised 1985) and the Dutch Law on Experimental Animal Care.

### Aorta transplantation

Aortic allografts (10–12 mm) were transplanted as described previously [9,16]. Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor rat and perfused with saline to remove blood cells. Subsequently, the graft was orthotopically transplanted into the recipient rat via end-to-end anastomosis using 9–0 nylon suture (Monosof 9–0; Tyco Healthcare, Zaltbommel, the Netherlands). Total ischemic time was consistently <30 min during which the grafts were kept in ice-cold saline. Transplantations were performed in the following MHC incompatible strain combinations: BN to Lew, PVG to AO and DA to WF.

### Experimental groups

To study the effect of timing of RCMV infection on the development of TA, Lew recipients received 5 Gy total body irradiation (TBI) 6 h before administration of virus in order to promote viral replication. Recipient rats were divided into six different treatment groups (groups 1–6) (Table 1). Allograft recipients were mock-infected (group 1) or RCMV infected shortly after transplantation (day 1 or day 1 + 5; groups 2 and 3) or late after transplantation (day 21 or day 21 + 25; groups 4 and 5). In addition, one group of recipient rats was infected 21 days before transplantation (group 6) to study the effect of chronic RCMV infection of the host on the development of TA.

To analyze transmission of RCMV via the graft after transplantation allografts were transplanted into irradiated hosts 5, 10, and 21 days postinfection (p.i.) of the donor (groups 7, 8 and 9, respectively) (Table 1). Recipient rats were killed 7 weeks after transplantation.

To analyze RCMV-enhanced TA development in other strain combinations, aortic transplantation and RCMV infection (+/− TBI) was performed in the PVG to AO (groups 10–13) and the DA to WF (groups 14 and 15) strain combinations. Grafts were removed 12 weeks after transplantation.

**Table 1.** Groups used for aorta transplantation to study the effect of RCMV infection of the recipient, and transfer of RCMV from the donor on the development of TA.

| Group | *N** | Graft donor | Graft recipient | 5 Gy TBI recipient | RCMV | | Day of infection‡ | Killing§ |
|---|---|---|---|---|---|---|---|---|
| | | | | | Recipient† | Donor† | | |
| 1 | 5 | BN | Lew | + | – | – | 1 (mock) | 7 |
| 2 | 5 | BN | Lew | + | + | – | 1 | 7 |
| 3 | 5 | BN | Lew | + | + | – | 1 and 5 | 7 |
| 4 | 5 | BN | Lew | + | + | – | 21 | 7 |
| 5 | 5 | BN | Lew | + | + | – | 21 and 25 | 7 |
| 6 | 5 | BN | Lew | + | + | – | –21 | 7 |
| 7 | 5 | BN | Lew | + | – | + | –5 | 7 |
| 8 | 6 | BN | Lew | + | – | + | –10 | 7 |
| 9 | 4 | BN | Lew | + | – | + | –21 | 7 |
| 10 | 6 | PVG | AO | – | – | – | – | 12 |
| 11 | 4 | PVG | AO | + | – | – | – | 12 |
| 12 | 6 | PVG | AO | – | + | – | 1 | 12 |
| 13 | 5 | PVG | AO | + | + | – | 1 | 12 |
| 14 | 4 | DA | WF | – | – | – | – | 12 |
| 15 | 5 | DA | WF | – | + | – | 1 | 12 |

*Number of animals analyzed.
†Animals were infected with $3 \times 10^5$ plaque forming units (pfu) RCMV salivary gland homogenate.
‡Animals were noninfected (–) or infected before or after transplantation (day 0).
§Weeks after transplantation.

At autopsy, grafts from all groups were removed and processed for morphometric analysis.

## Infection with RCMV

Infection with RCMV was achieved by i.p. injection of a salivary gland homogenate containing $3 \times 10^5$ plaque forming units (pfu) RCMV (Maastricht strain) as described elsewhere [17, 18]. To analyze whether irradiation is a prerequisite for RCMV-enhanced TA development recipients in some treatment groups (11 and 13) were sublethally γ-irradiated (5 Gy) 1 day after aortic transplantation but prior to infection in order to promote viral replication and dissemination [19].

### Transfer of infectious virus by aortic allografts

A sensitive infection transfer assay was used to analyze the transmission and dissemination of RCMV from aortic allografts into their recipients [20]. BN donors were 5 Gy γ-irradiated followed by i.p. injection of $3 \times 10^5$ pfu RCMV. After 5, 10, and 21 days p.i., allografts were transplanted into 5 Gy γ-irradiated Lew recipients. At the time of transplantation, parts of the graft and salivary glands (SG) were removed for RCMV detection (plaque assay, immunohistochemistry and PCR analysis). Allograft recipients were killed 7 weeks after transplantation and SG and aortic tissue (both the graft and autologous part) were removed and processed for RCMV detection.

### Quantification of transplant arteriosclerosis

At autopsy aortic allografts were transversally divided into two equally sized parts, fixed in 10% formalin (in PBS) and embedded in paraffin. Presence of viral antigens (see below) and TA was analyzed on 4 μm sections obtained from each part of the graft (not near the anastomosis). For quantification of TA, sections were stained with Lawson solution (Klinipath, Duiven, The Netherlands) to visualize elastin fibers. The surface of the media and neointima was calculated from two sections in each part and the mean was calculated using a computerized morphometric analysis system (QWin Software, Leica Microsystems B.V., Rijswijk, The Netherlands). The severity of TA was expressed as the ratio neointima/media.

### RCMV immunohistochemistry

Presence of viral antigens was analyzed by RCMV specific immunohistochemistry (mAb 8 [21]) on 4 μm formalin-fixed paraffin-embedded tissue sections. Briefly, sections were deparaffinized and incubated with mAb8 for 60 min. After subsequent incubation with an alkaline phosphatase-conjugated second step rabbit anti-mouse antibody (DAKO A/S, Glostrup, Denmark) for 30 min, antibody bound cells were visualized using the chromogen Fast Red. Sections were counterstained with Mayer's hematoxylin.

### RCMV plaque assay

Presence of infectious virus was quantified using a plaque-assay as described previously [17]. Briefly, SG and aortic tissue (graft as well as autologous part) were homogenized and sonicated in a 10% w/v ratio in EMEM medium. Subsequently, 10 and 100-fold dilutions of the supernatants were inoculated on RCMV-permissive confluent rat embryonal fibroblasts. After 7–10 days of incubation cells were formalin fixed and stained with methylene blue. Numbers of plaques were quantified microscopically and the virus titer was expressed as pfu.

### RCMV PCR

Presence of viral DNA in SG and aortic tissue was analyzed using a semiquantitative nested-PCR for the RCMV DNA polymerase gene (GenBank accession no. U50550) as described previously [22]. Briefly, total cellular DNA was extracted from SG and aorta using a DNA extraction kit (Gull Laboratories, Salt Lake City, UT, USA). Obtained DNA was serially diluted ($10^0$ to $10^{-8}$ μg) and subjected to a first and second round PCR resulting in amplicons of 536 and 431 nucleotides respectively. Primer sequences and PCR conditions are described elsewhere [22]. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

### Statistical analysis

To analyze differences for statistical significance between groups in the ratio neointima/media, the Kruskal–Wallis one-way ANOVA was performed. If this test revealed a significant difference, a Mann–Whitney *U*-test was performed to analyze differences between subgroups. Differences were considered to be statistically significant when $P < 0.05$.

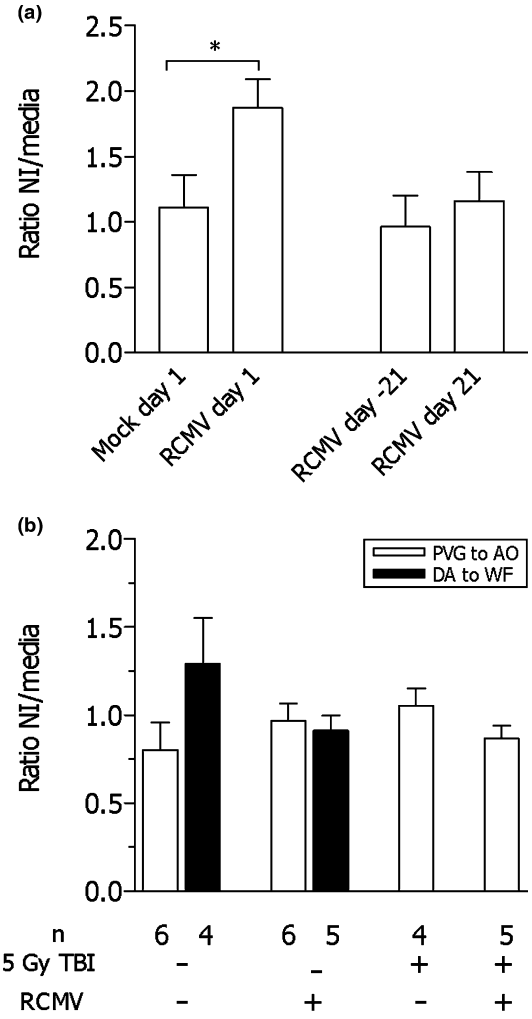


**Figure 1** Kinetics of the development of TA after allogeneic aorta transplantation in different strain combinations with or without additional RCMV infection. Neointima formation is expressed as the ratio surface neointima/media. (a) Effect of timing of RCMV infection on the development of TA in the BN to Lew combination 7 weeks after transplantation ($n = 5$ animals/group). (*$P < 0.05$, Mann–Whitney *U*-test). (b) Effect of RCMV infection (+/– 5 Gy TBI) on the development of TA after allografting in the PVG to AO and DA to WF strain combinations 12 weeks after transplantation. (*n* is the number of animals analyzed within each group). Data are expressed as mean ± SEM.

## Results

### RCMV-enhanced TA after BN to Lew allografting: effect of timing of infection

Only infection 1 day after transplantation showed a significant increase ($P < 0.05$) in TA development compared with mock infection (Fig. 1a). Infection 21 days after (group 4) or 21 days before (group 6) transplantation did not enhance TA. To analyze whether the absence of a virus-enhanced effect in group 4 (infection 21 days after transplantation) was due to a low viral load, recipients were infected twice shortly (days 1 and 5, group 3) or late (days 21 and 25, group 5) after transplantation. Again, only infection shortly after transplantation resulted in enhanced TA [ratio neointima/media: 1.68 ± 0.14 (days 1 and 5) vs. 1.17 ± 0.26 (days 21 & 25) ($p < 0.05$)]. These results indicate that the viral load is not responsible for the differences observed between groups 2 and 4. Thus, RCMV infection enhances TA only when infection is given shortly after engraftment.

### Effect of RCMV infection of the donor on the development of TA

To analyze whether aortic allografts are able to transfer RCMV following transplantation (causing systemic infection of the host) thereby possibly enhancing the development of TA, we determined (i) the presence of virus in aortic tissue at several time points p.i. (5, 10, and 21 days), (ii) the capability of this virus to disseminate after transplantation, (iii) and the possible effects of virus dissemination on TA development. Using RCMV-specific immunohistochemistry (mAb8) no infected cells could be detected in donor aorta and SG at all three time-points [5 days p.i. ($n = 4$), 10 days p.i. ($n = 4$) and 21 days ($n = 2$)]. However, RCMV plaque-assay revealed presence of infectious virus in aortic tissue 5 and 10 days p.i. (one of four animals at both time points) with the SG still being negative at these time-points. At 21 days p.i., however, the SG of the donor-rats contained infectious virus in both two animals. Although RCMV could not be detected by plaque-assay in aortic tissue transplanted 5 or 10 days p.i. in most animals, transplantation of such 'RCMV-negative' grafts resulted in transmission of virus to the recipient. Seven weeks after transplantation 100% (6/6) and 75% (3/4) of the recipient rats (transplanted 5 and 10 days p.i., respectively) showed signs of systemic infection as indicated by the presence of infectious virus in their SG (as detected by immunohistochemistry and plaque assay). These results suggest that the aortic grafts did contain infectious virus 5 and 10 days p.i. although the absolute amounts were very small. Fig. 2a and b show representative photomicrographs of RCMV-infected cells in SG of recipient rats transplanted with aortic allografts 5 days p.i. of the donor. RCMV was never detected by immunohistochemistry and plaque assay in both graft and autologous aorta tissue.

The results obtained by plaque assay were confirmed by a more sensitive PCR-based approach as shown in Fig. 2c, indicating that 21 days p.i. aortic tissue does no longer contain detectable levels of viral DNA and, as a result, also no viral transmission occurs after transplantation.

Infection of the donor 21 days before transplantation did not, as expected, influence TA development after transplantation [ratio neointima/media: $1.10 \pm 0.18$ (day –21 RCMV) vs. $1.11 \pm 0.25$ (day 1 mock)], as we showed that no virus was present in aortic tissue at this time point and no viral transmission occurred after transplantation. However, also no enhancing effect on neointima formation was observed after transplantation of grafts 5 and 10 days p.i. despite the fact that at these time-points aortic tissue contained virus and caused effective viral transmission after transplantation.

### RCMV-enhanced TA in other strain combinations

Only RCMV infection shortly after transplantation (1 and/or 5 days) enhanced TA development in the BN to Lew rat strain combination, suggesting that active RCMV infection and an acute alloresponse should occur simultaneously to get this potentiating effect of RCMV. To study whether this is a general effect which is also present in other strain combinations displaying weak TA in the absence of RCMV (like the BN to Lew combination), we performed similar experiments in the MHC incompatible PVG to AO (groups 10–13) and DA to WF (groups 14 and 15) strain combinations and results are shown in Fig. 1b. In contrast to the BN to Lew strain combination, RCMV did not enhance TA development in the PVG to AO combination measured at 12 weeks after transplantation (the optimal time-point for this combination). As irradiation-induced immunosuppression could be involved in the mechanism underlying RCMV-enhanced TA, the effect of RCMV in both the presence and absence of irradiation of the recipient was analyzed. However, no differences between the treatment groups in this strain combination were observed. Like the results in the PVG to AO combination, also in the DA to WF combination, no enhanced TA development was observed 12 weeks after transplantation following RCMV infection on day 1 after transplantation.

## Discussion

CMV is one of the pathogens that is most convincingly involved in the pathophysiology of TA in human cardiac allografts [4]. Although the exact underlying mechanism of CMV-enhanced TA is still elusive, mechanisms involved may include production of virus-encoded pro-inflammatory chemokines, general enhancement of T-cell-mediated alloreactivity, and direct viral effects on vascular endothelium resulting in e.g. changes in the eNOS system in favor of a pro-atherogenic environment. These possibilities are not mutually exclusive and may even reinforce each other.

The aortic transplant model in rats is commonly used as a model to study the mechanism underlying the development of TA after cardiac allografting [9], including the enhancing effects of CMV infection. In this transplant model, no immunosuppression is required to prevent acute rejection as is necessary for solid organ transplants. However, after transplantation a strong alloreactive response is induced which is characterized by massive perivascular inflammation that precedes the appearance of an occlusive neointima. Infection with RCMV has been shown to enhance perivasculitis in this model, reaching maximal levels approximately 1 month after transplantation [10,11].

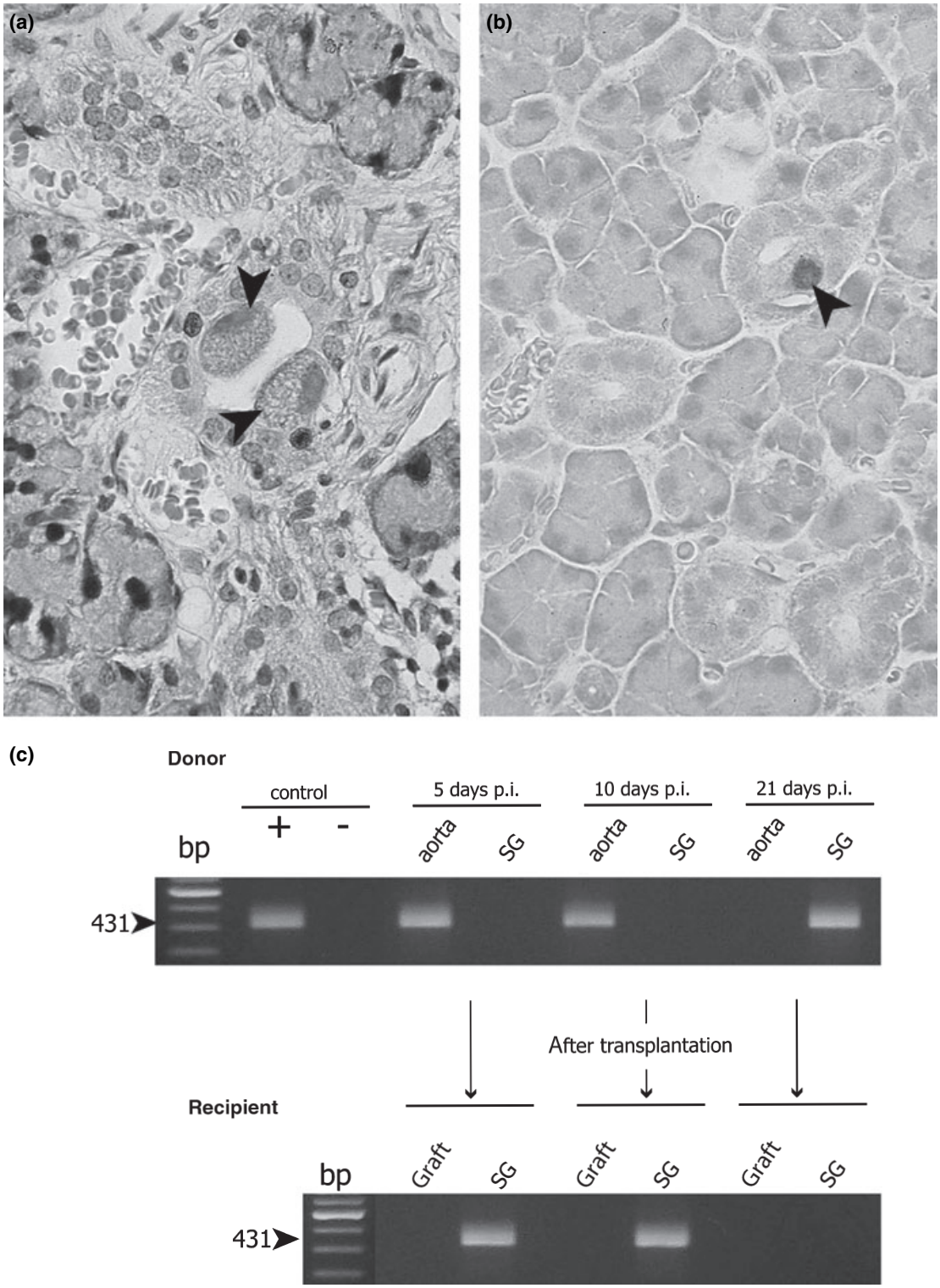


**Figure 2** Transfer of RCMV by transplantation of RCMV-infected allografts. (a) HE staining and (b) mAb8 (RCMV) staining of recipient salivary gland tissue 7 weeks after transplantation of an aortic allograft obtained from an RCMV infected donor 5 days p.i. Arrowheads indicate RCMV infected ductal epithelial cells. (magnification, ×400). (c) Nested RCMV PCR performed on donor aorta and salivary gland tissue at the time of transplantation, and on graft tissue and recipient salivary gland tissue 7 weeks after transplantation. Grafts were transplanted 5, 10, and 21 days p.i.

We now hypothesize that CMV increases TA development primarily by enhancing the alloreactive response. If this holds true, RCMV will only enhance TA when administered during the induction phase of the alloresponse, i.e. early after transplantation. In the current article we tested the proposed hypothesis. As appropriate immunosuppression is required for solid organ transplants to prevent acute rejection (thereby also promoting virus replication and dissemination), we decided to irradiate the aortic transplant recipients by sublethal TBI. Since the Lew strain, when used as a recipient, develops modest TA (compared with BN recipients, data not shown) we used the BN to Lew strain combination to study the effect of timing of RCMV infection on enhanced TA development. Our results indicate that indeed only infection 1–5 days (early) after transplantation results in enhanced neointima formation, suggesting that acute infection and the developing alloresponse should be present simultaneously in order to induce CMV-enhanced TA. Although we did not include histological analysis to characterize the inflammatory response in this study, it has been shown by others that, using the same strain combination, the rejection response starts to develop (appearance of invading $CD8^+$ T cells and macrophages in the adventitia) within the first week after engraftment [23]. In our experiments the recipients were irradiated 1 day after transplantation and we previously showed that this treatment in itself decreases the magnitude of the inflammatory response after transplantation; this effect is however largely overcome after RCMV infection [11]. The data presented in this study further strengthen our hypothesis that RCMV enhances TA development by direct effects on the alloreactive T-cell population. In line with this are our recent data showing that RCMV infection induces a strong anti-viral proliferative immune response with polyclonal characteristics (approximately 50% of the magnitude of the Concanavalin A response), including activation of bystander T cells. Although similar to the magnitude of superantigen-driven T-cell activation, no preferential skewing towards certain TCR Vβ was observed [24]. Based on these results, we now propose that RCMV enhances TA development by increased alloreactivity because of the activation/generation of RCMV-specific alloreactive T cells that is home to the graft and cause damage to the graft vasculature. More severe vascular damage will result in more severe TA eventually.

To mimick the clinical situation, in which CMV causes major problems after transplantation of a graft from a seropositive donor to a seronegative recipient [8,25] we transplanted aortic allografts from infected donors to uninfected recipients and determined virus transmission/dissemination and TA development. Surprisingly, infection of the donor 3 weeks before transplantation did not result in increased TA after transplantation. Retrospective analysis of donor aortic tissue for the presence of viral antigens and DNA as well as infectious virus revealed, however, that 3 weeks p.i. RCMV is no longer present in aortic tissue. We performed transfer experiments in both the acute phase (1–2 weeks p.i.) and chronic phase (3–12 weeks p.i.) of RCMV infection of the aorta donor [19]. Although it has been shown that kidney allografts can effectively transfer virus to recipient rats in the acute, chronic and even latent phase of infection [20], aortic allografts can obviously only transfer infectious virus in the acute phase. To test dissemination of RCMV by aortic allografts in the acute phase of infection, allografts were transplanted 5 and 10 days p.i. Although systemic infection was observed in virtually all recipients, the development of TA was not enhanced. Possibly, the viral load is not sufficiently high during the developing alloresponse to potentiate alloreactivity. Alternatively, the acute systemic infection phase may be somewhat delayed after transplantation of an infected aortic allograft compared with i.p. administration of RCMV. This may cause a delay in the development of the systemic infection resulting in a nonsynchronous occurrence of inflammation and acute infection.

Finally, we tested the generality of the potential of RCMV to enhance TA after aortic allografting. Therefore, similar experiments were performed in two other strain combinations (PVG to AO and DA to WF) that like the BN to Lew combination develop moderate TA in the absence of RCMV (data not shown). In both strain combinations, however, RCMV did not enhance TA when infected 1 day after transplantation. These results indicate that the enhancing effect of RCMV on TA development after aortic allografting is not a general phenomenon, and may reflect the variation as is observed in the human transplant population with respect to TA development and CMV infection. The observation that RCMV did not enhance TA in the DA to WF combination was rather unexpected as the first data reported on RCMV-enhanced TA in aortic allografts were described in the DA to WF combination using the same RCMV strain as we did [12]. So, our data suggest that even minor genetic differences and variations in microbiological status between the rat strains may account for the deviating results and caution is therefore warranted when designing experimental studies to dissect the pathogenesis of RCMV-enhanced TA after allogeneic transplantation.

In conclusion, RCMV infection enhances the development of TA in aortic allografts only when active RCMV infection and the developing alloresponse are present simultaneously, although this effect cannot be generalized. Our data favor for a direct potentiating effect of RCMV on the alloreactive T cells as the underlying mechanism.

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## References

1. Gao SZ, Schroeder JS, Alderman EL, *et al.* Prevalence of accelerated coronary artery disease in heart transplant survivors. Comparison of cyclosporine and azathioprine regimens. *Circulation* 1989; **80**: III100.
2. Costanzo MR, Naftel DC, Pritzker MR, *et al.* Heart transplant coronary artery disease detected by coronary angiography: a multi-institutional study of preoperative donor and recipient risk factors. *J Heart Lung Transplant* 1998; **17**: 744.
3. Kouwenhoven EA, IJzermans JN, de Bruin RW. Etiology and pathophysiology of chronic transplant dysfunction. *Transpl Int* 2000; **13**: 385.
4. Valantine HA. The role of viruses in cardiac allograft vasculopathy. *Am J Transplant* 2004; **4**: 169.
5. Grattan MT, Moreno-Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. *JAMA* 1989; **261**: 3561.
6. Fateh-Moghadam S, Bocksch W, Wessely R, Jager G, Hetzer R, Gawaz M. Cytomegalovirus infection status predicts progression of heart-transplant vasculopathy. *Transplantation* 2003; **76**: 1470.
7. Valantine HA, Gao SZ, Menon SG, *et al.* Impact of prophylactic immediate posttransplant ganciclovir on development of transplant atherosclerosis: a post hoc analysis of a randomized, placebo-controlled study. *Circulation* 1999; **100**: 61.
8. Weill D. Role of cytomegalovirus in cardiac allograft vasculopathy. *Transpl Infect Dis* 2001; **3**(Suppl. 2): 44.
9. Mennander A, Tiisala S, Halttunen J, Yilmaz S, Paavonen T, Häyry P. Chronic rejection in rat aortic allografts: an experimental model for transplant arteriosclerosis. *Arterioscler Thromb* 1991; **11**: 671.
10. Lemström K, Aho PT, Bruggeman CA, Häyry P. Cytomegalovirus infection enhances mRNA expression of platelet-derived growth factor-BB and transforming growth factor- β1 in rat aortic allografts: possible mechanism for cytomegalovirus- enhanced graft arteriosclerosis. *Arterioscler Thromb* 1994; **14**: 2043.
11. Li F, Grauls G, Yin M, Bruggeman CA. Correlation between the intensity of cytomegalovirus infection and the amount of perivasculitis in aortic allografts. *Transpl Int* 1996; **9**: S340.
12. Lemström KB, Bruning JH, Bruggeman CA, Lautenschlager IT, Häyry P. Cytomegalovirus infection enhances smooth muscle cell proliferation and intimal thickening of rat aortic allografts. *J Clin Invest* 1993; **92**: 549.
13. Bruning JH, Persoons M, Lemström K, Stals FS, De Clercq E, Bruggeman CA. Enhancement of transplantation-associated atherosclerosis by CMV, which can be prevented by antiviral therapy in the form of HPMPC. *Transpl Int* 1994; **7**: S365.
14. Li F, Yin M, van Dam JG, Grauls G, Rozing J, Bruggeman CA. Cytomegalovirus infection enhances the neointima formation in rat aortic allografts. Effect of major histocompatibility complex class I and class II antigen differences. *Transplantation* 1998; **65**: 1298.
15. Orloff SL, Streblow DN, Soderberg-Naucler C, *et al.* Elimination of donor-specific alloreactivity prevents cytomegalovirus-accelerated chronic rejection in rat small bowel and heart transplants. *Transplantation* 2002; **73**: 679.
16. Hillebrands JL, Klatter FA, van den Hurk BMH, Popa ER, Nieuwenhuis P, Rozing J. Origin of neointimal endothelium and α-actin-positive smooth muscle cells in transplant arteriosclerosis. *J Clin Invest* 2001; **107**: 1411.
17. Bruggeman CA, Meijer H, Dormans PH, Debie WM, Grauls GE, van Boven CP. Isolation of a cytomegalovirus-like agent from wild rats. *Arch Virol* 1982; **73**: 231.
18. Bruggeman CA, Grauls G, van Boven CPA. Susceptibility of peritoneal macrophages to rat cytomegalovirus infection. *FEMS Microbiol Lett* 1985; **27**: 263.
19. Bruggeman CA, Mijer H, Bosman F, van Boven CPA. Biology of rat cytomegalovirus infection. *Intervirology* 1985; **24**: 1.
20. Bruning JH, Bruggeman CA, van Boven CP, Breda Vriesman PJ. Passive transfer of cytomegalovirus by cardiac and renal organ transplants in a rat model. *Transplantation* 1986; **41**: 695.
21. Bruning JH, Debie WH, Dormans PH, Meijer H, Bruggeman CA. The development and characterization of monoclonal antibodies against rat cytomegalovirus induced antigens. *Arch Virol* 1987; **94**: 55.
22. Beuken E, Slobbe R, Bruggeman CA, Vink C. Cloning and sequence analysis of the genes encoding DNA polymerase, glycoprotein B, ICP18.5 and major DNA-binding protein of rat cytomegalovirus. *J Gen Virol* 1996; **77**: 1559.
23. Plissonnier D, Nochy D, Poncet P, *et al.* Sequential immunological targeting of experimental arterial allograft. *Transplantation* 1995; **60**: 414.
24. van der Werf N, Hillebrands JL, Klatter FA, Bos I, Bruggeman CA, Rozing J. Cytomegalovirus infection modulates cellular immunity in an experimental model for autoimmune diabetes. *Clin Dev Immunol* 2003; **10**: 153.
25. van der Bij W, Speich R. Management of cytomegalovirus infection and disease after solid- organ transplantation. *Clin Infect Dis* 2001; **33**(Suppl. 1): S32.