Effects of Acute Cytomegalovirus Infection on Rat Islet Allograft Survival

M. J. Smelt,* B. J. de Haan,* M. M. Faas,* B. N. Melgert,* A. de Haan,† and P. de Vos*

*Department of Pathology and Medical Biology, Division of Medical Biology, Section Immunoendocrinology,
University Medical Center Groningen and University of Groningen, Groningen, The Netherlands
†Department of Medical Microbiology, Division Molecular Virology, University Medical Center Groningen
and University of Groningen, Groningen, The Netherlands

Transplantation of pancreatic islets is a promising therapy for the treatment of type 1 diabetes mellitus. However, long-term islet graft survival rates are still unsatisfactory low. In this study we investigated the role of cytomegalovirus (CMV) in islet allograft failure. STZ-diabetic rats received an allogenic islet graft in combination with either an acute CMV infection or control infection. A third group received ganciclovir treatment in addition to the CMV infection. Graft function was assessed by measuring basal blood glucose levels. After sacrifice, the islet grafts were retrieved for analysis of infection and leukocyte infiltration. CMV-infected recipients demonstrated accelerated islet graft failure compared to noninfected controls. CMV infection of the graft was only observed prior to complete graft failure. Quantification of the leukocyte infiltration demonstrated increased CD8+ T-cell and NK cell infiltration in the CMV-infected grafts compared to the controls. This suggests that CMV infection accelerates immune-mediated graft destruction. Antiviral ganciclovir treatment did not prevent accelerated graft failure, despite effectively decreasing the grade of infection. Our data confirm the recently published CITR data, which state that CMV is an independent risk factor for failure of islet grafts. Also, our data demonstrate that new approaches for preventing virus-induced islet allograft failure may be required.

Key words: Islet transplantation; Cytomegalovirus (CMV) infection; Graft failure; CD8+; T cells; NK cells

INTRODUCTION

After more than three decades of research into the principle applicability of clinical islet transplantation (3,37), a major breakthrough was achieved in 2000 by Shapiro et al. (47), who demonstrated that insulin independence could be achieved by applying a glucocorticoid-free immunosuppressive regimen after grafting the islets into the liver of diabetic patients. This success has led to a tremendous growth in the number of clinical islet transplantations performed worldwide. In spite of this optimism, a number of critical issues remain to be solved. Islets from more than one donor are required to supply patients with sufficient graft volume (46). Another important issue is that graft function declines in the years after transplantation, with only 20% of the patients remaining insulin independent 5 years after transplantation (6,46). The exact causes of islet allograft failure remain to be identified, but a high metabolic pressure, recurring autoimmunity, alloimmunity, or a combination of these factors may lie at the basis of declining graft function (21,46).

It is surprising that the role of cytomegalovirus (CMV) has gained only minor attention as a contributing factor to islet graft failure. This is despite the strong correlation between this viral infection and solid organ graft rejection (1,13,17,24,32,48). CMV is a widely spread, persistent infection, which develops asymptomatic in healthy, immunocompetent individuals (16). In immunocompromized individuals, the virus may reactivate and cause mild to severe CMV disease. A secondary feature of the virus is that its reactivation is strongly associated with allograft rejection. In solid organ transplantation, this risk on CMV-induced graft failure highly depends on the serostatus of both donor and recipient. The highest risk on accelerated graft failure is observed in seronegative [non-CMV-carrying (R−)[recipients receiving a graft from a seropositive [CMV-carrying (D+)] donor (14). This process appears to develop independent of inhibition of viral replication by antiviral medication (24,49).

As for islet transplantation, the role of CMV in the failure of islet grafts was only recently recognized (6). The 2009 CITR report pointed to donor CMV seropositivity combined with recipient seronegativity as an independent risk factor for graft failure after islet transplantation. Before this report, studies that addressed the CMV issue in islet transplantation mostly focused on the low CMV seroconversion rates and the absence of CMV disease after transplantation (2,8,19,20,63). This absence of CMV transmission or de novo infection may be the result of adequate antiviral treatment given prophylactically in the peritransplant period. Only two studies addressed the role of CMV in islet graft survival. Warnock et al. (60) merely suggested the negative effect of the virus on islet graft survival, whereas Eckhard et al. (12) found high CMV transmission rates and a trend towards reduced graft function in CMV-positive recipients after combined islet–kidney transplantation, even when antiviral treatment was applied (12). Together with a high degree of CMV mismatches between islet donors and recipients, especially the R−/D+ combination (20), these studies clearly point to a role of CMV in islet graft failure.

Studies unarguably proving or excluding the deleterious effect of CMV on islet grafts and discussing its mechanism of action are lacking. At this point, interpretation of results from patient studies is difficult, as they show limitations in respect to size and characteristics of the experimental groups. Therefore, we decided to investigate the role of CMV in islet transplantation in an acute rat infection model (i.e., a model in which CMV is given directly after transplantation) (23,25,29). These models are generally accepted and well controlled in terms of the provided viral load and the lack of overshadowing and interfering secondary infections. Further, we aimed at identifying the mechanism by which CMV exerts its effect. In addition, we mimicked the clinical situation, in which prophylactic ganciclovir treatment is applied to prevent CMV viremia and disease.

MATERIALS AND METHODS

Study Design

Streptozotocin (STZ) diabetic Albino Oxford (AO) rats were transplanted with an islet allograft. To study the effects of CMV in islet graft survival we used a model that is generally accepted in solid organ transplantation (4,30), rather than the clinical immunosuppressive protocol, which has been demonstrated to induce severe insulin resistance and β-cell toxicity in rats (35). In our model, a 10-day low-dose cyclosporine A (CsA) immunosuppression protocol is applied, which is generally used to prevent acute rejection after solid organ transplantation (4,30). This protocol induces adequate immune suppression, since plasma CsA levels always exceeded the therapeutic 150 µg/L necessary for islet engraftment (43), but is sufficiently low to prevent CsA-induced islet toxicity (43).

RCMV infection develops in a similar manner as HCMV infection in humans (51,52). In immunocompetent animals, the infection develops asymptomatic, whereas in immunocompromised rats it develops in a generalized infection, characterized by hepatitis, splenitis, and thrombocytopenia (51,52). Since islet grafts are small in size, the transplanted viral load is considered to be low. To mimic this clinical situation, rats received a nonlethal low-dose CMV infection of 2×10^5 plaque-forming units (pfu).

The endocrine graft volume of 5 µl (57) was transplanted under the kidney capsule to allow full retrieval and histological study of the grafts after sacrificing the animals. Transplantation of an endocrine volume of 5 µl is generally accepted to study the effects of experimental treatments on islet graft survival (11,57). Transplantation was considered successful when nonfasting blood glucose concentrations reached levels below 10 mmol/L. One day after transplantation, the islet graft recipients received a CMV infection ($N = 10$). CMV infection at day 1 after transplantation, via intraperitoneal injection, is in solid organ transplantation models generally accepted to study the effects of the virus on graft survival (23,25,29). We chose to use this model, rather than infection via the islet graft, since it allows us to control the timing as well as the grade of infection. MOCK-infected, transplanted animals ($N = 11$) served as controls. CMV-infected and control animals were transplanted as pairs or two CMV-infected animals and one control. The necessary number of standardized islet grafts was prepared from one pool of isolated islets to ensure that both CMV-infected recipients and matching controls received identical islet grafts.

Recipients of islet grafts were followed and monitored by measuring blood glucose levels, until graft failure occurred. Graft failure was defined as two blood glucose measurements exceeding 20 mmol/L. Six of the CMV-infected animals were sacrificed at the time of complete graft failure. At the same time, the matching control animals were sacrificed ($N = 4$). Three control animals were followed up until complete graft failure. Moreover, four CMV-infected and four matching controls were sacrificed at 7 days after transplantation to gain more insight in the processes underlying islet graft failure. After sacrifice, the grafts, salivary glands, and native pancreata were retrieved for immunohistochemical staining.

In a third experiment, the effect of antiviral treatment on the CMV-induced graft failure was investigated. To this end, a group of transplanted recipients ($N = 5$) received the clinically applied antiviral ganciclovir treat-

ment in addition to a CMV infection. Ganciclovir treatment at a dose of 20 mg/kg/day has been demonstrated to be efficacious in suppressing lethal, generalized CMV infection in rats (53,54).

Experimental Animals and Diabetes Induction

Specified pathogen-free inbred male Albino Oxford rats (RT1u) weighing 280–300 g served as islet recipients. Pathogen-free inbred Lewis rats (RT1l) weighing 300–320 g served as islet donors. All experimental animals were obtained from Harlan (Horst, The Netherlands). The animals were fed standard rat chow and acidified water ad libitum. All animal experiments were performed after receiving approval of the institutional Animal Care Committee of the Groningen University and all animals received human care in compliance with the Dutch Law on Experimental Animal Care.

Diabetes was induced in the recipient rats by injection of 75 mg/kg streptozotocin (Zonasar, Upjohn Co., Kalamazoo, MI) in the tail vein. Animals received a second injection of 90 mg/kg streptozotocin when at day 10 after the first injection blood glucose levels were less than 20 mmol/L. Only animals showing weight loss and blood glucose levels exceeding 20 mmol/L over a period of 2 weeks served as islet graft recipients. The absence of functional β-cells in the native pancreas, defined as less than 5% of normal controls, was always confirmed after sacrifice.

Islet Transplantation

Islets were isolated from pancreata of Lewis rats as previously described (10), separated from exocrine tissue by centrifugation over a discontinuous dextran gradient (58) and further purified by handpicking. After this procedure, the purity of the islet graft was close to 100%. The total islet volume obtained by the isolation procedure was determined by measuring the diameters (57) of the islets in a 2% aliquot of the islet suspension. Subsequently, the total islet volume was calculated, assuming the islets to be perfect spheres. The graft volume (5 µl) was expressed in microliters, as previously reported by our group (15,57,62), which corresponds to approximately 11,000 IEQ/ kg body weight. Transplantation under the kidney capsule was performed at the upper pole by carefully expelling the islets from a polyethylene tube introduced at the lower pole of the kidney, immediately after isolation.

Immunosuppression and Antiviral Treatment

Rats received 5 mg/kg CsA (Sandimmune, Novartis, Basel, Switzerland) subcutaneously on a daily basis, starting directly after transplantation. To prevent CsA cytotoxicity on the islet grafts (43), CsA immunosuppression was stopped after 10 days. This protocol has been demonstrated to prevent long-term graft rejection in 75–100% of the transplanted animals (43). Plasma CsA levels were monitored, and exceeded the therapeutic 150 µg/L in all animals during the whole course of the experiment (43). Plasma CsA levels were determined by Liquid-Chromatography-Mass Spectometry-Mass-Spectometry. Rats received 20 mg/kg ganciclovir (Cymevene, Roche, Woerden, The Netherlands) intraperitoneally (IP) on a daily basis, for 10 days, starting from the day of CMV infection (34).

RCMV Infection

Rats received a RCMV infection by IP injection of 2×10^5 pfu of RCMV (Maastricht strain) at day 1 after transplantation. RCMV was obtained by homogenization of salivary glands of irradiated, acutely infected AO rats as described previously (5). MOCK-infected islet allograft recipients served as controls. MOCK infection was established by IP injection of virus-free salivary gland homogenate at day 1 after transplantation. Virus-free homogenate was obtained by homogenization of salivary glands of irradiated, noninfected AO rats.

Chemical Determinations

Nonfasting blood glucose levels were determined in blood sampled from the tail vein once every 2 days. The glucose concentration was determined using the Accu-Chek Sensor system (Roche). Two blood glucose levels exceeding 20 mmol/L in a 2-week period was considered as reestablishment of diabetes and complete islet graft failure.

Immunocytochemistry

At sacrifice, the kidney with the islet graft and the salivary glands were removed and snap frozen in liquid nitrogen. The native pancreas was removed and fixed in Bouins fixative for paraffin processing. The pancreas was sectioned at 5 µm and stained with aldehyde fuchsin to determine the presence or absence of functional β-cells.

The islet graft and the salivary glands were sectioned at 5 µm, fixed for 10 min in ice-cold acetone, and air dried for histological examination. Endogenous peroxidase was blocked for 20 min in methanol (Merck, Darmstadt, Germany) containing 1% hydrogen peroxide (kidney and islet graft) or in phosphate-buffered saline (PBS) containing 0.15% hydrogen peroxide (salivary glands). Primary antibodies were incubated for 1 h, and secondary antibodies were incubated for 30 min. The whole procedure was performed at room temperature. Primary monoclonal antibodies used were: mAb8 (1:8) (26) against RCMV R44-protein, OX35 (undiluted, ATCC) against CD4, OX8 (undiluted, ATCC) against CD8, and NKRP1A (1:50) (BD Pharmingen, San Jose, CA) against

NKRP1A-positive NK cells. Secondary antibodies used were: horseradish peroxidase-conjugated rabbit anti-mouse IgG1 or IgG2a (Dako, Glostrup, Denmark), and biotin-conjugated goat-anti-mouse IgG1 or IgG2a (Dako). Peroxidase activity was demonstrated by using 3-amino-9-ethyl-carbazole (Sigma), biotin labeling was demonstrated using horse-radish peroxidase conjugated Streptavidin (Dako), and subsequent 3,3-diaminobenzidine (DAB) staining (Sigma). In all control sections, PBS was used instead of the primary antibody. All control sections were consistently negative.

Measurement of Leukocyte Infiltration

Morphometry was used to quantify the infiltration of $CD4^+$ T cells, $CD8^+$ T cells, and $NKRP1A^+$ NK cells. Sections were stained as described above and analyzed using a Leica microscope with a 20× objective lens (Leica, Wetzlar, Germany) and Qwin software (Qwin 4.0, Leica Microsystems, Annandale, NY). The staining threshold was set manually, based on the negative control section, and subsequently, the total staining area was measured using Leica Qwin software. Leukocyte influx was expressed as the stained surface area as a percentage of the total surface area. Per section, the complete islet graft was analyzed, which generally comprised 8–10 microscopic fields. Non-islet graft tissue, such as kidney tissue, within the analyzed field, was excluded from analysis.

Flow Cytometry

Blood T cell subpopulations were harvested from heparinized blood, sampled from the tail vein, and in splenocytes harvested from the spleen after sacrifice. The blood sample (1 ml) was 1:1 diluted with PBS and centrifuged (20 min, 2,500 rpm, 4°C). The erythrocytes were lysed by incubation in NH_4Cl for 10 min at 4°C.

For four-color flow cytometry, 1×10^6 cells were resuspended in PBS containing 5% fetal calf serum (FCS) and 0.1% sodium azide (PBS/FCS/azide). Cells were incubated with a cocktail of primary antibodies for 1 h at room temperature in the dark. Primary antibodies used were: PerCP-conjugated R73 (1:100) against the rat T-cell receptor (BD Pharmingen), FITC-conjugated anti-CD4 (BD Pharmingen), PE-conjugated anti-CD25 (BD Pharmingen), APC-conjugated anti-FoxP3 (eBioscience, San Diego, CA), or corresponding isotype controls. The intracellular staining was performed according to the FoxP3 staining kit protocol (eBioscience). After staining, the cells were washed twice, resuspended in PBS/FCS/azide, and analyzed by flow cytometry (FACS Calibur, Beckton Dickinson).

During flow cytometry, at least 1×10^5 lymphocytes, based on the forward and side scatter pattern, were counted. During analysis, the percentage of $CD25^+$ and $FoxP3^+$ T cells was determined based on the samples stained with the isotype controls. The isotype controls were used to set the gate, in such a way that 99% of the cells were negative. Then, the gate was copied to the samples stained with anti-CD25 and anti-FoxP3, and the percentage of positive cells was determined. Data were analyzed using Winlist 5.0 software (Verity Software House).

Statistical Analysis

Values are expressed as median with quartiles. Statistical comparisons between the groups were performed by the Mann-Whitney U-test. Statistical significance in the Kaplan Meier survival curve was calculated using the log rank test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Restoration of Blood Glucose Levels Following Transplantation

After allogenic islet transplantation, complete normoglycemia, defined as blood glucose levels below 10 mM, was restored in 73% (8/11) of the MOCK-infected control recipients within 4 days after transplantation, while only 50% (5/10) of the CMV-infected animals completely restored normoglycemia within 3 days after transplantation. The time to reach normoglycemia was not significantly different between the CMV-infected and control animals. Body weights (MOCK 236.6 ± 18.47 g vs. CMV 248.3 ± 12.6 g) and plasma CsA levels (MOCK 1389 ± 27.97 µg/L vs. 1689 ± 302.1 µg/L) were not significantly different between the MOCK-infected and the CMV-infected animals at day 0 and day 7 after transplantation, respectively. The animals that did not reach the criteria for complete normoglycaemia did show a reduction of blood glucose levels, demonstrating the functionality of the transplanted islet grafts. All animals gained weight after islet transplantation and the general health status improved. The low-dose CMV infection had no direct consequences for the weight development or the appearance of the animals.

Islet Allograft Survival in the Presence and Absence of a CMV Infection

Six of the CMV-infected animals were followed up until complete graft failure, as well as three MOCK-infected controls. The CMV-infected animals demonstrated significantly accelerated graft failure ($p < 0.01$), compared to the MOCK-infected control recipients (Fig. 1A, B). Moreover, the four controls that were sacrificed at the same time of their respective CMV-infected partner did not show signs of graft failure at the time of sacrifice (e.g., blood glucose levels >20 mM). At day 13
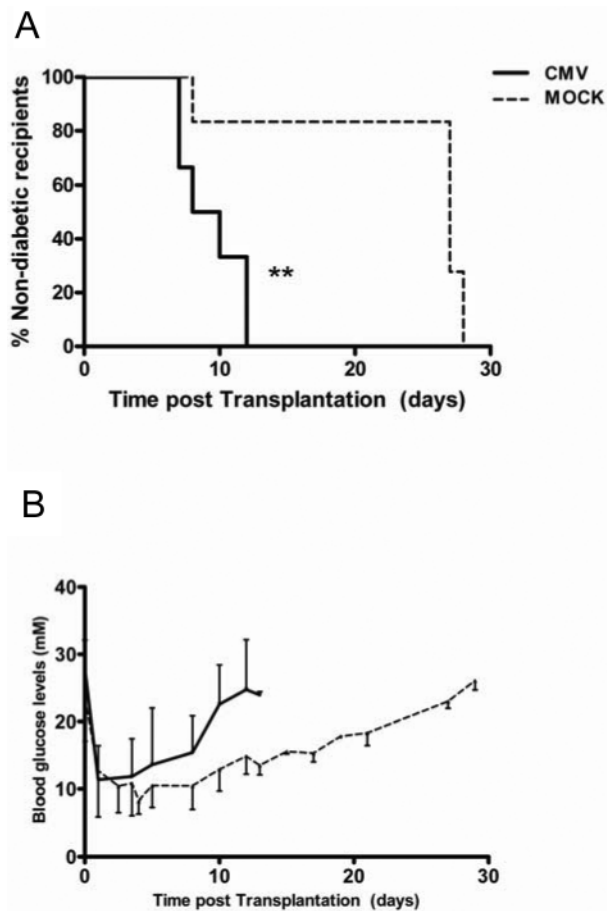
Figure 1. Islet graft function and survival CMV-infected recipients and MOCK-infected controls. Graft survival was followed up in all CMV-infected (solid line, $N = 6$) and MOCK-infected (dashed line, $N = 7$) animals that demonstrated functional islet grafts after transplantation. Graft failure was determined as two consecutive blood glucose measurements above 20 mmol/L. Results are presented as the percentage of nondiabetic islet allograft recipients. Statistical significance was calculated using the Kaplan Meier log-rank test (**$p < 0.01$) (A). After transplantation, blood glucose levels were reduced in both CMV-infected recipients (solid line) and MOCK-infected controls (dashed line). Blood glucose levels gradually increased, demonstrating gradual islet graft failure (B). Results are depicted as the mean ± SEM.

after transplantation (i.e., the time of complete graft failure in the CMV-infected animals), all animals demonstrated sufficient, nontoxic levels of CsA immunosuppression (i.e., above the therapeutic levels of 150 µg/ml) (43). This was not significantly different between the CMV-infected recipients and the noninfected controls (controls: 335 ± 58 µg/L vs. CMV-infected recipients 334.8 ± 48.54 µg/L). These results demonstrate that CMV infection impairs the functional survival of islet

allografts, despite adequate levels of CsA immunosuppression.

CMV Infection of Islet Grafts and Salivary Glands

To gain more insight into the mechanism of CMV-accelerated islet graft failure, we studied the CMV infection in the islet allograft tissue and the salivary glands. To study the processes preceding graft failure, four CMV-infected and four MOCK-infected recipients were sacrificed 7 days after transplantation (i.e., before complete graft failure).

Retrieved grafts and salivary glands were stained for the presence of CMV-R44 protein (Fig. 2). At day 7 after transplantation, as well as at the time of graft failure, all salivary glands of the CMV-infected animals demonstrated R44+ cells (Fig. 2A), demonstrating the establishment of a generalized CMV infection. CMV infection of the islet grafts was demonstrated by the presence of R44+ cells in the functioning grafts retrieved at day 7 after transplantation (Fig. 2C). R44+ cells were not found in the grafts retrieved at the time of complete graft failure (Fig. 2D). The salivary glands and grafts of noninfected control recipients were consistently negative (Fig. 2B, E, F).

Immune Responses in the Islet Graft

Since CMV infection has previously been associated with increased immune activation and accelerated immune-mediated solid organ graft rejection (13,24,32, 33,48,59), we studied the infiltration of the CD4+ and CD8+ T cells in the islet grafts of CMV-infected and MOCK-infected control recipients. Since NK cells are known to be important in the defense against viral infections, especially in the absence of functional T-cell responses (41,61), the infiltration of NK cells was also investigated.

In all grafts, CD4+ and CD8+ T cells were observed (Fig. 3A, B). T cells were found scattered throughout the graft, but in the CMV-infected and control grafts that were retrieved at day 13 after transplantation, T cells were also organized in dense clusters (Fig. 3A, B, depicted by the arrows). This was not observed in the grafts retrieved at day 7 after transplantation. Also, NK cells were found scattered throughout the islet grafts (Fig. 3C).

Morphometric analysis demonstrated that the number of infiltrating CD4+ T cells did not significantly change over time, nor did the number of infiltrating CD4+ T cells differ between the CMV-infected recipients and the controls (Fig. 3A). In the CMV-infected recipients, the number of CD8+ T cells and NK cells infiltrating the islet grafts increased in time and was significantly increased compared to the MOCK-infected controls at the time of
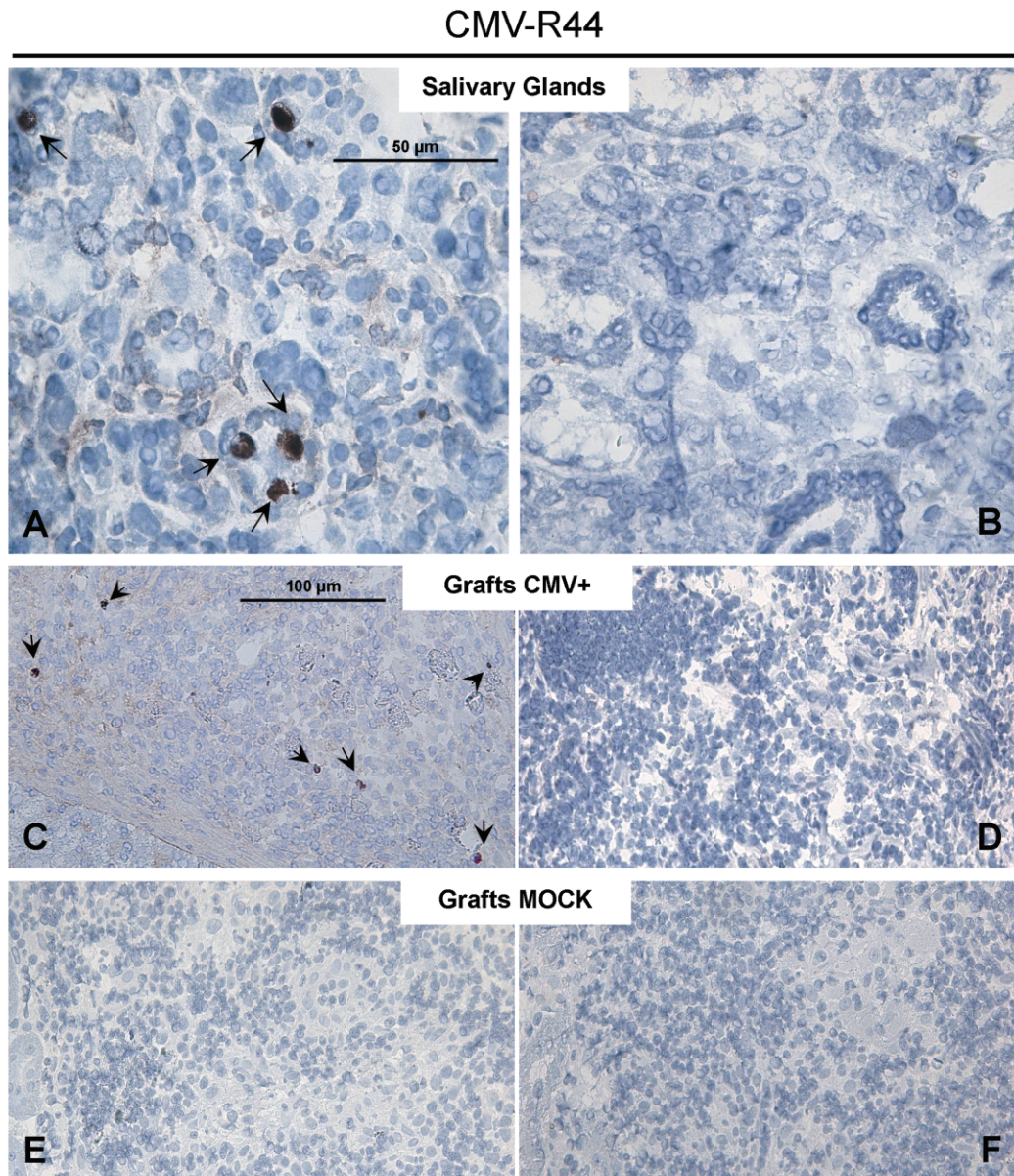
CMV-R44

Figure 2. CMV infection of islet grafts and salivary glands. Histological staining of the salivary glands showed the presence of R44 nuclear staining in the CMV-infected recipients (arrows, 40× original magnification, day 13) (A), but not in the salivary glands of MOCK-infected recipients (40× original magnification, day 13) (B). Histological staining of the islet allografts demonstrated R44$^+$ cells in the grafts retrieved at day 7 after transplantation (C), but not in the grafts retrieved at the day of complete graft failure (e.g., day 13 after transplantation) (20× original magnification) (D). Islet graft tissue of the MOCK-infected control animals was consistently negative (E, F) (20× original magnification). Histological panels show grafts representative for the analyzed groups.

complete graft failure (CD8 T cells, $p < 0.05$, NK cells, $p < 0.01$) (Fig. 3B and C, respectively).

Systemic Immune Activation

To investigate whether the observed differences in immune cell infiltration were also observed systemically, we determined the proportion of circulating effector cells and regulatory CD4 T cells in the blood at different time points after transplantation. As a measure for immune activation, the proportion of FoxP3$^-$CD25$^+$CD4$^+$ effector T cells was determined. As a measure for immune regulation the proportion of FoxP3$^+$CD25$^+$CD4$^+$ regulatory T cells was determined (44).

Although systemic CsA immunosuppression was applied, the peripheral balance between FoxP3$^-$CD25$^+$CD4$^+$ effector T cells and FoxP3$^+$CD25$^+$CD4$^+$ regulatory

T cells was shifted towards immune activation rather than to immune regulation. In the CMV-infected recipients, the ratio between CD4 effector T cells and regulatory T cells increased from 1.86 (0.59–2.58) at 1 day prior to transplantation to 4.00 (2.68–5.49) at day 13 after transplantation ($p < 0.05$). The ratio between CD4 effector T cells and regulatory T cells did not significantly change in the MOCK-infected recipients [2.37 (1.64–3.22) at day −1 vs. 2.67 (2.11–6.80) at day 13]. Although it did not reach statistical significance, the increased ratio between CD4 effector T cells and regulatory T cells in the CMV-infected recipients could be attributed to both a decreased frequency of regulatory T cells, as well as an increased frequency of effector T cells. These changes were not observed in the MOCK-infected recipients (data not shown).

Frequencies of effector T cells and regulatory T cells in the spleens at the time of complete graft failure did not differ between CMV-infected and MOCK-infected recipients (data not shown).

Antiviral Treatment Does Not Prolong Islet Allograft Function

Since it is thought that a generalized systemic CMV infection is a requirement for CMV-induced effects on islet grafts, we questioned whether antiviral treatment of
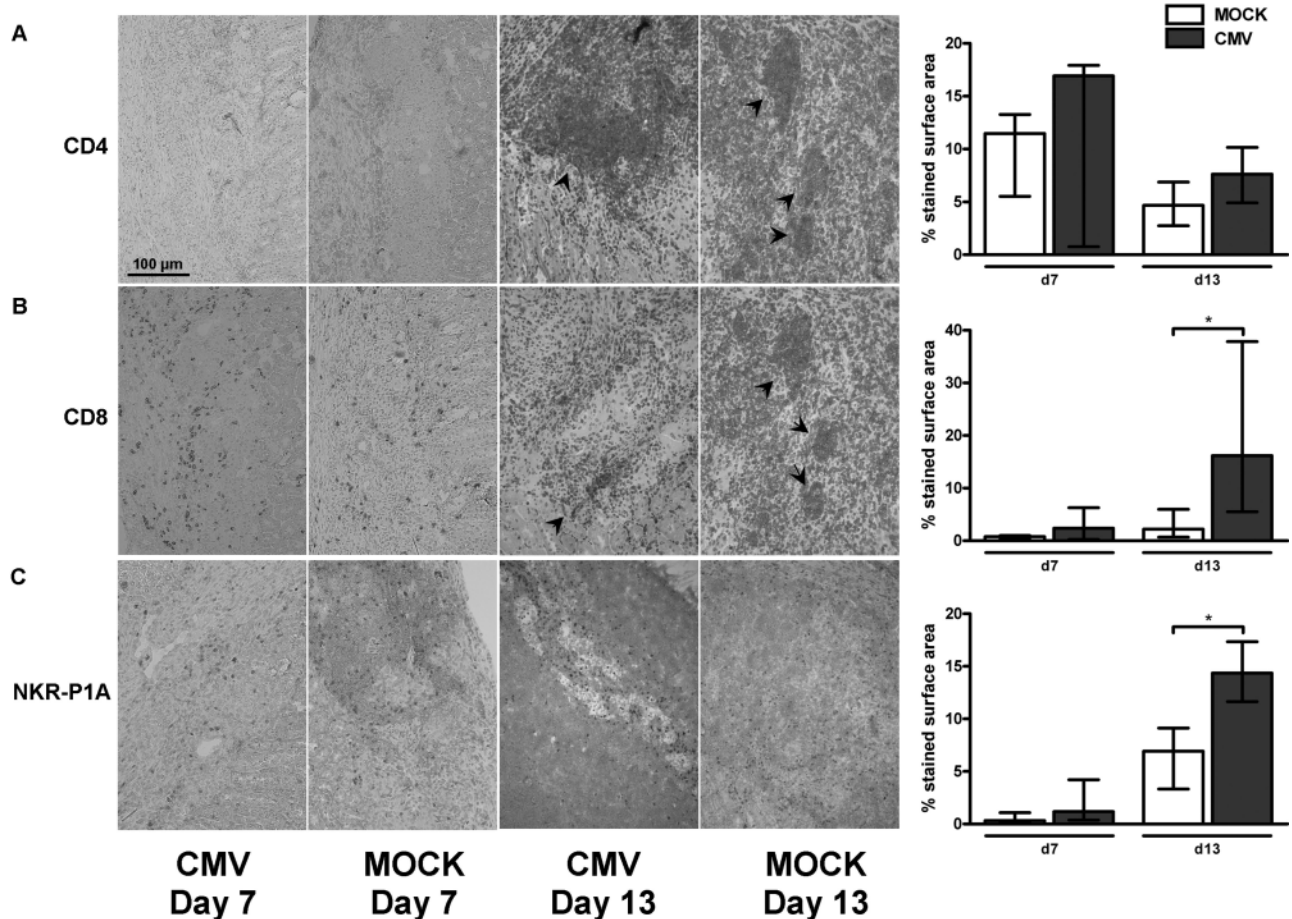

Figure 3. Immune cell infiltration of islet grafts. Islet grafts were stained for CD8, CD4, and NKRP1A. Both MOCK- and CMV-infected animals demonstrated the presence of CD4$^+$ T cells (A), CD8$^+$ T cells (B), and NKRP1A$^+$ NK cells (C), both at day 7 and day 13 after transplantation. Histological panels (all 20× original magnification) show grafts representative for the analyzed groups. Leukocyte infiltration was quantified by measuring the percentage of stained surface area within the islet allografts by morphometry. Infiltration of CD4$^+$ T cells was not significantly different between the analyzed time points or between CMV-infected animals and controls ($p = 0.2$) (A). Grafts demonstrated somewhat increased infiltration of CD8$^+$ T cells in CMV-infected animals (solid bars) compared to control animals (white bars) at day 7 after transplantation and significantly increased infiltration at day 13 after transplantation ($p < 0.05$) (B). Infiltration of NKRP1A$^+$ NK cells was significantly increased in the islet grafts of CMV-infected animals compared to control animals at day 13 after transplantation ($p < 0.05$) (C). Results are depicted as median and quartiles. Statistical significance was calculated using the Mann-Whitney U-test (E). *$p < 0.05$.

the CMV infection could prevent CMV-accelerated graft failure. To this end, an additional group of islet graft recipients was studied, which received ganciclovir treatment in addition to a CMV infection.

Following islet transplantation, 80% (4/5) ganciclovir-treated CMV-infected islet graft recipients restored normoglycemia. This was a higher survival rate compared to the nontreated CMV-infected group, but this did not reach statistical significance. The animals that successfully restored normoglycemia demonstrated similar islet graft failure rates as the nontreated CMV-infected recipients, which was significantly accelerated compared to the MOCK-infected controls ($p < 0.05$ ganciclovir-treated recipients vs. controls, CMV-infected recipients vs. ganciclovir-treated recipients not significant, log-rank test) (Fig. 4).

After sacrifice, R44$^+$ cells were observed in the salivary glands, but the number was low and in most animals absent, illustrating the efficacy of the treatment. R44$^+$ cells were not observed in the retrieved islet grafts (not shown). Systemically, ganciclovir treatment was not able to suppress CMV-induced immune activation, since the ratio between CD4 effector T cells and regulatory T cells increased in a similar manner as in the CMV-infected recipients that did not receive ganciclovir [from 3.50 (2.05–4.19) 1 day prior to transplantation to 5.26 (5.05–6.29) at day 13 after transplantation ($p < 0.05$)]. In contrast to the systemic circulation, we found an effect of the ganciclovir treatment on the CMV-induced infiltration of leukocytes in the graft. In all ganciclovir-treated recipients we found CD4$^+$ and CD8$^+$ T cells, as well as NK cells, but ganciclovir treatment decreased the infiltration of CD8$^+$ T cells to the levels observed in MOCK-infected controls (Fig. 4B). The infiltration of NK cells was even less than observed in the CMV-infected recipients ($p < 0.05$) or MOCK-infected controls ($p < 0.05$) (Fig. 4B).

In conclusion, these data demonstrate that ganciclovir treatment can prevent systemic CMV infection and direct infection of the islet graft, but it does not have an effect on CMV-accelerated islet graft failure or systemic immune activation.

DISCUSSION

In search of factors determining islet graft failure, we studied the effect of CMV on the functional survival of islet allografts. We applied a generally accepted model in solid organ transplantation (4,30). In the present study we demonstrate that this model is also very suitable for studying the deleterious effects of acute CMV infection on cellular grafts, such as pancreatic islet grafts. An advantage of this model is that the mechanisms involved in CMV-induced graft failure can be studied in a short time frame, in a controllable manner and that acute, crit-

ical effects can be identified, which could be missed in more latent, chronic infection models. In order to determine the deleterious effects of CMV infection on islet grafts, it is necessary to use a controllable infection model. For the present experiments, we therefore chose to induce the CMV infection systemically, rather than via the islet graft itself. It might be argued that, due to the systemic CMV infection, the infection-induced islet graft failure is not of any clinical relevance, since systemic viremia is rarely observed after clinical islet transplantation (2,6,8,19,20,63). However, the absence of systemic viremia after clinical islet transplantation may be directly attributed to the use of ganciclovir prophylaxis (2,6,19,20). However, when we studied the effects of ganciclovir in combination with a CMV infection, we also found a reduction in graft survival, despite the successful suppression of systemic infection. Thus, according to these results, systemic CMV infection is not an absolute requirement for CMV-induced accelerated islet graft failure. We therefore feel that our observations are of great clinical relevance.

Our experimental rat study demonstrated accelerated graft failure and enhanced immune activation and infiltration in the CMV-infected recipients compared to noninfected controls. This corroborates the results presented in the 2009 CITR report (6), which states that also human islet graft failure is accelerated in the presence of a CMV infection. Furthermore, we demonstrated that ganciclovir treatment, which is routinely applied after clinical islet transplantation (46), did not prolong the functional survival of islet allografts. The results from the present study suggest that several mechanisms of graft failure may be involved, which will be outlined below. Although the infectability of islet grafts was demonstrated by the presence of R44$^+$ cells in the grafts retrieved 6–7 days prior to complete graft failure, the number of infected cells appeared to be too low to support complete graft failure based on cytolytic infection alone. This finding suggests that CMV infection may have direct deleterious effects, but also that other mechanisms of graft failure are involved. This is substantiated by the observation that treatment of the infection remarkably reduced the number of infected cells, but could not prevent accelerated graft failure.

Many studies have demonstrated CMV-induced solid organ graft failure via intragraft inflammation and consequently accelerated graft rejection (27,36,42,55,56). Our study demonstrated that this not only holds true for solid organ grafts, but also for the failure of cellular islet allografts. In solid organ transplantation, the accelerated rejection is interpreted as the result of local CMV-induced changes, such as increased MHC, adhesion molecule, or chemokine expression, with increased immune cell infiltration and subsequent allograft rejection as a conse-
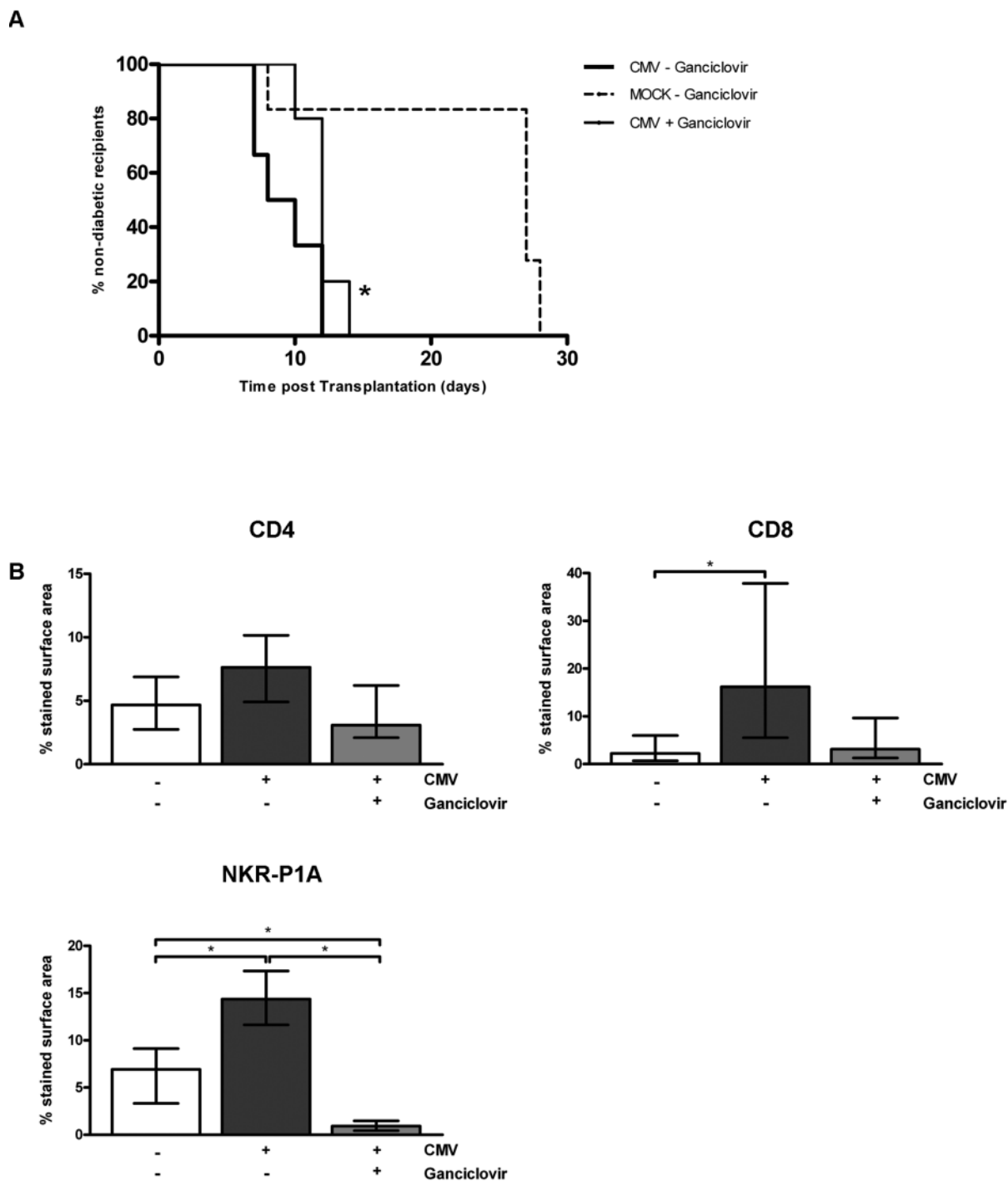
Figure 4. Ganciclovir treatment does not prolong islet graft survival. Graft survival was followed up in ganciclovir-treated CMV-infected recipients (thin solid line, $N = 5$) and compared to CMV-infected (thick solid line, $N = 6$) and MOCK-infected (dashed line, $N = 7$) animals. Graft failure was determined as two consecutive blood glucose measurements above 20 mmol/L. Results are presented as the percentage of nondiabetic islet allograft recipients. Statistical significance was calculated using the Kaplan Meier log-rank test. The asterix represents a statistical significant difference between ganciclovir-treated recipients and MOCK-infected controls ($p < 0.05$). The survival of ganciclovir-treated recipients and nontreated CMV-infected recipients was not significantly different (A). The number of infiltrating cells in the grafts of CMV-infected recipients (black bars), noninfected controls (white bars), and ganciclovir-treated recipients (gray bars) was determined in the grafts retrieved at day 13 after transplantation by quantifying the percentage stained surface area (B). Results are depicted as median and quartiles. Statistical significance was calculated using the Mann-Whitney U-test (E). *$p < 0.05$.

quence (27,36,42,55,56). The marked systemic immune activation, combined with the increased infiltration of CD8+ T cells and NK cells in the grafts of the CMV-infected recipients, suggests that also in islet transplantation, CMV-enhanced immune responses against the islet allograft may act in concert with direct CMV infection of the graft to induce islet graft failure. This is in line with our previous data demonstrating that rat pancreatic β-cells are not only directly susceptible to RCMV infection, but that the infection also markedly enhanced the cellular immunogenicity (50). This was characterized by the increased expression of both classical and nonclassical MHC I molecules and the adhesion molecules ICAM-1 and LFA-3 (50). In a study using HCMV and human β-cells, similar results were found (Smelt et al., submitted). Interestingly, in this study the upregulation of β-cell immunogenicity was observed in a large proportion of the cells, while only a small minority of the β-cells demonstrated apparent CMV protein expression (Smelt et al., submitted). This suggests that the presence of only a few CMV-infected cells may be enough to establish enhanced local immunogenicity, leading to immune cell activation and islet graft destruction.

The presence of elevated numbers of NK cells in the CMV-infected grafts warrants some further consideration. In the CMV-infected recipients, these NK cells may be primarily responding to the presence of CMV in the graft (31), but the presence of NK cells in the grafts of the noninfected controls suggests that these cells may also have a role in the immune response directed against the islet allograft. Recently, NK cells with a specific alloreactivity have been described in solid organ transplantation (28,38,40). Since NK cells are inhibited by "self" MHC I, the lack of "self" MHC I on the islet allografts may contribute to NK cell activation and proinflammatory and destructive NK cell effector functions. Increased infiltration of NK cells in the CMV-infected islet grafts, as a result of graft infection and/or infection-induced increased local immunogenicity (50), may subsequently further enhance the ongoing alloimmune response, accelerating the rejection process.

Our finding that antiviral treatment, using ganciclovir, does not prolong graft survival is corroborated by several studies that found similar results after solid organ transplantation (24,49). This suggests that the immediate-early stages of viral infection, which are not affected by ganciclovir (7,39), are highly contributing to accelerated graft failure. This may also hold true in this islet transplantation model, since Hayashi et al. (22) demonstrated that even low-grade infection of immunocompetent mice had direct effects on the β-cell insulin secretory capacity and inflammation of the native islets. Moreover, after islet transplantation in humans, systemic viremia may not be an absolute requirement for deleteri-

ous CMV-induced effects in the islet graft. This was demonstrated in the 2009 CITR report, which negatively correlated CMV infection to islet graft failure after transplantation. Interestingly, this effect of CMV was found using a transplantation protocol that routinely uses ganciclovir prophylaxis (46) to prevent systemic viremia and clinical signs of CMV infection. Treatment of clinically active CMV infection with ganciclovir may revert islet allograft dysfunction, as suggested by a case report (8). However, subclinical CMV infection may occur even during ganciclovir treatment (18), and may be interfering with islet graft function. That low-grade CMV infection may still affect islet graft survival is corroborated by our finding that ganciclovir is ineffective in preventing CMV-induced islet graft failure in rats. Furthermore, also Eckhard et al. (12) demonstrated a trend towards declining islet graft function in CMV-positive patients. Also these patients had been treated with antiviral medication and demonstrated no signs of systemic viremia at the time of declining graft function (12). Taken together, this suggests that, not only in the current model, but also in clinical islet transplantation, subclinical CMV infection may be an important factor contributing to islet graft loss.

In our experiments, all animals that demonstrated early graft loss (i.e., CMV-infected animals) demonstrated systemic immune activation. This activation was characterized by increased effector T-cell versus regulatory T-cell ratios. This systemic immunoactivation developed independent of viral inhibition by ganciclovir, suggesting that systemic CMV infection and subclinical CMV infection are equally able to skew the immune response towards activation. In what manner systemic immune activation affects islet graft rejection remains to be identified. The pronounced systemic immune response in the ganciclovir-treated animals in the absence of increased immune cell infiltration in the grafts is an observation we cannot explain. We speculate that ganciclovir may have direct effects on the islet graft by affecting local responses. Alternatively, ganciclovir may have more systemical effects, which may interfere with islet graft survival. Such effects, however, are not apparent from the literature, in which ganciclovir is routinely used after clinical islet transplantation. Future research efforts will focus on the effects of ganciclovir on islet graft function and survival. At this point, we interpret our observation as a suggestion that not only the "classical" immunological rejection pathway, but other also pathways [either immunological or non-immunological (9)] contribute to the early loss of islet grafts in these recipients.

Clinically, the leading dogma has been that CMV infection was a negligible factor contributing to islet graft failure. Only recently, with the publication of the 2009

CITR report (6), this opinion has started to change. It has always been assumed that islet grafts were relatively uninfectable and contained only low amounts or no virus particles at all. This led to the assumption that transmission of the infection after islet transplantation, as well as secondary CMV-induced effects on graft survival, was rare to nonexistent. The results from the present study argue against this clinical reasoning, since we demonstrated that at least several cell types within the islet grafts are able to support viral infection and replication. These may also be the pancreatic β-cells, since we have previously demonstrated that pancreatic β-cells are able to support all stages of viral replication in vitro (50). The susceptibility of islet grafts for the infection is further corroborated by several reports which demonstrate that viral transmission from infected islet grafts is possible (8,63). The reported absence of viral transmission after clinical islet transplantation (2,19,20) may be a direct consequence of the applied antiviral treatment given prophylactically in the peritransplant period (46). However, this absence of viral infection in these patients may not be used as an argument to rule out subclinical CMV infection as a factor in graft failure, since in the present study we demonstrated that ganciclovir treatment is not able to prolong islet graft survival.

Compared to solid organ transplantation, the deleterious effects of CMV on graft survival may be even more prominent. As for yet unknown reasons, the CMV seroprevalence of type 1 diabetes mellitus patients appears to be lower compared to the general population (20,45). Since in islet transplantation patients often receive islets from multiple donors (47), it is likely that the majority, if not all, of the currently transplanted islet grafts are actually CMV infected. This has serious consequences for the success of islet allografts, since it is well known from solid organ transplantation that the highest incidence of CMV transmission, reactivation, and most important, CMV-induced graft loss, is observed in uninfected recipients receiving grafts from CMV-infected donors (14). Since we also demonstrated that treatment of CMV using ganciclovir is not effective in prolonging islet allograft survival, it is not only mandatory to carefully monitor CMV transmission and reactivation after clinical islet transplantation, but also to investigate the local (e.g., in the graft) CMV-induced effects that may lead to early islet graft loss.

In conclusion, in the present study we demonstrated that CMV infection negatively influences the survival of islet allografts. This corroborates the recent findings presented in the CITR report (6). Further research is required to quantify whether the islet graft of an animal carrying CMV can infect a CMV-negative, chronically immunosuppressed recipient and if this would interfere with long-term graft function and survival. Furthermore, it would also be of interest to study the effects of a CMV infection derived from the recipient, rather than the donor. Also, the impact of a delayed CMV infection on graft function and survival should be established. The finding that ganciclovir did not prolong the survival of islet allografts, despite effectively reducing the magnitude of viral infection, suggests that also low-grade CMV infection should be considered as a serious deleterious factor in islet transplantation. Our data suggest that more effective suppression of CMV infections in islet allograft recipients may contribute to prolongation of islet graft function.

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