

Reduction of Transplant Arteriosclerosis After Treatment With Mycophenolate Mofetil and Ganciclovir in a Mouse Aortic Allograft Model

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Abstract

Objectives: Transplant arteriosclerosis is a major obstacle for long-term allograft survival in heart transplant. The aim of this study was to investigate potential synergistic effects of combined treatment with mycophenolate mofetil and ganciclovir on the development of transplant arteriosclerosis, presence of regulatory T cells, and expression of donor specific alloantibodies.

Materials and Methods: Donor aortas from C57BL/6 (H2b) mice that were fully mismatched to the major histocompatibility complex were transplanted into CBA (H2k) mouse recipients. Groups of mice received mycophenolate mofetil (100 or 300 mg/kg, oral), ganciclovir (10 or 72 mg/kg, intraperitoneal), or a mycophenolate mofetil and ganciclovir combination. Grafts were analyzed by histology and morphometry on day 30 after transplant. Numbers of regulatory T cells and donor-specific

alloantibodies were examined by fluorescence-activated cell sorting analysis of splenic tissue and peripheral blood.

Results: Mycophenolate mofetil (100 mg/kg) and ganciclovir (10 mg/kg and 72 mg/kg) did not show effects on transplant arteriosclerosis formation or alloantibody production. However, groups treated with mycophenolate mofetil (300 mg/kg) or a low- or high-dose mycophenolate mofetil and ganciclovir combination had significantly reduced transplant arteriosclerosis and alloantibody levels. Expression of regulatory T cells within the spleen was similar between all experimental groups and untreated controls.

Conclusions: The combination of mycophenolate mofetil and ganciclovir significantly reduced the development of transplant arteriosclerosis in a mouse abdominal aortic allograft model. This effect may be a result of decreased alloantibody production.

Key words: Vasculopathy, Immunosuppression, Chronic rejection, Alloantibodies

Introduction

Although immunosuppressive therapies have been well investigated and improved in recent years, they do not protect heart transplant patients from the formation of transplant arteriosclerosis, a hallmark feature of chronic rejection. This disease is a major limiting factor for long-term success and a leading cause of late mortality after heart transplant.¹ The typical characteristics of transplant arteriosclerosis include diffuse and progressive thickening of the arterial intima that affects minor and major coronary

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Acknowledgements: This work was supported by an unrestricted research grant from Roche Pharma AG, Grenzach-Wyhlen, Switzerland. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) via the SFB 643 Project B9 and the ADUMED-Foundation. The authors thank Katharina Porsche, Leonie Littmann, and Susanne Rössner for expert technical assistance. They also thank Professor von Hörsten and the staff of the animal facility of the University of Erlangen-Nürnberg for their expert care of animals used for this study. Julia Hoffmann and Martin Böhm contributed equally. Julia Hoffmann, Martin Böhm, Alexander Steinkasserer, and Stephan M. Ensminger designed the study. Julia Hoffmann, Martin Böhm, Martina Ramsperger-Gleixner, Bernd M. Spriewald, and Elisabeth Zinser performed the experiments and collected data. Julia Hoffmann, Martin Böhm, Silke Abele-Ohl, and Stephan M. Ensminger analyzed the data. Julia Hoffmann, Martin Böhm, Bernd M. Spriewald, Michael Weyand, and Stephan M. Ensminger wrote the paper.

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Experimental and Clinical Transplantation (2012) 6: 592-600

arteries of transplanted cardiac allografts. In contrast with general arteriosclerosis, transplant arteriosclerosis involves long segments of the affected arteries and is characterized by concentric intimal thickening.^{2,3} This ultimately leads to myocardial ischemia of the transplanted heart and subsequent organ failure.

Transplant arteriosclerosis may be caused by immune mediated vascular injury, inflammation of the vascular endothelium, ischemia reperfusion injury, *cytomegalovirus* infection, and metabolic risk factors.^{2,3} Inflammation is a primary mechanism in the development of chronic rejection, mediated mainly by T and B lymphocytes, migrating through the endothelial layer and triggering intimal proliferation by producing cytokines. A crucial enzyme for the proliferation of lymphocytes is inosine monophosphate dehydrogenase, which mediates de novo synthesis of purines. Mycophenolate mofetil is a selective inhibitor of inosine monophosphate dehydrogenase⁴ and is used for immunosuppressive therapy after heart transplant.

In addition to inhibiting T- and B-lymphocyte proliferation, mycophenolate mofetil decreases the maturation and antigen presenting capacity of dendritic cells, recruitment of monocytes and macrophages into the graft, and adhesion of leukocytes onto the vascular endothelium of the graft.⁴⁻⁶ The proliferation of vascular smooth muscle cells, another crucial step in the development of transplant arteriosclerosis, also can be decreased by mycophenolate mofetil in vitro and in vivo.^{7,8} Furthermore, experimental data and clinical trials have demonstrated that mycophenolate mofetil may decrease the production of alloreactive antibodies.^{9,10}

As a consequence of immunosuppression, heart transplant patients are more susceptible to infections, especially viral infections such as human *cytomegalovirus*. Therefore, ganciclovir is used commonly for prophylaxis against human *cytomegalovirus* and antiviral therapy. After phosphorylation by virus-induced cellular enzymes, ganciclovir intercalates with viral DNA, causes a chain termination, and blocks viral replication.¹¹ Synergistic effects of combination therapy of mycophenolate mofetil and ganciclovir for the antiviral activity of ganciclovir have been described.¹²

The role of regulatory T cells (Tregs) during the development of transplant arteriosclerosis has been subject of several recent studies. Targeted transfer of donor specific Tregs into donor naive recipients has

become a promising experimental approach to induce immune tolerance toward the graft.¹³ In an abdominal aortic allograft model of CD4⁺CD25⁻ effector T-cell-mediated rejection in Rag-/-y-/- mice, decreased levels of transplant arteriosclerosis were shown after application of donor-specific, ex vivo expanded Tregs.¹³ A further study performed in a clinically relevant chimeric humanized mouse model showed that ex vivo expanded human Tregs prevented the development of transplant arteriosclerosis.¹⁴ These results suggest a potential clinical application of Tregs, but studies about the interactions with immunosuppressive medications are necessary. The effects of mycophenolate mofetil on the proliferation and function of Tregs are controversial.¹⁵⁻¹⁷

The aim of the present study was to investigate the immune modulatory effects of combined therapy of mycophenolate mofetil and ganciclovir on the development of transplant arteriosclerosis. Another aim of the present study was to analyze the effect of mycophenolate mofetil on Tregs in the context of transplant arteriosclerosis. In addition, we were interested in the efficacy of mycophenolate mofetil in modulating the humoral immune response, especially the production of donor-specific alloantibodies. Mouse abdominal aortic allografts were used as an experimental model¹⁸ because they have similar vascular lesions as those observed in human coronary arteries that are affected by transplant arteriosclerosis and allow a precise analysis of the composition of vascular lesions.¹⁹

Material and Methods

Animals

C57BL/6 (H2b) and CBA.J (H2k) mice were purchased from Charles River (Sulzbach, Germany). C57BL/6 (H2b) mice were used as donors and CBA.J (H2k) mice as recipients of the aortic allografts. Mice (age, 6-12 weeks at the time of the experiments) were bred and maintained at the central animal facility of the University of Erlangen-Nürnberg (Franz-Penzoldt-Zentrum) under specific pathogen-free conditions. The health condition of the mice was assessed continuously during the course of the experiment with a scale based on criteria including weight loss, bad fur structure, changes in skin, and wasting syndrome.²⁰ This study was carried out in strict accordance with international guidelines for

animal care and use, guidelines of the Animal Care and Use Committee of the Government of Bavaria, and institutional guidelines of the University of Erlangen-Nuremberg. The protocol was approved by the Government of Bavaria (Regierung von Mittelfranken, Permit Number: 54-2531.31-17/07).

Medication

Mycophenolate mofetil was reconstituted with 5.0% glucose and administered by oral gavage at a daily dose of either 100 mg/kg or 300 mg/kg, divided into 2 daily applications. Ganciclovir was dissolved in 0.9% sodium chloride and administered by 2 daily intraperitoneal injections at a total daily dose of 10 mg/kg or 72 mg/kg. Reconstituted solutions of mycophenolate mofetil and ganciclovir were stored at 4°C for a maximum 7 days.

Dose and blood level monitoring

To evaluate the appropriate dose of mycophenolate mofetil, 2 groups (n=5) of nonoperated CBA mice were treated with mycophenolate mofetil, orally administered at a dose of 100 mg/kg/d or 300 mg/kg/d. Another group of 5 mice received 100 mg/kg mycophenolate mofetil orally and 10 mg/kg ganciclovir (intraperitoneal). The low dose was chosen according to published data.¹⁷ The high 300 mg/kg dose was calculated by using the LOWE formula,²¹ which helps to determine an adequate dose in small animals, considering their higher metabolic activity. To control the mycophenolic acid plasma level, blood samples (100 µL) were taken from the mouse retroocular venous plexus under ketamine and xylazine anesthesia and centrifuged immediately after extraction on days 7, 14, 21, and 28. Plasma levels of mycophenolic acid were assessed with liquid chromatography-mass spectrometry. Plasma calibrator and controls were purchased from Chromsystems (Munich, Germany).

Abdominal aortic transplant

Abdominal aortic transplant was performed using a modified technique previously described.¹⁸ In brief, the donor thoracic aorta was isolated, resected, and transferred to the recipient animal. The recipient aorta was clamped and then transected with sharp microvascular scissors. A proximal end-to-end anastomosis was performed. The aortic graft was repositioned and the anastomosis continued with single interrupted sutures.

Treatment protocol

All mice were weighed before and during treatment. After abdominal aortic transplant (day 0), mice received medication for 30 days starting on day 1 after transplant. Two groups of mice (5 mice each) received mycophenolate mofetil in a daily dose of 100 mg/kg (group M1) or 300 mg/kg (group M2), and 2 groups (5 mice each) were treated with ganciclovir, 10 mg/kg (group G1) or 72 mg/kg (group G2). Additional groups of mice (5 mice each) were treated with a combination of mycophenolate mofetil (100 mg/kg) and ganciclovir (10 mg/kg) (group MG1) or mycophenolate mofetil (300 mg/kg) and ganciclovir (72 mg/kg) (group MG2). Control groups did not receive any medication after allogeneic aortic transplant. Most mice (85%) undergoing aortic transplant were included in further analyses; exclusion criteria were major intraoperative or postoperative bleeding or premature death because of adverse events in the treatment protocol.

Analysis of the aortic graft

Aortic grafts were removed with the animal under anesthesia on day 30 after transplant. Grafts were perfused with saline and flash frozen in optimal cutting temperature medium (Tissue-Tek, Sakura, Netherlands) in liquid nitrogen for morphometric analysis of cryostat sections (7 µm).

Morphometry

Five sections from each graft were recovered at day 30, snap frozen, stained with Elastica van Gieson, and analyzed by 2 independent examiners (J.H. and M.B.) who were blinded to the experimental conditions, using a conventional light microscope (original magnification ×200). A digitized image of each section was captured and the areas within the lumen and the internal and external elastic lamina were circumscribed manually and measured as previously described.²² From these values, a quotient for the thickness of the intima (Q_{int}) was calculated. Q_{int} indicated the relative thickness (%) of the intima. Therefore, a Q_{int} value of 0% indicates no intimal thickening and a Q_{int} value of 100% indicates a total occlusion of the lumen. All image analyses were carried out on a color display monitor using image analysis software (ANALYSIS, Olympus, Hamburg, Germany).

Antibodies and fluorescence-activated cell sorting analyses for Foxp3

Recipient splenocytes were analyzed by fluorescence-activated cell sorting at the end of the experimental protocol on day 30 after transplant. The directly conjugated murine antibodies anti-CD4-FITC and anti-CD25-PerCP were purchased from BD Biosciences (Heidelberg, Germany). Cells were stained for 30 minutes on ice without light. The murine anti-Foxp3 PE staining kit was purchased from eBioscience (NatuTec, Frankfurt, Germany). After fixation and permeabilization of the cells, Foxp3 staining was performed as directed by the manufacturer. Cells were analyzed using a fluorescence-activated cell sorter (Canto II, Becton-Dickinson Biosciences, Heidelberg, Germany) and (FlowJo software version 7.5, Tree Star Inc., Ashland, OR, USA).

Alloantibody analyses

Blood of all recipient mice was collected on day 30 under anesthesia and centrifuged to obtain serum. Spleens of 2 untreated C57BL6 mice were collected under anesthesia. To extract the spleen cells, spleens were grinded, and phosphate buffered saline (PBS) was added. After centrifugation, cells were resuspended in PBS and supplemented with red cell lysis buffer. After incubation (10 min), PBS was added to stop the lysis. Samples were centrifuged again and the cell count was measured. The cell number was adjusted to 5 to 10 million cells/mL using bovine serum albumin. After blockade of Fc receptors, cell suspension (50 μ L) was transferred into a fluorescence-activated cell sorting tube and recipient serum (50 μ L) was added. After incubation (30 min) followed by 3 wash steps, bovine serum albumin buffer (50 μ L) was added. FITC-labeled anti-mouse Fc antibody (F2772, goat derived, Fc-specific FITC anti-mouse IgG F[ab]2 fragment, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (1 μ L) was added and incubated for 20 minutes at 4°C. After a final washing step with PBS, fluorescence-activated cell sorting analysis (BD FACSCanto II; BD Biosciences, Heidelberg, Germany) was done according to the manufacturer's directions.

Statistical analysis

Results were reported as mean per group \pm standard error of the mean (SEM). Statistically significant differences between the groups were determined using 1-way analysis of variance (ANOVA). To

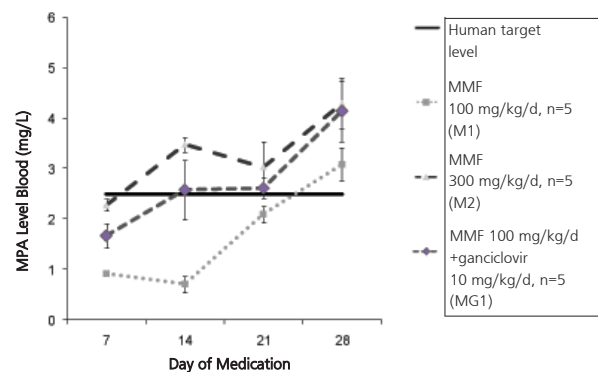
compare 2 particular groups within an experiment, a 2-tailed unpaired *t* test with a Bonferroni-Holm correction was performed. Statistical significance was defined by $P < .05$.

Results

Mycophenolate mofetil (300 mg/kg) leads to an adequate blood level of mycophenolic acid in mice

Serum samples from mice treated with 100 mg/kg mycophenolate mofetil (group M1) taken on day 7, 14, 21, and 28 showed mean mycophenolic acid plasma concentrations of 1.70 mg/L. In the 300 mg/kg group (group M2), mean mycophenolic acid plasma levels reached 3.27 mg/L. The combination of 100 mg/kg mycophenolate mofetil with 10 mg/kg ganciclovir (group MG1) increased plasma levels to 2.75 mg/L. Therefore, mycophenolic acid plasma levels in the M2 and MG1 groups were continuously kept above the human target plasma level for heart transplanted patients of 2.5 to 4.5 mg/L,²³ but plasma levels in the M1 group did not reach the target concentration until day 28 (Figure 1). No apparent degradation of the health status of the mice was observed in any of the groups.²⁰

Figure 1. Serum Levels of Mycophenolic Acid



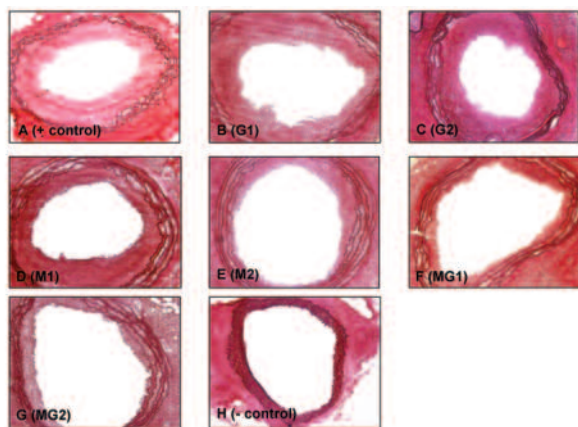
Serum levels of mycophenolic acid (MPA) determined in mice treated with mycophenolate mofetil (MMF). Serum levels were assessed with liquid chromatography - mass spectrometry on day 7, 14, 21, and 28 (each group, $n=5$).

High-dose mycophenolate mofetil (alone and in combination with ganciclovir) and low-dose mycophenolate mofetil in combination with ganciclovir significantly reduced the development of transplant arteriosclerosis

Aortic allografts from C57BL/6 (H2^b) mice transplanted into CBA/J (H2^k) recipients were

recovered 30 days after treatment, when distinctive changes of transplant arteriosclerosis are most evident. Histomorphometric analysis did not reveal a significant decrease of transplant arteriosclerosis in both groups receiving ganciclovir monotherapy (Q_{int} : group G1, $53\% \pm 9\%$; group G2, $62\% \pm 11\%$; control, $67\% \pm 1\%$) (Figure 2). In contrast, aortic grafts from recipients of group M2 and group MG2 showed a highly significant reduction of transplant arteriosclerosis compared with untreated controls (Q_{int} : group M2, $23\% \pm 7\%$; group MG2, $19\% \pm 8\%$; control, $67\% \pm 1\%$; $P \leq .001$) (Figure 2). In the low-dose mycophenolate mofetil group (group M1), a reduction of transplant arteriosclerosis on day 30 was not detected (group M1, $65\% \pm 12\%$; control, $67\% \pm 1\%$; not significant). However, treatment with low-dose mycophenolate mofetil and ganciclovir (group MG1) led to a significant decrease of luminal obliteration (intimal proliferation: group MG1, $32\% \pm 6\%$; control $67\% \pm 1\%$; $P \leq .001$) (Figure 2). Syngeneic controls (CBA/J [H2k] donors into CBA/J [H2k] recipients) had no evidence of transplant arteriosclerosis on day 30 after transplant (Figure 2). None of the recipient mice entering analysis had any hemorrhage or major postoperative bleeding.

Figure 2. Histomorphometric Evaluation of Fully Allogeneic C57BL/6 Aortic Grafts

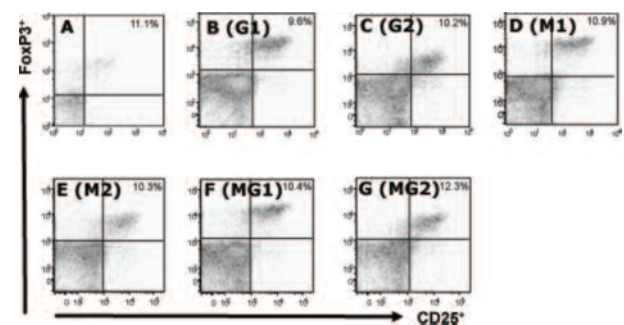


Histomorphometric evaluation of fully allogeneic C57BL/6 aortic grafts implanted into CBA recipients on day 30 after transplant. Significant intimal proliferation was observed in aortic allografts recovered from untreated CBA recipients (A). Treatment with 10 mg/kg ganciclovir (group G1) (B), 72 mg/kg ganciclovir (group G2) (C) and 100 mg/kg mycophenolate mofetil (group M1) (D) did not result in a significant reduction of intimal proliferation. In contrast, a significant reduction in intimal proliferation of the aortic allografts on day 30 after transplant was observed after treatment with 300 mg/kg mycophenolate mofetil (group M2) (E); a combination of mycophenolate mofetil 100 mg/kg and ganciclovir 10 mg/kg (group MG1) (F); and a combination of mycophenolate mofetil 300 mg/kg and ganciclovir 72 mg/kg (group MG2) (G). Syngeneic controls did not show any vascular lesions (H). For the morphometric analysis of the degree of intimal thickening, Elastica van Gieson stained sections were used (original magnification $\times 200$).

Unchanged numbers of CD4⁺CD25⁺Foxp3⁺ regulatory T cells after treatment with mycophenolate mofetil or ganciclovir

The presence of infiltrating Tregs was analyzed by fluorescence-activated cell sorting using murine splenic tissue on day 30 after transplant. The percentage of CD4⁺CD25⁺Foxp3⁺ cells within the population of CD4⁺ T cells was assessed. Numbers of CD4⁺CD25⁺Foxp3⁺ cells did not differ between the experimental groups treated with mycophenolate mofetil, ganciclovir, a combination of both drugs, or the untreated control group (percentage of CD4⁺CD25⁺FoxP3⁺ Tregs: groups M1 and M2, $10\% \pm 1\%$; groups G1 and G2, $10\% \pm 2\%$; group MG1, $11\% \pm 1\%$; group MG2, $11\% \pm 4\%$; control, 11% ; not significant) (Figure 3).

Figure 3. Splens of CBA Recipients



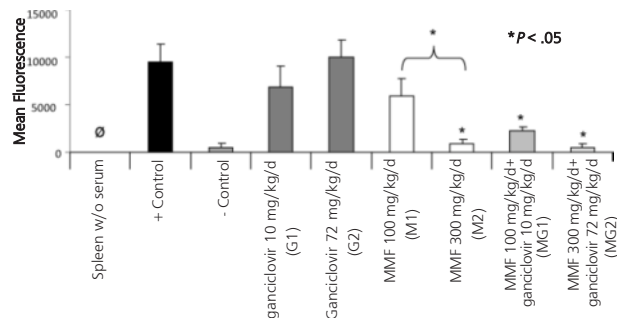
Splens of CBA recipients were collected on day 30 after transplant, and fluorescence-activated cell sorting analyses were performed to analyze the presence of CD4⁺CD25⁺Foxp3⁺ within the spleen in the different treatment groups. Results are given in percentage of Foxp3⁺ cells in relation to the total amount of CD4⁺CD25⁺ cells. Representative fluorescence-activated cell sorting plots are shown for an untreated control group (A), treatment with ganciclovir 10 mg/kg (group G1) (B); ganciclovir 72 mg/kg (group G2) (C); mycophenolate mofetil 100 mg/kg (group M1) (D); mycophenolate mofetil 300 mg/kg (group M2) (E); a combination of mycophenolate mofetil 100 mg and ganciclovir 10 mg/kg (group MG1) (F); and a combination of mycophenolate mofetil 300 mg/kg and ganciclovir 72 mg/kg (group MG2) (G). The average number of CD25⁺ T cells gated against CD4⁺ cells (grey) and the percentage of Foxp3⁺ cells (black) within the CD25⁺ fraction detected by fluorescence-activated cell sorting analysis in the spleen of transplanted recipients on day 30 after transplant are shown.

Mycophenolate mofetil alone or in combination with ganciclovir decreased the number of donor-specific alloantibodies

Donor-specific alloantibodies play a crucial role in the development of transplant arteriosclerosis.^{24,25} Therefore, on day 30 after aortic transplant, serum samples of the recipient mice were analyzed for the presence of donor specific alloantibodies. In comparison with untreated controls, the high-dose mycophenolate mofetil group (M2) and both

combination groups (MG1 and MG2) had a significant reduction of the mean fluorescence intensity (mean fluorescence intensity: group M2, 722 ± 201 ; group MG1, 2049 ± 437 ; group MG2, 586 ± 105 ; control, 9507 ± 1808 ; $P < .05$) (Figure 4). In addition, a significant difference was observed between the low- and high-dose mycophenolate mofetil groups (mean fluorescence intensity: group M1, 5720 ± 1807 ; group M2, 722 ± 201 ; $P < .05$). In contrast, ganciclovir monotherapy did not significantly influence alloantibody levels compared with controls (mean fluorescence intensity: group G1, 6940 ± 186 ; group G2, 9867 ± 1850 ; control, 9507 ± 1808 ; not significant).

Figure 4. Circulating Alloantibody Responses Against the Fully Mismatched Aortic Allograft



Circulating alloantibody responses against the fully mismatched aortic allograft were determined by FACS analyses on day 30 after transplant. IgG alloantibodies results are shown. (MFI = mean fluorescence intensity; n=5 animals per group / P values as indicated in the diagram).

Discussion

Transplant arteriosclerosis is a multifactorial process. T cells, macrophages, proinflammatory cytokines, adhesion molecules, growth factors, and alloantibodies trigger and maintain a chronic inflammatory response within the allograft, causing damage to the graft including vascular lesions. Mycophenolate mofetil, a selective inhibitor of inosine monophosphate dehydrogenase, is a well-established component of modern immunosuppression after heart transplant.^{26,27}

This study showed that high-dose mycophenolate mofetil alone (group M2) or in combination with ganciclovir (group MG2) effectively reduced development of transplant arteriosclerosis. The absence of efficacy of mycophenolate mofetil at a low dose (100 mg/kg, group M1) in our model might be due to significantly lower plasma levels of mycophenolic acid in this group. According to clinical data, minimal mycophenolic acid target

plasma levels should be ≥ 2.0 mg/L in heart transplanted patients or a target range of 2.5 to 4.5 mg/L.^{23,28} In the present study, levels of transplant arteriosclerosis in the low-dose mycophenolate mofetil group (group M1) did not significantly differ from untreated controls, possibly because the required target range of mycophenolate mofetil was not reached until day 28. In a similar model of aortic transplant in rats, a significant reduction of transplant arteriosclerosis was achieved using a lower mycophenolate mofetil dosage (20 mg/kg orally) that resulted in a mycophenolic acid plasma concentration of 2.0 mg/L.⁷ However, in that experimental model, mycophenolic acid levels gradually declined during the postoperative period and the protective effect of mycophenolate mofetil disappeared after 6 months.⁷ Levels of transplant arteriosclerosis in animals treated with mycophenolate mofetil did no longer differ from untreated controls after this time, suggesting a crucial correlation between mycophenolic acid plasma levels and efficacy of mycophenolate mofetil therapy in the prevention of transplant arteriosclerosis, as observed in our study.

Combination low-dose mycophenolate mofetil with 10 mg/kg ganciclovir led to a significant decrease of transplant arteriosclerosis compared with untreated controls or low-dose mycophenolate mofetil monotherapy. This effect could be explained by higher mycophenolic acid plasma levels with ganciclovir, because mycophenolic acid levels in the combined treatment group were > 2.5 mg/L throughout the experiment. Concomitant use of both drugs results in competitive renal excretion of ganciclovir and a mycophenolate mofetil metabolite (mycophenolic acid glucuronide), leading to augmented mycophenolic acid plasma levels. With high-dose mycophenolate mofetil, sufficient mycophenolic acid plasma levels were achieved throughout the experimental protocol and additional treatment with ganciclovir did not have an additive effect.

Monotherapy with ganciclovir did not have an effect on the development of transplant arteriosclerosis in the present model. However, ganciclovir may modulate the formation of transplant arteriosclerosis and prolong graft survival.²⁹ In a rat cardiac transplant model, ganciclovir reduced transplant arteriosclerosis in rats infected with *cytomegalovirus* to the level observed in uninfected

controls.²⁹ However, despite a moderate reduction of intimal proliferation, there was significant evidence of transplant arteriosclerosis in the ganciclovir-treated groups; this suggests that ganciclovir successfully eliminated *cytomegalovirus* as a possible risk factor for the formation of transplant arteriosclerosis, but did not influence other factors mediating the development of transplant arteriosclerosis. These data are consistent with the observation that, in our experimental model, ganciclovir monotherapy had no effect on the development of transplant arteriosclerosis in the absence of *cytomegalovirus* infection. In contrast, combined treatment with mycophenolate mofetil and ganciclovir seems promising, because mycophenolate mofetil may potentiate the antiherpesvirus activity of ganciclovir.¹² Significantly prolonged graft survival has been observed previously in renal transplant patients infected with *cytomegalovirus* who received a combination of mycophenolate mofetil and ganciclovir compared with treatment with azathioprine and ganciclovir.³⁰ In addition, ganciclovir significantly elevates mycophenolic acid plasma levels and may amplify its immunosuppressive efficacy.

The effect of mycophenolate mofetil on the development of Tregs is controversial.^{16,17,31} Treg numbers are increased after treatment with a combination of mycophenolate mofetil and vitamin D3.¹⁷ However, other experimental studies have not demonstrated relevant negative effects of mycophenolate mofetil on the development and function of Tregs.³¹ Furthermore, an experimental mouse study showed that mycophenolate mofetil at therapeutic doses may exert an inhibitory effect on Treg proliferation and function.¹⁶ In the current study, analysis of splenic tissue did not reveal significant differences in Treg counts between the different experimental groups. A correlation between Treg expression and levels of transplant arteriosclerosis was not observed, indicating that in our model additional mechanisms may have resulted in the reduction of transplant arteriosclerosis.

Allogeneic antibody responses are important in the development of transplant arteriosclerosis. The reduced antibody response in the groups treated with mycophenolate mofetil may have resulted from impaired B-cell activation. In a clinical study, administration of mycophenolate mofetil was associated with a reduction of B cells and down-regulation of activation markers on B cells in patients

after cardiac allograft transplant.³² In addition, in a mouse model of colitis, mycophenolate mofetil pretreatment improved experimental colitis by down-regulation of expanded B cells by apoptosis.³³ Furthermore, in a pig model of cardiac transplant, cardiac allograft vasculopathy was attenuated after treatment with mycophenolate mofetil, possibly because of decreased interferon- γ expression in the myocardium and prevention of the generation of alloantibodies.³⁴

Numerous reports have evaluated donor-specific alloantibodies, and in some experimental models an association was demonstrated. Alloreactive antibodies were transferred into immunodeficient, heart transplanted mice, and this caused significant vascular lesions, even in absence of cellular immunity.³⁵ These findings were recently confirmed in a humanized mouse aortic transplant model using severe combined immunodeficiency mice as recipients, which were treated with anti-HLA I antibody.³⁶ However, other studies with different murine models of experimental transplant did not show a significant contribution of alloantibodies to the rejection process of transplanted allografts.^{37,38} Recent reports suggest that donor-specific alloantibodies may induce the development of transplant arteriosclerosis by up-regulation of growth factors from endothelial and vascular smooth muscle cells; release of adhesion factors; consecutive induction of macrophages, lymphocytes, and dendritic cells; and activation of complement factors.^{24,25} Alloantibodies also are associated with collagen deposition and proliferation of smooth muscle cells. However, pronounced endotheliitis, characterized by a massive inflammatory intimal infiltrate, can be observed in the absence of alloantibodies.³⁹

Data from the current study demonstrated a strong correlation between the level of donor-specific alloantibodies and the formation of transplant arteriosclerosis throughout all the experimental groups. These data are in contrast to a previous study in which there was no correlation between antiendothelial antibodies and transplant arteriosclerosis in a rat aortic allograft model after treatment with mycophenolate mofetil.⁴⁰ However, several other experimental and clinical studies revealed a suppressive effect of mycophenolate mofetil on donor-specific alloantibodies and antivimentin antibodies and are consistent with the present data.^{10,34,41} Therefore, the suppressive effect

of mycophenolate mofetil on the secretion of donor-specific alloantibodies may be a major contributing effect for the mycophenolate mofetil induced reduction of intimal proliferation in our experimental model.

In conclusion, mycophenolate mofetil may effectively suppress transplant arteriosclerosis in a murine aortic allograft model. Concomitant application of ganciclovir raised the immunosuppressive capacity of mycophenolate mofetil, most likely by increasing mycophenolic acid plasma levels. After treatment with mycophenolate mofetil, a marked reduction of donor-specific alloantibodies was detected in our model correlating with reduced intimal proliferation, but the amount of Tregs remained unchanged within the different experimental groups.

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