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# CXCR4 Antagonist Reduced the Incidence of Acute Rejection and Controlled Cardiac Allograft Vasculopathy in a Swine Heart Transplant Model Receiving a Mycophenolate-based Immunosuppressive Regimen

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**Background.** CXC motif chemokine receptor 4 (CXCR4) blockade is pursued as an alternative to mesenchymal stem cell treatment in transplantation based on our previous report that burixafor, through CXCR4 antagonism, mobilizes immunomodulatory mesenchymal stem cells. Here, we explored the efficacy of combining mycophenolate mofetil (MMF)-based immunosuppressants with repetitive burixafor administration. **Methods.** Swine heterotopic cardiac allograft recipients received MMF and corticosteroids (control,  $n = 10$ ) combined with burixafor as a 2-dose (burixafor<sub>2D</sub>,  $n = 7$ ) or 2-dose plus booster injections (burixafor<sub>2D + B</sub>,  $n = 5$ ) regimen. The efficacy endpoints were graft survival, freedom from first acute rejection, and the severity of intimal hyperplasia. Each specimen was sacrificed either at its first graft arrest or after 150 days. **Results.** After 150 days, all specimens in the control group had died, but 28.5% of the burixafor<sub>2D</sub> group survived, and 60% of the burixafor<sub>2D + B</sub> group survived ( $P = 0.0088$ ). Although the control group demonstrated acute rejection at a median of 33.5 days, the burixafor<sub>2D + B</sub> group survived without acute rejection for a median of 136 days ( $P = 0.0209$ ). Burixafor administration significantly attenuated the incidence rate of acute rejection ( $P = 0.002$ ) and the severity of intimal hyperplasia ( $P = 0.0097$ ) at end point relative to the controls. These findings were associated with reduced cell infiltrates in the allografts, and modulation of C-reactive protein profiles in the circulation. **Conclusions.** The augmentation of conventional MMF plus corticosteroids with a CXCR4 antagonist is potentially effective in improving outcomes after heart transplantation in minipigs. Future studies are warranted into optimizing the therapeutic regimens for humans.

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With advances in immunosuppressive drugs (ISDs), acute myocardial rejection after heart transplantation has decreased. However, cardiac allograft vasculopathy

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(CAV) remains the leading cause of allograft failure 1 year after transplantation.<sup>1</sup> Cardiac allograft vasculopathy manifests as accelerated, diffuse coronary arteriosclerosis that has a different pathogenesis than conventional native coronary artery disease (CAD).<sup>2,3</sup> Cardiac allograft vasculopathy is characterized by progressive, concentric intimal thickening composed of proliferative smooth muscle cells and the extracellular matrix.<sup>2</sup> Cardiac allograft vasculopathy progression eventually leads to severe myocardial ischemia and graft failure.

In recent years, novel ISDs, other than calcineurin inhibitors (CNIs), have been developed for reducing the adverse effects of nephrotoxicity and hypertension. The mammalian target of rapamycin inhibitor has recently been demonstrated to reduce the frequency and severity of CAV in humans,<sup>4</sup> but this inhibitor is associated with hyperlipidemia,<sup>5</sup> a major risk factor for CAD and CAV. Mycophenolate mofetil (MMF), an inhibitor of inosine monophosphate dehydrogenase, suppresses purine synthesis and thus reduces the proliferation of T and B lymphocytes.<sup>5-7</sup> Moreover, MMF inhibits CAV progression<sup>8</sup> and does not impair renal function.<sup>9</sup> However, a MMF-based immunosuppressive regimen without CNIs is less efficacious in preventing acute rejection.<sup>10</sup> An optimized immunosuppressive regimen that protects cardiac allografts against vasculopathy without compromising the prevention of acute rejection is still warranted.

The application of mesenchymal stem cells (MSCs) has emerged as an immunomodulatory tool in solid organ transplantation.<sup>11,12</sup> Mesenchymal stem cells act synergistically with MMF in suppressing allogenic lymphocyte proliferation<sup>13</sup> and prolonging allograft survival.<sup>14-16</sup> Therefore, we hypothesized that MSCs or an MSC-mobilizing strategy in combination with MMF would not only reduce the requirement for CNI but also potentially prevent acute transplant rejection and CAV.

In our previous study, we demonstrated that a CXC motif chemokine receptor 4 (CXCR4) antagonist, burixafor, mobilized immunomodulatory MSCs,<sup>17</sup> and alleviated the impairment of cardiac function after myocardial infarction. The effect of burixafor are mediated in part by attenuating myocardial and systemic inflammation following ischemic injury.<sup>17</sup> In the present study, we evaluated the therapeutic effects of the concomitant administration of burixafor with a MMF-based immunosuppressive regimen in a porcine model of heterotopic heart transplantation.

## MATERIALS AND METHODS

See **Supplemental Materials and Methods** (SDC, <http://links.lww.com/TP/B621>) for detailed methods.

### Animals

Adult Taiwanese Lanyu miniature pigs (minipigs; aged 4-6 months and weighing 20-25 kg) were procured from the Animal Propagation Station of the Livestock Research Institute (Taitung, Taiwan) and maintained in the Laboratory Animal Center of National Taiwan University. Twenty-four minipigs (female = 18, male = 6) were used in accordance with the Animal Research: Reporting of In Vivo Experiment guidelines. The experimental protocol was approved by the Institutional Animal Care and Use Committee of National Taiwan University (approval numbers 20120420 and 20150260).

## Swine Model of Heterotopic Heart Transplantation

The selection of donor-recipient pairs was based upon major histocompatibility complex incompatibility by mixed lymphocyte reaction (MLR). The stimulation index (SI) was calculated through the following formula:  $(\text{mean}_{\text{cpm}} \text{ of allogeneic MLR}) / (\text{mean}_{\text{cpm}} \text{ of autologous MLR})$ . The donor heart was heterotopically transplanted into the recipient swine abdomen by infrarenal allografting (**Figure S1**, SDC, <http://links.lww.com/TP/B621>).

### Administration of Burixafor, a CXCR4 Antagonist

Burixafor is a selective CXCR4 small-molecule antagonist and was provided for the experiment by TaiGen Biotechnology Co., Ltd (Taipei, Taiwan). A burixafor dose of 2.85 mg/kg per pig was converted from the Food and Drug Administration's guidelines based on the dose of 3.14 mg/kg used in clinical studies. Healthy, age-matched, and MLR-screened minipigs were selected to receive burixafor intravenously as a 2-dose regimen (burixafor<sub>2D</sub>) or a 2-dose plus booster injection schedule (burixafor<sub>2D + B</sub>) (**Figures 1A and 6A**). Minipigs treated with ISDs alone served as controls (**Figures 1A and 6A**).

### CNI-free Immunosuppressant Regimen

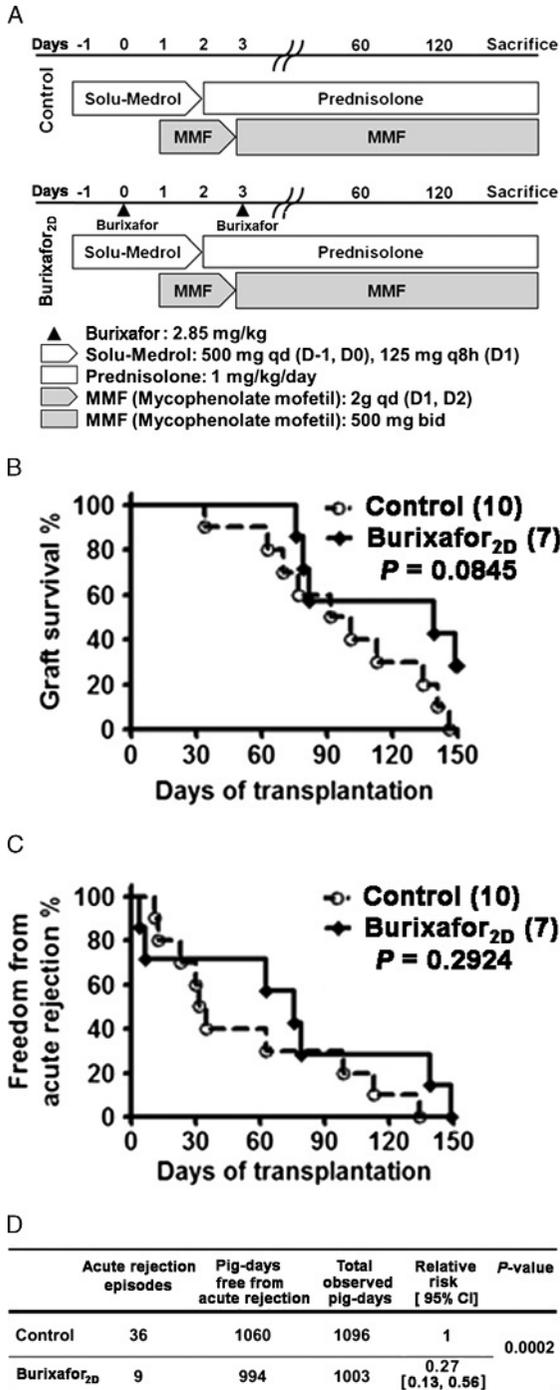
MMF (CellCept, Roche, Germany) was orally administered for 2 days at a loading dose of 2 g/d and 500 mg BID thereafter,<sup>18</sup> which was administered orally per day at 9:00 am and 5:00 pm. In addition, methylprednisolone (Solu-Medrol, Pfizer, Belgium) was given through an i.v. bolus at a dose of 500 mg 1 day before and on the day of transplantation, followed by a dose of 125 mg every 8 h on the first postoperative day (POD). Thereafter, prednisolone (1 mg/kg per day; Predonine, Sinphar, Taiwan) was administered orally until the end of the experiment (**Figures 1A, 6A**).

### Measurement of Mycophenolic Acid Through Liquid Chromatography and Tandem Mass Spectrometry Analysis

Plasma levels of mycophenolic acid (MPA) (**Figure 2A**), the active metabolite of MMF, was determined through a combination of high-performance liquid chromatography (HPLC) with tandem mass spectrometry using validated methods. To verify the absence of ion suppression or ion enhancement effects attributable to the matrix, blank pig plasma was extracted using ultrafiltration, followed by reconstitution through a mobile phase containing MPA at 3 concentrations. The results of matrix effect were calculated as  $100 \times (A_{\text{st}} - A_{\text{extr}}) / A_{\text{st}}$ , where  $A_{\text{extr}}$  is the peak area of MPA from the postextraction spiked sample (**Figure 2B**) and  $A_{\text{st}}$  is the peak area of MPA from the direct injection of the standard solution (**Figure 2C, left**). An interval of 85% to 115% was considered acceptable.

### Definition of Acute Rejection and Relative Risk of Acute Rejection

Based on autopsy findings of the minipigs at our preliminary study, each new onset of allograft bradycardia (<60 beats per minute) was defined as an episode of acute rejection.<sup>19-21</sup> The ratio of the risk in the burixafor-treated group to the risk in the control group was calculated to assess the relative risk of acute rejection using the equation  $[(E_{\text{burixafor}} / D_{\text{burixafor}}) / (E_{\text{control}} / D_{\text{control}})]$ , where E represents acute rejection episodes and D represents the total observed pig-days.



**FIGURE 1.** Two-dose regimen of burixafor (Burixafor<sub>2D</sub>) reduced the incidence of acute rejection and increased prolonged graft survival. A, Immunosuppressive regimen, survival estimates for (B) graft survival and (C) freedom from first acute rejection, and (D) incidence rate of acute rejection in the control and Burixafor<sub>2D</sub> groups. Times of immunosuppressive drug administration in relation to the day of transplantation (on day 0) are provided in panel A. The sacrifice was performed on day 150 or at the time of graft arrest. All graft recipients in each group received methylprednisolone intravenously (Solu-Medrol; Pfizer; 500 mg/d) on days -1, 0, and 1. Oral prednisolone (Sinphar, Taiwan; 1 mg/kg per day) was administered daily, from day 2 until the end of the study. MMF (CellCept, Roche, Germany) was orally administered for 2 days after transplantation at a loading dose of 2 g/d and continued with a minimum dose of 1 g/d (500 mg BID) during the subsequent days. Graft recipients in the burixafor<sub>2D</sub> group received burixafor intravenously (2.85 mg/kg) on days 0 and 3.

**Definition of Inaccessible Small Vessels**

Intravascular ultrasound (IVUS) is commonly used in the detection of CAV.<sup>22</sup> The Opticross catheter (Boston Scientific) is the IVUS catheter with the lowest entry profile (0.67 mm) currently available. Therefore, we defined graft vessels with a diameter of less than 0.67 mm as small vessels that were inaccessible through IVUS. The corresponding internal elastic laminae (IELAs) of the inaccessible small vessels were calculated through the following equation:  $\pi \times [(0.67 \text{ mm}/2)]^2 = 0.35 \text{ mm}^2$ .

**RESULTS**

**Baseline Characteristics of Porcine Heart Transplant Recipients**

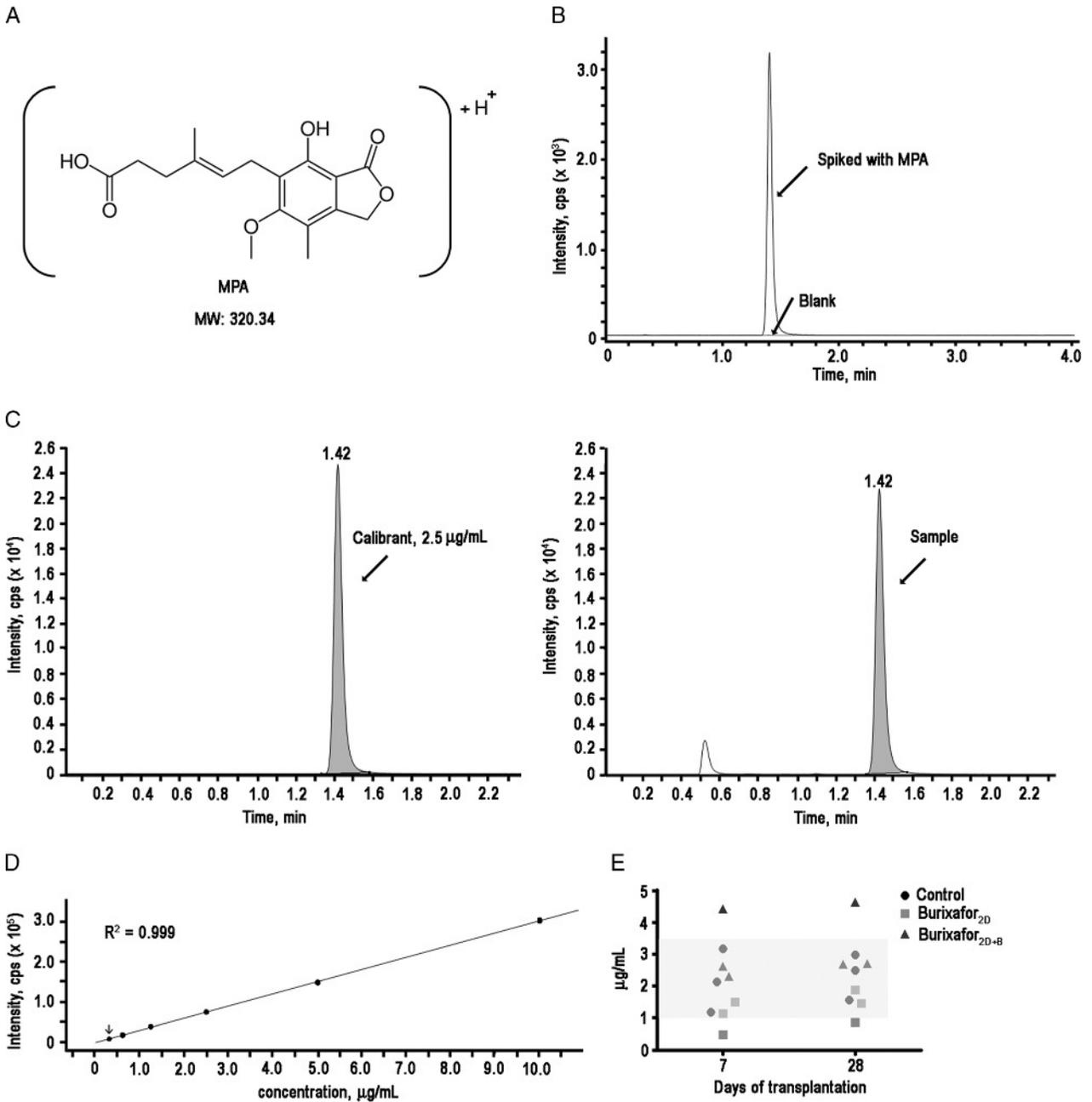
We enrolled 10 minipigs in the control group that received conventional ISDs (ie, corticosteroids plus MMF). Based on the observation that adventitial inflammation in graft coronary arteries occurred as early as 10 days after transplantation in the controls (Figure S2, SDC, <http://links.lww.com/TP/B621>), we enrolled 8 minipigs in the burixafor treatment group that received 2 doses of burixafor on the day of transplantation (POD 0) and POD 3 (designated burixafor<sub>2D</sub>) (Figure 1A). We subsequently included a third group of 6 minipigs that received 2 additional booster doses of burixafor on POD 60 and POD 120 after transplantation (designated burixafor<sub>2D + B</sub>) (Figure 6A). One burixafor<sub>2D</sub> recipient died of infection on POD 67 and 1 burixafor<sub>2D + B</sub> recipient died of acute pancreatitis on POD 56. In these 2 instances of early mortality, the monitoring of graft survival was unexpectedly terminated due to the recipients' disease statuses. Therefore, they were excluded from the graft survival analysis. The incidence rate of acute rejection or the severity of CAV might be underestimated in these 2 minipigs, and they were thus excluded from the assessment of graft outcomes. The antidonor activity levels of the remaining 22 minipigs in the 3 experimental groups were comparable (Table 1). The perioperative ischemia duration and the immediate postoperative beating rate of the allografts were not significantly different among the 3 groups of recipients (Table 1).

**Steady-state Plasma MPA Concentration in Porcine Heart Transplant Recipients**

Mycophenolate mofetil was administered at a fixed dose rather than a concentration-controlled dose, because no consensus on drug monitoring exists. However, we performed the retrospective analyses of plasma MPA concentration at 7 and 28 days after heart transplantation, when a steady state was achieved, to investigate the adequacy of the applied MMF dosing in the CNI-free regimen. Predose plasma concentrations (C<sub>0</sub>) should be maintained between 1.0 and 3.5 μg/mL based on HPLC data.<sup>23</sup> With the exception of 1 low trough level (0.47 μg/mL) observed in the burixafor<sub>2D</sub> group, we accomplished this goal (Figure 2E, gray area). MMF-treated pigs did not exhibit any noticeable anorexia, vomiting, or diarrhea.

**Burixafor<sub>2D</sub> Regimen Reduced the Incidence of Acute Rejection With a Trend Toward Prolonged Graft Survival**

The estimated graft survival rate was first compared between the control and the burixafor<sub>2D</sub> groups. The control



**FIGURE 2.** Plasma MPA concentration in porcine heart transplant recipients. A, Structure of the protonated adduct ion of MPA ( $m/z = 321.1$ ). B, Chromatograms of blank pig plasma extracted through ultrafiltration (blue line) compared with pig plasma spiked with MPA (black line). C, Representative chromatograms of a calibrant with 2.5  $\mu\text{g/mL}$  of MPA and a pig sample with comparable levels of MPA. D, Calibration curve and its linearity expressed as the correlation coefficient ( $R^2$ ). E, Plasma MPA concentration in different experimental groups at 7 and 28 days after heart transplantation. In panel E, the gray area indicates therapeutic range.

group exhibited a constant rate of graft loss from POD 30 to POD 150; only 20% survived at POD 130 and no hearts remained beating at POD 150. By contrast, 57% of the cardiac allografts in the burixafor<sub>2D</sub> group survived on POD 130, and 30% continued beating at POD 150 (Figure 1B). The median survival time for the control group was 96.5 days, compared with 139 days for the burixafor<sub>2D</sub> group. The graft survival time of the burixafor<sub>2D</sub> group was prolonged but did not reach statistical significance compared with the control group (log-rank test,  $P = 0.0845$ ). We then analyzed the occurrence of the first episode of acute rejection between these 2 groups. We observed no significant difference in the

acute rejection-free graft survival rate (Figure 1C). However, the burixafor<sub>2D</sub> group exhibited a lower risk of acute rejection than the control group (incidence rate = 8.9 per 1000 pig-days vs. 32.8 per 1000 pig-days; relative risk = 0.27,  $P < 0.002$ ) (Figure 1D).

**Burixafor<sub>2D</sub> Regimen Attenuated the Severity of CAV**

Because CAV is a limiting factor for the long-term survival of a cardiac allograft, we evaluated the efficacy of burixafor on CAV through comparing the burixafor<sub>2D</sub> and control groups. We demonstrated that CAV mimicking that in human cardiac allografts commonly occurred in minipigs

**TABLE 1.****Baseline recipient characteristics among experimental groups**

Variables	Control	Burixafor <sub>2D</sub>	Burixafor <sub>2D + B</sub>	P
Antidonor activity <sup>a</sup>	1.29 ± 0.14	1.27 ± 0.11	1.47 ± 0.21	0.0771
Ischemia time, min	64.7 ± 10.5	66.7 ± 4.7	58.7 ± 2.3	0.1894
POD 1 heart rate, bpm	97.7 ± 10.3	90.6 ± 6.8	96.9 ± 4.4	0.9599

<sup>a</sup> Antidonor activity is expressed as a stimulation index measured through a 1-way MLR. Burixafor<sub>2D</sub>, 2-dose regimen of burixafor (n = 7); Burixafor<sub>2D + B</sub>, 2-dose plus booster injections of burixafor (n = 5); control, conventional ISD (n = 10).

receiving conventional ISDs (control group) after heart transplantation (Figure S3, SDC, <http://links.lww.com/TP/B621>). Furthermore, multiple small infarcts were noted in the allograft, especially at the apical to midventricular levels of the left ventricle, which is consistent with diffuse arteriosclerosis of small vessels (black arrows, Figure S3A, SDC, <http://links.lww.com/TP/B621>). We analyzed intimal hyperplasia and luminal stenosis in small (IELA <0.35 mm<sup>2</sup>) and large vessels (IELA >0.35 mm<sup>2</sup>). In the allografts of the control group, small vessels were more susceptible to CAV compared with large vessels (Figure 3), similar to the findings of human cardiac allograft studies. Notably, the observed intimal hyperplasia of small vessels was significantly reduced after burixafor<sub>2D</sub> treatment (P = 0.0097, Figure 3). However, we observed no significant difference in the extent of intimal hyperplasia of large vessels between the burixafor<sub>2D</sub> and control groups (Figure 3).

Of the 10 control recipients, 7 (70%) had at least 1 episode of acute rejection, whereas 3 of the 7 (43%) minipigs in the burixafor<sub>2D</sub> group underwent acute rejection (Figure 4C). This finding is consistent with the lower incidence rate of acute rejection in the burixafor<sub>2D</sub> group (Figure 1D). We further evaluated the effect of burixafor treatment on CAV in recipients that developed 1 or more rejection episodes. Notably, of the 10 recipients that experienced at least 1 episode of acute rejection, those in the burixafor<sub>2D</sub> group had a significantly lower degree of intimal hyperplasia than did those in the control group (Figure 4). For the 7 recipients that were free from acute rejection, the benefit of burixafor treatment on intimal hyperplasia was not significant (Figure 4).

### Burixafor<sub>2D</sub> Regimen Reduced Inflammatory Cell Infiltration in Graft Vessels

The status of inflammatory cell infiltration was examined in the myocardium and coronary arteries of minipigs that received cardiac allografts. For the control group, dense mononuclear cell infiltrations were observed in the myocardium (Figure 5A) as well as in the different layers of the CAV artery (Figure 5B, Figure S3B, SDC, <http://links.lww.com/TP/B621>). Most of the infiltrating mononuclear cells in the controls were CD3<sup>+</sup> lymphocytes that were present in the adventitia and neointima, with an abundant present outside the vessel wall (Figure 5B). By contrast, the accumulation of inflammatory cells was less prominent in the burixafor<sub>2D</sub> group (Figures 5A, B). To elucidate the potential immunomodulatory effects of burixafor on CAV, we examined the recruitment and activation of inflammatory cells and local priming of the complement cascade. For the controls, accumulation of mononuclear cells

in the vascular intima is associated with C4d deposition. By contrast, CD3<sup>+</sup> lymphocytes were attenuated and C4d deposits were almost undetectable in the intimal and perivascular regions of cardiac allografts in the burixafor-treated group (Figure 5B). To test the hypothesis that the intimal changes associated with CAV are mediated by cellular activation and cytokine production, we next investigate the role for burixafor contributing to regulation of cytokine plasma levels. Although circulating TNF-α and IL-6 were at very low levels (data not shown), systemic C-reactive protein (CRP) levels, which can reflect the general inflammatory state associated with CAV development, were decreased by a burixafor<sub>2D</sub> regimen (Figure 5C). Furthermore, a quantitative analysis demonstrated that burixafor treatment resulted in a significant reduction in perivascular cell infiltration (P = 0.0411, Figure 5D).

### Addition of Booster Doses of Burixafor on POD 60 and POD 120 (burixafor<sub>2D + B</sub>) Delayed Acute Rejection and Prolonged Graft Survival

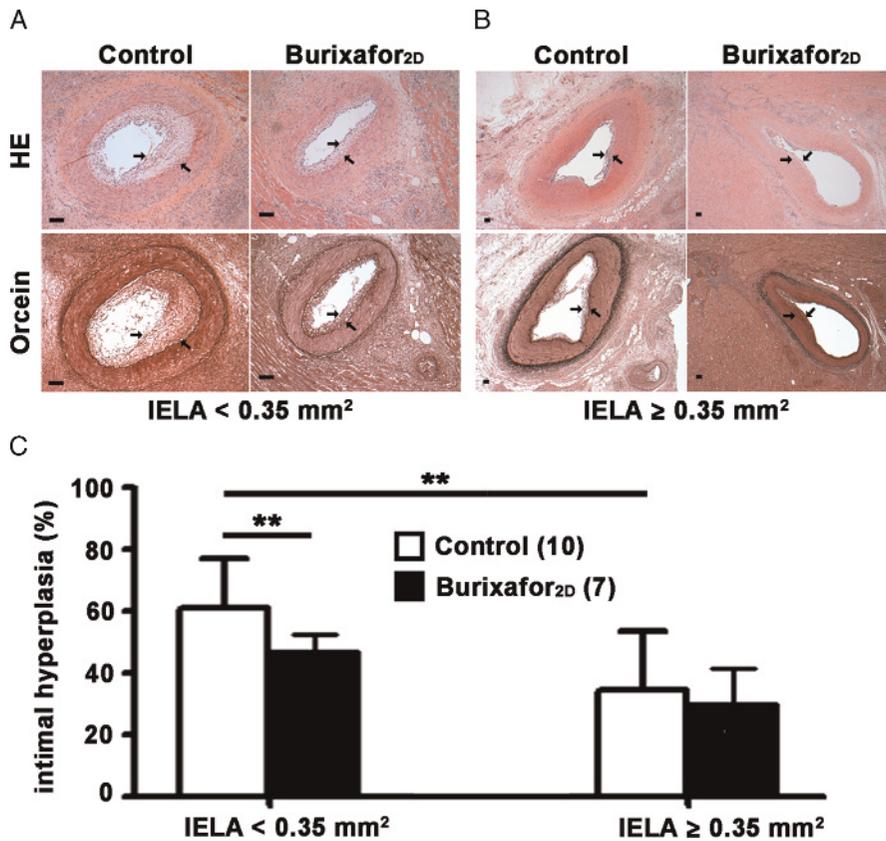
As mentioned, the acute rejection-free rate of allografts in the burixafor<sub>2D</sub> group dropped precipitously on POD 79 (Figure 1C), suggesting that the efficacy of burixafor administered on POD 0 and POD 3 was effective in controlling early rejection, but their efficacy was relative short and could not be observed beyond POD 60. Therefore, we evaluated the effect of further administration of burixafor at a later stage (ie, POD 60 and POD 120; burixafor<sub>2D + B</sub>; Figure 6A). The addition of these 2 booster doses improved the outcome significantly. All allografts survived until POD 130, with a mean graft survival rate of 60% on POD 150 (Figure 6B). Acute rejection-free graft survival was also significantly improved (P = 0.0209; Figure 6C). The median time to the development of the first episode of acute rejection was 136 days after transplantation in the burixafor<sub>2D + B</sub> group, and it was 33.5 days in the control group. These results confirmed our hypothesis that the booster doses at late period following transplantation could reinforce the efficacy of burixafor on controlling allograft rejection. In addition, we noted minor intimal hyperplasia with less dense cell infiltrates in allografts from the burixafor<sub>2D + B</sub> group compared with the control group, indicating attenuated CAV at a comparable time point after transplantation (Figure S4, SDC, <http://links.lww.com/TP/B621>).

### Multiple Doses of Burixafor Did Not Result in Renal or Hepatic Toxicity

Minipigs that received 2 doses and 4 doses of burixafor did not experience overt adverse side effects. Renal and liver function tests including measurements of blood urea nitrogen, creatinine, aspartate transaminase, and alanine transaminase did not reveal a significant difference between the burixafor<sub>2D + B</sub> and control groups (Figure S5, SDC, <http://links.lww.com/TP/B621>). These findings suggest that the burixafor<sub>2D + B</sub> regimen administered is safe in a swine model.

## DISCUSSION

The treatment options of CAV in humans remain limited. Studies that have demonstrated incremental benefits in terms of preventing or slowing the progression of CAV have been conducted on rodent models.<sup>24-27</sup> This pilot study evaluated the efficacy of a CXCR4 antagonist combined with CNI-free



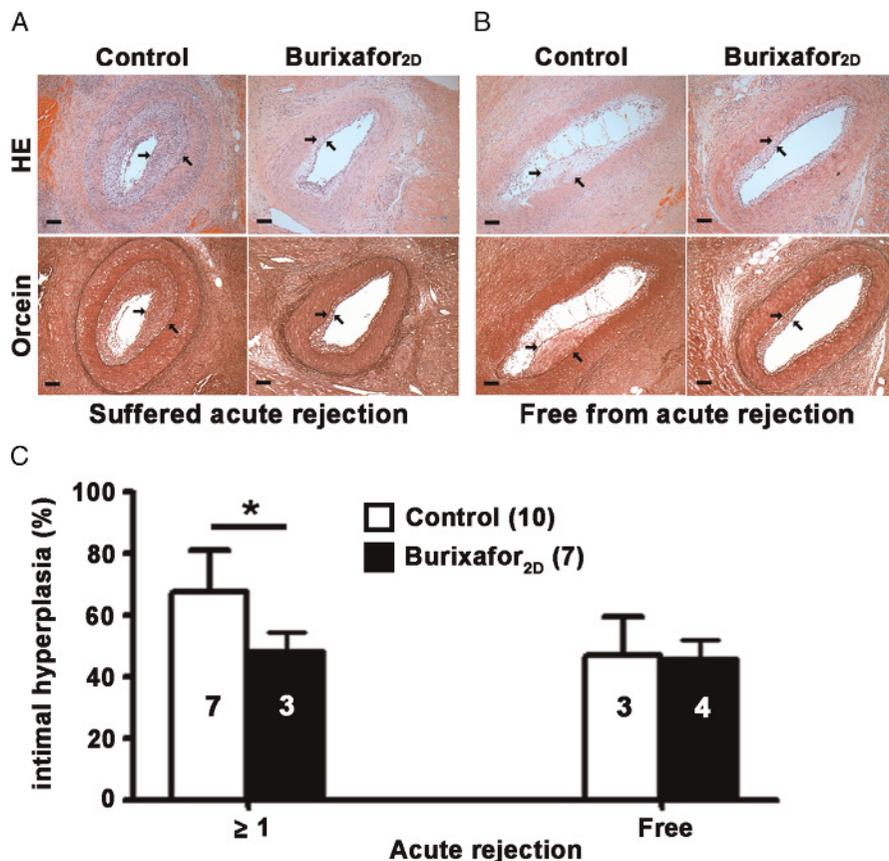
**FIGURE 3.** Two-dose regimen of burixafor (Burixafor<sub>2D</sub>) attenuated the severity of intimal hyperplasia. Representative histological images for small (IELA < 0.35 mm<sup>2</sup>, 100×) (A) and large vessels (IELA ≥ 0.35 mm<sup>2</sup>, 40×) (B) in the control and Burixafor<sub>2D</sub> groups. Scale bars indicate 100 μm. The area between the black arrows indicates an area developing intimal thickening. C, Quantitative analysis of the percentage of luminal stenosis. The numbers in parentheses and bar graphs indicate the sample size. \*\**P* < 0.01. HE, hematoxylin and eosin.

ISDs for controlling both acute rejection and CAV in a swine model of allogeneic heterotopic cardiac transplantation. A favorable survival trend was observed in the burixafor<sub>2D</sub> group, and a significantly prolonged graft survival was achieved through intensified treatment in the burixafor<sub>2D</sub> + B group. In addition, a lower incidence of acute rejection yielded benefits in the attenuation of CAV through burixafor treatment. Therefore, immunomodulation using a CXCR4 antagonist combined with a MMF-based regimen may be a safe and effective alternative to CNI therapy for clinical application in patients undergoing heart transplantation.

Inflammation and acute myocardial rejection are associated with the development of CAV.<sup>28-30</sup> Allorecognition promotes the infiltration of macrophages, T lymphocytes, alloreactive antibodies, and proinflammatory cytokines that contribute to endothelial dysfunction and smooth muscle proliferation. Similar to previous reports,<sup>31-33</sup> our data demonstrate that inflammatory cells migrated from the adventitia, disrupted the external lamina and IELA, and infiltrated the intima (Figure S3B, SDC, <http://links.lww.com/TP/B621>). The characteristic involvement of small vessels in CAV (Figure 3) may be partially attributed to the thin external elastic laminae in small arteries that form a weaker barrier to infiltrating cells than that formed in large vessels (Figure S3, SDC, <http://links.lww.com/TP/B621>). Burixafor treatment attenuated inflammatory infiltration (Figure 5) and reduced intimal hyperplasia in small vessels (Figure 3), especially in grafts that experienced acute rejection (Figure 4). Interestingly, C4d deposits was evident in the region of

mononuclear cell accumulations (Figure 5). Deposition of C4d has been recognized to be associated with granzyme B-positive T cells in transplant glomerulitis,<sup>34</sup> adding new dimensions to the regulatory effects of complement on cytotoxic T cell-derived damage. Moreover, allograft recipients who received burixafor treatment had lower plasma levels of CRP relative to the levels of those in the control group (Figure 5C). This modulatory effect of 2 doses of burixafor on CRP levels was temporally correlated with favorable survival (Figure 1B). Because CRP has been reported to be associated with endothelial intercellular adhesion molecule-1 expression and arteriosclerosis development in cardiac allografts,<sup>35,36</sup> the correlations among burixafor treatment, CRP levels, and graft survival offer some mechanistic clues for the efficacy of CXCR4 antagonist. These results suggest that burixafor is effective in reducing acute rejection-associated vasculopathy and prevents CAV by attenuating alloantigen-induced inflammatory reactions.

We previously reported that burixafor exerted therapeutic effects by mobilizing immunomodulatory MSCs to alleviate postinfarction myocardial and systemic inflammation.<sup>17</sup> We also provided evidence that the administration of MSCs could prevent transplant arteriosclerosis by inducing regulatory T type 1 (T<sub>R</sub>1)-like cells,<sup>37</sup> which can suppress allogeneic immune responses and act synergistically with prostaglandin E2 (PGE2).<sup>38</sup> In the current study, we demonstrated the therapeutic effects of burixafor on acute rejection, vasculopathy, and long-term survival of cardiac allografts. It is reasonable to attribute the therapeutic effects of burixafor, at least in part, to the mobilization of MSCs and the



**FIGURE 4.** Two-dose regimen of burixafor (Burixafor<sub>2D</sub>) attenuated the severity of intimal hyperplasia in recipients that experienced acute rejection. Representative histological images of recipients that experienced at least 1 episode of acute rejection ( $\geq 1$ , 100 $\times$ ) (A) or in those that were free of acute rejection throughout the follow-up period (free, 100 $\times$ ) (B). Scale bars indicate 100  $\mu$ m. The area between the black arrows indicates an area developing intimal thickening. C, Quantitative analysis of the percentage of luminal stenosis in small vessels. Numbers in parentheses and bar graphs indicate the sample size. \* $P < 0.05$ .

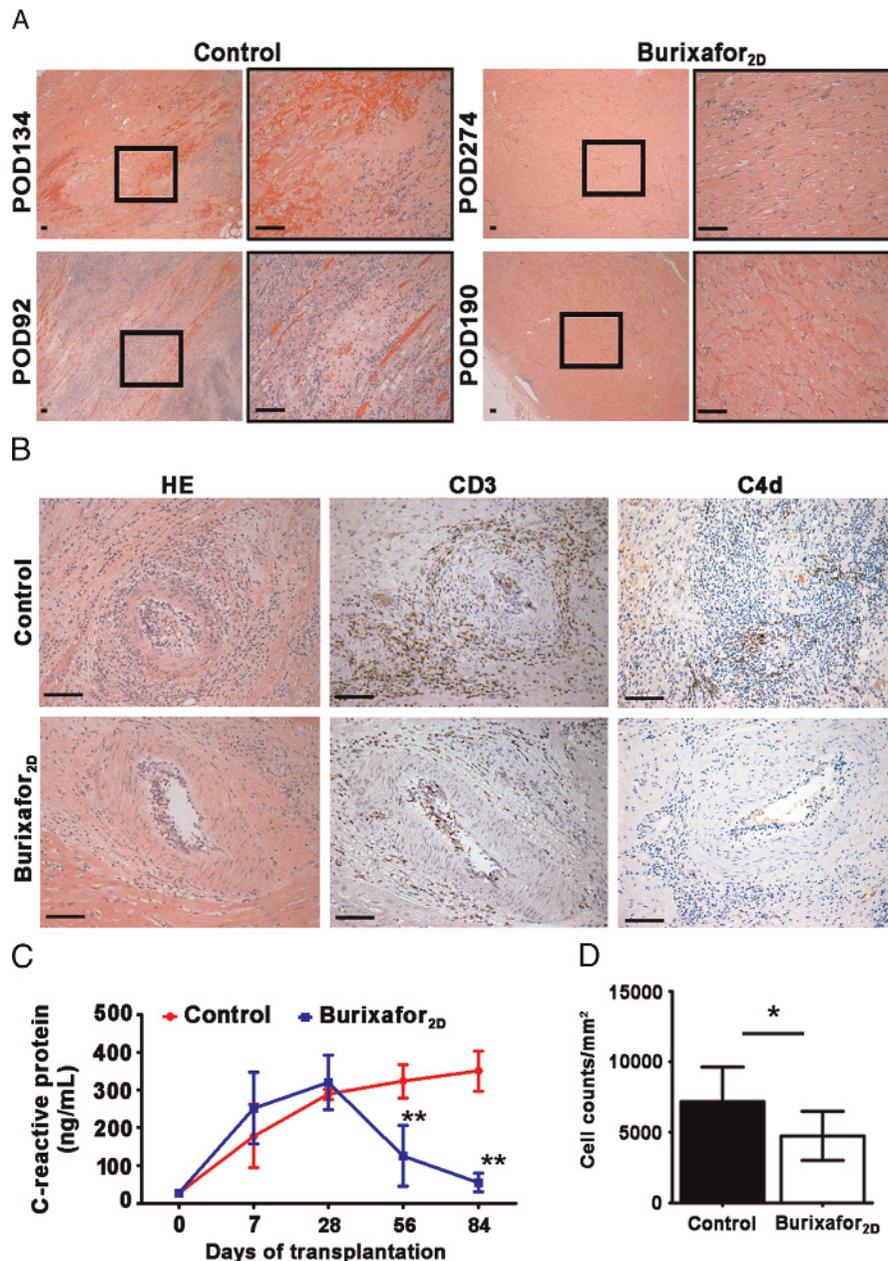
induction of immune tolerance to alloantigens (Figure S6, SDC, <http://links.lww.com/TP/B621>).

Burixafor delivered as a 2-dose regimen plus booster injections (burixafor<sub>2D</sub> + B) was more effective than that delivered as a 2-dose regimen (burixafor<sub>2D</sub>) in prolonging overall and acute rejection-free survival. These findings suggest that the early administration of 2 doses of burixafor after transplantation reduces acute rejection and improves graft survival but is ineffective in preventing late-onset graft rejection. MSC-induced mediators possibly act at different temporal sequences and operate in concert for immunomodulation. This assumption is supported by MLR findings that MSCs produce PGE<sub>2</sub> at the early stage and then induce T<sub>R</sub>1-like cells at a later stage.<sup>38</sup> Both PGE<sub>2</sub> and downstream interleukin-10 and interferon- $\gamma$  secreted by T<sub>R</sub>1-like cells are involved in the entire process of MSC-mediated immunomodulation. Therefore, following the mobilization of MSCs through the initial 2-dose administration of burixafor, booster injections may consolidate the immunomodulation at the chronic stage of allogeneic transplantation. For clinical application, the timing and dose frequency of burixafor administration should be further optimized.

Other mechanisms apart from MSC mobilization may be responsible for the therapeutic effects of burixafor. Several lines of evidence have suggested that stromal cell-derived factor 1 (SDF-1) and CXCR4 interaction can provoke neointima formation<sup>39,40</sup> and that SDF-1 neutralization or inhibition can

prevent transplant arteriosclerosis.<sup>41,42</sup> CXCR4 is involved in the basal trafficking of naïve lymphocytes.<sup>43</sup> The SDF-1 expression was reported to increase in cardiac allografts that had peritransplant ischemic injury, which is associated with poor graft survival and more severe CAV.<sup>44</sup> Similarly, in a murine model, SDF-1 was upregulated in the adventitia and media of aortic grafts with chronic rejection.<sup>41</sup> Therefore, burixafor may directly abolish SDF-1-attracted CXCR4<sup>+</sup> inflammatory cells and inhibit cell-mediated responses in allografts. CXCR4 is also expressed in smooth muscle progenitor cells (SPCs)<sup>45</sup> and vascular smooth muscle cells.<sup>46</sup> Circulating SDF-1 concentrations were demonstrated to increase with CAV severity and to be correlated with peripheral SPC counts.<sup>45</sup> In line with this finding, in the study of Sakihama et al, SDF-1 mediated the mobilization and local recruitment of SPCs in a mouse model of CAV, thereby contributing to neointima formation.<sup>41</sup> SDF-1 blockade resulted in a significant reduction in the proliferation of murine vascular smooth muscle cells in vitro and a decrease in the gene expression of profibrotic cytokines in cardiac allografts.<sup>42</sup> Taken together, the direct inhibition of the interaction between SDF-1 and CXCR4 to consequently prevent inflammatory infiltration or intimal proliferation in allografts might be another mechanism explaining the effects of burixafor in this study.

In our experiment, MMF dose selection was based on the explanation by a previous study that 500 mg twice daily is an appropriate maintenance dose for conventional pigs.<sup>18</sup> Under

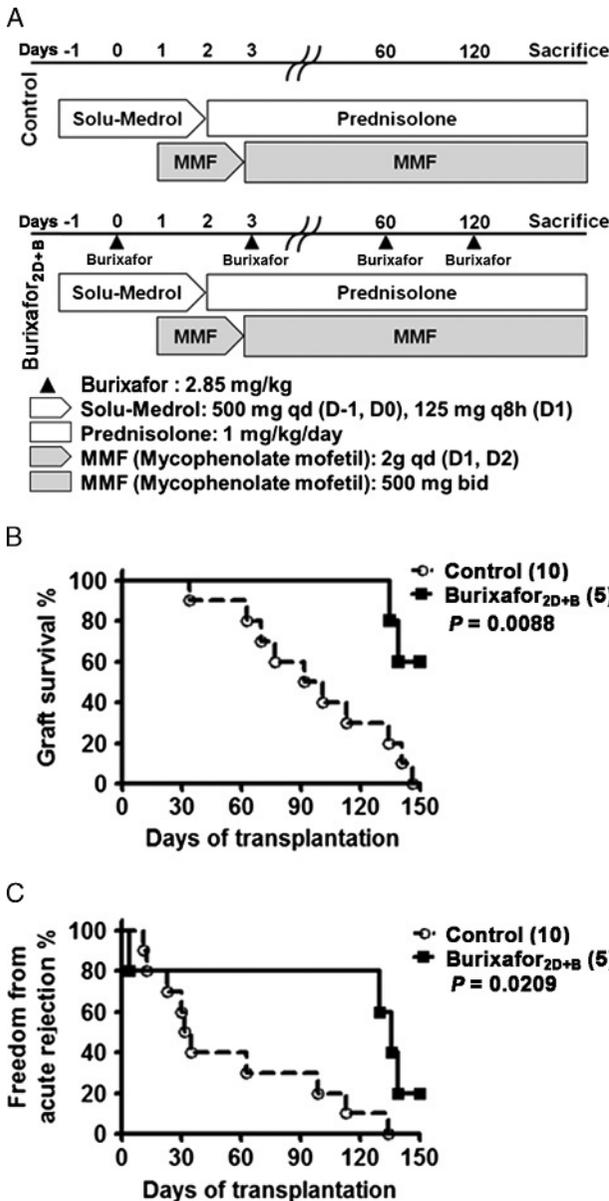


**FIGURE 5.** Two-dose regimen of burixafor (Burixafor<sub>2D</sub>) alleviated inflammatory cell infiltration in graft vessels. A, Histological images (40 $\times$ , left and 200 $\times$ , right) of myocardial sections from representative allografts harvested at different time points after heart transplantation in the control and Burixafor<sub>2D</sub> groups. B, Representative histological images (200 $\times$ ) of infiltrating cells in the control and Burixafor<sub>2D</sub> groups. HE staining of the coronary arteries and surrounding accumulations of mononuclear cells (left panel). Distribution of brown-stained CD3<sup>+</sup> T cells (middle panel) and C4d deposition (right panel) in immunohistochemical staining. C, Concentrations of CRP at baseline and specified time points ( $n \geq 3$ ) after cardiac transplantation. D, Quantitative analysis of the density of mononuclear cells in HE-stained sections. The numbers of infiltrating cells in the perivascular areas were counted in 3 fields per slide ( $n \geq 6$ ) in each group and averaged for statistical analysis. Scale bar = 100  $\mu$ m. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

this dosing regimen, plasma MPA levels were confirmed to achieve bioequivalent levels in pigs (Figure 2) compared with those in humans. However, concomitant ISDs influence the pharmacokinetics of MMF. Commencing MMF treatment in combination with cyclosporine at a dose of 600 mg/m<sup>2</sup> BID, in combination with tacrolimus at a dose of 300 mg/m<sup>2</sup> BID, and without a CNI at a dose of 500 mg/m<sup>2</sup> BID has been proposed for clinical application.<sup>47</sup> In our study, the pigs' body surface area ranged from 1.1-1.4 m<sup>2</sup>, as calculated according to body weight using a specific formula established by Deroth et al.<sup>48</sup> Therefore, the corresponding MMF dose for pigs was

550-700 mg BID in the CNI-free regimen, which is comparable to the dose used in our study. However, therapeutic drug monitoring of MMF is not generally accepted for the treatment of patients after a heart transplant because there is no consensus on whether it improves clinical outcomes. Therefore, whether titrating MMF doses based on plasma MPA levels can optimize the efficacy of a CXCR4 antagonist in combination with MMF-based ISDs warrants further investigation.

One major limitation of this study is the lack of histological documentation of cases of acute rejection. Endomyocardial biopsy, although specific, explores only a small portion of the



**FIGURE 6.** Two-dose plus booster regimen of burixafor (Burixafor<sub>2D+B</sub>) prolonged graft survival and delayed the onset of acute rejection. A, Immunosuppressive regimen, survival estimates for (B) graft survival and (C) freedom from first acute rejection in the control and Burixafor<sub>2D+B</sub> groups. Times of the burixafor regimen with 2 consecutive boosts are provided in (A). The sacrifice was performed on day 150 or at the time of graft arrest. All graft recipients in each group received methylprednisolone intravenously (Solu-Medrol; Pfizer, Belgium; 500 mg/d) on days -1, 0, and 1. Oral prednisolone (Sinphar, Taiwan; 1 mg/kg per day) was administered daily, from day 2 until the end of the study. MMF (CellCept, Roche, Germany) was orally administered for 2 days after transplantation at a loading dose of 2 g/d and continued with a minimum dose of 1 g/d (500 mg BID) during the subsequent days. Graft recipients in the burixafor<sub>2D+B</sub> group received burixafor intravenously (2.85 mg/kg) on days 0 and 3, 60, and 120.

myocardium and may thus exhibit a low sensitivity level. Serial echocardiography or cardiac magnetic resonance imaging may be useful for assessing cardiac function and the early detection of acute rejection. Another limitation was the small sample size of the burixafor<sub>2D+B</sub> group, and that recipients in this group did not routinely undergo

a regular IVUS examination. Increase in sample size and stratifying recipients in the burixafor<sub>2D+B</sub> group based on the incidence of acute rejection in the future study may elucidate further information. Finally, the efficacy of CXCR4 antagonists on CAV should be evaluated by including a serial volumetric IVUS analysis, which can stratify recipients into different posttransplantation periods (early, intermediate, and late) with increased statistical power.

For further mechanistic investigation, it would be important to elucidate whether SDF-1 $\alpha$ /CXCR-4 axis determines MSC migration to or retaining in allografts, and how the mobilized MSCs affect inflammatory events that undermine graft survival. To avoid the influences of temporal and regional expressions, a small animal model in which serial analyses of entire cardiac allografts at different time intervals after transplantation will be helpful.

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**REFERENCES**

- Lund LH, Edwards LB, Kucheryavaya AY, et al. The registry of the International Society for Heart and Lung Transplantation: thirty-second official adult heart transplantation report—2015; focus theme: early graft failure. *J Heart Lung Transplant.* 2015;34:1244–1254.
- Isobe M, Kosuge H, Suzuki J. T cell costimulation in the development of cardiac allograft vasculopathy: potential targets for therapeutic interventions. *Arterioscler Thromb Vasc Biol.* 2006;26:1447–1456.
- Jansen MA, Otten HG, de Weger RA, et al. Immunological and fibrotic mechanisms in cardiac allograft vasculopathy. *Transplantation.* 2015;99:2467–2475.
- Arora S, Andreassen AK, Andersson B, et al. The effect of everolimus initiation and calcineurin inhibitor elimination on cardiac allograft vasculopathy in de novo recipients: one-year results of a Scandinavian randomized trial. *Am J Transplant.* 2015;15:1967–1975.
- Lindenfeld J, Miller GG, Shakar SF, et al. Drug therapy in the heart transplant recipient: part II: immunosuppressive drugs. *Circulation.* 2004;110:3858–3865.
- Abadja F, Atemkeng S, Alamartine E, et al. Impact of mycophenolic acid and tacrolimus on Th17-related immune response. *Transplantation.* 2011;92:396–403.
- Allison AC, Eugui EM. Mechanisms of action of mycophenolate mofetil in preventing acute and chronic allograft rejection. *Transplantation.* 2005;80:S181–S190.
- Schwarze ML, Houser SL, Muniappan A, et al. Effects of mycophenolate mofetil on cardiac allograft survival and cardiac allograft vasculopathy in miniature swine. *Ann Thorac Surg.* 2005;80:1787–1793.
- Demirkiran A, Sewgobind VD, van der Weijde J, et al. Conversion from calcineurin inhibitor to mycophenolate mofetil-based immunosuppression changes the frequency and phenotype of CD4+FOXP3+ regulatory T cells. *Transplantation.* 2009;87:1062–1068.
- Kaczmarek I, Zaruba MM, Beiras-Fernandez A, et al. Tacrolimus with mycophenolate mofetil or sirolimus compared with calcineurin inhibitor-free immunosuppression (sirolimus/mycophenolate mofetil) after heart transplantation: 5-year results. *J Heart Lung Transplant.* 2013;32:277–284.

11. Roemeling-van Rhijn M, Weimar W, Hoogduijn MJ. Mesenchymal stem cells: application for solid-organ transplantation. *Curr Opin Organ Transplant*. 2012;17:55–62.
12. Plock JA, Schnider JT, Schweizer R, et al. The influence of timing and frequency of adipose-derived mesenchymal stem cell therapy on immunomodulation outcomes after vascularized composite allotransplantation. *Transplantation*. 2017;101:e1–e11.
13. Buron F, Perrin H, Malcus C, et al. Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: an in vitro study using human cells. *Transplant Proc*. 2009;41:3347–3352.
14. Eggenhofer E, Steinmann JF, Renner P, et al. Mesenchymal stem cells together with mycophenolate mofetil inhibit antigen presenting cell and T cell infiltration into allogeneic heart grafts. *Transpl Immunol*. 2011;24:157–163.
15. Popp FC, Eggenhofer E, Renner P, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. *Transpl Immunol*. 2008;20:55–60.
16. Eggenhofer E, Renner P, Soeder Y, et al. Features of synergism between mesenchymal stem cells and immunosuppressive drugs in a murine heart transplantation model. *Transpl Immunol*. 2011;25:141–147.
17. Hsu WT, Jui HY, Huang YH, et al. CXCR4 antagonist TG-0054 mobilizes Mesenchymal stem cells, attenuates inflammation, and preserves cardiac systolic function in a porcine model of myocardial infarction. *Cell Transplant*. 2015;24:1313–1328.
18. Jensen-Waern M, Kruse R, Lundgren T. Oral immunosuppressive medication for growing pigs in transplantation studies. *Lab Anim*. 2012;46:148–151.
19. DiBiase A, Tse TM, Schnittger I, et al. Frequency and mechanism of bradycardia in cardiac transplant recipients and need for pacemakers. *Am J Cardiol*. 1991;67:1385–1389.
20. Chan JB, Levi DS, Lai CK, et al. Cellular rejection of the conduction system after orthotopic heart transplantation for congenital atrioventricular block. *J Heart Lung Transplant*. 2006;25:1371–1375.
21. Blanche C, Czer LS, Fishbein MC, et al. Permanent pacemaker for rejection episodes after heart transplantation: a poor prognostic sign. *Ann Thorac Surg*. 1995;60:1263–1266.
22. Wu YW, Chen YH, Wang SS, et al. PET assessment of myocardial perfusion reserve inversely correlates with intravascular ultrasound findings in angiographically normal cardiac transplant recipients. *J Nucl Med*. 2010;51:906–912.
23. Weber LT, Shipkova M, Armstrong VW, et al. Comparison of the emit immunoassay with HPLC for therapeutic drug monitoring of mycophenolic acid in pediatric renal-transplant recipients on mycophenolate mofetil therapy. *Clin Chem*. 2002;48:517–525.
24. Chatur S, Wong BW, Carthy JM, et al. Inhibition of vascular endothelial growth factor reduces cardiac allograft vasculopathy. *J Heart Lung Transplant*. 2016;35:1124–1130.
25. Shirasugi N, Adams AB, Durham MM, et al. Prevention of chronic rejection in murine cardiac allografts: a comparison of chimerism- and nonchimerism-inducing costimulation blockade-based tolerance induction regimens. *J Immunol*. 2002;169:2677–2684.
26. Kwun J, Farris AB, Song H, et al. Impact of leukocyte function-associated antigen-1 blockade on endogenous allospecific T cells to multiple minor histocompatibility antigen mismatched cardiac allograft. *Transplantation*. 2015;99:2485–2493.
27. Sun Y, Feng J, Shi J, et al. Gene transfer of the S24F regulated on activation normal T-cell expressed and secreted-chemokine ligand 5 variant attenuates cardiac allograft rejection. *Transplantation*. 2014;97:1233–1239.
28. Jimenez J, Kapadia SR, Yamani MH, et al. Cellular rejection and rate of progression of transplant vasculopathy: a 3-year serial intravascular ultrasound study. *J Heart Lung Transplant*. 2001;20:393–398.
29. Soleimani B, Fu F, Lake P, et al. Development of a combined heart and carotid artery transplant model to investigate the impact of acute rejection on cardiac allograft vasculopathy. *J Heart Lung Transplant*. 2008;27:450–456.
30. Stoica SC, Cafferty F, Pauriah M, et al. The cumulative effect of acute rejection on development of cardiac allograft vasculopathy. *J Heart Lung Transplant*. 2006;25:420–425.
31. Clausell N, Molossi S, Rabinovitch M. Increased interleukin-1 beta and fibronectin expression are early features of the development of the postcardiac transplant coronary arteriopathy in piglets. *Am J Pathol*. 1993;142:1772–1786.
32. Sun H, Lu X, Wu S, et al. The effects of C-reactive protein, interleukin-6, and tumor necrosis factor-alpha in rat allograft adventitial inflammation and allograft arteriosclerosis. *Transplant Proc*. 2009;41:3909–3912.
33. van Loosdregt J, van Oosterhout MF, Bruggink AH, et al. The chemokine and chemokine receptor profile of infiltrating cells in the wall of arteries with cardiac allograft vasculopathy is indicative of a memory T-helper 1 response. *Circulation*. 2006;114:1599–1607.
34. Jin J, Li YW, He Q. C4d deposition is associated with immune cells infiltrating in kidney allograft glomerulitis and peritubular capillaritis. *Ren Fail*. 2015;37:791–797.
35. Hognestad A, Endresen K, Wergeland R, et al. Plasma C-reactive protein as a marker of cardiac allograft vasculopathy in heart transplant recipients. *J Am Coll Cardiol*. 2003;42:477–482.
36. Labarrere CA, Lee JB, Nelson DR, et al. C-reactive protein, arterial endothelial activation, and development of transplant coronary artery disease: a prospective study. *Lancet*. 2002;360:1462–1467.
37. Jui HY, Lin CH, Hsu WT, et al. Autologous mesenchymal stem cells prevent transplant arteriosclerosis by enhancing local expression of interleukin-10, interferon- $\gamma$ , and indoleamine 2,3-dioxygenase. *Cell Transplant*. 2012;21:971–984.
38. Hsu WT, Lin CH, Chiang BL, et al. Prostaglandin E2 potentiates mesenchymal stem cell-induced IL-10+IFN- $\gamma$ +CD4+ regulatory T cells to control transplant arteriosclerosis. *J Immunol*. 2013;190:2372–2380.
39. Gao C, Li Y. SDF-1 plays a key role in the repairing and remodeling process on rat allo-orthotopic abdominal aorta grafts. *Transplant Proc*. 2007;39:268–272.
40. Nemenoff RA, Horita H, Ostriker AC, et al. SDF-1 $\alpha$  induction in mature smooth muscle cells by inactivation of PTEN is a critical mediator of exacerbated injury-induced neointima formation. *Arterioscler Thromb Vasc Biol*. 2011;31:1300–1308.
41. Sakihama H, Masunaga T, Yamashita K, et al. Stromal cell-derived factor-1 and CXCR4 interaction is critical for development of transplant arteriosclerosis. *Circulation*. 2004;110:2924–2930.
42. Thomas MN, Kalnins A, Andrassy M, et al. SDF-1/CXCR4/CXCR7 is pivotal for vascular smooth muscle cell proliferation and chronic allograft vasculopathy. *Transpl Int*. 2015;28:1426–1435.
43. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med*. 2006;354:610–621.
44. Yamani MH, Ratliff NB, Cook DJ, et al. Peritransplant ischemic injury is associated with up-regulation of stromal cell-derived factor-1. *J Am Coll Cardiol*. 2005;46:1029–1035.
45. Schober A, Hristov M, Kofler S, et al. CD34+CD140b+ cells and circulating CXCL12 correlate with the angiographically assessed severity of cardiac allograft vasculopathy. *Eur Heart J*. 2011;32:476–484.
46. Shi X, Guo LW, Seecial S, et al. Local CXCR4 Upregulation in the injured arterial wall contributes to intimal hyperplasia. *Stem Cells*. 2016;34:2744–2757.
47. Filler G, Zimmering M, Mai I. Pharmacokinetics of mycophenolate mofetil are influenced by concomitant immunosuppression. *Pediatr Nephrol*. 2000;14:100–104.
48. DeRoth L, Bisailon A. Determination of body surface area in neonatal swine. *Lab Anim Sci*. 1979;29:249–250.