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ORIGINAL ARTICLE

Correlation between circulating endothelial progenitor cell function and allograft rejection in heart transplant patients

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Summary

Endothelial progenitor cells (EPCs) may contribute to rejection and cardiac allograft vasculopathy (CAV) by being intrinsically involved in the rejection process and causing neointimal hyperplasia. The mammalian target of rapamycin inhibitors (mTORi), sirolimus and everolimus, have been demonstrated to attenuate the progression of CAV and are cytotoxic to EPC. Thus, one mechanism by which mTORi may protect against CAV is by altering EPC function. Our study measured circulating EPC function and correlated this assessment with rejection episodes in heart transplant (HT) recipients. In addition, we examined the effect of mTORi on EPCs. Patients who received HT at our institution between 1995 and 2007 were included and stratified by International Society for Heart and Lung Transplantation (ISHLT) rejection grade. Group A ($n = 13$) consisted of patients with at least one moderate/severe rejection episode (grade ≥ 2). Group B ($n = 28$) patients had no moderate/severe episodes (grade < 2). Patients were also independently stratified based on exposure as mTORi ($n = 21$) vs. non mTORi ($n = 20$). To assess EPC functional capacity, we counted the number of colony-forming units (CFU) of EPCs in peripheral blood samples from HT recipients. There were no significant differences in baseline characteristics between groups. The mean EPC-CFU counts/plate for group A (rejecting) were 30 ± 6 vs.16 ± 3 for group B (nonrejecting) ($P = 0.03$). The EPC-CFU counts/plate in the mTORi group (15 ± 3) were lower compared to the non mTORi (27 ± 5) group ($P = 0.04$). We found that EPC colony-forming capacity was higher in HT patients who experienced moderate/severe rejection episodes. Patients on mTORi showed a reduced EPC colony count consistent with our previous findings of EPC cytotoxicity. Detection of circulating EPC function post-transplant may reliably identify patient risk level for subsequent allograft rejection and allow for appropriate adjustments to immunosuppression. Converting to mTORi therapy may reduce EPC function and provide a novel mechanism to prevent rejection and possibly attenuate the development of CAV.

Introduction

Cardiac allograft vasculopathy (CAV), widely regarded as a chronic form of vascular rejection, is a major cause of late phase morbidity following cardiac transplantation [1]. Cardiac allograft vasculopathy remains a leading cause of death between 1 and 3 years after transplantation according to the International Society for Heart and Lung Transplantation (ISHLT) [2]. Histologically, it is characterized by diffuse concentric neointimal hyperplasia along the length of epicardial and smaller intramyocardial coronary vessels [1,3]. Both immunologic and nonimmunologic factors may be involved in the development of CAV by causing endothelial dysfunction, leading to progressive

intimal thickening [1]. Treatment of the disease is limited and difficult, because of its diffuse nature, making re-transplantation the only option in many cases. Acute allograft rejection, an immune-mediated inflammatory response involving infiltration of the myocardium with mononuclear cells, is regarded by many as a precursor to chronic rejection and CAV. Attenuating allograft rejection and the subsequent progression of CAV is truly the most effective long-term option for patients requiring heart transplantation.

Endothelial progenitor cells (EPCs), bone marrow-derived cells that have endothelial reparative properties by localizing to sites of vascular injury, may contribute to allograft rejection and CAV [4]. Normally, the endothelium undergoes dynamic processes of degeneration and regeneration. While EPCs play an important role in this homeostatic mechanism, slight imbalances in the process may cause endothelial dysfunction [4]. During vascular injury, circulating EPCs adhere to the vascular wall and replace endothelial cells that have been shed, which promotes healing and prevents plaque formation [5–7]. This explains the widely accepted beneficial role of EPCs in the nontransplant setting. Numerous studies have demonstrated that increased EPC levels are associated with better cardiovascular outcomes and a reduction of neointimal hyperplasia [8–12].

In transplant physiology, as a result of persistent allograft antigenicity, this EPC homeostatic mechanism may be uncontrolled and pathological. Allograft rejection and CAV may be a result of defective EPC repair mechanisms secondary to allograft recognition [4]. A number of studies have demonstrated a detrimental role of EPCs post-transplant through contribution to allograft rejection and CAV [4,13,14]. Woywodt *et al.* [13] demonstrated an association between acute rejection and high EPC levels in renal transplant patients. Simper *et al.* [14] found that EPC colony counts were lower in heart transplant (HT) patients with established vasculopathy and illustrated EPC seeding at CAV plaque sites, suggesting that circulating EPCs contributed to plaque formation, thus depleting the systemic EPC pool. Endothelial progenitor cells may overwhelm the endothelium causing intimal hyperplasia, giving a plausible explanation for their role in the progression of CAV [4]. Also, EPCs may carry the ability to differentiate into both endothelial and smooth muscle cells, making it possible for them to re-endothelialize vessels and cause smooth muscle hyperplasia simultaneously [15,16]. This lends more plausibility to the proposed mechanism of CAV, suggesting that EPCs may contribute through preferential differentiation into smooth muscle cells as opposed to endothelial cells.

Until recently, it was believed that progenitor cells contributing to allograft rejection and CAV arose from the allograft itself, namely cells from the local vessel wall. However, recent evidence suggests that recipient-derived cells occupy allograft endothelium [14,17,18]. Hillebrands *et al.* [17] demonstrated the replacement of graft endothelial cells with circulating host-derived cells through the use of a sex-mismatched rat HT model. Utilizing a murine model, Hu *et al.* [18] showed that as allograft age increases, allograft cells are replaced with recipient-derived cells. Furthermore, Simper *et al.* [14] illustrated the seeding of recipient-derived endothelial progenitors in donor coronary arteries and areas of CAV in human HT patients. Therefore, the idea of a circulating host-derived progenitor cell that is responsible for allograft rejection and CAV development is well supported and could have numerous clinical implications.

Recent advances in immunosuppressive therapy have allowed for more effective treatment and prevention of allograft rejection. Though many agents have been successful in preventing acute rejection, few have been able to protect against late-stage transplant vasculopathy. Only mammalian target of rapamycin inhibitors (mTORi), such as sirolimus and everolimus, have been shown to attenuate CAV [1,19]. These agents prevent lymphocyte proliferation via inhibition of the cell-signaling molecule mammalian target of rapamycin (mTOR). In addition, we have previously shown that sirolimus is cytotoxic to EPCs *in vitro*; in contrast to cyclosporine and tacrolimus [20]. Other studies have also demonstrated mTORi cytotoxicity to EPCs as well as systemic lowering of EPC levels in patients receiving sirolimus-eluting stents [21–23]. Given that mTORi slow CAV progression and have a detrimental effect on EPCs, we hypothesized that decreasing EPC function may represent a mechanism by which mTORi protect against rejection and potentially CAV.

This study was designed to assess the correlation between circulating EPC function and allograft rejection episodes in HT recipients. We also elucidated the effect of mTORi (sirolimus and everolimus) on EPC functional capacity.

Methods

Patients

This retrospective study included 41 randomly selected patients who underwent heart transplantation at Toronto General Hospital during the period between 1997 and 2007, and was approved by the research ethics board from our institution. Each study participant had blood drawn for EPC functional assessment during routine endomyocardial biopsy procedures post-transplant. Patients were stratified into two different groups, depending on their cardiac allograft rejection profile [24]. Though each patient in our study had numerous biopsy

rejection scores at different times, only the rejection scores within 1 year prior to blood sample determination of EPC counts were used for analysis. Group A ($n = 13$) consisted of patients who suffered from at least one moderate or severe rejection episode (ISHLT grade ≥ 2) and were considered rejectors. Group B ($n = 28$) patients did not suffer from any moderate or severe episodes (ISHLT grade < 2) and were considered nonrejectors. None of the patients were suffering from an acute rejection episode at the time of sampling. Patients were also independently stratified based on exposure as mTORi ($n = 21$) vs. non mTORi ($n = 20$). All patients in the mTORi group were receiving the immunosuppressant (sirolimus or everolimus) at the time of EPC colony counting at standard clinical doses to achieve serum concentrations of 5–10 ng/ml.

Biopsy tissue sample

Biopsies obtained from the right interventricular septum of each patient during routine endomyocardial biopsy procedures were used for the assessment of rejection. All specimens had been fixed in 10% neutral buffered formalin, embedded in paraffin blocks and mounted onto slides to survey allograft rejection. Serial myocardial biopsies were performed every week during the first month, biweekly until month 3, monthly until month 6 and every 3 months until 1 year post-transplant. Cardiac allograft rejection scores were given to each heart biopsy sample and graded according to the International Society for Heart and Lung Transplantation [24]. Patients with poor biopsy tissue quality were excluded from the study. Biopsies were assessed for rejection independently of tissue quality of lesions and were, hence, appropriately graded. Screening for antibody-mediated rejection was only performed when clinically indicated. The cardiac pathologist was blinded to all patients' clinical information and EPC count. Importantly, regardless of rejection score, all biopsies were obtained as per the above protocol and none of these biopsy specimens were obtained in response to a clinical event.

Endothelial progenitor cell colony-forming units

To assess the functional capacity of circulating endothelial progenitor cells, we counted the number of colony-forming units (CFU) of EPCs from peripheral blood samples via the StemCell Technologies EndoCult® Liquid Medium CFU kit (Stem Cell Technologies, Vancouver, BC, Canada). Although controversial, the determination of CFUs is a widely utilized method of quantifying EPC function [8,10]. Briefly, 16 ml of peripheral blood was taken from each HT patient via a central line during a routine endomyocardial biopsy procedure. Blood was collected into sodium citrate-containing tubes to prevent coagulation, stored at room temperature, and analyzed within 1–3 h. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient, plated on 6-well fibronectin-coated dishes at 5 million cells per well, and incubated for 48 h. To deplete the sample of adherent macrophages and mature endothelial cells, nonadherent cells were collected and re-plated on 24-well fibronectin-coated dishes in duplicate at 1 million cells per well. After 3–7 days, CFUs emerged and could be counted accordingly. The phenotype of an EPC-specific colony was defined as a cluster of round cells with spindle-shaped cells at the periphery (Fig. 1). Colonies that did not meet these criteria were not included in the EPC count. Staining of a subset of colonies with VEGF-R2, CD34 and CD31 was done to confirm endothelial lineage. The assay itself did not permit the growth of mature endothelial cells, excluding the possibility of mature endothelial colonies contaminating the final count. The investigator counting the colonies was blinded towards patient rejection status and medication regimen. To assess reproducibility, intra- and inter-observer correlations

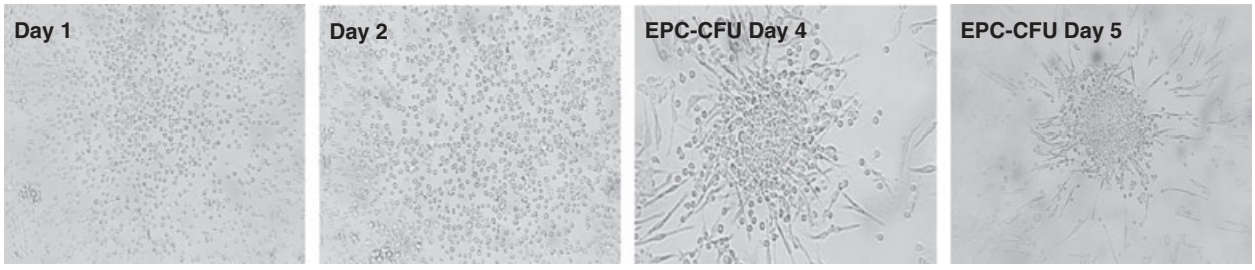

Figure 1 Plating of blood mononuclear cells via StemCell Technologies EndoCult® liquid medium CFU kit. Day 1 and 2, cells are dispersed and plated on 6-well fibronectin-coated dishes. After 48 h, adherent macrophages and mature epithelial cells are depleted by collecting nonadherent cells and re-plating on a 24-well fibronectin-coated dish. Day 4 and 5, EPC colony-forming units begin to form, defined as a cluster of round cells with spindle-shaped cells at the periphery. EPC, endothelial progenitor cell.

were calculated using 10 healthy controls (0.95 and 0.97 respectively). Once counts were gathered for all patients, the mean EPC-CFU counts between group A and group B as well as between the mTORi versus non mTORi groups were compared.

Statistical analysis

All values are expressed as mean ± standard error unless otherwise specified. For the purpose of determining differences in baseline characteristics between groups, independent *t*-tests were used for all continuous variables and chi-squared tests for all categorical variables. ANOVA was used for comparison of mean values between groups. In Fig. 4, Tukey *post hoc* analysis was used to further identify such groups as were significantly different from group A-non mTORi. Exact *P*-values are provided to enable the reader to determine statistical and clinical significance for each comparison.

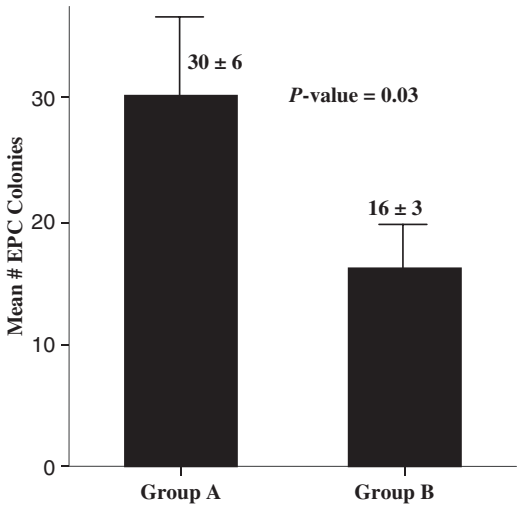

Figure 2 Comparison of mean EPC colony counts between groups A and B. Group A, Rejecting; Group B, Nonrejecting; EPC, endothelial progenitor cell.

Results

Among the 41 patients (31 male, 10 female) included in this study, cardiac transplantation had been performed as a result of idiopathic cardiomyopathy (46%), ischemic cardiomyopathy (29%) and other heart diseases (24%). Group A (rejecting) consisted of 13 patients (10 male, 3 female) with a mean age of 49 ± 15 years (range 20–69), while group B (nonrejecting) consisted of 28 patients (21 male, 7 female) with a mean age of 50 ± 15 years (range 19–74). Between the mTORi (17 male, 4 female) and non mTORi (14 male, 6 female) groups, mean age was 49 ± 15 years (range 19–74) and 50 ± 14 years (range 20–69) respectively. There were no significant differences in mean age, gender, indication for transplant, immunosuppressive regime, Cytomegalovirus (CMV) status, or time after transplant between groups. There was, however, a significant difference in tacrolimus therapy between the mTORi and non mTORi groups. No patients had hemodynamically decompensated cellular rejection and none developed left ventricular dysfunction measured by echocardiography following transplantation. As there were no clinical indications, no patient required screening for antibody-mediated rejection.

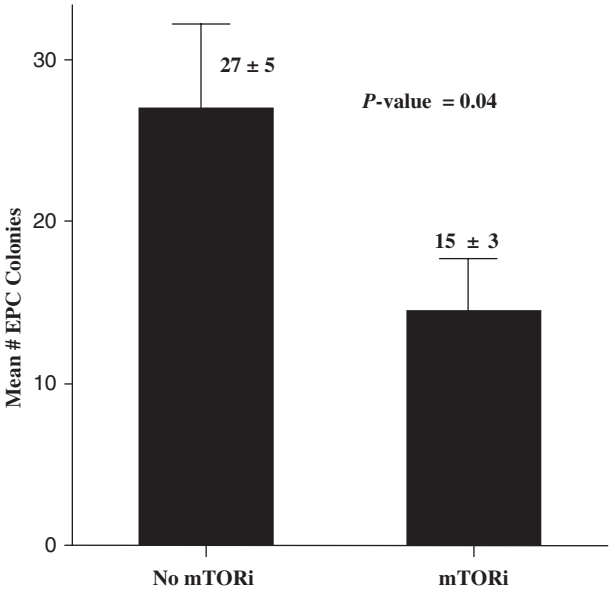

Figure 3 Comparison of mean EPC colony counts between non mTORi and mTORi groups. mTORi, mTOR inhibitors sirolimus or everolimus; EPC, endothelial progenitor cell.

When comparing between groups A (rejecting) and B (nonrejecting), we found significantly higher EPC colony counts in the rejecting group as predicted (Fig. 2), with mean EPC-CFU counts/plate being 30 ± 6 and 16 ± 3 respectively ($P = 0.03$). Furthermore, patients in the mTORi group had significantly lower EPC counts than the non mTORi group (Fig. 3) with mean EPC-CFU counts/plate of 15 ± 3 vs. 27 ± 5 respectively ($P = 0.04$), consistent with our previous *in vitro* investigations demonstrating that sirolimus has a detrimental effect on EPCs. Figure 4 shows the comparison of mean EPC-CFU counts between groups A and B, with the use of mTORi as a covariate. As shown, there is a significant difference in the mean values between groups ($P = 0.01$). Tukey *post hoc* analysis showed that only group B-mTORi was significantly different from group A-non mTORi.

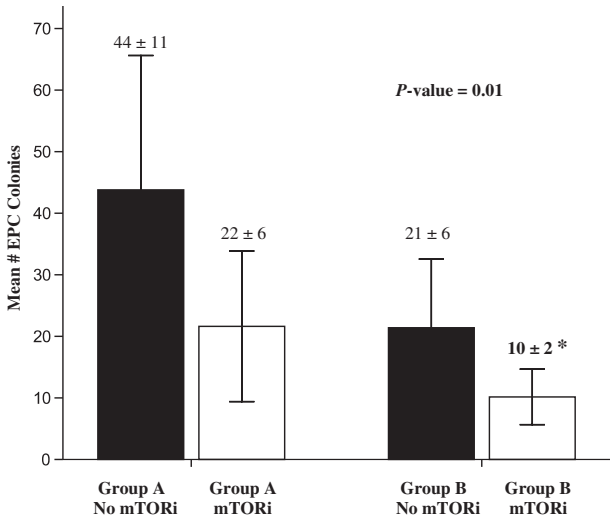

Figure 4 Comparison of mean EPC colony counts between group A-non mTORi, group A-mTORi, group B-non mTORi, and group B-mTORi. Group A, Rejecting; Group B, Nonrejecting; mTORi, mTOR inhibitors sirolimus or everolimus; EPC, endothelial progenitor cell. *Mean difference between group A-non mTORi and group B-mTORi is significant at the 0.05 level. *Post hoc* analysis with Tukey test used.

Discussion

This study demonstrates that EPC function, as measured by the number of colony-forming units, is higher in patients who experienced moderate/severe rejection episodes after heart transplantation, indicating that circulating EPCs may portend a high risk of allograft rejection and subsequent CAV. Thus, EPCs may reliably aid in identifying patients with a high immunologic risk of developing subsequent moderate or severe rejection episodes. Furthermore, patients on mTORi showed reduced EPC functional capacity; consistent with our previous findings that sirolimus is cytotoxic to EPCs [20]. This may represent a means by which mTORi attenuate CAV progression, providing us with a novel mechanism for the future prevention of CAV.

Allograft rejection and CAV are complex diseases with various etiologies. Animal studies have shown that cells found in CAV lesions and allograft endothelium are of recipient origin, paving the way for the idea of a circulating recipient-derived progenitor [14,17,18]. Though EPCs in healthy individuals may be part of a homeostatic mechanism whereby they are attracted to sites of vascular injury and aid in re-endotheliazation, this does not appear to be the case following heart transplantation. Allograft rejection and transplant vasculopathy may represent pathologic repair in response to continuous and persistent damage to the endothelium, eventually leading to thickening of the intima and smooth muscle hyperplasia [4].

Few studies have assessed the association between rejection and EPCs in the HT population. Our results are consistent with other studies showing higher EPC numbers and function in patients suffering acute rejection after renal transplantation [13]. Also, Simper *et al.* [14] demonstrated that the number of EPC outgrowth colonies were lower in HT patients with established vasculopathy as compared with those without. Although seemingly contrary to our hypothesis, this study found EPC seeding at plaque sites and hypothesized that circulating EPC numbers were lower in CAV patients as a result of ongoing EPC recruitment to areas of endothelial injury in the transplanted heart. As our study examined acute rejection, we did not expect the EPC pool to be depleted as of yet. Hence, we expected to see higher rejection risk in patients with a higher number of EPC outgrowth colonies. It is important to note that we did not directly assess CAV in this study, and hence cannot make any conclusions regarding higher EPC function and increased CAV risk. As mentioned above, evidence for a circulating recipient-derived EPC involved in the pathogenesis of CAV is abundant. On the basis of our study, we can only speculate that higher EPC functional capacity, shown in our study to be associated with allograft rejection, will lead to an increased risk of subsequent CAV. We have illustrated for the first time an association between EPCs and rejection episodes via heart biopsy grading. Heart biopsy is the current gold standard for allograft rejection analysis; making this association a potentially clinically applicable method of predicting the number and severity of rejection episodes in post-transplant patients.

Our study also illustrates an association between EPC and mTORi therapy, which has not been examined before in a clinical setting involving HT patients. We have previously demonstrated that sirolimus is cytotoxic to EPCs and alters their functional ability [20]. Doses far below clinically relevant levels were cytotoxic to EPCs. Significant effects were seen at concentrations as low as 0.01 ng/ml. Further, the effect of sirolimus on EPCs was not shared by other immunosuppressants such as cyclosporine and tacrolimus. In this study, the lower EPC counts in the mTORi-treated group suggest that sirolimus and everolimus may in fact protect against CAV via EPC mediation. It is possible that mTORi lower EPC numbers and functional ability, removing their contribution to neointimal hyperplasia. Table 1 demonstrates that there was no significant difference between groups A and B with regards to the use mTORi. However, it is difficult in a retrospective study to determine the indication for the use or nonuse of mTORi. All patients receiving everolimus were enrolled in a prospective randomized clinical trial; but the use of sirolimus may have been dictated by a prior history of malignancy, potential CAD in the

Table 1. Baseline characteristics and immunosuppressive regime.

	Group A (n = 13)	Group B (n = 28)	P-value	mTORi (n = 21)	Non mTORi (n = 20)	P-value
Demographics						
Gender (M/F)	10/3	21/7	0.89	17/4	14/6	0.10
Mean age (range)	49 ± 15	50 ± 15	0.95	49 ± 15	50 ± 15	0.76
Induction therapy						
Basiliximab/RATG	5/8	7/21	0.38	8/13	4/16	0.61
Immunosuppressive therapy (%)						
Cyclosporine	12 (92)	17 (61)	0.06	17 (80)	12 (60)	0.18
Tacrolimus	2 (15)	9 (32)	0.45	2 (10)	9 (45)	0.02*
Mycophenolate mofetil	9 (69)	17 (61)	0.73	10 (48)	16 (80)	0.06
Prednisone	13 (100)	25 (89)	0.54	20 (95)	18 (90)	0.61
Any mTORi	8 (62)	13 (46)	0.51	–	–	–

*$P < 0.05$. Group A, rejecting; Group B, nonrejecting; mTORi, mTOR inhibitors sirolimus or everolimus, RATG, rabbit anti-thymocyte globulin.

donor or renal impairment. Therefore, it is impossible to correlate the use of mTORi with either rejection or CAV. However, our results do demonstrate that mTORi consistently reduce EPC-CFU irrespective of underlying rejection and therefore we can speculate that mTORi should be beneficial in attenuating the future development of CAV.

It is unknown how EPC numbers and function are altered during and after HT. Prospective studies measuring EPC levels before and after transplant need to be conducted to further test this hypothesis. This will elucidate whether EPC colony counts in fact predict rejection episodes or if the rejection episode itself causes an increase in EPC levels. It is quite plausible that EPC functional capacity and numbers are increased as a result of the cytokine release intrinsic to allograft rejection or in an attempt to repair damaged endothelium as opposed to being a predisposing factor. This must be taken into account during interpretation of our results. Prospective studies would also allow EPC function to be measured before rejection has occurred, testing their true predictive value towards future rejection episodes. In our study, patients were grouped according to their highest rejection score within 1 year prior to when they had blood drawn for EPC functional analysis. This does not provide us with information regarding EPC function before rejection occurred. It is important to note that prior studies have shown a clear link between chronic CMV infections and CAV. Though we did not examine CAV in this study, we did show that there was no significant difference in CMV status between groups (Table 2). However, there was a significantly higher number of EPC-CFU in CMV positive patients (25 ± 4 vs. 12 ± 3, $P = 0.02$). Given that there was no difference in CMV status between rejecters and nonrejecters, the significance of this finding is questionable. In regards to the sirolimus- and everolimus-treated group, a prospective study would allow us to examine EPC colony counts before and after therapy. This would allow us to determine whether there is in fact a decrease in EPC function after the induction of therapy.

Future studies can also expand by using different parameters to distinguish and enumerate EPCs. Flow

Table 2. Transplant characteristics.

	Group A (n = 13)	Group B (n = 28)	P-value	mTORi (n = 21)	Non mTORi (n = 20)	P-value
Reason for transplant (%)						
Idiopathic cardiomyopathy	5 (38)	14 (50)	0.38	9 (43)	10 (50)	0.65
Ischemic cardiomyopathy	5 (38)	7 (25)	0.49	6 (29)	6 (30)	0.92
Other	3 (23)	7 (25)	0.89	6 (29)	4 (20)	0.52
Time after transplant (%)						
<1 year	1 (8)	8 (29)	0.13	4 (19)	5 (25)	0.65
1–4 years	7 (54)	12 (43)	0.51	9 (43)	10 (50)	0.65
>4 years	5 (38)	8 (29)	0.53	8 (38)	5 (25)	0.37
CMV status						
Positive/negative	9/4	17/11	0.60	14/7	12/8	0.66

Group A, rejecting; Group B, nonrejecting; mTORi, mTOR inhibitors sirolimus or everolimus; CMV, cytomegalovirus.

cytometry is a valuable option, yet a consensus on EPC-specific cell markers has not yet been reached. Debate is ongoing as to whether VEGF-R2, CD133, and CD34 or any combination of the three represents a true EPC. There has been tremendous controversy as to whether or not EPC-CFUs are truly derived from EPCs and whether flow cytometrical analysis is a better option. Recent studies have shown that EPC-CFU may in fact be derived from monocytic and macrophage cells, as opposed to endothelial progenitor cells [25,26]. This would alter the conclusions of our study significantly, as the colonies would represent markers of inflammation and immune-mediated response as opposed to endothelial dysfunction. Nevertheless, these studies are controversial and the EPC-CFU method of measuring EPC function is used widely and has been linked to endothelial dysfunction and cardiovascular outcomes in numerous studies [8,10,13,14].

In summary, detection of EPC function post-transplant may reliably identify patient risk for subsequent allograft rejection and allow for appropriate adjustments to immunosuppression. Elucidating the mechanism by which mTOR inhibitors alter EPC survival and/or function may result in enhanced protection against CAV in the future. More detailed study of the role of EPCs in allograft rejection and the development of CAV will allow for more targeted therapy and prolonged survival post-transplant.

Authorship

CS: designed study, collected data, performed study, analyzed data, wrote the paper. RS and DD: designed study, performed study. JP: designed study, collected data. LT: contributed important reagents, collected data. HR: designed study. VR: designed study, collected data, analyzed data, wrote the paper.

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