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Long-term survival of rat cardiac allografts by intrathymic plus portal venous injections of donor bone marrow cells and short-term tacrolimus immunosuppression

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Abstract Intrathymic (IT) or portal venous (PV) injection of donor antigens has been shown to prolong organ acceptance in low responder rat strain combinations. We determined whether a combination of these strategies would prolong cardiac allograft survival in high responder combinations. Wistar Furth rats received 1×10^8 ACI rat bone marrow cells (BMCs) via IT, intravenous (IV), PV, IV + PV, IT + IV or IT + PV route at the time of ACI cardiac transplantation. Without tacrolimus (FK), all grafts were acutely rejected. With FK immunosuppression (1.5 mg/kg per day, I.M., days 0–4), single BMC injection did not increase graft survival beyond 93 days, whereas 70% of grafts survived indefinitely (> 150 days) when IT and PV BMCs were combined. Animals receiving IT and PV BMCs also had less allograft vasculopathy. Thus, IT and PV injections of donor BMCs under a brief course of FK synergistically improve cardiac allograft survival.

Keywords Rat cardiac allograft · Intrathymic injection · Portal venous injection · Bone marrow cells · Allografts vasculopathy

Abbreviations *BMCs* Bone marrow cells · *CAV* Coronary artery vasculopathy · *CPM* Counts per minute · *FK* Tacrolimus · *IT* Intrathymic · *IV* Intravenous · *LEW* Lewis · *MLR* Mixed leukocyte reaction · *MST* Mean graft survival time · *PV* Portal venous · *WF* Wistar Furth

Introduction

Despite recent refinements in immunosuppressive agents, side effects associated with the lifetime use of non-specific immunosuppression, including organ toxicity, allograft vasculopathy, post-transplant lymphoproliferative disease, and opportunistic infection, remain a barrier to successful clinical solid organ transplantation [24]. The ultimate goal of clinical transplantation, therefore, is to induce a permanent state of donor-specific tolerance that will allow weaning the recipient from long-term use of immunosuppressive agents. Strategies that promote graft acceptance, such as intrathymic (IT) [1, 17, 20, 21, 25, 29, 30, 33], intravenous (IV)[2] [32], and por-

tal venous (PV)[3] injections [5, 9, 11, 12, 13, 26, 35, 36] of donor alloantigens, or creation of mixed allogeneic chimerism [3, 6, 7, 8], have been extensively studied in rodents, however, none have been successful in humans. Obstacles that hinder the clinical application of these strategies include the timing of the pre-treatment which, in some models, requires 1–3 weeks prior to transplantation in order to promote a tolerant state [1, 13, 20, 21, 29, 30], the limited animal strain combinations in which tolerance could be promoted [33, 1, 13], and the risk associated with conditioning regimens. Although strategies utilizing either IT or PV injection of donor antigens have resulted in long-term graft acceptance in certain rat strain combinations, these strategies have not proven successful in high responder strains. Furthermore, combining these two treatments has not, to date, been reported. In the present study, we report for the first time that a combination of IT and PV injections of donor bone marrow cells (BMCs) at the time of transplantation, along with a short course of tacrolimus (FK), results in long-term acceptance of rat cardiac allografts in a high responder strain combination.

Materials and methods

Animals

Four to five-week-old male ACI ($RT1A^a$) and Wistar Furth (WF^6; $RTIA^u$) rats were used as donors and recipients, respectively. This strain combination involves a complete disparity in both major and minor histocompatibility antigens. Lewis (LEW^7; $RT1A^l$) rats were used as third-party donors. Rats were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA) and housed in a pathogen-free facility at the Biomedical Science Center at the University of Pittsburgh. All animals received human care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985).

Cardiac transplantation

Heterotopic cardiac transplantation was performed as described by Ono and Lindsey [22]. Donor ACI or third-party LEW hearts were procured and stored in a cold saline bath. Recipient (WF) rats were anesthetized with methoxyflurane and the aorta and inferior vena cava were exposed through median laparotomy. Arterial and venous anastomoses were constructed with 8–0 monofilament sutures. Allograft survival was assessed by daily palpation. Graft rejection was defined as complete cessation of ventricular contraction, and further confirmed by histological examination.

Preparation of BMCs

Fresh BMCs were harvested from femurs, tibias, and humeri of ACI or WF rats and resuspended in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA), using sterile techniques. BMC suspensions were adjusted to 1×10^9 cells/ml in medium for IT injection and 1×10^8 cells/ml in medium for IV and PV injections. Cell viability was over 95% as determined by trypan blue dye exclusion.

IT injection of BMCs

After cardiac transplantation, the thymus of the intubated WF recipient was widely exposed by upper median sternotomy. With the aid of an operating microscope, a total of 1×10^8 cells in 0.1 ml of medium were meticulously injected into both lobes of the thymus with a 30-gauge needle. Animals that received the same volume of medium alone served as controls. All animals in whom intralobular hemorrhage or leakage of BMCs into- or out of the capsule occurred were excluded from the study.

PV or IV injection of BMCs

Under methoxyflurane anesthesia, a total of 1.0×10^8 cells in 1 ml of medium or 1 ml of medium alone (for control) were injected into superior mesenteric (PV route) or penile (IV route) veins with a 30-gauge needle, respectively.

Immunosuppression

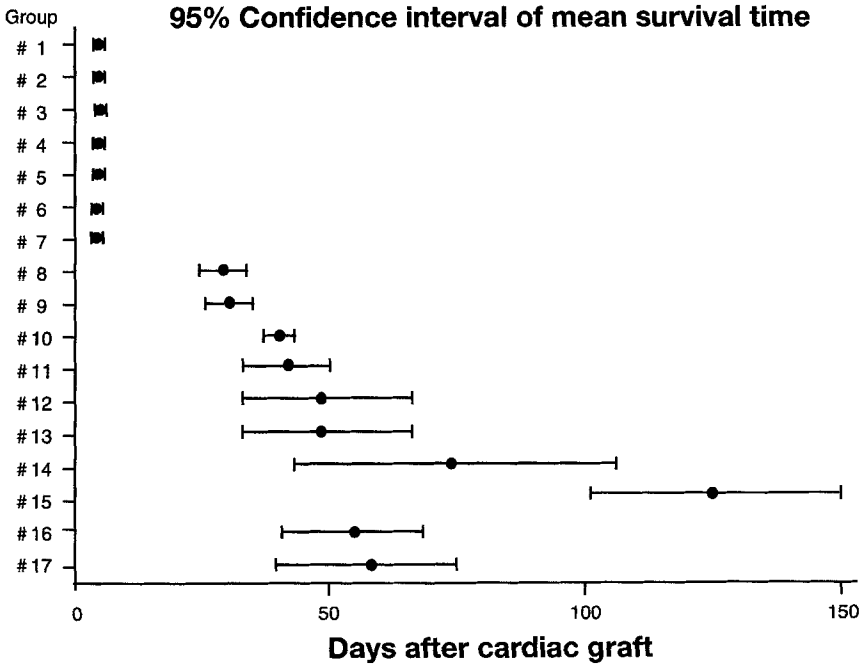
A short course of FK (generously provided by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was administered at a dose of 1.5 mg/kg per day, intramuscularly, for a period of 5 days, starting on the day of transplantation. Treatment groups are depicted in Figure 1.

Mixed leukocyte reaction (MLR)

Three animals from each group were tested for in vitro alloresponsiveness to donor antigens using one-way MLR assay. Gamma irradiated splenocytes (2000 rads from ^{137}Cs source) obtained from naive ACI, WF, or LEW, were used as stimulators. Responder cells were harvested from cervical and mesenteric lymph nodes of the WF recipients. Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden) purified mononuclear cells were washed and resuspended at a concentration of 1×10^6 cells/ml in a medium consisting of DMEM (Life Technologies) supplemented with 0.5% fresh normal ACI serum, 2 mM L-glutamine (Life Technologies), 25 mM HEPES buffer solution (Life Technologies), and 56 ng/ml gentamycin (Life Technologies). Responders (10^5) and stimulator cells (10^5) were co-cultured in a total volume of 200 µl of medium in 96-well, round-bottomed microtiter plates (Costar, Cambridge, MA, USA). Cultures were incubated at 37 °C in 10% CO_2, pulsed on the fourth day with 1.0 µCi of [^3H] thymidine, harvested on the fifth day with an automated cell harvester (Tomtec, Hamden, CT, USA), and were counted in a beta scintillation counter (Wallac, Gaithersburg, Mass. USA). All assays were performed in triplicate. Results were expressed as counts per minute (CPM) ± standard deviation. The stimulation index is the ratio of the CPM generated in response to a given stimulator over the baseline CPM generated in response to the host [3].

Fig. 1 Survival of donor ACI or third-party LEW cardiac allografts in WF recipients. Animals were killed when the cardiac graft ceased to beat, or at 150 days after transplantation (top). Kaplan-Meier analysis with the 95% confidence interval of mean graft survival time was applied to compare graft survival. Non-overlapping intervals indicate $P < 0.05$ (bottom).

Group	Treatments		Heart donors	*n*	Days of cardiac allograft survival		
	Cellular inoculum	FK				Median	Mean
1	None	–	ACI	4	6X2, 7X2	6.5	6.5
2	ACI BMCs IT	–	ACI	4	6X2, 7X2	6.5	6.5
3	ACI BMCs IV	–	ACI	4	5X2, 6, 7	6.5	5.8
4	ACI BMCs PV	–	ACI	4	5, 6X2, 7	6.0	6.0
5	ACI BMCs IV+PV	–	ACI	4	4, 5, 6X2	5.5	5.3
6	ACI BMCs IT+IV	–	ACI	4	5X3, 7	5.0	5.5
7	ACI BMCs IT+PV	–	ACI	4	4, 5, 6X2	5.5	5.3
8	None	+	ACI	8	24X3, 28, 29, 31, 33, 39	28.5	29.0
9	WF BMCs IT+PV	+	ACI	5	25, 27, 28, 32, 36	28.0	29.6
10	ACI BMCs IT	+	ACI	8	32, 33, 40X3, 41X2, 42	40.0	38.6
11	ACI BMCs IV	+	ACI	8	29, 31, 33, 35, 41, 44, 56, 59	38.0	41.0
12	ACI BMCs PV	+	ACI	8	28, 30, 31, 36, 44, 63, 69, 93	40.0	49.3
13	ACI BMCs IV+PV	+	ACI	5	27, 33, 53, 58, 72	53.0	48.6
14	ACI BMCs IT+IV	+	ACI	10	29, 36, 37, 38, 42, 48, 64, >150X3	45.0	74.4
15	ACI BMCs IT+PV	+	ACI	10	52, 65, 77, >150X7	>150.0	124.4
16	None	+	Lewis	5	40, 45, 51, 67, 78	51.2	56.2
17	ACI BMCs IT+PV	+	Lewis	5	39, 42, 58, 70, 86	58.0	59.0



Histological analysis

Cardiac allografts were taken from killed rats and fixed in 10% neutral buffered formalin. Sections were made and stained with hematoxylin-eosin and submitted for a blind evaluation under light microscopy. The overall severity of inflammation in the endocardium, pericardium, interstitium, and periarterial spaces, and that of fibrosis, were semi-quantitatively graded on a scale of 0–4 as none, minimal, mild, moderate, or severe. All arteries larger than 80 μm at their shortest external diameter in one cross-section were evaluated for the severity of coronary artery vasculopathy (CAV). The grading of CAV was as follows: grade 0, normal artery with no intimal thickening, grade 1, < 10% luminal narrowing; grade 2, 10–25%; grade 3, 25–50%; grade 4, 50–75%; and grade 5, > 75% luminal narrowing, as described by Demetris et al [4]. Vessels with a luminal narrowing greater than grade 2 (more than 10%) were defined as diseased vessels.

Statistical analysis

Kaplan-Meier analysis with the 95% confidence interval of mean graft survival time (MST) was applied to compare graft survival; intervals that do not overlap imply $P < 0.05$ (Fig. 1). Severities of inflammation, fibrosis, and CAV were compared with Mann Whitney's U test. The chi square test was applied to compare percentage of diseased vessels. Differences were considered to be significant at the $P < 0.05$ level.

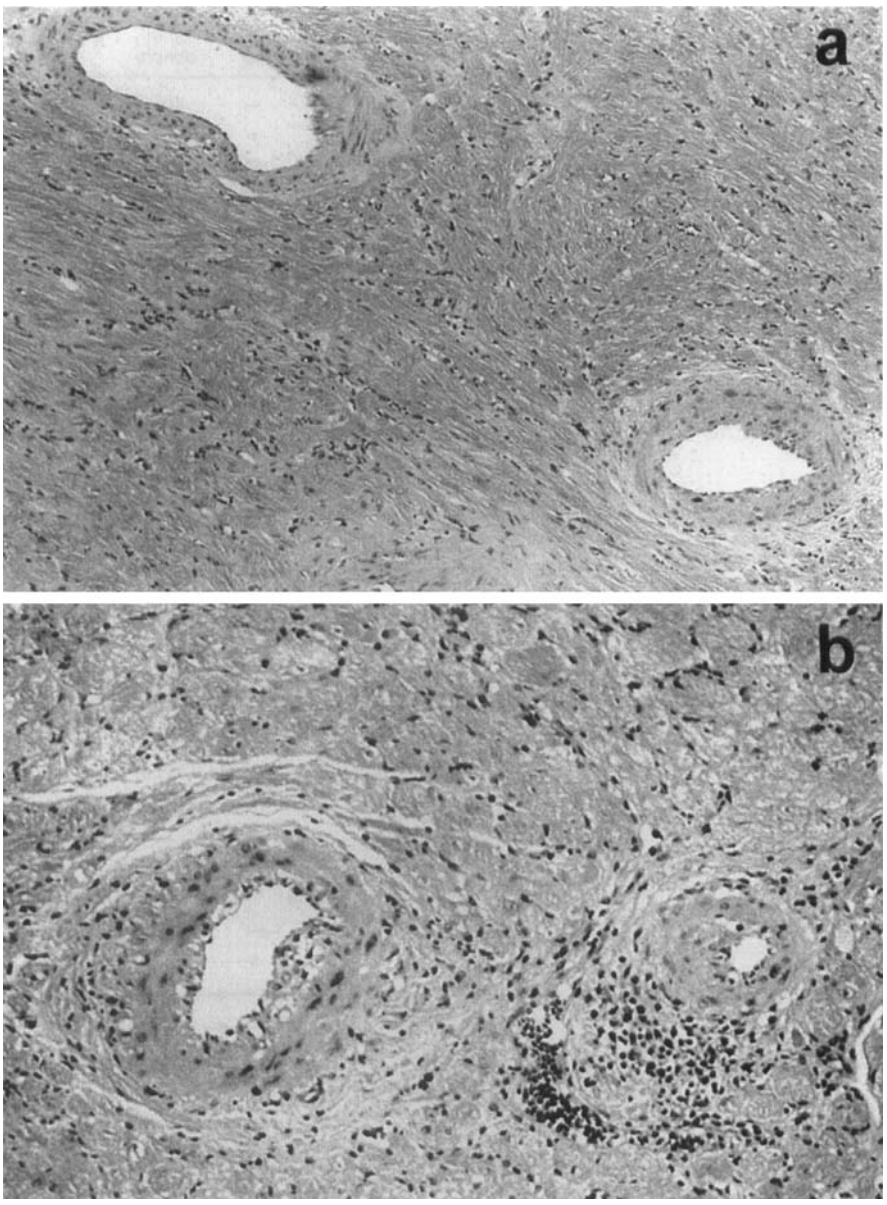


Fig. 2 Histological appearance of ACI cardiac allografts that survived more than 150 days after transplantation (hemotoxylin-eosin); **a** IT + PV donor BMCs with FK group had minimal myointimal thickening, and sparse interstitial lymphocytic infiltrate (× 100). **b** In contrast, IT + IV donor BMCs with FK group had pronounced obliterative lesions in the coronary arteries, and multifocal inflammatory infiltration within the myocardium (× 200)

Results

Graft-versus-host disease

IT, IV and PV injections of BMCs and FK treatment had no apparent effect on rat body weight after transplantation, compared with age-matched naive WF rats (data not shown). None of the animals had clinical evidence of graft-versus-host disease. Animals in all treatment groups appeared healthy throughout the study.

Graft survival

Figure 1 depicts the graft survival of all treatment groups. Animals that received either IT, PV or both in the absence of a short course of FK treatment, rejected cardiac allografts at the same tempo as untreated rats (groups 1–7). All grafts were invariably rejected within a week of their transplantation. However, when FK was administered without BMC injections, the survival was significantly prolonged (MST = 29 days, group 8). Injection of syngeneic (WF) BMCs by both IT and PV routes did not enhance graft survival in the FK-treated group (MST = 29.6 days, group 9). Injection of donor-specific allogeneic (ACI) BMCs via IT, IV, or PV route along

Table 1 Severity of CAV and myocardial rejection

Variables	Treatment		*P* value
	IT + IV BMCs + FK (Group 14)	IT + PV BMCs + FK (Group 15)	
Number of cardiac grafts graded [a]	3	7	
Number of vessels graded	50	184	
Average grade of CAV lesion	3.5 ± 1.2	1.4 ± 1.1	< 0.01[b]
Percent of diseased vessels	92%	40%	< 0.01[c]
Average grade of inflammation score	2.3 ± 0.9	1.0 ± 0.6	< 0.01[b]
Average grade of fibrosis score	1.0 ± 0.0	0.3 ± 0.5	< 0.05[b]

[a]Only animals with long-term (> 150 days) cardiac allograft survival were analyzed
[b]Mann-Whitney's U test
[c]Chi square test

with FK treatment resulted in a modest prolongation of graft survival time (MST = 38.6, 41.0, and 49.3 days, groups 10, 11, and 12, respectively). A combination of IT + IV injections of BMCs plus FK treatment further increased the graft survival (MST = 74.4 days, group 14) with 3 of 10 grafts surviving more than 150 days (endpoint of this experiment). The longest graft survival time was achieved in animals that received FK in combination with IT + PV injections of donor BMCs. Seventy percent (7/10) of the animals had grafts that survived more than 150 days (MST = 124.4 days with a median survival time > 150 days, group 15). The specificity of the hyporesponsive state induced by IT + PV BMCs was confirmed when WF recipients that were pretreated with IT + PV ACI BMCs and FK exhibited no improvement in third-party LEW cardiac allograft survival, compared with WF recipients that were treated with FK alone (MST = 56.2 and 59.0 days, groups 16 and 17).

Histopathology of cardiac allograft

All grafts recovered at the time of rejection or at 150 days after transplantation were sectioned and stained with hemotoxylin-eosin and examined histologically. All rejected grafts (survival < 50 days) showed evidence of acute rejection with severe lymphocytic infiltration and extensive myocyte damage. Grafts that survived longer than 150 days after transplantation (day of killing) were also assessed for the severity of CAV and myocardial rejection. These grafts consisted of 3 from group 14 (FK and IT + IV BMC injections), and 7 from group 15 (FK and IT + PV BMC injections). The prevalence of CAV in grafts from group 15 was significantly lower than that of group 14 (Table 1, Fig. 2). Only 40% of the coronary vessels from group 15 developed moderate myointimal thickening, compared to 92% from group 14 (Table 1). Mononuclear inflammation and fibrosis in the functioning grafts of group 14 (Fig. 2b) was more frequent and obvious than those observed in group 15 (Fig. 2a). Moderate lymphocyte infiltration (average grade: 1.0 ± 0.6) was seen in the myocardium in 4 of 7 long-surviving grafts in group 15, while the score was much higher (average grade: 2.3 ± 0.9) in group 14 (Table 1).

MLR proliferation responses

Only groups receiving FK either alone or with donor BMCs were evaluated. This test was performed at 21 days (i.e. 17 days after completion of FK treatment), and 150 days only in animals from group 14 and 15 that had functioning grafts after transplantation. In all groups, regardless of the treatment, MLR responses against third-party LEW stimulator cells were invariably high (Fig. 3). Interestingly, MLR reactivities against donor-specific stimulator cells at 3 weeks after transplantation were almost inversely proportional to allograft survival time. It was highest in animals that received FK alone (group 8) and lowest in animals that were treated with FK and IT + PV injections of donor BMCs (group 15, Fig. 3). This immune modulation by IT + PV injections of donor BMCs persists up to 150 days after transplantation (group 15, Fig. 3).

Discussion

In the present study, we demonstrated a synergistic effect of IT and PV injections of donor BMCs in prolonging the survival of cardiac allografts in a high responder rat strain combination (ACI to WF). Grafts in recipients treated with IT + PV donor BMCs and a short course of FK have lower incidence of acute and chronic rejection (coronary allograft vasculopathy) than grafts of recipients receiving IT, IV, PV, or IT + IV donor BMCs and FK treatment (Fig. 1, Table 1). Donor-specific hyporesponsiveness was also confirmed by *in vitro* MLR assays. Lymphocytes obtained from rats treated with IT + PV injections of donor BMCs and FK were unable to proliferate against donor-specific cells, yet exhibited normal response against third-party stimulator cells at 3 weeks, and at 150 days after transplantation (Fig. 3). One unique feature in the design of this study is that

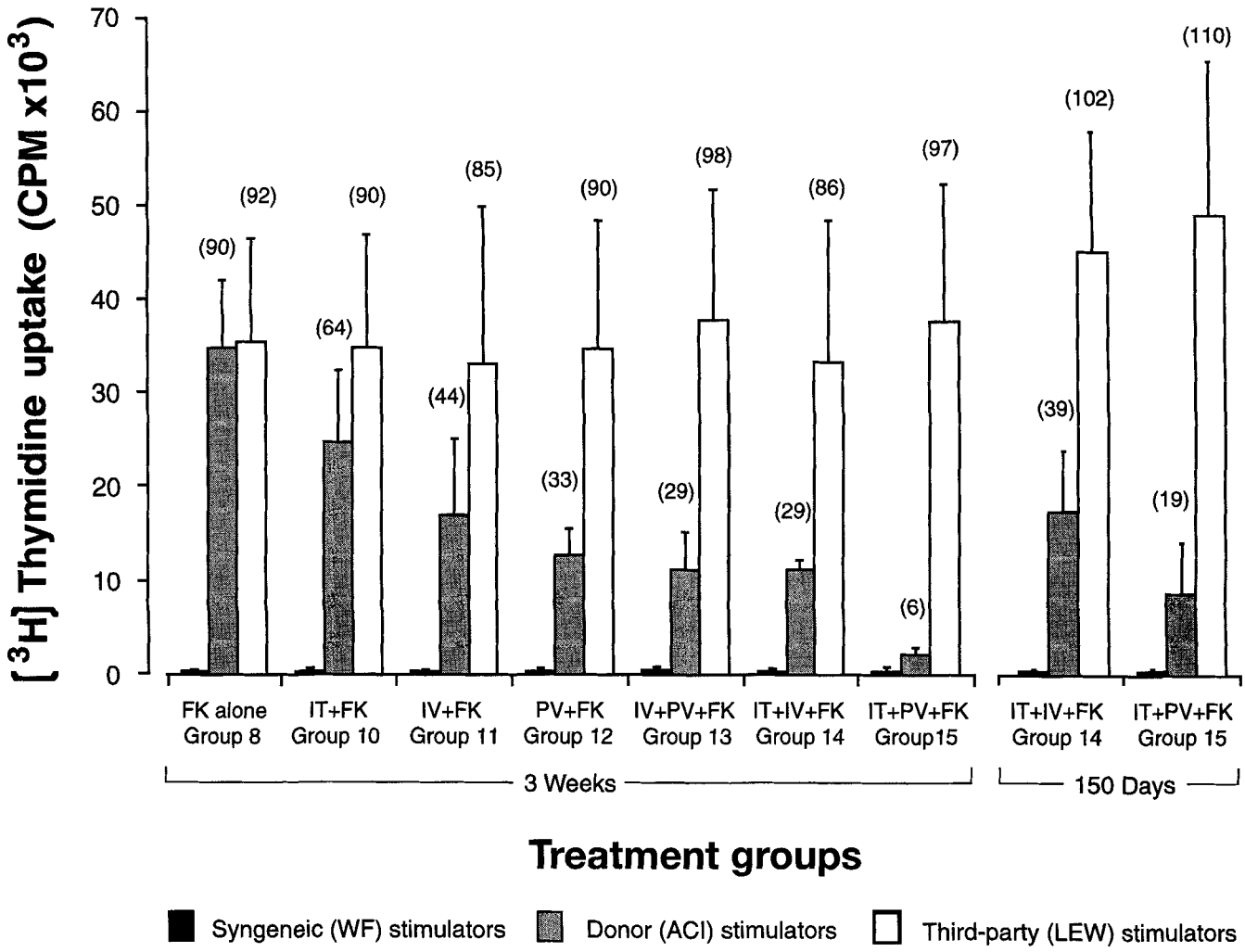


Fig. 3 MLR. Each value represents the mean of three rats tested at 21 or 150 days after cardiac transplantation. In the latter, only animals that received IT + IV or IT + PV donor BMCs with FK were evaluated. Responder leukocytes from WF recipients were co-cultured with irradiated WF, ACI and LEW stimulator cells. Values are expressed as CPM ± standard deviation of triplicate cultures. Stimulation indexes are shown above each bar in *parentheses*

BMCs were administered immediately after cardiac transplantation rather than 1–3 weeks earlier, as other groups have done, because we thought our protocol might be more appropriate for clinical application.

Another important aspect of the present study is that a short course of FK did not inhibit, but promoted long-term acceptance of the cardiac allograft. Although Perico and associates [23] have shown that immunosuppressive agents abrogate unresponsiveness to renal allografts induced by thymic inoculation of donor antigens, several other investigators were able to achieve donor-specific unresponsiveness by using a short course of FK or cyclosporine immediately after thymic inoculation of donor antigens. Matsuura et al. reported that donor- and organ-specific unresponsiveness to Brown Norway (BN) heart allografts was achieved in Lewis (LEW) rats by giving intrathymic donor bone marrow cells and immunosuppression with either ALS or FK at the time of transplantation [15]. Klatter and associates demonstrated that tolerance induction for cardiac allografts could be achieved by simultaneous cardiac transplantation and intrathymic injection of donor splenocytes and treatment with antilymphocyte serum, provided that low doses of cyclosporine were administered perioperatively [14]. It is possible that in our model the short course of FK during the perioperative period is beneficial because it protects both the cardiac allograft and the infused BMCs from alloimmune attacks.

The ACI to WF rat strain combination is known to be a high responder combination in which transplantation tolerance cannot be achieved by IT injection of donor antigens [30]. We and others have demonstrated that donor-specific tolerance can be achieved in this combination with mixed hematopoietic chimerism [3, 6, 7, 8].

However, the morbidities associated with myeloablative conditioning required to achieve mixed chimerism have limited the clinical application of this strategy. IT injection of donor antigens has been advocated as a safer alternative to promote transplantation tolerance [1, 17. 20, 21, 25, 29, 30, 33]. However the success of this strategy has been limited mainly to the "low responder" rat strain combinations in which histocompatibility barriers are weak [1, 33]. Furthermore, the "tolerance" achieved by this thymic approach is not complete because it fails to prevent the development of allograft vasculopathy [31].

Another experimental approach to induce long-term acceptance of organ allografts is the injection of donor antigens into the recipient's portal vein [5, 9, 11, 12, 13, 26, 35, 36]. This strategy takes advantage of the tolerogenic property of the liver. The liver has been considered to play a central role in the acquisition of immune tolerance [4, 10, 27]. It has been demonstrated that the tolerogenic effect of oral feeding is readily abolished upon portacaval transposition [2]. In organ transplantation, it has been shown that in mice and in some rat strain combinations, liver allografts are accepted by the host without the need for immunosuppression while other organs are not [10, 27]. Moreover, other donor-specific, but not third-party, organs that are transplanted into the same host after liver transplantation will be accepted as well [27]. This tolerogenic effect is attributed, in part, to the non-parenchymal components (mainly the dendritic cell progenitors) of the liver graft [28]. This concept is further supported by the fact that infusion of donor cells via PV route results in prolongation of allograft survival in rodents [5, 9, 11, 12, 13, 26, 35, 36]. The immediate draining organ, i.e. the liver in which the injected cells home, likely contributes to the tolerizing effect of PV injection. Kenick et al. [12] demonstrated that most allogeneic lymph node cells infused via PV were trapped within the liver, while very few cells could be detected intrahepatically when IV route was used. The nature of PV injected cells also plays a role in the induction of tolerance. In a study that compared injections of BMCs versus lymph node cells, Wood et al. [34] reported that the former were superior in prolonging allograft survival in mice. One of the mechanisms that may account for this difference is the fact that donor BMCs can suppress the regeneration of donor-reactive CTL [16, 19]. Another explanation may be due to the perpetuation of donor-specific allogeneic BM cells (microchimerism) in the recipient's liver. Zhang et al. [37] reported that in mice that were PV preimmunized with donor splenocytes or BMCs, did not reject the subsequent PV or IV injected donor-specific splenocytes. These investigators reported that the tolerance (assessed by delayed-type hypersensitivity assays) could be maintained for more than 49 days by PV injection plus IV injection (at intervals of 2 weeks) of hematopoietic stem cells (HSCs). The cells responsible for the tolerance induction were found to be HSCs trapped in the liver. These findings indicate that the perpetuation of donor HSCs in the recipient's liver play a crucial role in the induction and maintenance of tolerance. Recently, Morita et al. were able to achieve long-term (> 350 days) survival of MHC- and minor-disparate skin grafts in mice by preimmunizing the recipients with donor splenocytes followed by IV injection of donor bone marrow cells 5 days later [18]. In addition, these investigators also demonstrated a persistence of donor-derived cells, mainly in the recipient's liver, for more than 350 days. The mechanism by which long-term acceptance of skin grafts was achieved in this model is via clonal anergy, as the in vitro data from this study indicated that the unresponsiveness by MLR assay was reversed by interleukin-2.

Collectively, currently available data suggest that, besides the thymus, the liver plays an important role in the acquisition of immune tolerance, and that the perpetuation of allogeneic HSCs in the liver, after portal injection of donor BMCs, is essential for the induction and maintenance of tolerance. It is conceivable that in our experiment, IT injection of donor BMCs may protect the donor BMCs that were injected to the recipient's portal vein, allowing them to perpetuate within the liver, thus promoting graft acceptance. The trafficking of donor bone marrow cells and how the IT + PV injections of donor bone marrow induce prolonged acceptance of cardiac allografts in our model are ongoing studies at our laboratory.

While the mechanism by which IT + PV injections of donor BMCs promote long-term acceptance of cardiac allografts remains to be elucidated, this strategy holds great promises for prolonging graft acceptance in clinical transplantation.

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