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ORIGINAL ARTICLE

Comparison of the effect of bone marrow cells infusion through the portal vein and inferior vena cava combined with short-term rapamycin on allogeneic islet grafts in diabetic rats

Qingzhen Gao*, Xiaoping Wang, Ruibin Zhang, Pu Wang, Yongsheng Jing, Wanjun Ren, Bin Zhu

Department of Nephrology, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong Province, China

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***Correspondence**
Qingzhen Gao
Tel.: +86-0531-8569-5758
Fax: +86-0531-8569-5759
E-mail address: qingzhengaogao@163.com

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ABSTRACT

Aims/Introduction: The study aimed to compare the impact of allogeneic bone marrow cells (BMCs) infusion through the inferior vena cava (IVC) and portal vein (PV) combined with rapamycin on allogeneic islet grafts in diabetic rats.

Materials and Methods: Recipient diabetic Wistar rats were infused with islets from Sprague–Dawley rats through the PV. PKH26-labeled BMCs of Sprague–Dawley rats were infused to recipients through the PV or IVC, followed by administration of rapamycin for 4 days. Blood glucose level was measured to evaluate the survival time of the islets. Lymphocytes separated from blood, BMCs, thymus, liver, spleen and lymph node were analyzed by flow cytometry. The peripheral blood smear, BMCs smear and frozen sections of tissues were observed by a fluorescence microscope.

Results: The survival time of the islets was significantly prolonged by the BMCs infusion combined with rapamycin. The rats receiving BMCs infusion through the PV induced a significantly longer survival time of the islets, and increased mixed chimeras of allogeneic BMCs in the thymus, liver, spleen and lymph node compared with the rats receiving BMCs infusion through the IVC. The amount of the mixed chimeras on day 14 was lower than that on day 7 after islet transplantation. Furthermore, PV transplantation had significantly more mixed chimera than IVC transplantation in all analyzed organs or tissues.

Conclusions: BMCs infusion combined with rapamycin prolongs the islets survival and induces mixed chimeras of BMCs. PV infusion of BMCs might be a more effective strategy than IVC infusion of BMCs.

INTRODUCTION

Diabetes mellitus is a chronic disease, resulting from either a lack of insulin production or resistance to insulin. This disease increases the risk of ischemic heart disease, atherosclerosis and nephropathy, along with various complications, such as blindness, renal failure, cardiovascular disease and amputation[1–4]. It has been considered a major threat to health worldwide, and now affects approximately 25.8 million people of all ages, with the prevalence rising with age[5]. In addition, the global figure is speculated to increase to 380 million people by 2025[6], and 430 million by 2030[7]. Effective therapies mainly include diet, insulin and oral hypoglycemic agents; however, these treatments fail to maintain the normal range of blood glucose levels. Management of diabetes mellitus, therefore, still remains complex and challenging.

In recent years, pancreas transplants or islet transplantation have received increasing attention[8–10]. Pancreas transplants that use cadaveric donor organs could reduce insulin dependence, but bring the risk of chronic immunosuppression, surgical complications and graft rejection[11]. For islet transplantation, islets from donor pancreases are intravenously infused to the recipients[12], which offers a minimally invasive option for β-cell replacement[13].

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This technique requires no surgery, and can make use of the islets isolated from pancreases unsuitable for whole organ transplantation. This treatment achieves better glucose control and substantially improves the quality of life. Successful transplantation of allogeneic islets, however, is difficult because of the strong antigenicity and high sensitivity to graft rejection[5,14]. Despite continuous use of immunosuppressants, the development of acute and chronic rejection still remains a major obstacle[15,16]. Additionally, standard steroid regimens might be poisonous to β-cells[17]. Furthermore, immunological graft loss is one of the obstacles leading to a low success rate of islet grafts[18]. Fortunately, these immunological problems could be conquered by an induction of mixed lymphocyte chimerism. A growing body of evidence has suggested that the donor-specific tolerance induced by bone marrow (BM) chimerism can conquer the problem of rejection[6,13,15,19,20]. Furthermore, portal vein (PV) administration of alloantigens can induce immune tolerance. However, rare studies focus on comparing BM cells (BMCs) infusion through the PV plus short-term rapamycin with BMCs infusion through the inferior vena cava (IVC) plus short-term rapamycin for the survival of allogeneic islet grafts in diabetic rats.

In the present study, recipient diabetic Wistar rats induced by streptozotocin were infused with islets from Sprague–Dawley (SD) rats through the PV, and labeled BMCs from SD rats through the PV or IVC, followed by administration of rapamycin. The results might provide a base for the development of improved treatment of diabetes mellitus.

MATERIALS AND METHODS

Experimental animals

A total of 30 male donor SD rats (age 12 weeks, weighing 240–260 g and specific pathogen-free grade) and 50 male recipient Wistar rats (age 12 weeks, weighing 240–260 g and specific pathogen-free grade) were purchased from the laboratory animal center, Shandong University of Traditional Chinese Medicine, Jinan, China. Before the experiment, all animals were raised in a single clean cage in an air-conditioned room (22 ± 2°C), and were with food and water *ad libitum*. All rats were fasted for 8–12 h before the experiment, but had free access to water. The protocols of animal use complied with the Principles of Laboratory Animal Care (NIH Publication 85–23, revised 1995).

BMCs preparation and PKH26 labeling

Under anesthesia (pentobarbital 30 mg/kg), normal SD rats were immersed in 75% alcohol for 5 min. The rats were then killed by bleeding, and femurs, tibias and humerus were immediately collected to harvest BM. The BM was washed by cold phosphate-buffered saline. Flushing liquor was collected and placed in a sterile Petri dish to prepare cell suspension. Cells were sieved with a 100-μm cell strainer (BD Falcon, Palo Alto, CA, USA). Filtering medium was then put into a 15-mL sterile centrifuge tube, and centrifuged for 2 min at 251.6 *g*. Tris–NH_4Cl solution (5 mL) was added after standing for 5 min, and then the supernatant was discarded. The mixture was washed twice with 10 mL serum-free medium (RPMI-1640; GIBCO/BRL, Grand Island, NY, USA). After washing, the resuspended cells were counted. The isolated BMC (1×10^8) were centrifuged for 2 min at 251.6 *g* and suspended in 1 mL of diluent C. After that, 4×10^{-6} mol/L PKH26 (Sigma Chemical Co., St. Louis, MO, USA) was added and the cells were incubated for 3 min at 25°C. The staining reaction was stopped with serum-supplied medium (10 mL). The cells were then centrifuged at 251.6 *g* for 2 min, and the liquid supernatant was discarded. Furthermore, 10 mL complete medium was added to the mixture that was then centrifuged at 251.6 *g* for another 2 min. After discarding the liquid supernatant, the cell suspension was prepared, with a total volume of 2 mL remaining. The cell suspension was then stored at 4°C until use.

Isolation of pancreatic islets

Pancreatic islets were isolated by a modified method of ductal collagenase distention[21]. Healthy SD rats were anesthetized by intraperitoneal injection with 10% pentobarbital (40 mg/kg), and laparotomy was carried out on the rats. The pancreatic duct close to the intestinal wall was ligated with number 1 silk thread, and the common bile duct was inserted with a scalp needle (5-G) and ligated. The rats were then killed by bleeding. Collagenase type V (6 mL; Sigma Chemical Co.) was injected to distend the pancreas. After that, the pancreas was harvested and mixed with 6 mL of Hanks balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA), followed by a water bath at 37°C for 15–20 min.

The pancreatic tissue was filtered with 600 μm stainless steel wire mesh and then washed twice. The cell suspension was centrifuged at 111.8 *g* (4°C) for 1–2 min, and the liquid supernatant was discarded. The islets were purified by discontinuous 25% Ficoll-400 (Sigma-Aldrich) density gradient (23, 20 and 11%), and centrifuged at 1006.2 *g* for 20 min at 4°C. The islets in an interface of 23–20 and 20–11% were moved to 4°C Hank's solution, washed and then centrifuged twice. After centrifugation, the isolated islets were harvested with a pipette, stained with dithizone (Sigma-Aldrich) and then counted. The number of islets was assessed using an optical graticule attached to the eyepiece of a dissecting microscope and then converted to the standard islet equivalent, which was calculated based on volumetric assumptions.

Pharmacological induction of diabetes

Diabetes was induced in healthy Wistar rats by a single intraperitoneal injection of streptozotocin (Sigma), which was dissolved in 0.1 mol/L citrate buffer (pH 4.5) at a dose of 60 mg/kg bodyweight. After 1 week, diabetes was verified by measurement of tail vein blood glucose levels using blood glucose test strips (SanNuo, ChangSha, China). Diabetic rats were considered successful when fasting blood glucose level was >14 mmol/L. Finally, diabetes was successfully induced in 51 rats, 21 of which had glucose levels ≥20 mmol/L.

Islet transplantation and BMCs infusion

For BMCs infusion and islet transplantation, the abdominal cavity of rats under general anesthesia was opened on the abdominal median line, and the PV and IVC were exposed. Islet transplantation (number 800 each rat) was randomly carried out from a single donor to a single recipient through the PV. Based on treatments, the 51 recipient diabetic rats were randomly divided into three groups ($n = 17$ in each group): control group, IVC group and PV group.

For the control group, islets were injected into the PV, but without BMC; whereas in the IVC group, islets were injected into the PV and the BMCs labeled with PKH26 (0.5 mL, 5×10^7) were injected into the IVC; whereas in the PV group, both islets and the PKH26-labeled BMCs (0.5 mL, 5×10^7) were injected to the PV (Table 1). A 6–0 atraumatic suture was placed around the PV and IVC. Intraperitoneal injection of penicillin (100,000 U) was given to the rats in each group. Then the incisions were sutured and the rats were kept under supervision. Immediately after the infusion of islets and BMCs, the rats of each group received an intraperitoneal injection of rapamycin (0.4 mg/kg; North China pharmaceutical Group Corporation, Shijiazhuang, China) for 4 consecutive days.

Evaluation of survival time of islets and criteria for rejection

Tail vein blood glucose was evaluated daily from the second day after the transplantation of islets. The continuous level of random blood glucose <11.2 mmol/L was considered as the criteria for survival of islets. Graft rejection was considered when the random blood glucose was ≥11.2 mmol/L for ≥3 consecutive days.

Distribution and chimerism features of allogeneic BMCs in different organs

Under anesthesia, five recipient rats in every group were randomly selected from each group and killed on day 7 after islet transplantation. Another five rats were randomly selected from each group and killed on day 14. The peripheral blood, BM, thymus, liver, spleen and para-aortic lymph node were collected. The peripheral blood was layered with lymphocyte separation medium (Lympholyte-Mammal; Cedarlane Laboratories Ltd, Hornby, Ontario, Canada), and the BM was prepared for BMC suspension, respectively. Then percoll (GE Healthcare, Piscataway, NJ, USA) medium was used to isolate lymphocytes from the thymus, spleen and para-aortic lymph node. Ficoll medium was used to isolate lymphocytes from the liver. After isolation, the mixture was centrifuged at 1006.2 *g* for 20 min. The lymphocyte liquid was selected, washed and counted. Flow cytometry (Beckman Coulter Epics XL, Krefeld, Germany) was used to assess the chimerism features of allogeneic BMCs in the aforementioned organs with an excitation wavelength of 551 nmol/L and an emission wavelength of 567 nmol/L.

In addition, the peripheral blood smear, the BMCs smear, and frozen sections (6 μm) of the liver, spleen, thymus and paraaortic lymph node were observed by a FSX100 fluorescence microscope (FM; Olympus Corporation, Tokyo, Japan) to determine distribution of allogeneic BMCs. Fluorescence images and phase-contrast images were collected under the same field. Hematoxylin–eosin staining was also applied after microscope.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analyses were carried out using SPSS 13.0 for windows (SPSS, Chicago, IL, USA). Student's *t*-test was used for comparison of means between two groups. The χ^2-test was used for comparison of percentages (%) between two groups. A *P*-value <0.05 was considered statistically significant.

RESULTS

Measurement of blood glucose and survival time of islets

The blood glucose levels of all rats were monitored every day. As shown in Figure 1a, a sharp decrease in the blood glucose level was observed on the second day after islet transplantation in all groups. The difference in the blood glucose level was insignificant between the three groups in the first 3 weeks after islets transplantation ($P > 0.05$). The blood glucose levels were significantly more decreased in the IVC and PV group than that in the control group from week 4 to week 6 ($P < 0.05$). The PV group had a significantly lower blood glucose level than the IVC group from week 5 to week 8 ($P < 0.05$).

The survival time of the islets of different groups is shown in Figure 1b. The survival time of the islets of the IVC and PV groups (IVC 35.3 ± 7.4 days, PV 56.2 ± 10.3 days) was significantly longer compared with that of the control group (20.3 ± 3.6 days, $P < 0.05$). Furthermore, the PV group had a markedly longer survival time of the islets than the IVC group ($P < 0.05$).

Chimerism features of allogeneic BMCs

The results of flow cytometry showed that the level of mixed chimeras of allogeneic BMCs in the peripheral blood and BM

Table 1 | Design of animal experiments

Groups	Number of diabetic rats	Rat age	Injection of islets	Dose of BMCs	BMCs transplantation route	Rapamycin
Control group	17	12 week	800 islets; PV	No	No	0.4 mg/kg; 4 days
PV group	17	12 week	800 islets; PV	5×10^7 (0.5 mL)	PV	0.4 mg/kg; 4 days
IVC group	17	12 week	800 islets; PV	5×10^7 (0.5 mL)	IVC	0.4 mg/kg; 4 days

PV, portal vein; IVC, inferior vena cava; BMCs, bone marrow cells.

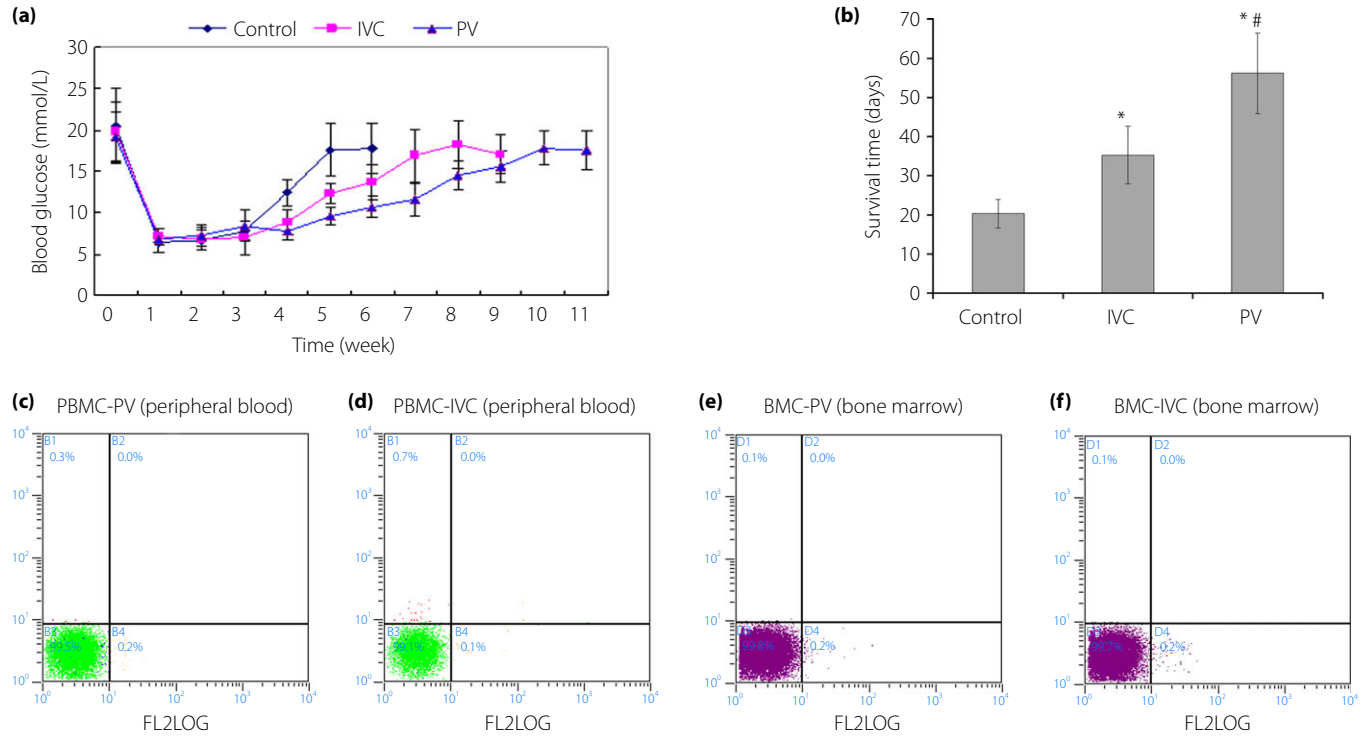

Figure 1 | Survival time of islets and mixed chimeras of bone marrow cells (BMCs) in the peripheral blood and BM. (a) Blood glucose level of all rats in different groups. (b) Survival time of islets in different groups. (c) Mixed chimeras of BMCs in the peripheral blood (PBMC) of rats receiving an infusion of BMCs through the portal vein (PV). (d) Mixed chimeras of BMCs in the PBMC of rats receiving an infusion of BMCs through the inferior vena cava (IVC). (e) Mixed chimeras of BMCs in the BM of rats receiving an infusion of BMCs through the PV. (f) Mixed chimeras of BMCs in the BM of rats receiving an infusion of BMCs through the IVC. *$P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the IVC group.

was very low (0.1–0.2%) in rats of both PV and IVC groups on day 7 and day 14 after allogeneic BMCs infusion (Figure 1c–f). Similarly, PKH26-labeled cells in the peripheral blood and BM were not found under a FM.

The percentage of mixed chimeras of allogeneic BMCs was remarkably higher in the thymus than those in the spleen, liver, and lymph node in both the PV and IVC groups on day 7 ($P < 0.001$; Table 2). The difference in the percentage of mixed chimeras was not significant among the thymus, spleen, liver and lymph node in the PV group on the day 14 ($P > 0.05$), whereas the percentages of the mixed chimeras were significantly higher in the thymus, spleen and liver than that in the lymph node in the IVC group on day 14 ($P < 0.001$).

In the thymus, the percentage of mixed chimeras in the PV group was significantly elevated than that in the IVC group on day 7 ($12.6 \pm 3.5\%$ vs $3.0 \pm 0.8\%$, $P < 0.01$) and day 14 ($3.0 \pm 0.8\%$ vs $1.1 \pm 0.3\%$, $P < 0.01$). The percentage of mixed chimeras in the thymus on day 14 was significantly decreased than that on day 7 in both the PV and IVC groups ($P < 0.01$; Table 2). Consistent changes of chimerism of allogeneic BMCs in the thymus were observed under a FM (Figure 2a–d). There were more PKH26-labeled cells in the PV group than that in the IVC group both on day 7 and day 14. The PKH26-labeled cells were more decreased on day 14 than that on day 7.

Table 2 | Percentage of mixed chimerism of the labeled BMCs in different lymphoid organs

Tissue	Day 7		Day 14	
	PV (%)	IVC (%)	PV (%)	IVC (%)
Thymus	12.6 ± 3.5	8.5 ± 1.5*	3.0 ± 0.8*	$1.1 \pm 0.3^{\#\$}$
Spleen	5.5 ± 1.2	2.1 ± 0.6*	2.4 ± 0.6*	$0.9 \pm 0.2^{\#\$}$
Lymph node	3.4 ± 1.1	1.9 ± 0.4*	2.1 ± 0.8*	$0.2 \pm 0.07^{\#\$}$
Liver	7.1 ± 1.9	2.7 ± 0.6*	2.0 ± 0.8*	$0.7 \pm 0.1^{\#\$}$

PV, portal vein; IVC, inferior vena cava; BMCs, bone marrow cells. *$P < 0.01$ compared with PV on day 7; $^{\#}P < 0.01$ compared with PV on day 14; $^{\$}P < 0.01$ compared with IVC on day 7.

Similarly, the PV group had a significantly higher percentage of mixed chimeras in the spleen than the IVC group on day 7 ($5.5 \pm 1.2\%$ vs $2.1 \pm 0.6\%$, $P < 0.01$) and day 14 ($2.4 \pm 0.6\%$ vs $0.9 \pm 0.2\%$, $P < 0.01$). The percentage of mixed chimeras in the spleen was more markedly decreased on day 14 than that on day 7 in both the PV and IVC groups ($P < 0.01$; Table 2). Consistent changes of chimerism of allogeneic BMCs in the spleen were found under a FM (Figure 2e–h). The PKH26-labeled cells were more increased in the PV group than that in

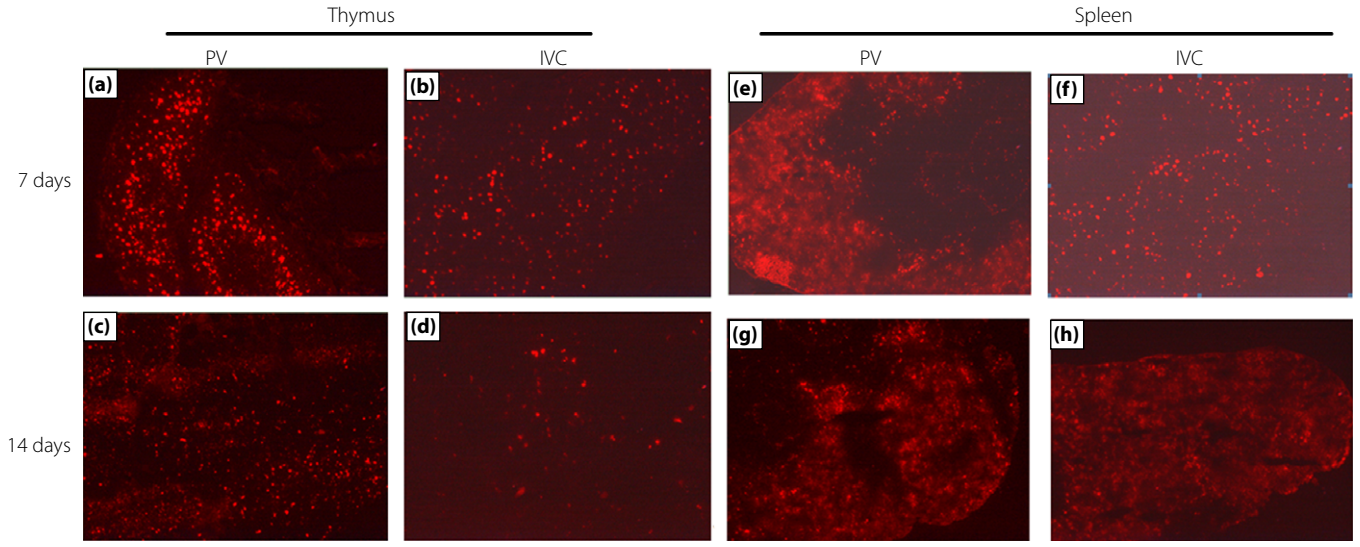

Figure 2 | Mixed chimeras of allogeneic bone marrow cells (BMCs) in the thymus and spleen. (a) Mixed chimeras of BMCs in the thymus of rats receiving an infusion of BMCs through the portal vein (PV) on day 7 after islets transplantation. (b) Mixed chimeras of BMCs in the thymus of rats receiving an infusion of BMCs through the inferior vena cava (IVC) on day 7. (c) Mixed chimeras of BMCs in the thymus of rats receiving an infusion of BMCs through the PV on day 14. (d) Mixed chimeras of BMCs in the thymus of rats receiving an infusion of BMCs through the IVC on day 14. (e) Mixed chimeras of BMCs in the spleen of rats receiving an infusion of BMCs through the PV on day 7. (f) Mixed chimeras of BMCs in the spleen of rats receiving an infusion of BMCs through the IVC on day 7. (g) Mixed chimeras of BMCs in the spleen of rats receiving an infusion of BMCs through the PV on day 14. (h) Mixed chimeras of BMCs in the spleen of rats receiving an infusion of BMCs through the IVC on day 14.

the IVC group, and more decreased on day 14 than that on day 7.

Likewise, mixed chimeras of allogeneic BMCs in the liver and lymph node showed similar changes to those found in the thymus and spleen (Table 2 and Figure 3). These results showed that the mixed chimeras in the thymus, spleen, liver and lymph node on day 7 was remarkably higher than that on day 14 after BMCs infusion ($P < 0.01$). BMCs infusion through the PV caused a higher percentage of mixed chimeras compared with BMCs infusion through the IVC.

Distribution characteristics of PKH26-labeled BMCs in different organs

Distribution of PKH26-labeled BMCs in the thymus, spleen, lymph node and liver are presented by fluorescence images, phase-contrast images and hematoxylin–eosin stain images in Figure 4. With regard to the thymus (Figure 4a–c), PKH26-labeled BMCs were mainly distributed in the thymic cortex, and rarely distributed in the inner cortex. The PKH26-labeled cells in the outer cortex were bigger than those in the inner cortex. The cells were distributed around the medulla of the cortex and were rarely observed in the medulla. In the spleen (Figure 4d–f), the mixed chimera was primarily distributed in the red pulp parts, and rarely located in the white pulp. Furthermore, PKH26-labeled BMCs were also observed around the periarterial lymphatic sheath in the white pulp parts.

With reference to the lymph node, there were few PKH26-labeled BMCs in the peripheral cortex. Big labeled cells were presented in the paracortical area and medullary cord (Figure 4g–i). Additionally, PKH26-labeled BMCs were predominately distributed in the portal area of the liver, and just a few cells were observed among the hepatic plates (Figure 4j–l).

These findings showed that the donor islets in rats of the PV group had a markedly longer survival time of than that of the rats of the IVC group. There were significantly higher percentages of mixed chimeras of allogeneic BMCs in the thymus, spleen, liver and lymph node of the rats receiving BMCs infusion through the PV compared with the rats receiving BMCs infusion through the IVC. The increased mixed chimeras of BMCs in the thymus, spleen, liver and lymph node might contribute to a longer tolerance to the islets, thus prolonging the survival time of the islets. PV infusion of BMCs might be more effective in inducing tolerance to donor islets in diabetic rats than IVC infusion of BMCs.

DISCUSSION

In the present study, PV and IVC administration of BMCs combined with short-term use of rapamycin were compared for the survival of allogeneic islet grafts, and the percentage and distribution of mixed chimera of BMCs in diabetic rats. The results showed that the survival time of the islets was prolonged by the combination of rapamycin and BMCs. PV infusion of BMCs resulted in a markedly longer survival time of the islets than IVC infusion of BMCs. PV infusion of BMCs induced a significantly elevated percentage of mixed chimeras of BMCs in the thymus, spleen, liver and lymph node than IVC infusion of

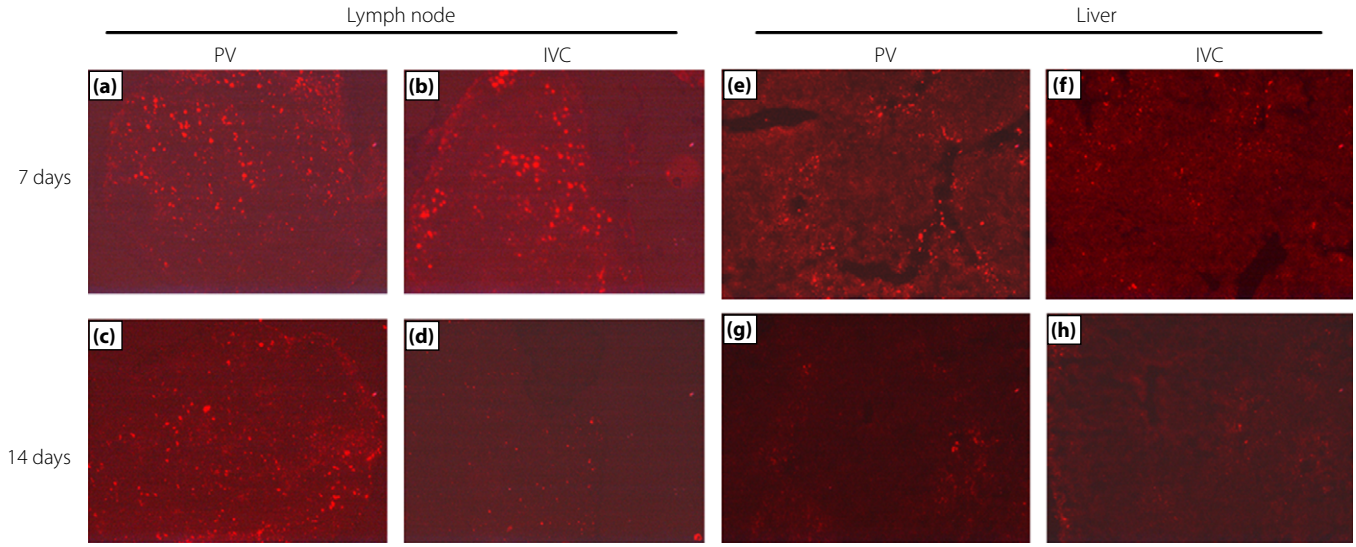

Figure 3 | Mixed chimeras of allogeneic bone marrow cells (BMCs) in the lymph node and liver through the portal vein (PV) and inferior vena cava (IVC). (a) Mixed chimeras of BMCs in the lymph node of rats receiving an infusion of BMCs through the PV on day 7. (b) Mixed chimeras of BMCs in the lymph node of rats receiving an infusion of BMCs through the IVC on day 7. (c) Mixed chimeras of BMCs in the lymph node of rats receiving an infusion of BMCs through the PV on day 14. (d) Mixed chimeras of BMCs in the lymph node of rats receiving an infusion of BMCs through the IVC on day 14. (e) Mixed chimeras of BMCs in the liver of rats receiving an infusion of BMCs through the PV on day 7. (f) Mixed chimeras of BMCs in the liver of rats receiving an infusion of BMCs through the IVC on day 7. (g) Mixed chimeras of BMCs in the liver of rats receiving an infusion of BMCs through the PV on day 14. (h) Mixed chimeras of BMCs in the liver of rats receiving an infusion of BMCs through the IVC on day 14.

BMCs. Transplantation of the isolated islets is technically a more simple method than whole pancreatic transplantation. Islets transplantation through the PV drainage is a more physiological approach than systemic drainage. In addition, previous studies have shown that the PV administration of allogeneic cells could induce donor-specific tolerance[22], and that the BMCs infusion through PV followed by transient use of an immunosuppressant could induce persistent tolerance[23]. Similarly, the isolated islets were injected to recipient rats by intraportal administration in the present study. Rapamycin, an immunosuppressant, was also administrated for 4 days after islets transplantation.

Mitsuoka *et al.*[23] found that portal infusion with donor-derived BMCs in rat small bowel transplantation in combination with tacrolimus decreased graft- infiltrating lymphocytes and enhanced Th2-type response in small bowel transplantation grafts. Luo *et al.*[24] found that BMCs could increase islet survival and function with eventual formation of pancreatic endocrine tissue capable of sustaining β-cell function. In line with the aforementioned studies, the present results showed that the survival time of the islets in the rats receiving BMCs infusion was significantly prolonged compared with the rats without BMCs infusion. One potential mechanism might be that BMCs could reduce the release of interleukin-1β by islets, thus the apoptotic process in cultured islets is abridged[13,24]. Furthermore, it has been suggested that BMCs transplantation might improve the function of injured pancreatic tissue by promoting the transfer of very small embryonic-like cells[25]. The anti-oxidant function of BMCs has been found in a mouse model of type 2 diabetes by upregulated Sirt1 and HO1[26]. Additionally, BMCs transplantation is closely related to inflammation[27]. These findings provide more insights regarding possible mechanisms of BMCs. Furthermore, in the current study, the survival time of the islets in the rats receiving BMCs infusion through the PV was much longer than the survival time of the islets in the rats receiving BMCs infusion through the IVC. The results showed that PV administration might be a more effective method than IVC administration for improving the therapeutic efficacy of islet transplantation in diabetic rats.

The fluorescent dye, PKH26, was used in the present study to identify the BMCs in the recipients. PKH26 is a cell-membrane dye, which sends out red fluorescence. In the present study, the BMCs were efficiently labeled in recipients without leaking out or transferring to other cells. Furthermore, the PKH26-labeled BMCs in recipients were mainly concentrated in the thymus, followed by the spleen, lymph node and liver. Immune tolerance might contribute to the results. However, the mechanism of tolerance induction by BMCs infusion is not clear. An alternative explanation was the clonal deletion theory of tolerance induction. This theory states that BMCs contain a large number of hematopoietic stem cells and immature dendritic cells. These cells will enter into the thymus by intravenous infusion and produce specific cell subsets, namely dendritic cells that are effective mediators in the thymus (clonal

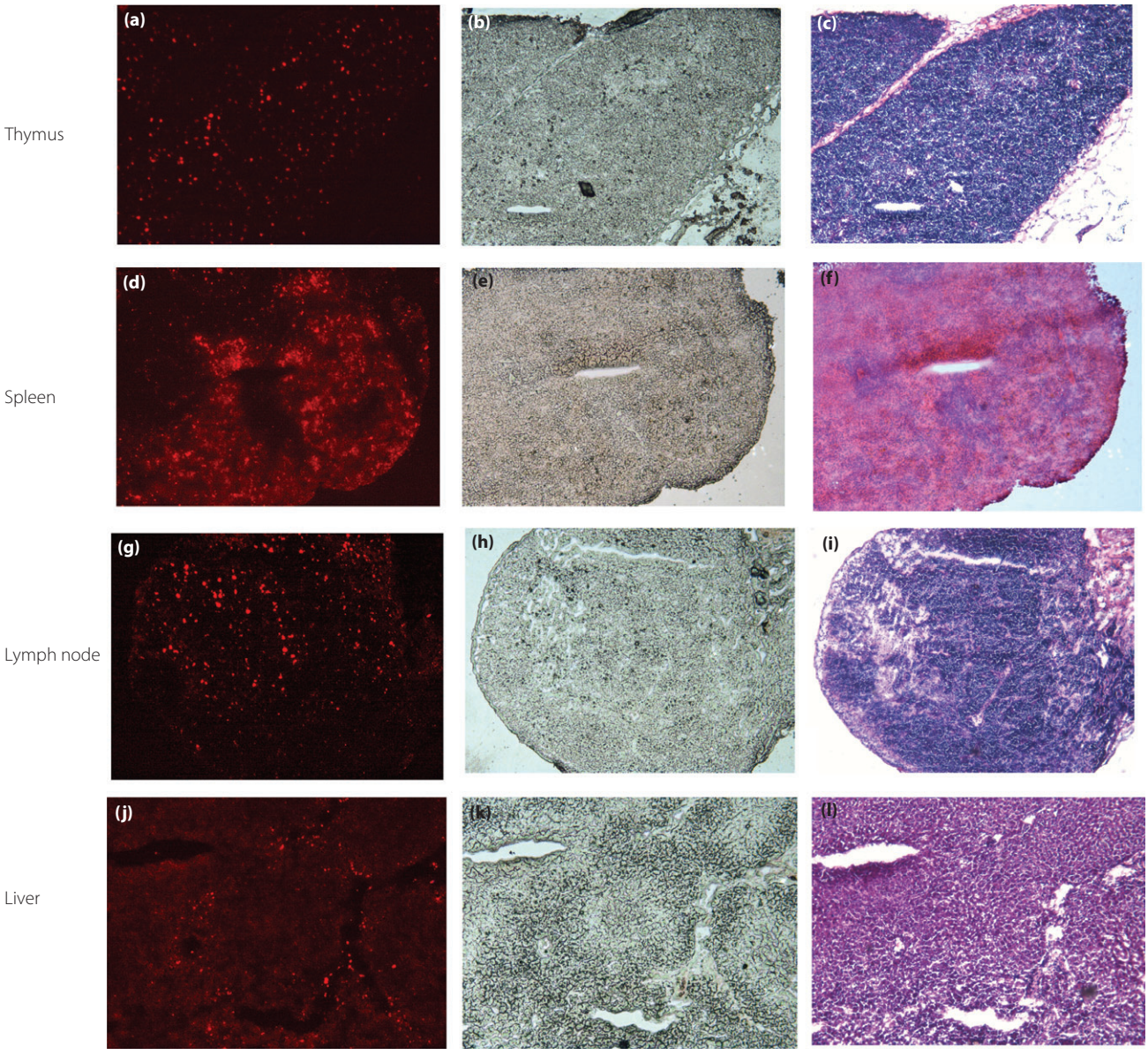

Figure 4 | Distribution of allogeneic bone marrow cells (BMCs) in different organs under the same field (scale bar, 50 μm). (a) Distribution of allogeneic BMCs in the thymus of fluorescence images. (b) Distribution of allogeneic BMCs in the thymus of phase-contrast images. (c) Distribution of allogeneic BMCs in the thymus of hematoxylin–eosin (HE) stain images. (d) Distribution of allogeneic BMCs in the spleen of fluorescence images. (e) Distribution of allogeneic BMCs in the spleen of phase-contrast images. (f) Distribution of allogeneic BMCs in the spleen of HE stain images. (g) Distribution of allogeneic BMCs in the lymph node of fluorescence images. (h) Distribution of allogeneic BMCs in the lymph node of phase-contrast images. (i) Distribution of allogeneic BMCs in the lymph node of HE stain images. (j) Distribution of allogeneic BMCs in the liver of fluorescence images. (k) Distribution of allogeneic BMCs in the liver of phase-contrast images. (l) Distribution of allogeneic BMCs in the liver of HE stain images.

deletion)[28]. In addition, the percentage of mixed chimeras of allogeneic BMCs was significantly reduced on day 14 than that on day 7. One possible reason is that more BMCs are broken down as the time passes.

In the present study, the PKH26-labeled cells mainly gathered in the thymus, indicating that the BMCs primarily underwent development and differentiation in the thymus after allogeneic BMCs infusion. In addition, the BMCs mainly distributed in the thymic cortex, and the size of labeled cells in the outer cortex was bigger than the cells in the inner cortex. The distribution of the labeled cells was consistent with the differentiation and development of lymphocytes in the thymus[29]. Therefore, the BMCs from donors and the thymus cells presented coexistence and mutual chimera, and both of them

underwent differentiation and maturation. During the process of differentiation and maturation in thymus cells, negative selection of T cells was inducted. The mature T cells in recipients finally produced specific immune tolerance to the donors. Thus, it was speculated that the survival time of the islets was prolonged mainly as a result of the mixed chimerism induced by BMCs infusion. Similarly, there is evidence that BM chimerism induces donor-specific tolerance, and suppresses allograft rejection[30]. A stable level of chimerism is a marker of transplantation tolerance[31]. In the current study, a significantly elevated percentage of mixed chimeras of BMCs was found in the rats receiving PV infusion of BMCs in comparison with the rats receiving IVC infusion of BMCs. PV infusion of BMCs might prolong the survival time of islets by increasing the mixed chimeras of allogenic BMCs.

The spleen is an organ for lymphocytes' settlement and removal. We found that the labeled cells were mainly distributed in the red pulp parts. The observation showed that the BMCs were removed in the red pulp parts. Furthermore, there were few labeled cells in the white pulp parts, which might be relatively mature cells. However, the characteristics and functions of these cells need to be investigated in further research. In addition, we found that there were few labeled cells in the peripheral cortex of the lymph node, and the size of labeled cells presented in the paracortical areas and medullary cord was larger. The results showed that the BMCs underwent coexistence and mutual chimera with the B cell of the recipients. Therefore, the BMCs might not only play a part in negative selection of T cells, but also in negative selection of B cells. In the liver species, the labeled cells were centered on the portal area, whereas just a few labeled cells were distributed among the hepatic plates. The labeled cells might be hepatic sinusoidal endothelial cells, Kupffer cells or large granular lymphocytes produced by allogeneic BMC[32], but others hold a conflicting view[33].

In addition to the BMCs infusion, chimerism might be affected by other causes. It has been shown that the dose of cells, conditioning regimen and application of chemotherapy were associated with the kinetics of chimerism in recipients of allogeneic hematopoietic cell transplantation[34]. Similarly, Sugita *et al.*[35] found that conditioning regimens and stem cells sources might influence donor-type chimerism. This was a preliminary study. More experiments are required to validate and expand the results. Hypertrophic and fibrotic changes have been observed in the islets tissue transplanted into the liver of type 2 diabetic rats[36]. Thus, histological analysis of islet grafts in the liver is necessary to evaluate whether changes of islet histology are in accordance with blood glucose levels and survival time of islets. The underlying mechanism of BMCs infusion in diabetic rabbit models is one primary direction of our future studies. Insulin-producing cells derived from BM have been used for treatment of type 1 diabetes[37]. Furthermore, it has been reported that BM-derived stem cells could induce proliferation of pancreatic cells[20,38]. Histological analysis of the pancreas should also be carried out to determine whether BMC infusion induced pancreatic islet regeneration in the present study.

In conclusion, the combination of allogeneic BMCs infusion and short-term use of rapamycin elongates the survival time of islets, and induces mixed chimeras of allogenic BMCs and decreased rejection response. In addition, PV infusion of BMCs might be a more effective strategy than IVC infusion of BMCs for improving the therapeutic effect of islet transplantation on diabetic rats.

DISCLOSURE

The authors declare no conflict of interest.

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