public health issues, these attempts yielded important information about modifying donor characteristics, including intensive pretreatment of transgenic (Tg) donors [9].

Donor bone marrow-derived cells (BMDCs) persist in human transplant recipients with functioning allografts; this is referred to as microchimerism [10]. Stable chimerism in a liver transplant that overcomes immunological rejection may enhance the feasibility of xenotransplantation. For example, compared with xenogeneic liver grafts, engineered chimeric livers comprising autologous hepatocytes and a xenogeneic scaffold reduce rejection [11]. However, rejection was still strong, suggesting that replacement of additional nonhepatocyte components may be necessary for the successful development of less immunogenic liver grafts.

Bone marrow cells (BMCs) recruited into the liver become functioning hepatocytes, endothelial cells (ECs), Kupffer cells (KCs), oval cells, stromal cells, or cholangiocytes [12–18]. We hypothesized that transplanting prospective donors with BMCs isolated from prospective recipients would enhance graft tolerance by replacing donor liver components. Therefore, in the present study, we used a rat model of LT and preoperatively transplanted

recipient BMCs into allogeneic liver transplant donors to evaluate whether this strategy reduced damage to liver grafts.

Material and methods

Animals

Wild-type male Lewis (LEW) ($RT1^{l}$) and Dark Agouti (DA) ($RT1^{ab}$) rats were purchased from Charles River Japan (Yokohama, Kanagawa, Japan) and Japan SLC (Hamamatsu, Shizuoka, Japan), respectively. LacZ-Tg LEW and green fluorescence protein transgenic LEW (GFP-Tg LEW) rats were kindly provided by E. Kobayashi (Division of Organ Replacement Research, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Tochigi, Japan) [19]. The LacZ-Tg and GFP-Tg LEW rats (age, 8–14 weeks) served as BMC donors, DA rats (age, 10–12 weeks) served as BMC recipients and liver transplant donors, and LEW rats (age, 7–9 weeks) served as liver transplant recipients.

All animals were housed in a specific pathogen-free animal facility at Kyoto University under the following conditions: 50% ± 10% relative humidity, 12 h/12 h light-dark cycle, and 24°C ± 2°C. Rats were fed a standard diet and tap water *ad libitum*. Animal handling and care met the institutional guidelines for animal welfare. The institutional ethics committee of Kyoto University (Medkyo-13290) approved the experimental protocol, which met the ethical guidelines of the Declaration of Helsinki.

Study design

The study design is summarized in Fig. 1. We divided the rats into two groups as follows: bone marrow-cell transplanted (BMC) and control (CTR). In the BMC group, liver transplant donors (DA rats) underwent total body irradiation (TBI) and received BMCs from LacZ-Tg LEW rats the next day. Six days later, the LEW rats received whole-liver grafts. These rats were not treated with immunosuppressants after BMC transplantation or LT. In the CTR group, LEW rats received whole-liver grafts from untreated DA rats. We monitored

the survival of five recipients each in the BMC and CTR groups. We sacrificed five rats from each group at 7 d after LT and conducted serological and histopathological analyses.

Bone marrow extraction and BMC transplantation

BMCs were obtained from LacZ-Tg LEW rats by flushing their tibias and femurs with phosphate-buffered saline (PBS). Unfractionated BMCs (4×10^7 cells per rat) with >90% viability determined using the trypan blue dye exclusion assay were intravenously injected into DA rats at 1 d after they were irradiated with 10 Gy TBI (Gammacell® 40 Exactor; Best Theratronics, Ltd., Ottawa, Canada). To confirm BMC engraftment, GFP-Tg LEW rats were used as BMC donors, and 6 d after BMC transplantation, the numbers of GFP-positive peripheral blood mononuclear cells (PBMCs) were determined using flow cytometry (BD Accuri™ C6 Flow Cytometer; Becton Dickinson Japan, Akasaka, Tokyo, Japan). Of these PBMCs, 62.0% expressed GFP, indicating that BMCs were effectively engrafted (Supplementary figure).

Surgical procedures for orthotopic LT

Rat LT without hepatic arterial reconstruction is an established and widely used model [20]. The detailed surgical procedures and postoperative care for rats undergoing LT have been described [21–23]. Liver grafts were maintained in histidine-tryptophan-ketoglutarate solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) at 4°C for 90 min. The anhepatic time for all grafts was less than 20 min [21].

Histopathological assessments and biochemical profiles

Liver graft samples were obtained by perfusing the abdominal aorta with 30 mL of PBS followed by 50 mL of 4% paraformaldehyde. A section of the middle lobe of the liver was excised before perfusion with 4% paraformaldehyde and was frozen in liquid nitrogen for analysis of messenger RNA (mRNA) expression levels. A part of each liver graft was embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen in liquid nitrogen. The remaining material was fixed overnight in 4%

paraformaldehyde, embedded in paraffin, and sectioned. The sections were stained with hematoxylin-eosin (HE), and acute allograft rejection was scored using the rejection activity index according to the Banff criteria of hepatic allograft pathology [24]. The graft damage score, modified from initial quantitative scores [24,25], is described elsewhere [23]. Scores were evaluated in ten random fields (magnification, ×100) per slide and averaged for each slide. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations and total bilirubin (T-Bil) levels were measured as standard indicators of hepatocyte injury, and serum hyaluronic acid (HA) concentrations were measured as an indicator of injury to liver sinusoidal ECs (LSECs).

Real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from liver tissues using an RNA isolation reagent (TRIzol; Invitrogen, Tokyo, Japan). The amount of RNA was measured spectrophotometrically (NanoDrop ND-2000c; NanoDrop Technologies, Wilmington, DE). Messenger RNA expression was quantified using TaqMan two-step qRT-PCR with primers for the genes

encoding interleukin-2 (IL-2), interleukin-10 (IL-10), interferon-γ (IFN-γ), transforming growth factor β1 (TGF-β1), and β-actin (ACTB). Total RNA was reverse transcribed to complementary DNA (Omniscript RT kit; Qiagen, Valencia, CA, USA), and the abundance of target genes normalized to the level of *Actb* was determined using a standard curve. The primers used for PCR were purchased as an Applied Biosystems Assays-on-Demand™ product (Nieuwekerk a/d IJssel, The Netherlands) and were as follows: *Il-2* (Rn00587673_m1), *Il-10* (Rn00563409_m1), *Ifn-γ* (Rn00594078_m1), and *Tgf-β1* (Rn00572010_m1). *Actb* (4352931E, Applied Biosystems) served as an internal control.

Immunohistological analyses

Frozen samples were cut into 6-μm-thick sections and assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) as described previously [11, 26]. Briefly, frozen sections were fixed with 0.2% glutaraldehyde (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2 mM $MgCl_2$ (Wako), and 5 mM EGTA (Dojindo Molecular Technologies, Inc., Kamimashiki, Kumamoto, Japan) for 5 min at room temperature and

washed in 2 mM $MgCl_2$, 0.01% sodium deoxycholate (Wako), and 0.02% NP40 substitute (Wako). Sections were then incubated with X-gal staining solution containing 1 mg mL^{-1} X-gal (Takara Bio Inc., Otsu, Shiga, Japan), 2 mM $MgCl_2$, 5 mM potassium ferrocyanide (Wako), and 5 mM potassium ferricyanide (Wako). The sections were washed and counterstained with Contrast Red Solution (Kirkegaard & Perry Laboratories, Inc., Washington, D.C., USA).

X-gal-positive cells were characterized by simultaneous X-gal and immunohistochemical staining using the Catalyzed Signal Amplification System (K150011; Dako, Carpinteria, CA, USA) with minor modifications of the manufacturer's protocol. Briefly, 6-μm-thick frozen sections were stained with X-gal without fixation and incubated overnight at 4°C with a 1:100 dilution of a monoclonal antibody against the vascular and sinusoidal endothelium marker CD31 (PECAM-1, BD PharMingen; BD Biosciences San Jose, CA, USA) and then incubated with Alexa Fluor® 488-conjugated donkey anti-mouse antibody (Invitrogen, Carlsbad, CA, USA). The sections were boiled in sodium citrate buffer (pH 6.0) for 20 min to remove all immunological reagents and then incubated with a 1:100 dilution of a monoclonal antibody against the KC marker CD68 (FA-11; Abcam, Cambridge, UK) or CD163 (ED2;

AbD Serotec, Kidlington, UK), and Alexa Fluor® 568-conjugated donkey anti-mouse antibody (Invitrogen),. Sections of at least three portal tracts and perivenular areas were analyzed using confocal laser-scanning microscopy (BX50 and AX80TR; Olympus, Tokyo, Japan) (magnification ×400).

Survival of liver transplant donor rats treated with gadolinium chloride ($GdCl_3$)

To evaluate the effects of inhibiting KC function, liver transplant donor rats that received preoperative BMC transplantation were intraperitoneally administered 10 mg/kg body weight $GdCl_3$ (Wako) at 24 h before LT.

Statistical analysis

Results have been expressed as the mean ± standard deviation values in the following order: BMC group vs CTR group. Differences in unpaired discontinuous and continuous variables between the two groups were analyzed using the Student *t* test. Survival curves were

calculated using the Kaplan-Meier method and compared using the log-rank test. All statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

Results

Survival analysis

The survival rate of rats in the BMC group was significantly higher than that of the CTR group ($P = 0.0027$) (Fig. 2A). Treating rats with $GdCl_3$ abrogated prolonged survival of the BMC group after LT ($P = 0.2416$) (Fig. 2B).

Histopathological analysis

HE-stained sections of CTR and BMC rat tissues are shown in Fig. 3A–F (Fig. 3 A–C, CTR; Fig. 3D–F, BMC). In the CTR groups, severe mononuclear cell infiltration was observed, and

the infiltration extended locally with bridging fibrosis at the adjacent periportal area (Fig. 3A). Infiltration into bile ducts was severe (Fig. 3B). In the BMC groups, the extent of infiltration was attenuated (Fig. 3D, E). In both groups, hepatocytes were not severely injured, and ballooning or vacuolation was rarely observed (Fig. 3C, F). The Rejection Activity Index (0.40 ± 0.26 vs 1.32 ± 0.33 points, $P = 0.0011$) (Fig. 3G) and graft damage scores (1.54 ± 0.34 vs 3.58 ± 0.26 points, $P < 0.0001$) (Fig. 3H) were significantly lower in the BMC group than in the CTR group.

Conventional biochemical profiles

The serum concentrations of AST (245.0 ± 49.2 vs 277.6 ± 124.3 U L^{-1}, $P = 0.6005$) (Fig. 3I) and ALT (108.2 ± 21.8 vs 81.2 ± 19.3 U L^{-1}, $P = 0.0716$) (Fig. 3J) were not significantly different. In contrast, the serum concentrations of T-Bil (2.28 ± 1.81 vs 5.82 ± 1.66 mg dL^{-1}, $P = 0.0121$) (Fig. 3K) and HA (437.78 ± 375.95 vs 1503.68 ± 777.81 ng mL^{-1}, $P = 0.0247$) (Fig. 3L) of the BMC group were significantly lower than those of the CTR group.

Messenger RNA levels in the liver graft after LT

RT-PCR analysis revealed that the mRNA levels of IL-2 and INF-γ (Th1-related cytokines) were significantly lower in the BMC group than in the CTR group. In contrast, the mRNA levels of IL-10 and TGF-β1 (Th2-related cytokine) showed no significant difference between the two groups (Fig. 4).

Engraftment of transplanted BMC cells in liver allografts

Engraftment of transplanted BMDCs in the liver allografts was confirmed by the detection of β-galactosidase activity (Fig. 5A, B). The BMDCs, which were distributed in the sinusoidal space, did not differentiate into hepatocytes (Fig. 5B).

Differentiation of engrafted cells in liver allografts

Immunohistochemical analysis of the liver revealed that $CD31^+$ and $CD68^+$-cells were not colocalized and represented two distinct populations (Fig. 5F). Analyses of liver grafts using β–galactosidase staining (Fig. 5C), single staining with either CD31 (Fig. 5D) or CD68 (Fig. 5E), or double-staining with CD31 and CD68 (Fig. 5F) showed that transplanted BMDCs differentiated into $CD68^+$ but not $CD31^+$ cells (Fig. 5G, H). KCs represented the majority of the $CD68^+$ cells. These results indicate that engrafted cells differentiated into KCs but not ECs. To evaluate the differentiation of BMDCs with higher specificity, We again analyzed the liver grafts using β–galactosidase staining (Fig. 5I) and immunostaining with CD31 (Fig. 5J) or CD163 (Fig. 5K), more specific marker for KCs. Transplanted BMDCs were positive for CD163 (Fig. 5L).

Discussion

Immunological ignorance of the recipient of the graft is ideal for LT and is mainly achieved successfully using immunosuppressants. However, immunosuppressants cause generalized immunosuppression, which increases the risk of opportunistic infections. The recognition of

and response to donor-specific antigens may induce graft rejection [27], which may be prevented if the graft tissue is replaced by recipient-derived cells. Therefore, we hypothesized that pretreatment of the donor graft with recipient-derived cells would result in a novel interaction between the graft and the recipient. For example, extrahepatic progenitor cells are recruited into transplanted liver grafts where they differentiate into hepatic tissues [14], and bone marrow-derived progenitor cells contribute to the regeneration of LSECs [12, 28–30]. Therefore, we used an allogeneic rat model of LT with strong rejection potential, in which recipient BMCs are transplanted preoperatively into liver transplant donors, assuming that the transplanted BMDCs replace the liver cells.

LSECs that line the sinusoidal space are major targets for rejection of liver transplants [31, 32]. Replacing donor cells with recipient-derived LSECs may attenuate rejection and increase the survival of liver allografts. However, previous reports indicate the difficulty of replacing LSECs with BMDCs. For example, using a mouse model, Follenzi et al. [33] found that BMDCs correct hemophilia in recipients after bone marrow transplantation; however, only a few BMDCs differentiated into hepatocytes or endothelial cells. Stolz et al. [34] also

reported that BMDCs make a limited contribution to the repopulation of LSECs after cold ischemia/warm reperfusion injury (CIWRI) in LT.

Here, we found that the serum HA concentrations at 7 d after LT were significantly lower in the BMC group than in the CTR group, although there were no significant differences in the serum concentrations of AST and ALT. These results indicate reduced injury to the LSECs, and we assume that transplanted recipient BMCs differentiated into LSECs. However, immunohistochemical analysis revealed that this did not occur. HA is a sensitive marker for organ rejection after LT [31, 35]. Therefore, these findings suggest that the lower serum concentration of HA in our BMC group may be a secondary effect of attenuated rejection. A possible explanation for the discrepancy in the results of AST/ALT and HA may be the timing of the measurement. The histopathology of the liver at 7 day after LT also showed that the damages to the hepatocytes were not severe in both groups.

Because LSECs are relatively radiation-resistant [36], replacement of LSECs may fail because of the nonlethal effect of 10 Gy TBI. Bone marrow-derived progenitors of LSECs contribute to regeneration after liver injury but not to normal LSEC turnover [30, 37], suggesting instead that ECs may replace LSECs in the grafts [38, 39].

We have shown here that the survival of liver transplant recipients was prolonged and that liver graft damage was attenuated by transplantation of recipient BMCs into liver transplant donors. Further, replacement of KCs by recipient BMDCs was observed in the liver graft, and functional inhibition of KCs by $GdCl_3$ abrogated prolonged survival. Analysis of mRNA expression levels in liver grafts showed a shift of the Th1/Th2 balance toward reducing rejection in the BMC groups. Replacement of KCs by recipient BMDCs may play an important role in this mechanism of immunological regulation that inhibits rejection. In contrast to the findings for other organs, liver allografts offer immunological advantages mediated by several mechanisms that include antigen overload [40]. For example, hepatic antigen-presenting cells (APCs) generally regulate systemic inflammatory responses against gut-derived antigens. These APCs include dendritic cells, KCs, and hepatic stellate cells, which are involved in the molecular regulation of tissue damage caused by CIWRI and transplant immunity after LT [40,41]. KCs are particularly involved in reducing allograft rejection or inducing tolerance after LT [40, 42–46].

One interesting aspect of our model is that KCs were not deleted from the liver graft but were replaced by recipient KCs. Functional deletion of KCs from liver grafts by $GdCl_3$ before

LT abrogated the prolonged survival observed in the BMC group. This result indicates that attenuated rejection may be attributed not to the deletion of allogeneic (i.e., DA rat-derived) KCs originally present in the liver graft but to their replacement by transplanted BMC-derived syngeneic (i.e., LEW rat) KCs. Generally, in allogeneic rat LT models of rejection or tolerance, KCs in the liver graft are replaced by recipient-derived KCs by day 7 after LT [47]. KCs from DA rats, which are allogeneic with liver transplant recipients, were targets of the immune system of liver transplant recipients. We found that KCs derived from transplanted BMCs were not replaced by KCs derived from the liver transplant recipient by day 7 in the BMC group. This result suggests that KCs derived from the transplanted syngeneic BMCs escaped strong allograft rejection.

Our protocol for preoperative treatment of donors would not be acceptable for human patients, including those undergoing living- and deceased-donor LT. However, pretreatment of donors would be acceptable for xenotransplantation. In xenotransplantation, porcine KCs directly recognize a sialic acid motif on human glycophorin A and destroy human erythrocytes [48, 49]. We speculate that the strategy of KC replacement might be useful in

making clinical xenotransplant trials feasible. We must therefore determine whether our strategy can be applied to xenotransplantation.

In conclusion, we have shown here that preoperative transplantation of BMCs isolated from rats syngeneic to liver transplant recipients into liver transplant donors attenuated the strong rejection of allogeneic rat liver transplants via engraftment of KCs from recipient-derived BMCs. These results may provide clues for developing a novel approach to recipient tolerance to a transplanted organ.

Acknowledgments: This work was supported by governmental grants to S. Uemoto (no. 20249058) and K. Endo (no. 245821) from the Japan Society for the Promotion of Science.

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