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Rainer W. G. Gruessner
Kristine Y. Zhang
Michele Dunning
Raouf E. Nakhleh
Angelika C. Gruessner

Bone marrow augmentation in kidney transplantation: a large animal study

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R.W.G Gruessner (✉) · K.Y. Zhang
M. Dunning · R.E. Nakhleh
A.C. Gruessner
Department of Surgery,
University of Minnesota,
Mayo Mail Code 90, 420 Delaware St. SE,
Minneapolis, MN 55455, USA
Tel.: + 1-612-625 1485
Fax: + 1-612-624 7168

Abstract Specific immunomodulatory strategies are required to eliminate the need for lifelong dependence on debilitating immunosuppressants. One proposed strategy is to simultaneously transplant the kidney and infuse donor-specific bone marrow cells. We prospectively studied the effect of *unmodified* donor-specific bone marrow infusion (DSBMI) on rejection, infection, graft-versus-host disease (GvHD), and graft survival. We performed 57 kidney transplants in mixed lymphocyte culture (MLC)-reactive, outbred pigs. The groups of recipient pigs differed according to the use of (1) indefinite versus short-term tacrolimus-based immunosuppression, (2) DSBMI, and (3) recipient preconditioning (RPC: whole body irradiation with 400 rads on day 0 and horse anti-pig thymocyte globulin (ATG) on days –2, –1, and 0). In all, we studied eight groups: group 1, nonimmunosuppressed control pigs ($n = 8$); group 2, nonimmunosuppressed DSBMI pigs ($n = 7$); group 3, nonimmunosuppressed RPC + DSBMI pigs ($n = 5$); group 4, tacrolimus (indefinite) pigs ($n = 11$); group 5, tacrolimus (10 days only) pigs ($n = 5$); group 6, DSBMI + tacrolimus (indefinite) pigs ($n = 8$); group 7, DSBMI + tacrolimus (10 days only) pigs ($n = 6$); and group 8, RPC + DSBMI + tacrolimus (indefinite) pigs ($n = 7$). DSBMI alone (group 2) or in combination with RPC (group 3) did not prolong graft survival, as compared with nonimmunosuppressed controls (group 1). In groups 1, 2, and 3, all but one pig died from rejection; in group 3 only, 45 % of the pigs died from concurrent infection or GvHD, indicating that RPC in combination with DSBMI aggravated the risk of generalized infection and GvHD. Post-transplant immunosuppression – irrespective of indefinite or short-term administration – was required for prolonged graft survival. With indefinite use of immunosuppression, graft survival rates and death rates from rejection were not different for pigs with (group 6) versus without (group 4) DSBMI; however, the death rate from infection was higher in group 6, suggesting that the bone marrow inoculum increased the risk of systemic infection. With short-term use of immunosuppression, graft survival rates were higher and death rates from rejection lower for pigs with (group 7) versus without (group 5) DSBMI. But DSBMI and short-term immunosuppression (group 7) failed to prolong survival beyond that achieved with indefinite immunosuppression (groups 4 and 6). Although the combination of DSBMI and short-term immunosuppression (group 7) reduced the risk of infection, it did not avert severe rejection. The addition of RPC to DSBMI and indefinite immunosup-

pression (group 8) significantly decreased graft survival, as compared with groups 4, 6, and 7. It also increased the incidence of death from rejection, GvHD, and infection, or a combination thereof. Unmodified DSBMI did not prolong graft survival after kidney transplantation, nor did it decrease the incidence of rejection. But it aggravated the risk of GvHD and infection. Short-term immunosuppression with DSBMI reduced the incidence of death from infection or GvHD, but it resulted in a higher incidence of death from rejection (as compared with indefinite use of immunosuppression). RPC, combined with DSBMI and indefinite immunosuppression, increased the death rate from rejection, GvHD, infection, or a combination thereof. In this large animal study, the effect of unmodified DSBMI has been disappointing. The search continues for the optimal way to successfully perform bone marrow augmentation in solid organ transplants.

Keywords Kidney transplantation · Bone marrow augmentation · Donor cell augmentation

Abbreviations *ATG* Antithymocyte globulin · *DSBMI* Donor-specific bone marrow infusion · *GvHD* Graft-versus-host disease · *MLC* Mixed lymphocyte culture · *PBMC* Peripheral blood mononuclear cell · *RPC* Recipient preconditioning · *SLA* Swine leukocyte antigen

Introduction

For nearly half a century, since the days of Medawar and Billingham, donor cell augmentation (with and without cytoablation) of nonvascularized and vascularized grafts has been used in an attempt to induce tolerance [2, 14, 29]. In the *precyclosporine era*, Monaco et al. were the first to use donor bone marrow augmentation in clinical kidney transplantation [20]. In the *cyclosporine era*, Barber et al. conducted the first prospective study investigating the effect on outcome of donor bone marrow augmentation after cadaveric kidney transplantation [1]. No significant improvement in clinical outcome resulted from any of these studies. But interest in bone marrow augmentation was revived in the *tacrolimus era*, in particular by the Pittsburgh transplant group. Their rationale for using donor-specific bone marrow augmentation was based on the chimeric concept: evidence of donor cells in the peripheral blood, skin, and lymph nodes was documented in kidney transplant recipients with long-term (> 25 years) graft function [32]. Subsequently, it was hypothesized that augmenting microchimerism by *unmodified* donor-specific bone marrow infusion (DSBMI) improves (long-term) graft acceptance [5, 31]. This hypothesis prompted a number of transplant centers to develop protocols to augment microchimerism by infusing unmodified donor bone marrow cells at the time of kidney transplantation.

Yet to date, not a single prospective, randomized clinical study has clearly shown a statistically significant improvement in kidney graft survival when unmodified DSBMI is used. Despite encouraging studies in small animals [22, 23, 36], very few preclinical studies using large animals have investigated the usefulness of unmodified DSBMI after kidney transplantation. To mimic the clinical setting, we used a large animal model to assess various combinations of tacrolimus-based immunosuppression, unmodified DSBMI, and recipient preconditioning (RPC) by whole body irradiation. Using outbred pigs, we compared not only graft survival rates, but also the incidence of rejection, infection, and graft-versus-host disease (GvHD) after kidney transplantation.

Materials and methods

Animals

We used 86 outbred, nonrelated Yorkshire-Landrace pigs, randomized to serve as kidney donors ($n = 29$) or recipients ($n = 57$). Mean donor weight was 27.3 ± 0.9 kg; mean recipient weight was 29.8 ± 1.1 kg. Only mixed lymphocyte culture(MLC)-reactive donor-recipient pairs were used. We studied eight groups of recipients according to the use of (1) immunosuppression, (2) DSBMI, and (3) RPC. Only recipients surviving for 3 days or more were included in our analysis.

Group 1 comprised nonimmunosuppressed control pigs ($n = 8$); group 2, nonimmunosuppressed DSBMI pigs ($n = 7$); and group 3, nonimmunosuppressed RPC and DSBMI pigs ($n = 5$). In group 4, tacrolimus was given indefinitely ($n = 11$); in group 5, for only 10 days ($n = 5$). In group 6, DSBMI was combined with indefinite use of tacrolimus ($n = 8$); in group 7, with a 10-day course ($n = 6$). In group 8, RPC and DSBMI were combined with indefinite use of tacrolimus ($n = 7$) (Table 1).

Mixed lymphocyte culture

To determine which pigs were MLC-reactive donor-recipient pairs, we irradiated unfractionated peripheral blood mononuclear cells (PBMCs) from donor and recipient pigs with 3000 rads. We then cultured the irradiated cells for 5 days in a humidified 5 % CO_2 environment in 96-well round-bottomed plates (Costar Corporation, Cambridge, Mass.) with unirradiated, unfractionated recipient or donor PBMCs. Cultures were pulsed with 0.2-μCi-tritiated thymidine per well, 5 days after initiation, and then harvested onto glass-fiber filter 8 h later. The incorporation of tritiated thymidine into the DNA of responding lymphocytes, as assessed by liquid scintillation counting, was used as a measure of cellular proliferation. Only reactive donor-recipient pairs were used in our analysis. Reactivity was defined as the tritiated thymidine incorporation value of 10,000 cpm or more.

Table 1 Study groups (*DSBMI* donor-specific bone marrow infusion, *RPC* recipient preconditioning [nonlethal whole body irradiation], *TAC* tacrolimus [*i* indefinite, *10* 10-day course only], *ATG* antithymocyte globulin)

	n	Comments
1. Control	8	Nonimmunosuppressed
2. DSBMI	7	
3. RPC + DSBMI	5	ATG pretransplant
4. TAC-i	11	
5. TAC-10	5	
6. TAC-i + DSBMI	8	
7. TAC-10 + DSBMI	6	
8. TAC-i + RPC + DSBMI	7	ATG pretransplant

Animal preparation, surgical technique, and post-transplant care

For all surgical procedures, pigs were premedicated with atropine (0.2 mg/kg i.m.) and thiopental sodium (30 mg/kg i.v.); general anesthesia was maintained with 3% isoflurane. Donor and recipient pigs were fasted for 24 h preoperatively, but maintained on intravenous fluids.

Donor and recipient operations are detailed elsewhere [10]. In brief, the donor renal artery was anastomosed end-to-side to the recipient distal aorta, and the donor renal vein end-to-side to the recipient distal vena cava. Bilateral native nephrectomies were done at the end of the transplant so serum creatinine levels could be used to monitor kidney graft function.

After transplantation, recipient pigs were given buprenorphine hydrochloride (0.3 mg/ml q · 6 h) for analgesia. Antibiotic prophylaxis was with cephalothin (500 mg/day) for 7 days. All pigs used in this study were handled in compliance with the University of Minnesota Research Committee Guidelines for the Care and Use of Laboratory Animals.

Bone marrow preparation

Fresh donor bone marrow was obtained from the exsanguinated donor at the time of kidney procurement. Bilateral long bones (tibiae, femora, and humeri) served as donor bones for marrow collection. After removing the bone fragments and debris by centrifugation, we prepared single-cell suspensions by multiple pipetting. Subsequently, mononuclear cells were isolated from the bone marrow cell suspensions by density gradient separation on Ficoll-Hypaque, as previously reported for human bone marrow graft preparations in clinical bone marrow transplant settings [35]. Mononuclear cells were washed in minimal essential medium, checked for viability by trypan blue exclusion, and counted. Bone marrow mononuclear cells (5×10^8 cells/kg) were infused intravenously into recipient pigs within 2 to 4 h after transplantation.

Recipient preconditioning

Pretransplant RPC entailed whole body irradiation with 400 rads (day 0). To administer this irradiation, we used a Phillips orthovoltage machine. In addition, using a randomization protocol, we gave horse anti-pig thymocyte globulin (pig ATG) on days –2, –1, and 0. The preparation of our pig ATG has been detailed elsewhere [11].

Post-transplant immunosuppression

Tacrolimus was started at 0.2 mg/kg per day, and then adjusted to maintain trough levels (determined by a microparticle enzyme immunoassay, ABBOTT IMX, Abbott Laboratories, Abbott Park, Ill.) of 8 to 20 ng/ml after transplantation. Tacrolimus was given indefinitely in groups 4, 6, and 8; for only 10 days post-transplant, in groups 5 and 7. Prednisone, used for induction and maintenance in groups 4, 5, 6, 7, and 8, was started at 2 mg/kg per day, then reduced by 50% at 7 days and discontinued at 10 days. Immunosuppressants were given intravenously. Tacrolimus was infused daily over 3-h periods; prednisone was given daily as a single injection. Rejection episodes were not treated in any group.

Postoperative follow-up

Pigs were observed daily for clinical signs of GvHD, such as erythema of the ears and extremities, skin body rashes, anorexia, diarrhea, and lethargy. They were weighed weekly. Pigs were also observed daily for clinical signs of infection, such as pneumonia or peritonitis.

Post-transplant biopsies and autopsies

Kidney graft biopsies were taken weekly to assess for interstitial and vascular rejection. Skin samples were obtained weekly to assess for cutaneous graft-versus-host reactions. At autopsy, the following tissues were examined histologically: the transplanted kidney, the native small bowel, the colon, the liver, the lungs, and the skin. Autopsy studies were done to determine the cause of death: rejection, GvHD, infection, or a combination thereof.

Tissue samples were fixed in 10% buffered formalin. Paraffin-embedded tissues were sectioned at 4 μm and stained with hematoxylin and eosin. We used our previously published scoring system to grade the extent of both interstitial and vascular rejection of the kidney (Table 2). We applied our scoring system to the Banff criteria as follows: borderline (Banff) changes correspond to mild interstitial rejection; mild (Banff) rejection corresponds to moderate interstitial rejection; moderate (Banff) rejection corresponds to severe interstitial or moderate interstitial and mild vascular rejection; severe (Banff) rejection corresponds to severe interstitial rejection and moderate or severe vascular rejection. Histologic studies were done by a single pathologist (R.E.N.) in a blinded fashion.

Cause of death

For each recipient pig, the cause of death (rejection, infection, GvHD, other) was defined by the clinical course, as well as by microscopic and macroscopic findings. *Death due to rejection* was defined by the presence of at least grade 2 (moderate) interstitial rejection in the kidney at autopsy. *Death due to infection* was defined by the clinical course or by the histologic features of infection (pneumonia, peritonitis). *Death due to GvHD* was defined by the typical clinical course (lethargy, cachexia, skin rashes) and by the typical histologic features of GvHD: in the liver, by a pronounced mononuclear infiltrate within the portal tracts, with invasion and damage to bile ducts; in the native intestine (small bowel, colon), by inflammation of the lamina propria, with individual necrosis of enterocytes in the crypts; and in the skin, by dermal infiltration by mononuclear cells and keratinocyte necrosis.

Table 2 Grading of interstitial and vascular rejection

Interstitial rejection		Vascular rejection	
Mild	Patchy lymphoplasma-cellular infiltrate with tubulitis present focally	Mild	Endotheliitis
Moderate	Patchy lymphoplasma-cellular infiltrate with easily identifiable tubulitis	Moderate	Vasculitis
Severe	Prominent lympho-plasmacellular inflammatory infiltrate (may also contain eosinophils and neutrophils). Edema may also be present. Tubulitis is widespread	Severe	Vasculitis with fibrinoid necrosis

Statistical analysis

Deaths from rejection, infection, and GvHD were analyzed according to the method of Kaplan-Meier. The log-rank test was used to determine late differences. The Wilcoxon test was used to determine early differences.

Results

Overall survival

In group 1 (control pigs), survival rates at 7, 14, 21, and 28 days after transplantation were 50%, 0%, 0%, and 0%; in group 2 (DSBMI pigs), 67%, 8%, 0%, and 0%; in group 3 (RPC + DSBMI pigs), 73%, 9%, 9%, and 9%; in group 4 (indefinite tacrolimus pigs), 100%, 80%, 40%, and 30%; in group 5 (10-day tacrolimus pigs), 80%, 40%, 0%, and 0%; in group 6 (DSBMI + indefinite tacrolimus pigs), 100%, 83%, 56%, and 46%; in group 7 (DSBMI + 10-day tacrolimus pigs), 100%, 100%, 67%, and 33%; in group 8 (RPC + DSBMI + indefinite tacrolimus pigs), 77%, 26%, 9%, and 9% ($P = 0.0001$) (Fig. 1a, 1b).

In the nonimmunosuppressed pigs (groups 1, 2, and 3), overall survival was not different between groups 1 and 2 ($P = 0.38$), groups 1 and 3 ($P = 0.7$), or groups 2 and 3 ($P = 0.7$). In the tacrolimus-only pigs (groups 4 and 5), overall survival was higher with indefinite use (group 4) versus a 10-day course (group 5) (log-rank $P = 0.05$, Wilcoxon $P = 0.07$). In the DSBMI + tacrolimus pigs (groups 6 and 7), no difference in overall survival was noted with indefinite tacrolimus (group 6) versus a 10-day course (group 7) ($P \geq 0.21$). Our comparison between the tacrolimus-only pigs (groups 4 and 5) and the DSBMI + tacrolimus pigs (groups 6 and 7) showed the following: with indefinite tacrolimus, overall survival was not different between groups 4 and 6 (log-rank $P = 0.067$; Wilcoxon $P = 0.08$); with a 10-day course, overall survival was significantly higher in group 7 versus group 5 ($P \leq 0.009$). Of note, overall survival was not statistically different between group 5 (10-day tacrolimus pigs) and groups 1, 2, or 3 (nonimmunosuppressed pigs). In the RPC + DSBMI + tacrolimus group (group 8), overall survival rates were significantly lower than in group 4 ($P = 0.002$), group 6 ($P = 0.02$), or group 7 ($P = 0.005$).

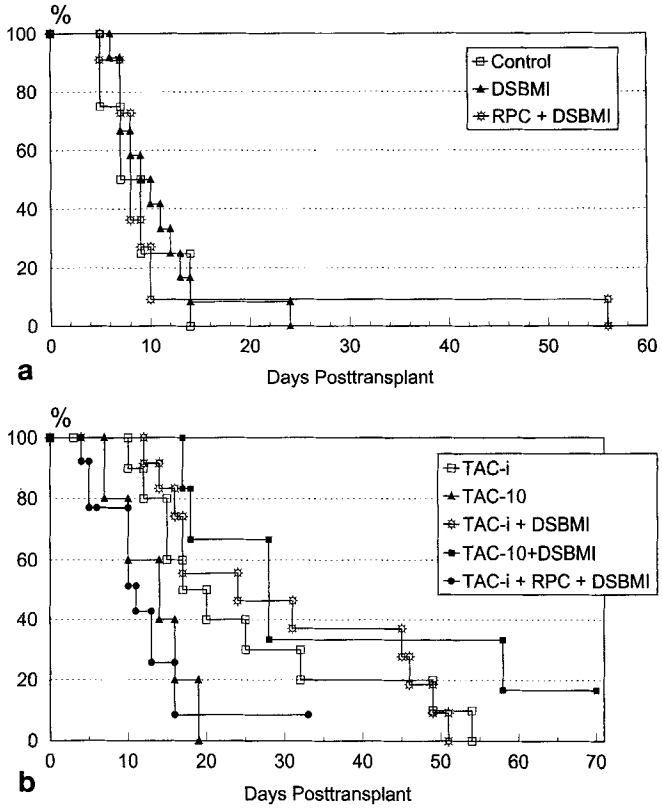

Fig. 1 Overall pig survival in the (**a**) nonimmunosuppressed groups (1–3) and in the (**b**) immunosuppressed groups (4–8) (*DSBMI* donor-specific bone marrow infusion, *RPC* recipient preconditioning [nonlethal whole body irradiation], *TAC* tacrolimus [*i* indefinite, *10* 10-day course only])

Death from rejection

For this analysis, only deaths from rejection were counted as graft failures (Fig. 2a, b). In group 1 (control pigs), the death rate from rejection at 7, 14, 21, and 28 days was 50%, 100%, 100%, and 100%; in group 2 (DSBMI pigs), 33%, 92%, 92%, and 100%; in group 3 (RPC + DSBMI pigs), 27%, 91%, 91%, and 91%; in group 4 (indefinite tacrolimus pigs), 0%, 20%, 60%, and 70%; in group 5 (10-day tacrolimus pigs), 20%, 60%, 100%, and 100%; in group 6 (DSBMI + indefinite tacrolimus pigs), 0%, 17%, 44%, and 54%; in

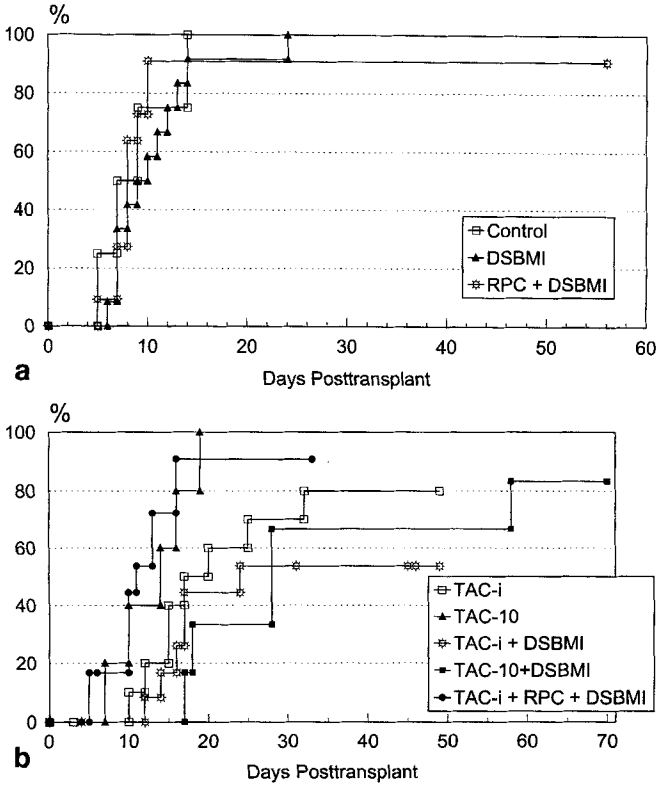

Fig. 2 Death from rejection in the (**a**) nonimmunosuppressed groups (1–3) and in the (**b**) immunosuppressed groups (4–8) (*DSBMI* donor-specific bone marrow infusion, *RPC* recipient preconditioning [nonlethal whole body irradiation], *TAC* tacrolimus [*i* indefinite, *10* 10-day course only])

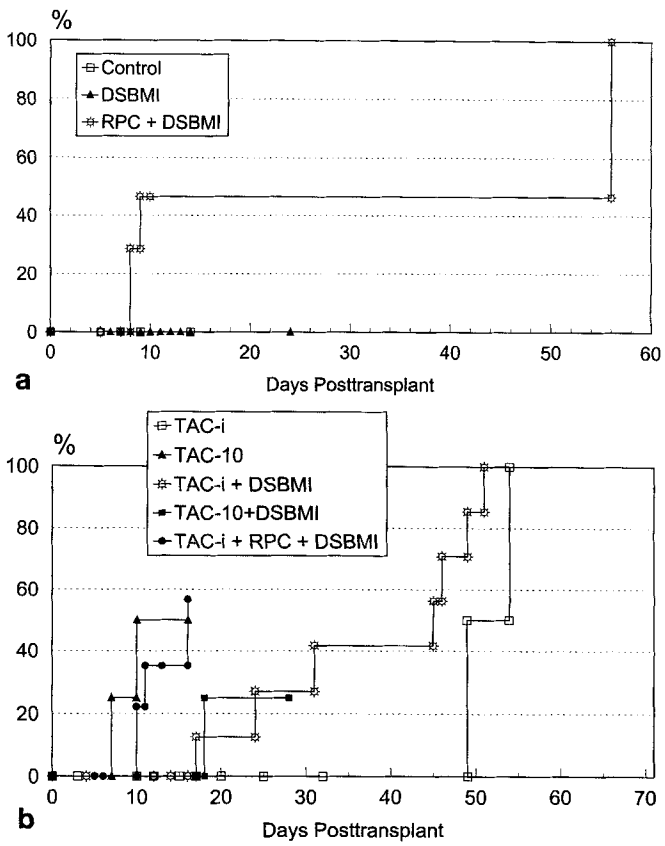

Fig. 3 Death from infection in the (**a**) nonimmunosuppressed groups (1–3) and in the (**b**) immunosuppressed groups (4–8) (*DSBMI* donor-specific bone marrow infusion, *RPC* recipient preconditioning [nonlethal whole body irradiation], *TAC* tacrolimus [*i* indefinite, *10* 10-day course only])

group 7 (DSBMI + 10-day tacrolimus pigs), 0%, 0%, 33%, and 67%; and in group 8 (RPC + DSBMI + indefinite tacrolimus pigs), 17%, 72%, 91%, and 91% ($P = 0.0001$).

In the nonimmunosuppressed pigs (groups 1, 2, and 3), the death rate from rejection was not different between groups 1 and 2 ($P = 0.4$), groups 1 and 3 ($P = 0.8$), or groups 2 and 3 ($P = 0.7$). In the tacrolimus-only pigs (groups 4 and 5), we noted a tendency toward a lower rate of death from rejection with indefinite tacrolimus (group 4) versus a 10-day course (group 5) (log-rank $P = 0.05$, Wilcoxon $P = 0.07$). In the DSBMI + tacrolimus pigs (groups 6 and 7), we found no difference in the death rate from rejection with indefinite tacrolimus (group 6) versus a 10-day course (group 7) ($P \geq 0.62$). Our comparison between the tacrolimus-only pigs (groups 4 and 5) and the DSBMI + tacrolimus pigs (groups 6 and 7) showed the following: with indefinite tacrolimus, the death rate from rejection was not different between groups 4 and 6 ($P = 0.4$); with a 10-day course, the death rate from rejection was significantly lower in group 7 versus group 5 ($P \leq 0.009$). In the RPC + DSBMI + tacrolimus pigs (group 8), the death rate from rejection was significantly higher than in group 4 ($P = 0.03$), group 6 ($P = 0.001$), or group 7 ($P = 0.006$). Of note, the death rate from rejection was not different between groups 8 and 1 ($P = 0.1$).

Death from infection

For this analysis, only deaths from infection were counted as graft failures (Fig. 3a, 3b). In group 1 (control pigs), the death rate from infection at 7, 14, 21, and 28 days was 0%, 0%, 0%, and 0%; in group 2 (DSBMI pigs), 0%, 0%, 0%, and 0%; in group 3 (RPC + DSBMI pigs), 0%, 46%, 46%, and 46%; in group 4 (indefinite tacrolimus pigs), 0%, 0%, 0%, and 0%; in group 5 (10-day tacrolimus pigs), 25%, 50%, 50%, and 50%; in group 6 (DSBMI + indefinite tacrolimus pigs), 0%, 0%, 13%, and 27%; in group 7 (DSBMI + 10-day tacrolimus pigs), 0%, 0%, 25%, and

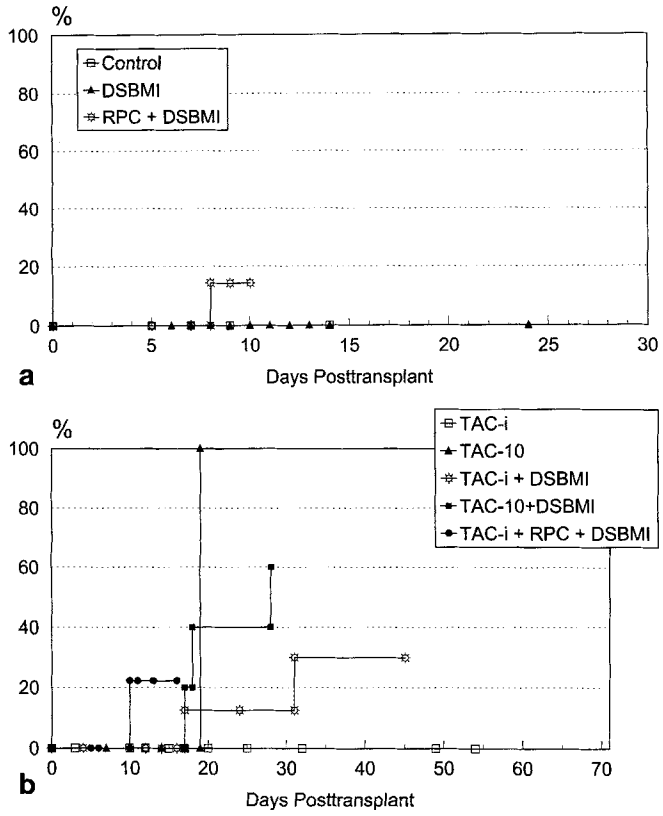

Fig. 4 Death from graft-versus-host disease in the (**a**) nonimmunosuppressed groups (1–3) and in the (**b**) immunosuppressed groups (4–8) (*DSBMI* donor-specific bone marrow infusion, *RPC* recipient preconditioning [nonlethal whole body irradiation], *TAC* tacrolimus [*i* indefinite, *10* 10-day course only])

25%; and in group 8 (RPC + DSBMI + indefinite tacrolimus pigs), 0%, 35%, 57%, and 57% ($P = 0.005$).

There were no deaths from infection at any time point in groups 1 and 2. In the nonimmunosuppressed pigs (groups 1, 2, and 3), the death rate from infection was not different between groups 1 and 2 ($P = 0.9$), groups 1 and 3 ($P = 0.1$), or groups 2 and 3 ($P = 0.1$). In the tacrolimus-only pigs (groups 4 and 5), the death rate from infection was lower with indefinite tacrolimus (group 4) versus a 10-day course (group 5) ($P = 0.016$). In the DSBMI + tacrolimus pigs (groups 6 and 7), we found no difference in the death rate from infection with indefinite tacrolimus (group 6) versus a 10-day course (group 7) ($P \geq 0.22$). Our comparison between the tacrolimus-only pigs (groups 4 and 5) and the DSBMI + tacrolimus pigs (groups 6 and 7) showed the following: with indefinite tacrolimus, there was a tendency toward a lower death rate from infection in group 4 versus group 6 ($P = 0.07$); with a 10-day course, the death rate from infection was not different between groups 5 and 7 ($P \geq 0.15$). In the RPC + DSBMI + tacrolimus pigs (group 8), the death rate from infection was significantly higher than in group 4 ($P = 0.02$) and group 6 ($P = 0.02$). Of note, the death rate from infection was also significantly higher in group 8 than in group 1 ($P = 0.04$).

Death from GvHD

For this analysis, only deaths from GvHD were counted as graft failures (Fig. 4a, b). In group 1 (control pigs), the death rate from GvHD at 7, 14, 21, and 28 days was 0%, 0%, 0%, and 0%; in group 2 (DSBMI pigs), 0%, 0%, 0%, and 0%; in group 3 (RPC + DSBMI pigs), 0%, 14%, 14%, and 14%; in group 4 (indefinite tacrolimus pigs), 0%, 0%, 0%, and 0%; in group 5 (10-day tacrolimus pigs), 0%, 0%, 100%, and 100%; in group 6 (DSBMI + indefinite tacrolimus pigs), 0%, 0%, 13%, and 13%; in group 7 (DSBMI + 10-day tacrolimus pigs), 0%, 0%, 20%, and 60%; and in group 8 (RPC + DSBMI + indefinite tacrolimus pigs), 0%, 22%, 22%, and 22% ($P = 0.29$).

There were no deaths from GvHD at any time point in groups 1 and 2. In the nonimmunosuppressed pigs (groups 1, 2, and 3), the death rate from GvHD was not different between groups 1 and 2 ($P = 0.4$), groups 1 and 3 ($P = 0.6$), or groups 2 and 3 ($P = 0.6$). In the tacrolimus-only pigs (groups 4 and 5), the death rate from GvHD was lower with indefinite tacrolimus (group 4) versus a 10-day course (group 5) ($P = 0.025$). In the DSBMI + tacrolimus pigs (groups 6 and 7), we found no difference in the death rate from GvHD with indefinite tacrolimus (group 6) versus a 10-day course (group 7) ($P \geq 0.21$). Our comparison between the tacrolimus-only pigs (groups 4 and 5) and the DSBMI + tacrolimus pigs (groups 6 and 7) showed the following: with indefinite tacrolimus, the death rate from GvHD was not different between groups 4 and 6 ($P = 0.3$); with a 10-day course, the death rate from GvHD was not different between groups 5 and 7 ($P \geq 0.58$). In the RPC + DSBMI + tacrolimus pigs (group 8), the death rate from GvHD did not differ from group 4 ($P = 0.1$), group 6 ($P = 0.1$), or group 7 ($P = 0.47$).

Long-term survival (> 4 weeks)

There were no long-term survivors in group 1 (longest survival: 14 days), group 2 (longest survival: 24 days), or group 5 (longest survival: 19 days). In the nonimmunosuppressed pigs (groups 1, 2, and 3), only one pig in group 3 survived more than 4 weeks (56 days). Thus, all but one long-term survivor received tacrolimus after transplantation. In the tacrolimus-only pigs (groups 4 and 5), long-term survivors were found only in group 4: 27% of those pigs survived for more than 28 days (range, 32–54 days); two of them died from pneumonia,

one from rejection. In the DSBMI + tacrolimus pigs (groups 6 and 7), the percentages of long-term survivors were highest: in group 6, 38% (range, 31–51 days); in group 7, 67% (range, 28–182 days). In group 6, all pigs died from infection (including one with concurrent GvHD). In group 7, all but one pig died from rejection (including one with concurrent GvHD); that remaining pig was euthanized due to its increased weight in the absence of rejection, infection, or GvHD. In the RPC + DSBMI + tacrolimus pigs (group 8), only one pig survived for more than 4 weeks (33 days).

Autopsy results and simultaneous immunologic events

In the nonimmunosuppressed pigs, all in groups 1 and 2 had evidence of moderate or severe rejection in the absence of concurrent infection or GvHD. In group 3, the most common cause of death was rejection, either alone (55%) or with concurrent infection (27%); infection alone or GvHD alone occurred in 9% each.

In the tacrolimus-only pigs (groups 4 and 5), the causes of death differed by duration of tacrolimus administration. In group 4 (indefinite), all pigs died from one condition only (rejection or infection), with rejection the most common cause (73%). In group 5 (10-day course), all pigs died from rejection, but 40% died with concurrent infection and 20% with concurrent GvHD.

In the DSBMI + tacrolimus pigs (groups 6 and 7), rejection, GvHD, and infection were not mutually exclusive. In group 6, simultaneous immunologic events were found at autopsy in 24% (rejection, infection, and GvHD in 8%; rejection and infection in 8%; infection and GvHD in 8%); rejection alone accounted for 31% of the deaths. In group 7, simultaneous immunologic events were found at autopsy in 25% (rejection, infection, and GvHD in 8%; rejection and GvHD in 17%); all but one pig died from rejection.

In the RPC + DSBMI + tacrolimus pigs (group 8), simultaneous immunologic events were found at autopsy in 30% (rejection, infection, and GvHD in 15%; rejection and infection in 15%); all but three pigs died from rejection.

Discussion

Current success in organ transplantation is based on the permanent use of potent but nonspecific immunosuppression. But transplant recipients experience not only the side effects of these drugs, but also the consequences of overimmunosuppression (infections and neoplasms) or of underimmunosuppression (chronic rejection). Since the early days of transplantation, it has therefore been the goal to modulate the immune response in such a way that tolerance can be achieved – without the need for permanent immunosuppression. One proposed strategy for facilitating development of graft hyporesponsiveness (or tolerance) has been to infuse donor-specific bone marrow cells, with or without cytoablation.

The concept of simultaneous bone marrow cell infusion and solid organ transplantation is based on a successful human kidney transplant from a cadaveric donor more than 20 years ago by Monaco et al. [20]. The recipient was given antilymphocyte serum during the first 14 days after transplantation and 11×10^9 donor bone marrow cells on post-transplant day 25. Kidney graft function was normal for the first 8 months after transplantation, but the patient depended on immunosuppressive therapy (prednisone and azathioprine). The patient eventually died from peritonitis secondary to perforated sigmoid diverticulitis; autopsy showed minimal evidence of kidney allograft rejection.

In their early experimental work on donor cell augmented transplants, Monaco and Wood made several important observations: (1) the bone marrow inoculum proved to be superior to other lymphoid cells (e.g., thymus, spleen, nodes) in inducing tolerance; (2) the timing of bone marrow infusions appeared to be critical (the effect was optimal 1 week after antilymphocyte serum treatment and grafting; there was no effect if bone marrow was given before grafting); (3) a dose-dependent effect was noted (progressive doses of bone marrow did not give progressive tolerance); and (4) pretreatment with anti-T-cell agents appeared to facilitate induction of tolerance by the bone marrow inoculum [13, 18, 19, 22, 36]. But a clinical study in the precyclosporine era by Monaco et al. involving four living-related kidney transplant recipients – who underwent DSBMI after discontinuing ATG treatment – failed to show a salutary effect [21]. Nevertheless, in the Monaco study, the bone marrow cells were well tolerated, with no laboratory or clinical evidence of GvHD.

In the cyclosporine era, Barber et al. did the first controlled prospective study using DSBMI in cadaveric kidney allograft recipients [1]. Seven days after their last dose of a 10- to 14-day induction course of antilymphocyte globulin, their patients received cryopreserved $2–3 \times 10^8$ viable donor bone marrow cells. Although the 1-year graft survival rate was higher in the DSBMI vs control group (90% vs 71%), the incidence of rejection episodes was similar in both groups during the induction period. Completely withdrawing immunosuppressive therapy in the DSBMI group was not possible. Incidentally, the study's authors reported persistence of donor-type lymphoid cells (chimerism) by polymerase chain reaction in the DSBMI group [1].

In a follow-up study 4 years later, Diethelm et al. [4] reported on 74 cadaveric and 11 living-related kidney allograft recipients with DSBMI. The control group comprised 64 recipients of the contralateral (cadaver) kid-

ney without DSBMI. Graft survival was not different at 1 and 3 years (88% and 74% with DSBMI, and 83% and 69% without). As it turned out, DSBMI had no effect on the frequency of acute rejection episodes and did not prevent development of chronic rejection.

In the clinical studies by Monaco and Barber, cryopreserved or cultured bone marrow cells were infused several days after transplantation. In contrast, the Pittsburgh transplant group, in the tacrolimus era, has propagated infusion of unmodified donor-specific bone marrow cells without cytoablation *at the time of transplantation* [5]. In their initial series of 36 kidney transplant recipients who received $3–5 \times 10^8$ unmodified bone marrow cells, graft survival (mean follow-up: 11 ± 6 months) was 92% [19]. In the control group without DSBMI, graft survival was 85%. All patients received tacrolimus-based therapy without either RPC (e.g., radiation, cytoreduction) or induction therapy. Of note, this was a nonprospective, nonrandomized study. And the control group was older, both in terms of recipient and donor age, and had a longer cold ischemia time. The incidence of rejection, delayed graft function, and cytomegalovirus infections was not different between the two groups. The only difference was the rate of chimerism: 97% in the group with DSBMI, 64% in the group without. Interestingly, donor-specific hyporesponsiveness was noted in only 21% of patients with DSBMI, but in 29% without [27].

The Miami transplant group reported their initial experience with 40 recipients of first cadaveric kidney transplants and DSBMI, as compared with 100 control recipients without DSBMI [8]. Cryopreserved donor bone marrow was infused at two planned intervals on postoperative days 1 to 4 and 10 to 14 (coinciding with initiation and completion of anti-T-cell induction therapy). At 2 years after transplantation, graft survival rates were 97% in the control and 86% in the DSBMI group. The frequency of rejection episodes was not different, and no grafts were lost because of rejection in the DSBMI group. But the incidence of clinically significant infections and of death from infection was significantly higher in the DSBMI (versus the control) group. The investigators speculated that the 10% mortality rate from infection in the DSBMI group might have been the result of overimmunosuppression (anti-T-cell therapy for induction; combined use of tacrolimus and mycophenolate mofetil for maintenance). In a follow-up study, they found no difference in patient and graft survival rates between the DSBMI ($n = 58$) and the control ($n = 188$) group at 36 months [9]. Of note, the control group experienced graft loss from rejection, but not the DSBMI group. The DSBMI group also had a more depressed cellular and humoral immune capacity – indicating an immunologic trade-off that resulted in a lower rate of rejection but a higher rate of infection. More recently, Miller et al. [17] reported a significantly lower incidence of chronic (but not acute) rejection in the DSBMI ($n = 63$) versus the control group ($n = 220$). Yet the study was not designed in a prospective or randomized fashion.

Spitzer et al. [30] recently succeeded in inducing allograft tolerance through mixed lymphohematopoietic chimerism. They performed a combined HLA-identical matched donor bone marrow and renal transplant for multiple myeloma with end-stage renal disease. Besides using an HLA-identical donor, a nonmyeloablative regimen for the induction of mixed lymphohematopoietic chimerism was used.

To date, some of these clinical studies have raised more questions than they have answered with respect to the effect of bone marrow augmentation on kidney transplant outcome. In our randomized large animal study, we prospectively investigated the effect of unmodified DSBMI, not only on kidney graft survival, but also on the incidence of rejection, GvHD, and infection. We used tacrolimus-based immunosuppression, with tacrolimus levels ranging from 8 to 20 ng/ml. We studied the effect of unmodified DSBMI on kidney transplant outcome in three different protocols: (1) DSBMI in combination with indefinite immunosuppression; (2) RPC to "make space" for cotransplanted bone marrow cells in the presence of indefinite immunosuppression; (3) DSBMI in combination with only short-term immunosuppression to abrogate potentially unwarranted immune responses (i.e., infection or GvHD) secondary to overimmunosuppression. Despite all these modifications in protocol, our overall experience with unmodified DSBMI was disappointing: it did not significantly prolong survival, nor did it avert rejection, infection, or GvHD.

Not surprisingly, DSBMI alone (without concurrent immunosuppression) did not prolong graft survival, as compared with our nonimmunosuppressed control group. Nor did the combination of RPC and DSBMI prolong graft survival. Of note, all pigs in the control and DSBMI-only groups died from rejection; autopsy showed no evidence of infection or GvHD. In the RPC and DSBMI group, all but one pig died from rejection; however, 36% of those pigs died with concurrent infection and 9% with GvHD. Thus, RPC in combination with DSBMI (and without immunosuppression) aggravated the risks of generalized infection and GvHD. In our study, the RPC protocol entailed nonlethal whole body irradiation with 400 rads and anti-pig ATG. Done shortly before transplantation, this protocol is suitable for human cadaveric organ recipients. We found that post-transplant immunosuppression – whether indefinite or short-term – was required for prolonged graft survival. With indefinite tacrolimus-based immunosuppression, graft survival was not significantly different between the group with versus without DSBMI. Only the percentage of pigs surviving for 4 weeks or more

was slightly higher in the group with DSBMI (38% with DSBMI vs 27% without). The death rates from rejection and GvHD were not different between the two groups. But there was a tendency toward a higher death rate from infection in the group with (versus without) DSBMI – suggesting that the bone marrow inoculum increased the risk of systemic infection.

Hoping to diminish or even abrogate unwarranted DSBMI-induced immune responses by both host and graft, we hypothesized that discontinuing post-transplant immunosuppression might decrease the risks of infection and GvHD and prolong survival. So we arbitrarily discontinued tacrolimus-based immunosuppression at 10 days after transplantation in one group with DSBMI and one group without. We anticipated that pigs with short-term immunosuppression only (no DSBMI) would not have extended graft survival. And indeed, survival rates for such pigs were significantly lower than with indefinite immunosuppression; their survival rates were no different as compared with non-immunosuppressed control pigs or nonimmunosuppressed DSBMI pigs.

But the addition of DSBMI to short-term immunosuppression significantly prolonged survival in pigs with short-term immunosuppression. It failed, however, to significantly prolong survival beyond that achieved with indefinite immunosuppression (with or without DSBMI). Interestingly, the percentage of pigs surviving for 4 weeks or more was the highest (67%) in the group with short-term immunosuppression and DSBMI. But 83% of those pigs had evidence of moderate or severe rejection at autopsy versus only 46% in the group with indefinite immunosuppression and DSBMI. Yet the death rate from infection was three times higher in the group with short-term immunosuppression and DSBMI, as compared to the group with short-term immunosuppression but no DSBMI. Obviously, the combination of DSBMI and short-term immunosuppression reduced the risk of infection, but was not sufficient to avert severe rejection. One possibility to decrease the risk of rejection in this group would be to recycle immunosuppression at certain intervals, which might decrease the death rate from rejection; however, theoretically, it might again increase the death rates from infection and GvHD.

The addition of RPC to DSBMI and indefinite immunosuppression significantly decreased graft survival (as compared with indefinite immunosuppression with and without DSBMI, and short-term immunosuppression with DSBMI). In fact, graft survival rates were no different from the nonimmunosuppressed groups. Clearly, RPC in combination with DSBMI and indefinite immunosuppression increased the incidence of death from rejection, GvHD, and infection; of all groups, the incidence of simultaneous immunologic events was highest in this group, as shown on autopsy.

Arguably, our DSBMI protocol could have been different with regard to dosing (multiple vs single), timing (delayed vs immediate), and composition (subpopulations vs whole bone marrow) [17]. In our opinion, the most promising of these approaches appears to be the infusion of only a subpopulation of donor-specific bone marrow cells – as shown in large animal studies by Thomas et al. [33, 34] and small animal studies by Ildstad and Kaufman [6, 15, 16]. Unmodified bone marrow obviously contains cellular elements that are capable of inducing GvHD and sensitizing the recipients. Thus, detailed knowledge of the phenotype of tolerizing and immunizing cells is crucial to eliminate these risks. If professional antigen-presenting cells are removed, the bone marrow inoculum might better facilitate specific immune tolerance. T-cell-depleted bone marrow may further reduce the risks of GvHD. Eventually, stem cell or hematopoietic progenitor cell infusion might be successful, as shown in a mouse model of allogeneic heart transplantation in which tolerance was achieved with purified allogeneic hematopoietic stem cells [7]. But in our study, unmodified bone marrow cells infused in the systemic circulation served more as immunogens than tolerogens, irrespective of RPC before, or the amount of immunosuppression after, transplantation.

We did not assess the degree of chimerism in our study. All of our transplants were performed with male donors and female recipients, offering the possibility of detecting male chromosomes in female tissue. But at the time of our study, no probes specific to the pig Y-chromosome were available to us. In our outbred Yorkshire-Landrace pigs (unlike in inbred swine), swine leukocyte antigen (SLA) specificities were not known. Thus, we were not able to use specific SLA probes for identifying donor and recipient antigens, assessing persistence of these cells, or detecting chimerism. We believe, however, that development of GvHD is direct evidence of engraftment of donor-derived cells [24].

Although DSBMI facilitates development of chimerism, most evidence relating chimerism to tolerance is circumstantial. Moreover, chimerism does not represent a stable immunologic state: it has been demonstrated in human recipients of solid organ transplants with rejection or GvHD [3, 26, 28].

In summary, the effect of unmodified donor-specific bone marrow infusion has been disappointing in this pig model of kidney transplantation. Donor bone marrow augmentation did not prolong survival, nor did it decrease the incidence of rejection. Instead, it aggravated the risks of GvHD and infection. In contrast to our previous large animal studies of intestinal transplantation [12, 25], adding DSBMI did not reduce graft and recipient survival in this kidney transplant model. But the combination of DSBMI, RPC, and indefinite immuno-

suppression clearly increased the risks of death from rejection, infection, GvHD, or a combination thereof. The optimal way to perform bone marrow augmentation after kidney transplantation remains to be determined.

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