Comparative Medicine
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Vol 62, No 6
December 2012
Pages 487–494

Original Research

Effects of Mobilization Regimens in Donors on Outcomes of Hematopoietic Cell Transplantation in Miniature Swine

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Toxicities and complications associated with hematopoietic cell transplantation currently limit this potentially curative therapy for malignant and nonmalignant blood disorders. Miniature swine provide a clinically relevant model for studies to improve posttransplantation outcomes. Miniature swine recipients of high-dose haploidentical hepatopoietic cell transplantation after reduced-intensity conditioning consisting of low-dose (100 cGy) total-body irradiation, partial T-cell depletion by using a CD3 immunotoxin, and a 45-d course of cyclosporine A typically successfully engraft without graft-versus-host disease. We recently observed broad variability in engraftment outcomes that correlates with the occurrence of adverse reactions in donors after cytokine treatment to mobilize hematopoietic progenitor cells from the bone marrow to the peripheral blood for collection. Haploidentical recipients (*n* = 16) of cells from donors remaining healthy during cytokine treatment engrafted with multilineage chimerism, did not develop graft-versus-host disease, and did not require any blood products. In comparison, identically conditioned recipients of cells from donors that had severe reactions during cytokine treatment had adverse outcomes, including the development of clinically significant thrombocytopenia requiring blood product support in 8 of 11 swine. Furthermore, all 11 recipients lost peripheral blood myeloid chimerism (indicating lack of engraftment of donor stem cells). These data suggest that posttransplantation complications in swine are influenced by the health status of the donor before and during the collection of hematopoietic cells by leukapheresis.

Abbreviation: GvHD, graft-versus-host disease.

Allogeneic hematopoietic cell transplantation is the treatment of choice for many malignant and nonmalignant hematologic disorders.[4] During the last 15 y, collection of cytokine-mobilized peripheral blood stem cells has emerged as the preferred technique by which hematopoietic stem cells are harvested, in part as an alternative to the invasiveness of bone marrow donation.[1] Generally, both procedures are well-established and considered extremely safe for the donor, with any morbidity being transient and self-limiting and mortality being very rare.

Recently, concerns have arisen that stem cell mobilization with granulocytic colony-stimulating factor may be associated with long-term complications, specifically hematologic malignancy.[2] Multicenter studies have assessed these risks.[9,20] In these series, the incidence of hematologic malignancies (3.92 per 10,000 transplants) or of death (0.98 per 10,000 transplants) after mobilization with granulocytic colony-stimulating factor and leukapheresis was low. However, one study[9] reported an incidence rate of "severe adverse events," including cardiovascular, thromboembolic, and pulmonary events, of 10.76 per 10,000 in peripheral blood donors, and another[20] reported that 15 of 2408 (0.6%) peripheral blood donors experienced a "serious short-term adverse event," and 25% of donors were said to "have significant headache, nausea, or citrate toxicity". The effect of these donor adverse events during cytokine mobilization and aphaeresis on the outcome of transplantation in recipients has yet to be evaluated.

Previously, we reported a model of stable multilineage chimerism without graft-versus-host disease (GvHD) after nonmyeloblative haploidentical hematopoietic cell transplantation in miniature swine.[3,5,10,21] Here we report retrospectively on the effect of the donor's health status during stem cell mobilization and aphaeresis on the outcome of hematopoietic cell transplantation in recipients conditioned with regimens that were reduced in intensity. We noted a striking correlation between donors that experienced severe adverse reactions during stem cell mobilization and leukapheresis and poor posttransplantation outcomes. Extrapolating from these observations in miniature swine, the clinical effects of transplanting aphaeresis products from similarly adversely affected human donors to patients receiving nonmyeloablative conditioning merit careful evaluation.

Received: 07 Apr 2012. Revision requested: 29 Apr 2012. Accepted: 11 Jul 2012.
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Materials and Methods

Transplant donors and recipients were selected from the herd of partially inbred, MHC-defined miniature swine of Massachusetts General Hospital. A breeding pair, one pig from the Andes and the other from the Rocky Mountains, was selected for the creation of the herd.[21] The swine (*Sus scrofa domestica*) herd is a closed SPF (pseudorabies, porcine reproductive and respiratory syndrome, transmissible gastroenteritis, and brucellosis) herd, which is defined at the major histocompatibility complex. The immunogenetic characteristics of this herd, which is heterogeneous for minor antigenic differences, have been reported previously.[3,5,10,21] Approximately 50 miniature swine of various ages and weights are housed in our large animal facility, which is AAALAC-accredited. Swine undergoing hematopoietic cell transplantation are housed in conventional steel cages with HEPA filters.

The ages of donor pigs ranged from 6 mo to 1 y; recipients were 8 to 12 wk old. Donors and recipients were chosen to differ by single haplotypes at class I and II loci. All donors were positive for pig allelic antigen, a nonhistocompatibility cell-surface antigen that is present on all differentiated hematopoietic cells in animals that express this allele.[8] All recipients were chosen to be negative for this allele so that chimerism after hematopoietic cell transplantation could be monitored by using by FACS. All transplants were approved by the Massachusetts General Hospital IACUC.

Preparatory regimen. Recipient SLA^{ad} miniature swine (age, 8 to 12 wk; weight, 8 to 12 kg) were pretreated with low-dose (100 cGy) total-body irradiation on day −2, partial T-cell depletion by using a CD3-immunotoxin delivered intravenously just prior to transplantation, and a 45-d course of oral cyclosporine A. On days −4 through −1 (relative to transplantation), recipients received recombinant CD3-immuntoxin (pCD3-rIT; 50 μg/kg)[24] twice daily and 8 h apartSwine were premedicated with 2 mg/kg diphenhydramine, and then the pCD3-rIT was administered by intravenous push, followed by flushing with PBS. Cyclosporine was administered through a gastrostomy tube twice daily beginning on day −1 and concluding on day 45. Target (therapeutic) levels were 400 to 800 ng/mL from day −1 to 30, followed by steady tapering until day 45, at which point cyclosporine levels were less than 200 ng/mL and considered to be subtherapeutic.

Donor cytokine mobilization and hematopoietic cell transplantation. Donor SLA^{ac} miniature swine (age, 6 to 12; weight, 40 to 60 kg) underwent hematopoietic stem cell mobilization with IL3 and porcine stem cell factor at doses of 100 μg/kg for the first 30 kg of body weight and 50 μg/kg for the remaining body weight. Porcine-specific cytokines were developed by Biotransplant (Charlestown, MA), and supplied either through BioTransplant or the DF-HCC Recombinant Protein Expression and Purification Core facility at Massachusetts General Hospital. The effects of these reagents on the mobilization of hematopoietic progenitor cells in pigs have been discussed previously.[16] Cytokines were administered beginning on day −5 and concluding on day 2 or until the animal was deemed clinically unfit to continue. Animals (n = 6) were sedated with Telazol (1 to 2 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) prior to subcutaneous administration of pIL3 and porcine stem cell factor. Peripheral blood mononuclear cells were harvested during 8 to 10 h of leukapheresis beginning on day 0. Donor pigs were leukapheresed while awake thereafter on day 1 and day 2 until the target cell dose of 15×10^9 cells/kg was achieved or until pigs were clinically unfit to continue. After each leukapheresis collection, cells were infused into conditioned recipient animals at a rate of 20 mL/kg/h. Outcomes of cell mobilization regimens in donors and subsequent hematopoietic cell transplantation of recipients were compared retrospectively with those previously published.[3] Any additional blood products (that is, platelets, packed RBC) were administered intravenously at a rate of 20 mL/kg hourly.

Tests. Data on PT and PTT parameters from naive pigs were obtained from IDEXX Veterinary Diagnostic Laboratories (Westbrook, Maine) for comparison with experimental values. The normal range for PT in pigs is 8.6 to 15.0 s, for PTT is 5.8 to 18.6 s, and for D-dimers is 149 to 1586 μg/L.[22]

PCR assay. PCR analysis was performed according to previously established methods.[18] Negative control DNA was extracted from the peripheral blood mononuclear cells of SLA^{DD} and SLA^{AA} swine, and DNA for positive controls was obtained from SLA^{CC} pigs. The primers used in the PCR amplification were: primer 136, 5′ CAC TCC CTG AGC TAT TTC 3′); primer 138, 5′ GCT CTG GTT GTA GTA GCC 3′; and primer 146, 5′ GTG TCC CTT TGT ATC TGT GTC 3′. Primers 136 and 138 amplified a 254-bp segment of the SLA class I gene common to the A, C, and D SLA haplotypes; this fragment served as a positive control. The primer pair of 136 and 146 amplified a 199-bp segment of the SLA class I gene unique to the SLA^{C} haplotype (SLA class I^{C}), which was present only on donor cells.

PCR amplification was performed by using a programmable thermal cycler (PTC100, MJ Research, Watertown, MA) with template denaturation at 94 °C for 15 min, followed by 45 cycles of melting at 94 °C for 15 s, annealing at 53 °C for 15 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Southern blot analysis of amplification products of primers 136 and 146 used the internal oligonucleotide probe no. 162 (5′ TAC GTC GAC GAC ACG CAG TTC G 3′), which is specific for Class I of SLA^{C}.

Results

Donor swine that experienced adverse reactions after cytokine-induced mobilization of peripheral hematopoietic progenitor cells. Donor swine that underwent mobilization regiments in the current study had abnormal coagulation parameters, including PT (Figure 1 A), PTT (Figure 1 B), and D-dimers (Figure 1 C), all of which correlated with the number of doses of IL3 and porcine stem cell factor that they received. After cytokine treatment to mobilize hematopoietic progenitor cells and leukapheresis, donor pigs exhibited signs and symptoms compatible with disseminated intravascular coagulation, including petecchia and ecchymoses (Figure 2 A through C), severe hemorrhagic diarrhea, thrombocytopenia, and the presence of shistocytes by peripheral blood smear. The first signs of sickness were loss of appetite and general lethargy. These objective signs typically occurred after the second day of cytokine injections and were not noted in previous donor pigs.[3] Initial symptoms presented after the fourth or fifth dose of cytokines. Physical exam of donors demonstrated generalized erythema that variably progressed to petechia and purpura. These changes affected the inguinal and axial areas and ears (Figure 2 A through C). In addition, all swine developed diarrhea or soft stools prior to aphaeresis.

After apheresis, the pigs' clinical condition worsened; pigs began to exhibit severe ecchymoses, and all developed hematochezia and sloughing of the gastrointestinal lining. At this point, apheresis was discontinued, and supportive care was initiated

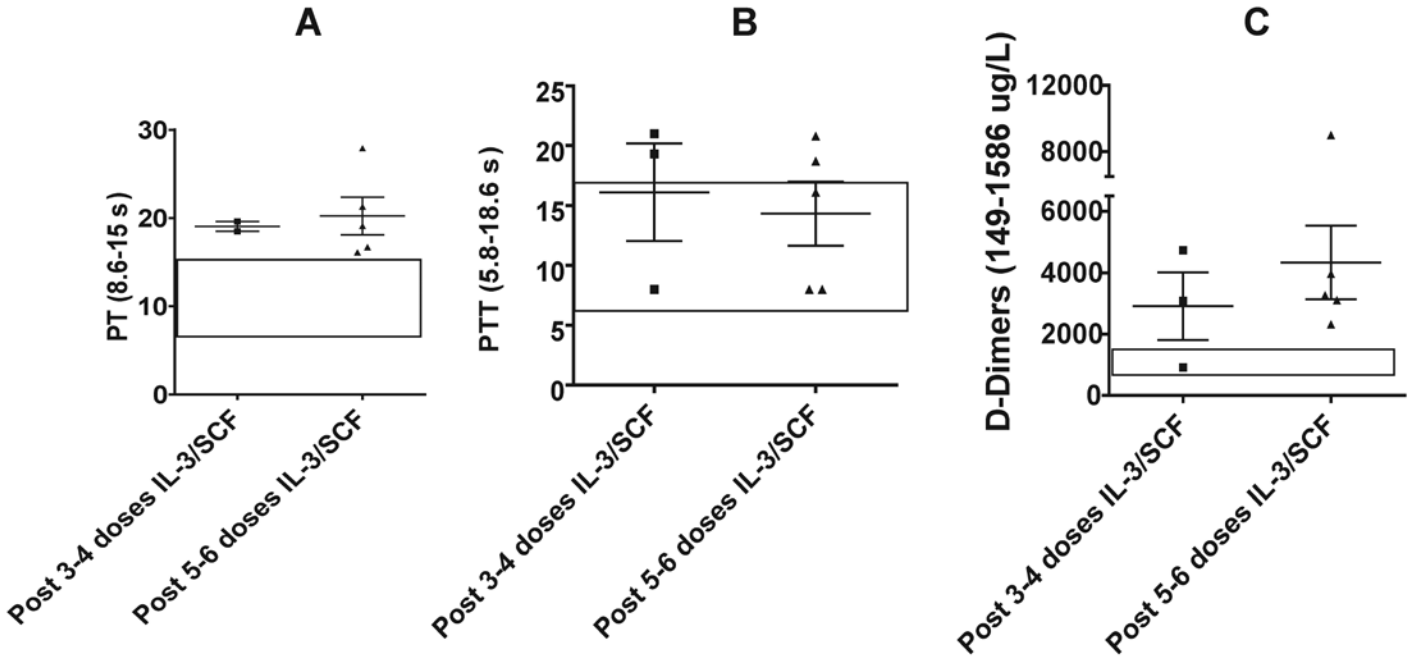

Figure 1. Coagulation parameters in swine that experienced severe adverse reactions during cytokine treatment. (A) PT. (B) PTT. (C) D-Dimers. Open rectangles indicate normal levels for PT (8.6 to 15.0 s), PTT (5.8 to 18.6 s), and D-dimers (149 to 1586 µg/L) in swine.

and included prophylactic antibiotic treatment (metronidazole and ceftriaxone) and fluid therapy. Some swine required colloidal support due to hypoproteinemia. Blood cultures taken from animals prior to antibiotic therapy were negative. No animals required euthanasia due to these adverse effects, and all were closely followed by the veterinary staff. Previous donor pigs[3] did not experience any adverse reactions after cytokine treatment and leukapheresis; instead, those pigs exhibited only a generalized erythema, a well-documented effect of treatment with IL3 and porcine stem cell factor (Figure 2 D and E).

Effects in recipients of cells from donors with adverse reactions. All recipient pigs (n = 11) in the current study developed marked hematologic abnormalities. The most prominent finding was pronounced thrombocytopenia: all 11 recipients of grafts from donors experiencing adverse reactions displayed platelet counts below 100,000 per microliter. Furthermore, 8 of these 11 pigs developed severe and clinically significant thrombocytopenia, with platelet levels below 20,000 per microliter (Figure 3 A) and necessitating support with blood products. Some swine demonstrated severe petechiation and ecchymosis; several had scleral hemorrhage, melena, or hematochezia; and schistocytosis was observed in blood smears, supporting a consumptive coagulopathy. Swine with platelet counts below 20,000 per microliter or with evidence of described clinical signs were transfused. Thrombocytopenia in recipients typically began 7 d after transplantation, and transfusion support usually was required by 14 d after transplantation. Recipients were transfused with platelet-rich plasma from the original donor or donor-matched haplotype pigs. In addition, some recipients developed clinically relevant anemia requiring transfusion with irradiated whole blood or packed RBC. Transfusions were discontinued after platelet levels stabilized above 20,000 per microliter, when petechiae or ecchymoses did not worsen, and when there was no clinical evidence of bleeding. Platelet levels in all pigs returned to normal (above 100,000 per microliter), on average, 21 d after transplantation and remained at that level for the duration of the studies. In contrast, only 4 of the 16 recipients of cells from donors without adverse reactions to cytokine treatment (Figure 3 B shows 3 representative animals) developed thrombocytopenia, with platelet counts below 100,000 per microliter; none of these animals required blood products.

Complications after haploidentical transplantation of cells from donors with adverse reactions to cytokine treatment. The 11 recipients in the current study received cell doses that ranged between 4.0 to 15.0 × 10^9 cells per kilogram (Table 1). Of these 11 swine, 5 died or required euthanasia before week 14 due to serious posttransplantation complications. Two of these 5 pigs died in the early posttransplantation period, including one (18860) on day 23 after transplantation from acute respiratory distress syndrome that was suspected to be secondary to engraftment syndrome or idiopathic pneumonia syndrome. The other pig that died during the early posttransplantation period (18861) was euthanized on day 44 due to severe airway blockage from posttransplantation lymphoproliferative disorder. One pig (18432) developed severe GvHD on day 76 after transplantation and was euthanized. The remaining 2 recipient swine that died before the end of the study died after donor leukocyte infusion on day 57 after transplantation: one (19561) from sepsis secondary to severe bone marrow failure; the other pig (19560) was euthanized after developing severe GvHD.

The remaining 6 of the 11 recipient pigs survived beyond 14 wk and were assessed for transplantation outcomes. All 6 recipients lost peripheral blood myeloid chimerism, indicating a lack of stem cell engraftment in the bone marrow. To assess long-term engraftment, colony-forming-unit assays were performed on bone marrow biopsies at least 14 wk after transplantation; PCR assays for class I^c antigen were used to detect donor-derived cells (Figure 4).

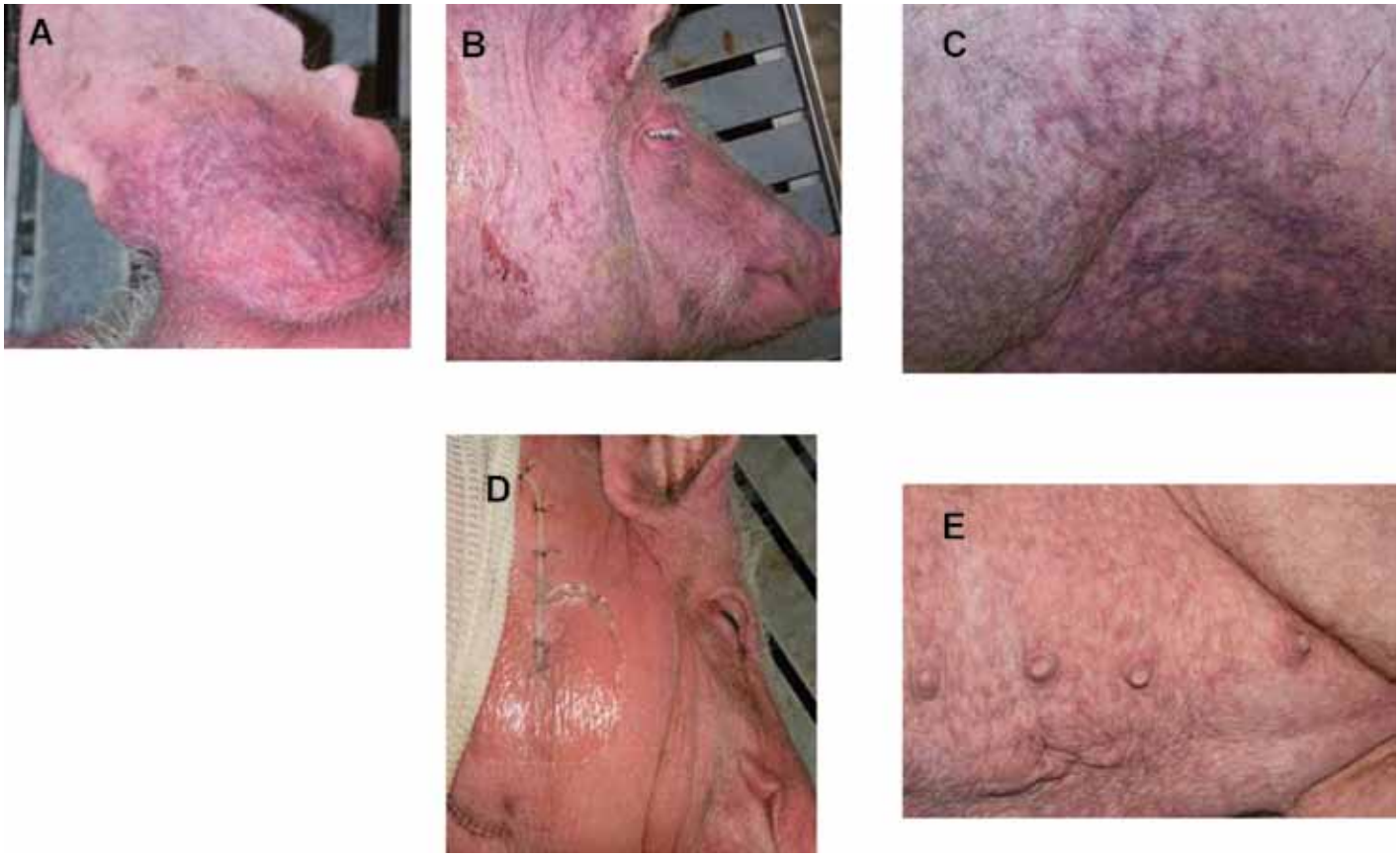

Figure 2. Donor pig that experienced a severe adverse reaction to cytokine treatment. (A) Severe petechiae on ears. Severe petechiae in (B) head and neck region and (C) abdomen. (D and E) Erythema in a pig that did not have any adverse effects.

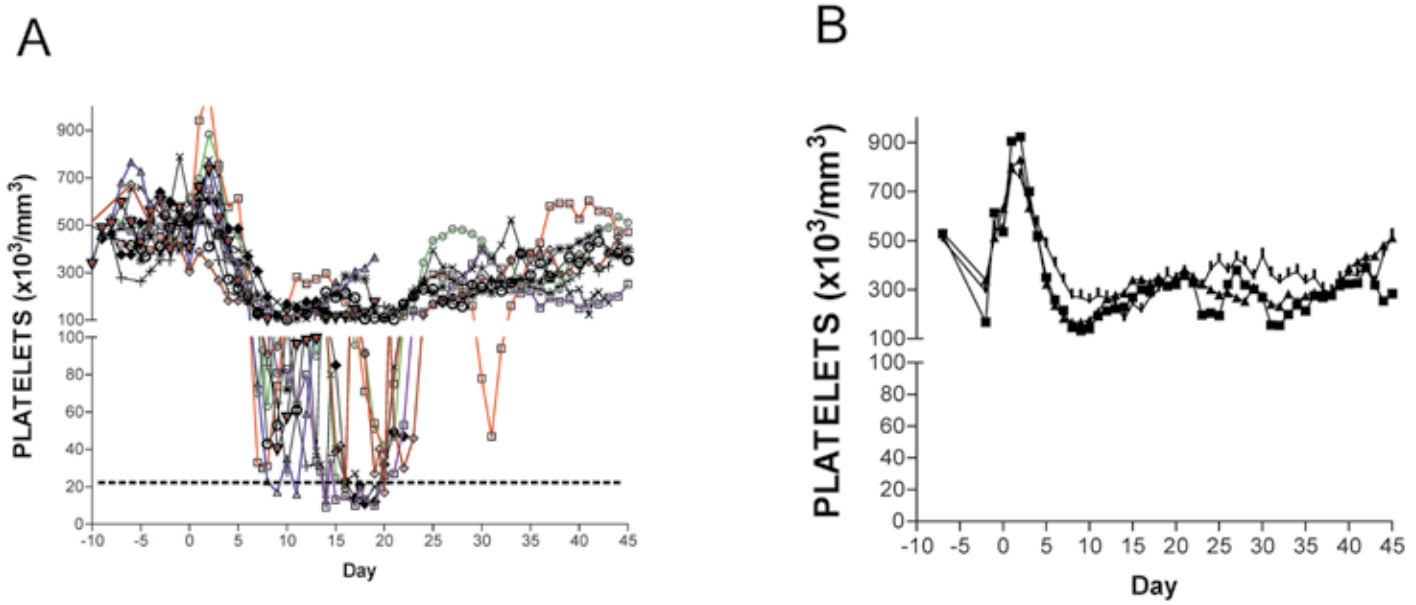

Figure 3. Platelet counts in recipient pigs that received stem cell grafts from (A) donors experiencing adverse reactions during cytokine treatment and (B) donors that did not have any adverse reactions.

One (19141) of these 6 animals maintained donor lymphoid chimerism at low levels and was unresponsive to the original donor by mixed-lymphocyte reaction and cell-mediated lympholysis. The remaining 5 (19138, 19139, 19140, 19925, and 19926) of these 6 recipients eventually lost peripheral blood chimerism and regained in vitro antidonor responses (Figure 5).

Table 1. Summary of recipients receiving hematopoietic cell transplants from donors experiencing adverse reactions during cytokine treatment

Pig	No. of hematopoietic cells received (per kg)	Day of death (after transplantation)	Chimerism at week 14 or death	Stem cell engraftment?	Posttransplantion complications
18860	9×10^9	23	lymphocytes, monocytes, granulocytes	No	Thrombocytopenia; acute respiratory distress syndrome
18861	9×10^9	44	lymphocytes, monocytes, granulocytes	No	Thrombocytopenia; posttransplantion lymphoproliferative disorder
18432	15×10^9	76	lymphocytes, monocytes, granulocytes	No	Thrombocytopenia; GvHD
19560	4×10^9	93	–	No	Thrombocytopenia; GvHD after donor leukocyte infusion
19561	4×10^9	60	lymphocytes (approximately 40%)	No	Thrombocytopenia; sepsis; bone marrow failure after donor leukocyte infusion
19138	12×10^9	long-term survival	–	No	Thrombocytopenia
19139	12×10^9	long-term survival	–	No	Thrombocytopenia
19140	7.5×10^9	long-term survival	–	No	Thrombocytopenia
19141	7.5×10^9	long-term survival	lymphocytes (<5%)	No	Thrombocytopenia
19925	5×10^9	long-term survival	–	No	Thrombocytopenia
19926	5×10^9	long-term survival	–	No	Thrombocytopenia

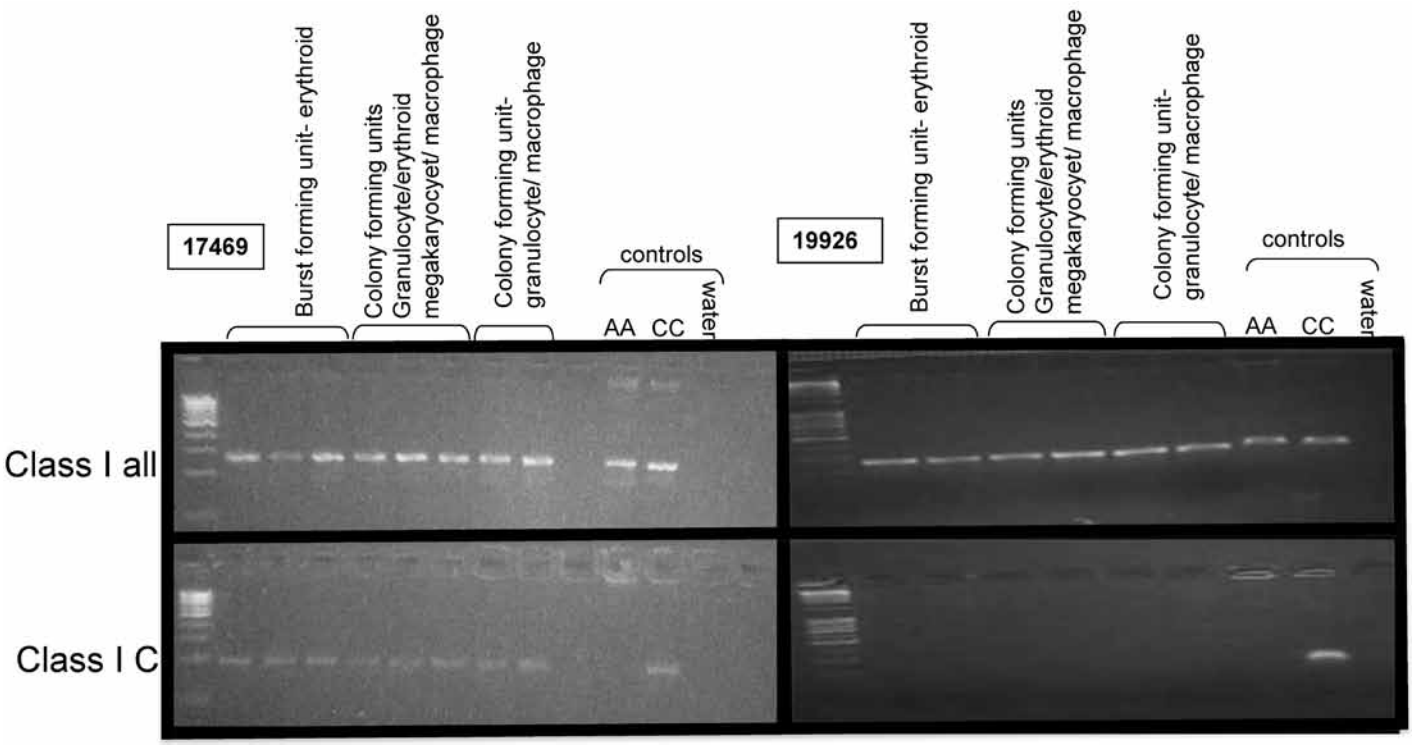

Figure 4. Donor MHC-I^c colony-forming units by PCR analysis. PCR analysis of pigs that received a haploidentical hematopoietic cell transplant from a donor that was healthy (17469, left) at week 56 posttransplantation and a donor that had an adverse reaction (19926, right) at week 14 posttransplantation. The class I-all PCR assay (top) was used to control for quantity and quality of DNA template. Pig 17469 had evidence of donor-derived SLA^c PCR product; this animal engrafted and maintained long-lasting mixed chimerism. In contrast, pig 19926 did not engraft, had no evidence of SLA^c, and lost peripheral blood chimerism.

Conversely, recipient swine whose donors did not experience adverse reactions and maintained multilineage chimerism long-term, were unresponsive to donor stimulators by mixed-lymphocyte reaction, and maintained a third-party response.[3]

Discussion

Allogeneic hematopoietic cell transplantation has broad clinical applications, ranging from the treatment of hemoglobinopathies and leukemias to the induction of tolerance to solid organs.

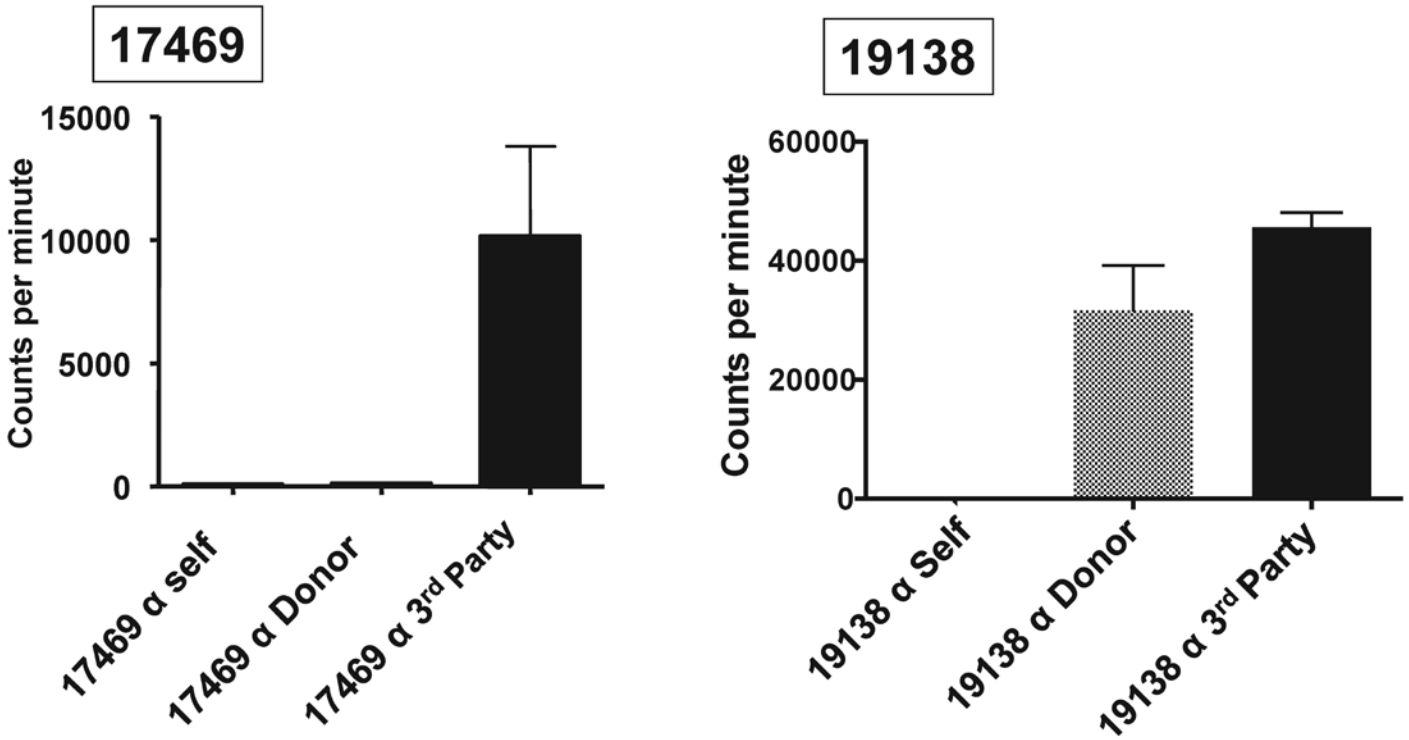

Figure 5. Pig 17469 (left panel) received a hematopoietic cell transplant from a healthy donor. The pig engrafted, was tolerant to the donor (as indicated by the absence of proliferative responses to the donor, middle bar) at 9 mo after transplantation, and responsive to a third-party stimulator (black bar). In comparison, pig 19138 (right panel) received a hematopoietic cell transplant from a donor that had an adverse reaction; proliferative responses were observed to both the donor and third party at 4 mo after transplantation.

Donor health during cytokine treatment to mobilize hematopoietic progenitor cells from the bone marrow and aphaeresis to collect them from peripheral blood recently has come under investigation due to concerns over potential short- and long-term adverse effects of these procedures. However, the effects of adverse events in donors on the outcomes of hematopoietic transplants in recipients have not yet been well studied.

Further investigation into the possible changes from our previous protocol,[3] in which donors tolerated mobilization well and all recipients engrafted without significant GvHD, revealed that the route of cytokine injection and the exclusion of diphenhydramine as a premedication differed between the 2 cohorts. We found that subcutaneous injection of IL3 and porcine stem cell factor in the inguinal area of the older pigs in the current study resulted in severe reactions including fever, loss of appetite, bloody diarrhea, shedding of the GI lining and development of disseminated intravascular coagulopathy in these donor animals prior to leukapheresis. In contrast, intramuscular injection of the same cytokines into the back of the neck or the flank was well tolerated. Donor swine injected with cytokines intramuscularly after premedication with diphenhydramine remained clinically healthy, and all conditioned recipients of mobilized cells from these donors developed stable multilineage chimerism in the peripheral blood.[3] Of note, the chimeric recipients described previously received pCD3–CRM9 immunotoxin.[15] Moreover, none of these previous recipients required blood product support. In contrast, all 11 recipients of hematopoietic cells collected from donors in the current study developed hematologic and coagulation abnormalities failed to engraft, and 8 of these 11 recipients required blood product support as a result of clinically significant thrombocytopenia (fewer than 20,000 platelets per microliter) during the early posttransplantation period (Figure 3). Contaminated cytokine preparations were ruled out as the cause of the adverse reactions in that concentrations of LPS and *Pichia pastoris* were below the maximal levels permitted by the Food and Drug Administration (data not shown) and were comparable to those of previous lots used in donors that did not react negatively.

Preliminary results suggest that subcutaneous compared with intramuscular administration of cytokines may affect subsequent inflammatory responses.[13] Although there is no conclusive evidence indicating that subcutaneous administration of cytokines was the primary cause of the donor reactions, we have reverted to our previous administration protocol consisting of premedication with intravenous diphenhydramine prior to intramuscular administration of cytokines. Overall, these subsequent donor swine have remained clinically healthy during cytokine treatment and leukapheresis, and the recipients have maintained trilineage chimerism. We confidently rule out differences in the manufacturers of the cytokines and in T-cell depletion reagents as contributing factors to the different hematopoietic cell transplantation outcomes, given that the same cytokines and T-cell–depletion agents have been used in subsequent studies with successful outcomes. Although we consider the differences between the mobilization protocols as the likely cause of the differences in outcome, we cannot rule out the possible involvement of other factors.

Overall, the current data suggest that engraftment outcomes and posttransplantation complications are influenced by many factors that affect the health status of the donor before and during

the collection. We offer 2 hypotheses that may explain these findings. First, as part of the pathophysiology of inflammation, proinflammatory cytokines, including TNFα, IL1, and IL6, increase and toll-like receptors are activated, leading to abnormal coagulation.[17] Several studies have implicated these inflammatory cytokines in contributing to negative transplantation outcomes.[6,11,12,19] One study demonstrated a correlation between serum TNFα levels and the development of acute GvHD after bone marrow transplantation.[11] TNFα levels were increased 2-fold in patients that exhibited grade II acute GvHD and nearly 3-fold in those patients with more severe GvHD.[22] These data suggest the importance of a quiescent immunologic state before and immediately after donor cell infusion for achieving successful transplantation outcomes. In the context of our studies, the transplanted grafts likely contained proinflammatory cytokines that were released as part of the physiologic reaction to cytokine treatment and leukapheresis. Given the role of cytokines in increasing cell-surface expression of leukocyte adhesion molecules and histocompatibility antigens, cells from donors that experienced adverse reactions likely exhibited increased antigenicity.[7] In the recipients of grafts from donors experiencing adverse reactions, increases in proinflammatory cytokines and alloreactivity of T cells could favor a state resulting in the failure of donor stem cell engraftment and loss of donor chimerism.[9] This negative outcome may be more likely in a nonmyeloblative setting, in which host and donor T cells are not fully depleted.

Second, as established in a retrospective analysis of organ recipients from deceased donors with or without disseminated intravascular coagulation,[23] a correlation exists between donors with disseminated intravascular coagulation and recipients that develop thrombocytopenia during the early posttransplantation period. Our current findings support these clinical observations. The mechanism underlying this phenomenon is poorly understood; however, in the present study, proinflammatory cytokines combined with activated alloreactive T cells from donor grafts may have targeted the lymphohematopoietic system of recipients, thereby inducing thrombocytopenia. Donor swine with adverse reactions also exhibited severe mucohemorrhagic diarrhea, possibly resulting in bacterial translocation into the circulation and contributing to the systemic posttransplantation inflammatory response after cell transfer into an immunocompromised recipient. Consequently, multiple blood product transfusions may have affected transplantation outcomes. Repeated exposure to donor antigen, albeit irradiated, may have provoked a sensitization and rejection process, explaining why those recipients eventually lost donor chimerism.

In summary, this study evaluated the outcome of haploidentical hematopoietic cell transplantation in which donor animals experienced adverse reactions at the time of cell donation and retrospectively compared results with previous transplantations in which donors did not have complications. Recipients showed a clear difference in posttransplantation complications and outcomes depending on whether donors experienced adverse reactions after cytokine treatment. Overall, the occurrence of adverse reactions to cytokine treatment in donor swine correlated with early posttransplantation complications and loss of donor chimerism in surviving recipients. These findings in swine indicate that the use of a stem-cell graft from a donor that experiences an adverse reaction during mobilization or harvest merits careful assessment.

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