Adoptive Immunotherapy against Allogeneic Kidney Grafts in Dogs with Stable Hematopoietic Trichimerism

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ABSTRACT

Dogs given nonmyeloablative conditioning and marrow grafts from two dog leukocyte antigen-(DLA) identical littermate donors developed stable trichimerism and stably accepted a subsequent kidney graft from one of the marrow donors without the need for immunosuppression. Here, we used trichimeras to evaluate strategies of adoptive immunotherapy to solid tumors, using the kidney as a tumor surrogate. Three DLA-identical trichimeric recipients were established by simultaneously infusing marrow from two DLA-identical donor dogs into a DLA-identical recipient conditioned with 2 Gy total body irradiation and given a short course of postgrafting immunosuppression. After confirming stable hematopoietic engraftment, a kidney was transplanted from one of the two marrow donors into each respective trichimeric recipient. Peripheral blood lymphocytes (PBL) from each kidney donor were then used to sensitize the alternate marrow donor. Donor lymphocyte infusions (DLI) from the sensitized dogs were given to the trichimeric recipients, whereupon chimerism, graft-versus-host disease (GvHD), and kidney rejection were monitored. After DLI, we observed both prompt rejection of the transplanted marrow-donor kidney and disappearance of corresponding hematopoietic chimerism. Presumably, owing to shared minor histocompatibility antigens, host chimerism also disappeared and GvHD in skin, gut, and liver developed. The native kidneys, while showing lymphocytic infiltration, remained functionally normal. The current study demonstrated that under certain experimental conditions, the kidney, an organ ordinarily not involved in graft-versus-host reactions, can be targeted by sensitized donor lymphocytes.

INTRODUCTION

Graft-versus-tumor effects against hematological malignancies after major histocompatibility complex (MHC) identical allogeneic hematopoietic cell transplantation (HCT) are the result of donor lymphocyte activity against minor histocompatibility (H)-antigens expressed on hematopoietic cells [1,2]. Target minor H-antigens include both ubiquitously expressed antigens and those specific for hematopoietic cells. Given these observations with blood cancers, clinicians have been quick to explore whether allogeneic graft-versus-tumor effects might exist and could be therapeutically exploited in a variety of metastatic solid tumors. Malignancies tested include colon, breast, prostate, and renal cancers among others [3-7]. Outcomes have been variable. Convincing graft-versus-tumor effects have been reported in a minority of patients with colon and renal cell cancer and possibly breast cancer, while patients with other types of malignancy showed no responses.

Previous work in our laboratory evaluated whether graft-versus-host reactions, typically directed against hematopoietic cells, skin, gut, and liver, could be diverted to reliably include metastatic solid malignancies [8]. In these experiments, we used a canine HCT model, in which the kidney served as a surrogate tumor target. The experiment was designed to determine whether adoptive immunotherapy could result in rejection of a specific organ not ordinarily involved in graft-vs.-host reactions. Stable mixed chimerism in this model has been maintained by regulatory T-cells [9,10] and could not be dislodged by infusion of naïve donor lymphocytes [11]. In order to shift mixed to all-donor chimerism and induce graft-vs.-host disease (GvHD), donor lymphocytes were sensitized to host minor H-antigens [8]. Accordingly, after establishing mixed chimerism in DLA-identical littermate recipients, the marrow donors were given kidney

transplants from their respective mixed chimeric recipients, which they rejected within 3 to 5 weeks. When marrow donor lymphocytes, harvested after kidney graft rejection, were injected into the recipients, mixed hematopoietic chimerism converted to full donor chimerism. However, while 2 of 5 dogs developed GvHD, the residual native kidneys which expressed the same minor antigens that were used to sensitize the donor were not targeted by the adoptively transferred lymphocytes. Thus, while marrow donors readily rejected their recipients' kidneys and thereby became sensitized to the mixed chimeras' ubiquitously expressed minor H-antigens as evidenced by elimination of host hematopoiesis and GvHD, the level of sensitization was apparently insufficient to induce immunologic damage to the recipients' remaining kidneys.

To further investigate this question, we developed a trichimeric model in which marrows from each of two DLA-identical littermate donors were simultaneously transplanted into a third littermate conditioned with 2 Gy total body irradiation (TBI) and given a short course of postgrafting immunosuppression [12]. A kidney heterotopically transplanted from one of the two marrow donors into the groin of each trichimera was stably accepted and then served as a solid organ surrogate for graft-versus-"tumor" reactions. To this end, the other marrow donor was sensitized against H-antigens of the marrow/kidney donor, and sensitized lymphocytes were subsequently infused into the respective trichimeras. The heterotopic location of the kidney grafts allowed for easy histological monitoring for lymphocyte infiltration and rejection. Additionally, we postulated that transplantation of the kidney would give rise to danger signals [13], thereby increasing the likelihood of becoming targets for lymphocytes sensitized to minor H-antigens.

MATERIALS AND METHODS

Dogs

Litters of beagles and beagle/mini-mongrel mixes were raised at the Fred Hutchinson Caner Research Center (FHCRC) and assessed for disease. They underwent a preventive medicine program against worms, distemper, parvovirus, adenovirus (type 2), parainfluenza virus, corona virus, rabies and canine papilloma virus. Dogs were 7–9 (median 8) months old and weighed 7.2–12.3 (median 8.5) kg. The study was approved by the Institutional Animal Care and Use Committee at the FHCRC (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International). Selection of donor/recipient triplets included typing of litters and parents to determine identity of triplets for highly polymorphic microsatellite markers within DLA class I and class II regions [14] which was confirmed by DLA-DRB1 gene sequencing [15].

Marrow Grafts

On day 0, recipients were treated with 2 Gy TBI and subsequent i.v. infusion of marrow from donors 1 and 2 as previously described (Fig. 1A) [12,16]. Bone marrow was aspirated from humeri of anesthetized donors by vacuum pump aspiration. Briefly, the skin over the humeral heads was surgically prepared. Using sterile techniques, a long aspiration needle was inserted into the marrow cavity of the shoulder joint. The needle was connected to a vacuum flask containing heparin via surgical tubing. Approximately 150 ml of blood-marrow mixture were collected per donor. The mixture was passed through 0.307 and 0.201-mm diameter stainless steel mesh screens. Nucleated marrow cell counts were corrected for white blood cell content. Marrows from both donors were infused into the recipients via the cephalic vein with

approximately a 2-hour delay between infusions. The marrow grafts from individual donors contained a median of 4.6 (range 3.6-6.1) x 10^8 nucleated cells/kg. Dogs were given supportive postgrafting care. Immunosuppression consisted of oral cyclosporine (CSP), 15 mg/kg orally twice daily (BID) from days -1 to 35, and mycophenolate mofetil (MMF), 10 mg/kg BID injected subcutaneously from days 0 to 27 [17]. MMF dosing was adjusted according to clinical toxicity which consisted of gastrointestinal distress.

Chimerism Analysis

Chimerism analyses were done on PBMC and granulocytes following separation of blood on Ficoll (density =1.074), on marrow cells after buffered NH_4Cl lysis of red cells [12], and on sections of kidney allografts collected at necropsy. For the latter, infiltrating lymphocytes were collected from the minced sections of resected kidneys after 16 hours of incubation (37^oC, 5% CO_2) using methods similar to those described [18] but without the use of enzymatic digestion. After overnight incubation of resected minced kidney tissue in RPMI medium (Gibco/Invitrogen, Carlsbad, CA) + 10% heat inactivated dog serum, the cells were layered over Ficoll (density 1.074) and centrifuged at 1100 x g for 40 minutes. Cells at the interface were collected and washed in phosphate buffered saline by centrifugation. The contributions of recipient and donor cells to peripheral blood and other hematopoietic tissues and kidney were quantified by fluorescent variable number of tandem repeat (VNTR) *PCR* analysis, as described [12].

Kidney Transplantation

Kidney allografts from one of the marrow donors (donor 2) to the respective trichimeric marrow recipients were performed as described [19]. Briefly, donor 2 was anesthetized, a midline laparotomy performed, and the left kidney exposed. The ureter, renal vein, and renal artery were secured and transected. The kidney was removed from the body cavity and perfused with 4^{o}C saline containing 10 U/ml heparin. The kidney was maintained in cold heparinized saline while the recipient was prepared for surgery. The kidney was subsequently transplanted into the trichimeric recipient's right anterior thigh, the renal artery and vein anastamosed to the femoral artery and vein respectively, and the ureter implanted into the bladder [8]. Kidney graft recipients received no immunosuppressive therapy. The vascularity of the transplanted kidney was confirmed by an Ultrasonic Doppler Flow Detector (Parks Medical Electronics, Aloha, OR). At least two biopsies of transplanted kidneys were performed before completion of the study. Tissue was fixed in 10% buffered formalin and stained using hematoxylin and eosin for evaluation by microscopy. Kidney volume was calculated based on techniques for measuring xenograft tumor volume using the formula: $0.1667 (L \times W^2)$ where L (length) and W (width) were orthogonal measurements of kidney diameter [20].

Donor Sensitization and DLI

Marrow donor 1 was sensitized to minor H-antigens from donor 2 (marrow + kidney donor) using a mean of 4.6 (range 1.6-8.5) x 10^7 PBMC (Fig. 1B). To that end, 50 ml of blood were collected from the kidney donor on each of three occasions, 10 days apart. Half the volume was injected intravenously and half was treated with ammonium chloride lysing solution (155 mM ammonium chloride, 10 mM sodium bicarbonate, 0.1 mM EDTA), washed, and injected

subcutaneously into the flank of the first marrow donor. Ten days after the third injection, leukopheresis was performed on donor 1 via a central venous catheter in the external jugular vein using a COBE 2997 continuous flow centrifuge (COBE BCT, Inc., Blood Component Technology, Lakewood, CO) [21,22].

RESULTS

Three stable hematopoietic trichimeric dogs received kidney transplants from one of their marrow donors 25 to 40 weeks after marrow grafting without postgrafting immunosuppression [12]. The hematological profiles and blood chemistries were in the normal range. Table 1 shows that host cell contributions among peripheral blood granulocytes at the time of kidney transplant and DLI ranged from 1–20% and from 16–50% among lymphocytes. Hematopoietic chimerism contributions from marrow donor 1 ranged from 18–96.9% and 5–53% among the granulocytes and lymphocytes, while those of donor 2 (marrow + kidney) ranged from 0.1–68% and 19–56% for the two respective populations.. Two of the trichimeric recipients (G362 and G643) did not receive immunosuppressive therapy since day 35 after marrow grafting. Dog G513 required 15 weeks of cyclosporine (7.5 mg/kg with taper to 2.5 mg/kg) before successful resolution of GvHD; in this dog an additional 14 weeks elapsed between discontinuing CSP and kidney transplantation. Kidneys were negative for lymphocytic infiltration for 17 to 23 weeks before DLI as assessed by histologic examinations of needle biopsy specimens and measurements of kidney volume (Fig. 2, left column). Trichimerism remained stable and unchanged between kidney transplantation and DLI.

Effect of DLI on Kidney Allografts and Native Trichimeric Kidneys

In each of the three sets of littermates, marrow donor 1 was sensitized against marrow/kidney donor 2 by three injections of PBMC administered 10 days apart. Ten days after the last injection, a mean of 4.2 (range 2.5-5.1) $x10^8$ PBMC was harvested from sensitized donor 1 and injected into the respective trichimeric recipient.

Within a mean of 7 (range 3-15) days after DLI, the transplanted kidney volumes began to increase in the three trichimeric recipients. For example, kidney volume in one of the dogs increased 162% in 28 days (data not shown). Biopsies of transplanted kidneys showed lymphocytic infiltration at various time points after DLI (data not shown). Samples taken at necropsy indicated extensive tubulitis and interstitial lymphocytic infiltration of transplanted kidneys consistent with acute severe rejection (Fig. 2, middle column). Periglomerular lymphocytic infiltration was observed in the trichimeric recipients' native kidneys, although these infiltrations were by far less pronounced than those in the allogeneic kidneys (Fig. 2, right column). Seventy-three percent of cells obtained from cultured transplanted kidney tissue in dog G643 were from donor 1, and 27% were from donor 2, the latter possibly due to contaminating kidney cells (data not shown). Serum chemistries after DLI showed normal serum creatinine and blood urea nitrogen levels indicating normal function of the recipients' native kidneys (data not shown).

Chimerism

Until DLI, all three dogs had trichimerism which had been stable for more than 9 months (Table 1). After DLI, a rapid shift from mixed to nearly 100% donor 1 chimerism occurred in all

three dogs within a median of 14 (range 9-28) days. This is illustrated for dog G643 in Fig. 3. Before DLI, donor 1 (G641) contributed the least to the three hematopoietic systems in recipient dog G643, but after DLI, both granulocytes and mononuclear cells from donor 1 dominated the hematopoietic system. The shift from trichimerism to all donor 1 chimerism was also observed in nucleated cells collected from marrow and lymph node at necropsy (data not shown). Peripheral blood cell counts did not change significantly during the shift in chimerism (data not shown).

GvHD after DLI

All three dogs developed liver GVHD, as indicated by serum levels of alkaline phosphatase (1,646, 2004, and 3165 U/L), aspartate aminotransferase (355, 862, and 1,201 U/L), alanine aminotransferase (3,380, 5,440, and 5,618 U/L), and bilirubin (5.0, 7.3, and 7.3 mg/dL). Further, skin GvHD, characterized by inflammatory rashes of the skin of the ears, abdomen, nose, or oral mucosa was observed within 30 (G362), 23 (G513), and 32 (G643) days of DLI. All dogs developed diarrhea and lost weight. The diagnoses of GvHD were confirmed by histopathology following euthanasia (Figure 4). The skin showed changes ranging from minimal infiltration of mononuclear cells to the presence of apoptotic bodies. The liver showed lymphocytic infiltration in central venous regions and bile duct lesions confirming the diagnosis of GvHD. Atypical cell shapes within bile ducts of G513 indicated cell regeneration. The jejunum showed infiltration of mononuclear cells, while the ileum of dog G362 revealed multiple exploding crypts and crypt abscesses. Thus, all three dogs eventually developed three-system acute GvHD and were euthanized because of worsening condition.

DISCUSSION

The current study was undertaken with the aim of targeting the kidney for allogeneic immune reactions, an organ that is not among the typical targets for graft-verus-host reactions; these usually include the hematopoietic system and epithelial cells from skin, gut, liver, and mucous membranes. The study was prompted by the desire to understand how graft-versus-tumor effects, typically observed for hematological malignancies, could be extended to include metastatic solid tumor targets. Several clinical studies have used marrow transplantation to treat a variety of metastatic solid tumors but with very limited success [3,7]. In these studies, no tumor- or tissue-associated minor H-antigens were used to sensitize donors against the patients' tumors. Therefore, it would seem reasonable to explore adoptive immunotherapy with specifically sensitized lymphocytes to achieve successful treatment of metastatic solid malignancies. Because no transplantable tumors exist for random bred dogs, a functioning kidney allograft with a unique set of minor antigens was chosen as a surrogate target since impairment of kidney function could easily be assessed by serum creatinine levels and histopathological examinations of percutaneous kidney biopsies.

In an earlier study, we sensitized canine marrow donors by transplanting kidneys from their respective mixed hematopoietic chimeric recipients, which they promptly rejected [8]. Subsequent lymphocyte infusions from marrow donors into chimeras caused conversion of mixed to all donor chimerism and, in some cases, GvHD in skin, gut, or liver; however, the remaining native kidneys did not come under immunological attack. What explained this lack of graft-versus-kidney effect, a finding which was in striking contrast to the speed with which the marrow donors rejected kidneys from their chimeric recipients? We hypothesized that native

kidneys lacked the appropriate “danger signals” [13] to attract lymphocytes and that these signals were present in the transplanted kidneys. Under this model, danger signals may be either constitutively expressed or, in the case of transplantation, induced upon stress, hypoxia, or trauma.

Here, we attempted to address the hypothesis in stable trichimeras which had received marrow grafts from two DLA-identical littermates and a kidney graft from one of the marrow donors. The marrow donor kidney was grafted heterotopically as a putative target for lymphocytes that had been sensitized to minor H-antigens. In all three cases, the kidneys were stably engrafted in the trichimeric recipients due to the induction of tolerance established by dual HCT. After immunizing the first marrow donors against minor H-antigens of the second (marrow and kidney) donors and then infusing sensitized lymphocytes into the marrow recipients, an immunological chain of events occurred that supported, in part, our experimental assumption that an organ, which is ordinarily not a target for GvHD reactions, might be targeted under appropriate experimental conditions. In all three cases, the allografted kidneys became promptly infiltrated with lymphocytes from the sensitized donors and were eventually rejected.

Consistent with the kidney allograft rejection after DLI, the previously stable contribution of the marrow/kidney donor to the trichimeric hematopoiesis disappeared. Further, and perhaps to be expected owing to sharing of minor H-antigens among recipients and kidney/marrow donors, host hematopoiesis also disappeared along with the appearance of GvHD in classical target organs, skin, gut, and liver. Somewhat contrasting with the widespread immunological damage in allografted kidneys, host skin, gut, and liver, and host and donor 2 hematopoiesis, the hosts’ native kidneys showed mild to moderate lymphocytic infiltration (Figure 2), but renal function was not impaired. These findings are consistent with the “danger signal” hypothesis

[13]. The basis of this model was that antigen presenting cells are activated by signals from injured cells that had been exposed to toxins, pathogens, and mechanical damage [23]. Also falling into this category were transplanted organs in which surgical and or ischemic damage occurred resulting in activation of antigen presenting cells. Thomas et al. [24] showed that long-term renal allografts were established in histocompatibility complex mismatched macaques treated with deoxyspergualin with or without immunotoxin (anti-CD3-CRM9, a mutant diphtheria toxin). The proposed mechanism of action of deoxyspergualin was the prevention of antigen presenting cell maturation, suppressed expression of costimulatory molecules, and establishment of a state of tolerance. Although the transplanted kidneys in our trichimeric model showed stable engraftment, undetected molecular events might have played roles in the graft versus host reaction following DLI. Park et al. [25] found in transcriptional profiles of histologically normal living donor kidney allografts an ongoing injury response and inflammation at 1 year posttransplant with upregulated genes associated with inflammation, immunity or response to injury.

Despite this evidence of susceptibility to rejection in the transplanted kidney, we nonetheless observed limited infiltration of lymphocytes within the native kidneys. Perhaps more time might have been required for renal function impairment to occur; however, the observation time was cut short by the development of life-threatening severe GvHD in skin, gut and liver. Nevertheless, it is clear that we successfully induced an immune response against a kidney tissue not normally targeted in GvHD. Further experiments with this model may shed light upon immunological mechanisms averting GvHD in many normal tissues, and may ultimately lead to methods for specifically targeting these responses against solid tumors. Next-generation sequencing studies are currently underway to identify coding nonsynonymous single nucleotide

polymorphisms (nsSNP) in breeders and their offspring that are unique to HCT recipients and expressed on tissues. Some of these polymorphisms may display ubiquitous tissue expression, while others may be restricted to hematopoietic cells or individual organs such as kidneys and, therefore, define sets of minor antigens that could be used to sensitize the marrow donor against a candidate target organ. Accordingly, subsequent DLI would result in immune reactions restricted to the desired target organ, e.g. marrow or kidney, without the development of GvHD.

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REFERENCES

1. Frederik JH, van de Corput L, Marijt EWA, Willemze R. Minor histocompatibility antigens in human stem cell transplantation. *Exp Hematol*. 2003;31:743-751.

2. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. *Nature Reviews Cancer*. 2004;4:371-380.

3. Carnevale-Schianca F, Cignetti A, Capaldi A, et al. Allogeneic nonmyeloablative hematopoietic cell transplantation in metastatic colon cancer: tumor-specific T cells directed to a tumor-associated antigen are generated in vivo during GVHD. *Blood*. 2006;107:3795-3803.

4. Bishop MR. Allogeneic hematopoietic stem cell transplantation for metastatic breast cancer (Review). *Haematologica*. 2004;89:599-605.

5. Ringden O, Le Blanc K. Allogeneic hematopoietic stem cell transplantation: state of the art and new perspectives (Review). *APMIS*. 2005;113:813-830.

6. Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. *N Engl J Med*. 2000;343:750-758.

7. Tykodi SS, Warren EH, Thompson JA, et al. Allogeneic hematopoietic cell transplantation for metastatic renal cell carcinoma after nonmyeloablative conditioning: toxicity, clinical response, and immunological response to minor histocompatibility antigens. *Clin Cancer Res*. 2004;10:7799-7811.

8. Junghanss C, Takatu A, Little M-T, et al. Adoptive immunotherapy against kidney targets in dog-leukocyte antigen-identical mixed hematopoietic canine chimeras. *Transplantation*. 2003;75:268-274.

9. Taranova AG, Georges GE, Yunusov M, Storb RF, Nash RA. Breaking tolerance in stable mixed chimeric dogs with low-dose TBI and donor or recipient lymphocyte infusion. *Blood.* 2003;102 (Part 1):76a, #256 [abstr.]

10. Lesnikova M, Nikitine A, Mason N, Nash RA, Georges GE. Ex vivo expanded T regulatory (Treg) cells block conversion of mixed chimeras to complete donor chimerism. *Blood.* 2006;108 (Part 2):382b, #5168 [abstr.]

11. Georges GE, Storb R, Thompson JD, et al. Adoptive immunotherapy in canine mixed chimeras after nonmyeloablative hematopoietic cell transplantation. *Blood*. 2000;95:3262-3269.

12. Graves SS, Hogan W, Kuhr CS, et al. Stable trichimerism after marrow grafting from 2 DLA-identical canine donors and nonmyeloablative conditioning. *Blood*. 2007;110:418-423.

13. Fuchs EJ, Matzinger P. Is cancer dangerous to the immune system? (Review). *Semin Immunol*. 1996;8:271-280.

14. Wagner JL, Burnett RC, DeRose SA, Francisco LV, Storb R, Ostrander EA. Histocompatibility testing of dog families with highly polymorphic microsatellite markers. *Transplantation*. 1996;62:876-877.

15. Wagner JL, Works JD, Storb R. DLA-DRB1 and DLA-DQB1 histocompatibility typing by PCR-SSCP and sequencing (Brief Communication). *Tissue Antigens*. 1998;52:397-401.

16. Ladiges WC, Storb R, Thomas ED. Canine models of bone marrow transplantation. *Lab Anim Sci*. 1990;40:11-15.

17. Storb R, Yu C, Wagner JL, et al. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood*. 1997;89:3048-3054.

18. Belldegrun A, Muul LM, Rosenberg SA. Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity. *Cancer Res*. 1988;48:206-214.

19. Kuhr CS, Allen MD, Junghanss C, et al. Tolerance to vascularized kidney grafts in canine mixed hematopoietic chimeras. *Transplantation*. 2002;73:1487-1493.

20. Li GP, Zhang H, Zhu CM, Zhang J, Jiang XF. Avidin-biotin system pretargeting radioimmunoimaging and radioimmunotherapy and its application in mouse model of human colon carcinoma. *World Journal of Gastroenterology*. 2005;11:6288-6294.

21. Sandmaier BM, Storb R, Santos EB, et al. Allogeneic transplants of canine peripheral blood stem cells mobilized by recombinant canine hematopoietic growth factors. *Blood*. 1996;87:3508-3513.

22. Lupu M, Gooley T, Zellmer E, Graves SS, Storb R. Principles of peripheral blood mononuclear cell apheresis in a preclinical canine model of hematopoietic cell transplantation. *J Vet Intern Med*. 2008;22:74-82.

23. Matzinger P. The danger model: a renewed sense of self. *Science*. 2002;296:301-305.

24. Thomas JM, Contreras JL, Jiang XL, et al. Peritransplant tolerance induction in macaques: early events reflecting the unique synergy between immunotoxin and deoxyspergualin. *Transplantation*. 1999;68:1660-1673.

25. Park W, Griffin M, Grande JP, Cosio F, Stegall MD. Molecular evidence of injury and inflammation in normal and fibrotic renal allografts one year posttransplant. *Transplantation*. 2007;83:1466-1476.

TABLE 1. Chimerism levels and GvHD

Trichimeric Recipient	Percent Chimerism at Time of (weeks after transplant)						GVHD after	
	Kidney Transplant		DLI		Euthanasia		Kidney Trans-plant	DLI
	Grans	PBMC	Grans	PBMC	Grans	PBMC		
G 362	(25)*		(48)		(56)			
Donor 1	33	28	30	28	100	99		
Donor 2†	66	43	68	56	0	1		
Recipient	1	29	2	16	0	0	No	S,G,L
G 513	(40)		(56)		(59.5)			
Donor 1	96.9	53	87.4	39	91	86		
Donor 2†	0.1	19	0.2	34	6	1		
Recipient	3	28	12.4	27	3	13	No	S,G, L
G 643	(26)		(44)		(49)			
Donor 1	28	5	18	17	95	94		
Donor 2†	55	45	62	49	4	5		
Recipient	17	50	20	34	1	1	No	S,G, L

Abbreviations: DLI, donor leukocyte infusion; Grans, granulocytes; PBMC, peripheral blood mononuclear cells.
*Weeks after hematopoietic cell transplant.
†Donor 2 donated one kidney to the trichimeric recipient. Donor 1 was apheresed following three injections of peripheral blood donated by donor 2. The apheresis product was injected i.v. into the trichimeric recipient dog. Percent chimerism of each of the trichimeric dogs was determined prior to kidney transplantation, DLI, and at the time of euthanasia. Blood for serum chemistries was collected before and after kidney transplantation and within 1 week before euthanasia of the trichimeric kidney recipients. GvHD was diagnosed for skin (S), gut (G), and liver (L) at necropsy.

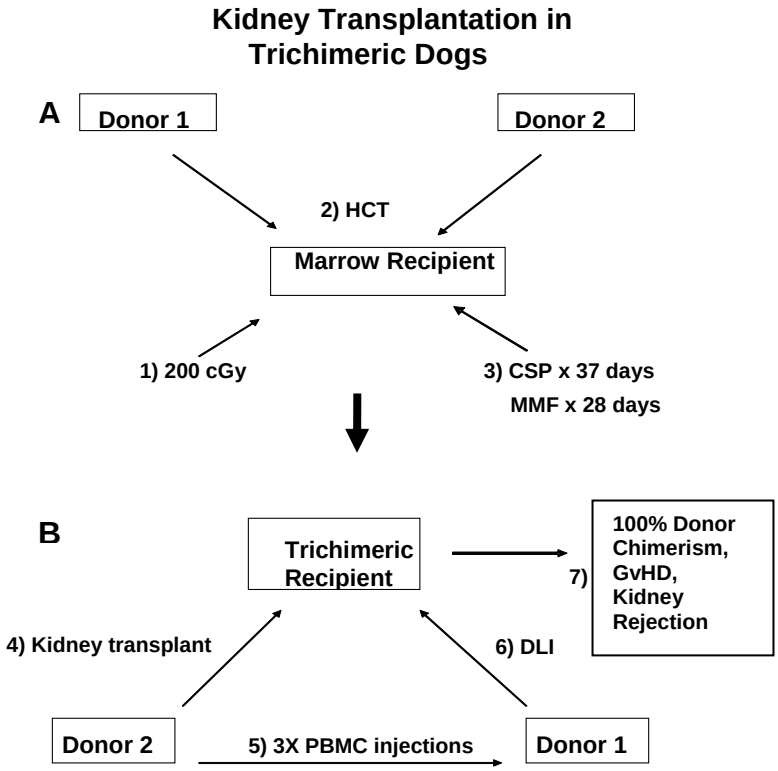

FIGURE 1. A) Schema for generating trichimeric marrow recipients and kidney transplant rejection. After 2 Gy TBI (1), marrow from two DLA-identical donors was simultaneously injected into the recipient (2). Postgrafting immunosuppression with CSP and MMF followed for 35 and 28 days respectively (3). B) After stable trichimerism was established, a kidney was transplanted from donor 2 into the recipient (4). Once stable kidney engraftment was verified, donor 1 was sensitized against minor H-antigens of donor 2 with three PBMC injections (5). Following DLI from sensitized donor 1 into the trichimeric recipient (6), the dogs were monitored for a shift in donor chimerism, GvHD, and kidney graft rejection (7).

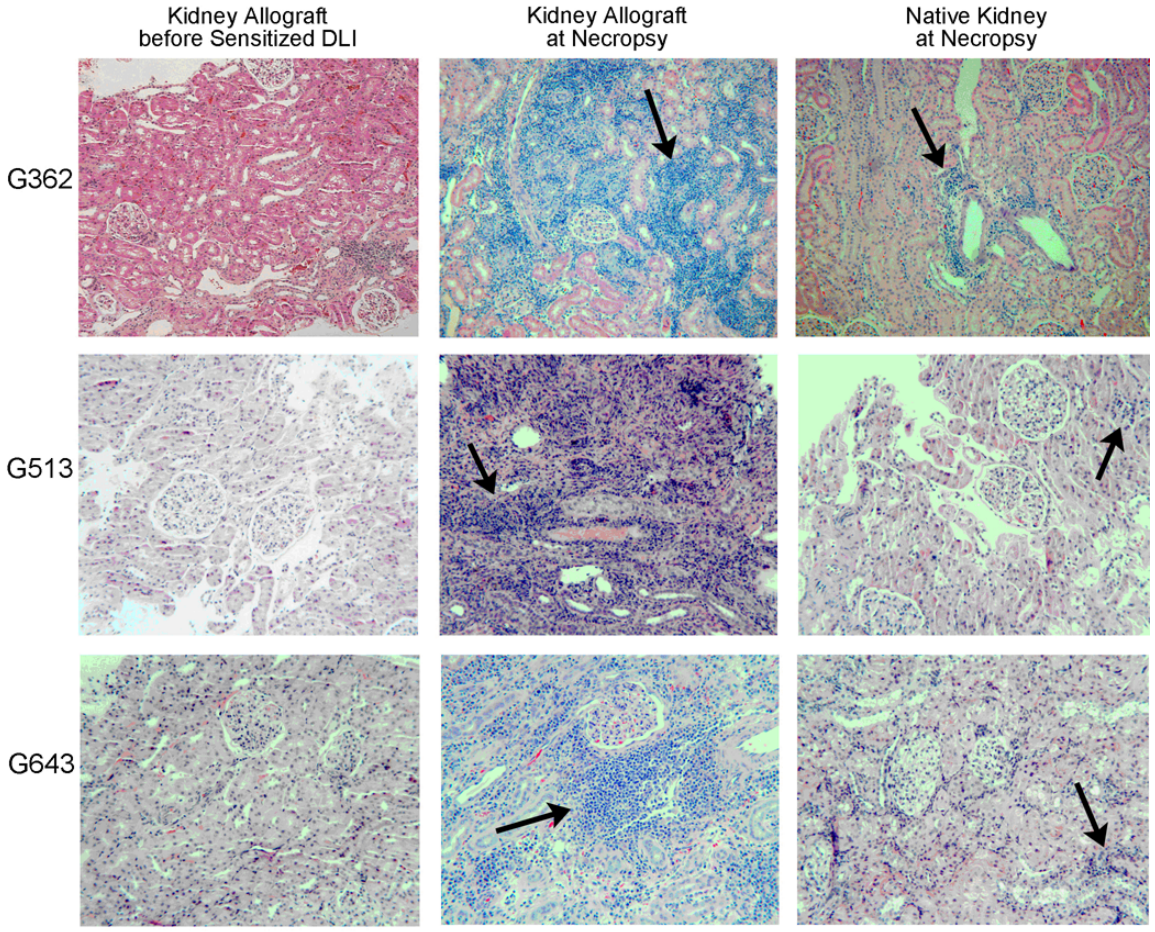

FIGURE 2. Kidney histology of dogs G513, G362, and G643 before sensitized DLI, and at necropsy. Biopsies of kidney allografts were done using a percutaneous biopsy needle within 1 week before DLI (left column); sections of both allografted (middle column) and native kidneys (right column) were collected at necropsy. Tissues were fixed in 10% buffered formalin, fixed, and stained with hematoxylin-eosin and photographed at 100x magnification.

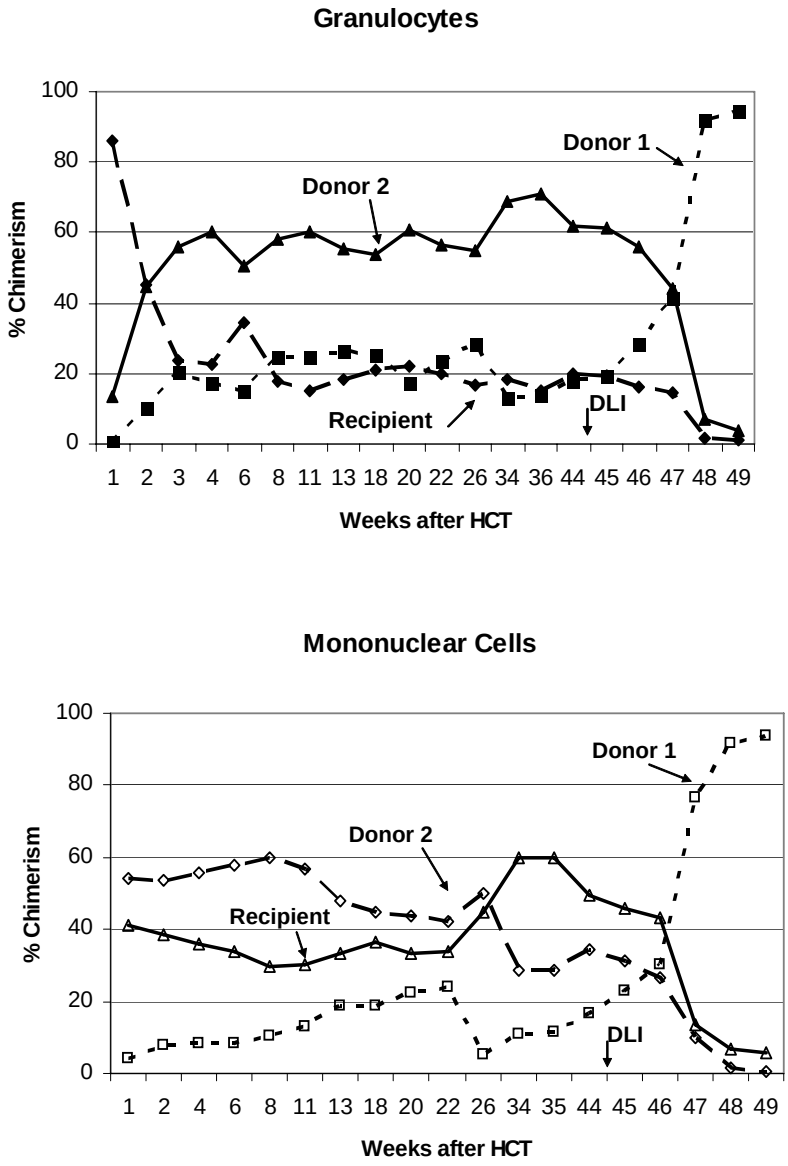

FIGURE 3. Granulocyte and peripheral blood mononuclear cell chimerism in dog G643 before and after sensitized DLI from donor 1. Stable long-term granulocyte and mononuclear cell trichimerism in the HCT recipient (diamonds), donor 1 (boxes) and donor 2 (triangles) was interrupted following an infusion of sensitized lymphocytes from Donor 1at week 44. Data points were obtained by VNTR-PCR analysis.

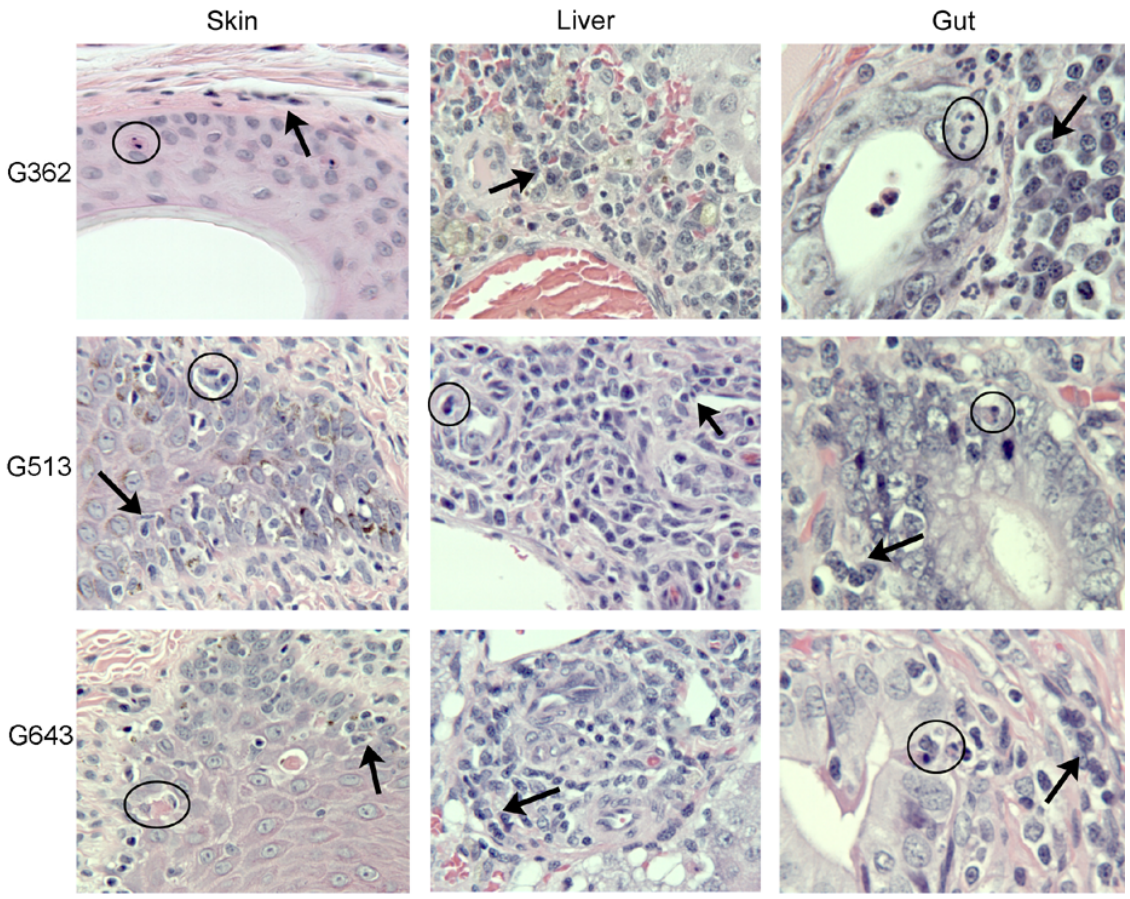

FIGURE 4. Histology of skin, liver and gut of dogs G513, G362, and G643 at necropsy. Tissues were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin-eosin and photographed at 250x magnification. Mononuclear cell infiltrates (arrows) and apoptotic epithelial cells or damaged obliterated duct structures (circles) are indicated.