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INDUCTION OF DONOR-SPECIFIC TRANSPLANTATION TOLERANCE TO SKIN AND CARDIAC ALLOGRAFTS USING MIXED CHIMERISM IN (A + B → A) IN RATS

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Abstract

Mixed allogeneic chimerism (A + B → A) was induced in rats by reconstitution of lethally irradiated LEW recipients with a mixture of T-cell depleted (TCD) syngeneic and TCD allogeneic ACI bone marrow. Thirty-seven percent of animals repopulated as stable mixed lymphopoietic chimeras, while the remainder had no detectable allogeneic chimerism. When evaluated for evidence of donor-specific transplantation tolerance, only those recipients with detectable allogeneic lymphoid chimerism exhibited acceptance of donor-specific skin and cardiac allografts. Despite transplantation over a major histocompatibility complex (MHO)- and minor-disparate barrier, animals accepted donor-specific ACI skin and primarily vascularized cardiac allografts permanently, while rejecting third party Brown Norway (BN) grafts. The tolerance induced was also donor-specific in vitro as evidenced by specific hyporeactivity to the allogeneic donor lymphoid elements, yet normal reactivity to MHC-disparate third party rat lymphoid cells. This model for mixed chimerism in the rat will be advantageous to investigate specific transplantation tolerance to primarily vascularized solid organ grafts that can be performed with relative ease in the rat, but not in the mouse, and may provide a method to study the potential existence of organ- or tissue-specific alloantigens in primarily vascularized solid organ allografts.

Keywords

Bone marrow; Transplantation tolerance; Chimerism; Skin

INTRODUCTION

In clinical transplantation, nonspecific immunosuppressive agents are required to control the rejection response. In their action, these agents suppress rejection of genetically different grafts, but they also suppress other immune responses to unrelated antigens such as virus or opportunistic organisms. In order to overcome these limitations, specific tolerance to allogeneic donor grafts with preservation of host immunocompetence has become a goal of transplantation today. In recent years, a number of methods have been developed to induce donor specific

transplantation tolerance using bone marrow transplantation (1,4,13,14,16,18). One approach, using bone marrow transplantation (BMT) with a mixture of syngeneic and allogeneic bone marrow (BM) to prepare mixed allogeneic chimeras ($A + B \rightarrow A$), has been shown to be effective and useful to achieve long-term donor-specific transplantation tolerance to alloantigens in mice (8). This regimen is advantageous over fully allogeneic ($A \rightarrow B$) transplantation models, because mixed chimeras exhibit superior immunocompetence to eliminate virus or produce antibody when compared with fully allogeneic chimeras due to the presence of requisite host-type antigen presenting cells (APCs) for T-cell activation and function (8,17).

Transplantation of vascularized solid organ grafts in mice has experienced limited success due to technical difficulties based upon vessel size. In contrast, transplantation of organs such as heart, small bowel, and liver can be performed with relative ease in the rat. Therefore, we adapted the model of mixed chimerism to the rat, which has allowed us to investigate the response of mixed allogeneic chimeras to skin and primarily vascularized solid organ.

The initial reports using allogeneic chimerism to induce donor-specific transplantation tolerance in the mouse were performed using congenic strain combinations disparate only in major histocompatibility complex (MHC), but matched for the minor region (9). When noncongenic strain combinations were utilized ($B10 + C3H \rightarrow B10$), donor-specific skin grafts underwent a slow chronic rejection (9). In spite of this, animals remained chimeric. In addition, when congenic skin matched to the MHC of the donor ($H-2^k$), but the minor antigens of the recipient (B10) was placed, it was permanently accepted (9). This was attributed to skin-specific antigens encoded in the minor region of the genetic locus present on the skin, but not on the bone marrow cells to which the recipient was rendered tolerant, similar to those previously described for fully allogeneic chimeras (3,21,22). The induction of mixed chimerism in the rat has now allowed us to evaluate whether skin-specific antigens may also exist in this species.

In our present experiments we report that stable mixed lymphopoietic chimerism can be established in the rat. Moreover, donor-specific skin and cardiac graft acceptance occurred over an MHC and non-MHC barrier to skin and primarily vascularized cardiac allografts without evidence of chronic rejection for up to 150 days. This tolerance was donor-specific in vitro when evaluated by mixed lymphocyte reaction (MLR). The model of allogeneic chimerism using the rat will provide a method to study donor-specific transplantation tolerance to other primarily vascularized allogeneic grafts, and to evaluate for potential tissue or organ-specific alloantigens.

MATERIALS AND METHODS

Animals

Male LEW (RT1^l), ACI (RT1^a), and BN (Rt1^{bn}) rats (225–250 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN), and maintained in a laminar flow caging system (Thoren Caging Systems, Hazelton, PA). Animals were given acidified water containing neomycin sulfate, 10 mL per liter.

Mixed Bone Marrow Reconstitution

(LEW + ACI $\rightarrow$ LEW) Recipient LEW animals were lethally irradiated with 100 rad total body irradiation (TBI) delivered from a ¹³⁷Cs source. BM was harvested from tibias and femurs of ACI and LEW donor rats, washed, and resuspended in Hanks' balanced salt solution (HBSS), supplemented with gentamicin 30 μ L/mL. T-cell depletion was performed using magnetic polymer beads (Dynabeads M4501, Bioproducts for Science, Indianapolis, IN) coated with

MAb OX-19 (anti-CD5). Beads were prepared by incubating the magnetic particles with fetal bovine serum (FCS) (Gibco, Grand Island, NY), at 290 μ L/FCS/mL of beads for 24 h, followed by OX-19 (29 μ L/mL of beads) for 24 h. ACI and LEW BM inocula were separately T-cell depleted using a ratio of beads/T cell of 1:20 for ACI and 1:40 for LEW cells. The beads/marrow mixture was incubated at room temperature for 30 min with constant gentle rotation. Separation of the beads/T cell complex from the depleted marrow was achieved by placing the beads/marrow mixture in a magnetic field. Flow cytometric analysis on spleen controls were performed routinely to assess successful depletion of OX-19 (anti-CD5) positive T cells. Mixed allogeneically reconstituted animals received 7×10^6 syngeneic TCD bone marrow cells and 43×10^6 allogeneic TCD bone marrow cells. Such animals will be referred to as mixed allogeneic, or LEW + ACI $\rightarrow$ LEW. Syngeneically reconstituted controls received 50×10^6 TCD syngeneic bone marrow cells (LEW $\rightarrow$ LEW). Radiation controls were performed simultaneously to confirm the adequacy of the lethal dose of irradiation.

Assessment of Chimerism by Flow Cytometry

Four weeks after reconstitution, rats receiving a mixed syngeneic + allogeneic BMT were assessed for the presence of allogeneic donor type (ACI) cells. In typing for chimerism, mAb were used to detect MHC Class I antigens. Monoclonal antibody 163 (Ig G2b) is specific for the RT1.A^l antigen on LEW cells, whereas mAb 211 (Ig G2b) is specific for the RT1.A^a antigen on ACI cells (7,11). MAb 42 (RT1.Aⁿ) was used as an irrelevant control (mAb 163, 211, and 42 were kindly provided by H.W. Kunz, University of Pittsburgh, Department of Pathology, Pittsburgh, PA). All three antibodies were tested against each strain used with no detectable cross reactivity. For surface marker analysis, 1×10^5 cells obtained from Ficoll Hypaque gradient centrifugation of peripheral blood, were placed in 100 mL of HBSS. The MAbs 163, 211, and 42 were added as primary biotinylated antibodies at 1:50 dilution, and incubated for 60 min at 4°C. The cells were washed twice with HBSS and 0.1 % gelatin, and resuspended in fluorescein isothiocyanate (FITC) as a secondary marker. After 1 h at 4°C, cells were washed three times with HBSS and 0.1% gelatin, and resuspended in 1% paraformaldehyde.

Skin Grafting

Full thickness skin grafts were prepared using a modification of the method of Billingham (2). Full thickness tail skin of donor (ACI), third party (BN) and host-type (LEW), were placed on the panniculus carnosus of the posterior and lateral thoracic wall. Rejection was diagnosed when 90% of the graft was necrotic.

Cardiac Transplantation

A heterotopic abdominal heart transplantation model was used in these experiments (15). Briefly, ACI donor animals were anesthetized and heparinized with 150 units heparin IV. The thoracic cavity was opened, and the IVC and the SVC were ligated and divided. The aorta and pulmonary artery were transected 3–5 mm distally to their origins. A mass ligature was placed around the pulmonary vein, and the heart was then removed and stored at 4°C in cold Ringers lactate. The recipient vena cava and abdominal aorta were occluded, and an aortotomy and venotomy were performed. End-to-side anastomosis of the aorta to the recipient abdominal aorta, and of the pulmonary artery to the recipient vena cava were completed. Survival of recipient animals, and skin and cardiac grafts were calculated by the life table method (6).

Mixed Lymphocyte Reaction (MLR)

MLR assays were performed by a modified method as previously described (9). Briefly, lymph nodes were removed under sterile conditions, and an ACK lysed single cell suspension prepared in RPMI medium containing 0.75% fresh normal rat serum, 1 mM glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 1 mM Na-pyruvate, and 0.27% gentamicin

(all from GIBCO). 2×10^5 stimulator cells (2000 rad) and 1×10^5 responder cells, each in a volume of 100 μ L were placed in the 96-well, round bottom Linbro plate (Flow Lab., McLean, VA). Cultures were incubated at 37° in 5% CO₂ for 4 days, pulsed on the third day with 1 μ Ci of [³H] Thymidine (New England Nuclear, Boston, MA) harvested 18 h later, and counted in a beta scintillation counter (Beckman, Palo Alto, CA).

RESULTS

T-Cell Depletion Using Beads

Titration was performed to determine the optimal number of beads required to achieve TCD of the rat bone marrow of both syngeneic (LEW) and allogeneic (ACI) donors. Complete removal of OX-19 positive T cells was achieved when the bead/T cell ratio was 20:1 for ACI and 40:1 for LEW cells. Adequacy of depletions was confirmed using flow cytometry with monoclonal antibody staining.

Titration of T-Cell Depleted Syngeneic and Allogeneic Bone Marrow Cells to Achieve Survival

Syngeneic and Allogeneic reconstitutions were performed using titrations of TCD bone marrow to determine the minimum number of infused cells to allow 100% survival of lethally irradiated recipient rats. One hundred percent survival (5/5) of syngeneic recipients (LEW → LEW) was achieved when a minimum of 7×10^6 TCD LEW bone marrow cells per animal were infused. Fifty-seven percent (4/7) of animals which received 40×10^6 allogeneic bone marrow cells survived. Survival was similar with increasing cell number, because 50% (3/6) or 57% (4/7) of recipients survived when 75×10^6 or 150×10^6 allogeneic cells per recipient were given respectively. Therefore, in consecutive experiments a mixture of a total of 50×10^6 cells in a ratio of 6:1 (7×10^6 syngeneic LEW and 43×10^6 allogeneic ACI cells) were given to each recipient.

Survival Of Mixed Allogeneically Reconstituted Rats

Survival of recipients of the mixed syngeneic + allogeneic bone marrow inoculum was 96% at 150 days (Fig. 1). Out of this group, 37% of animals (19/52) re-populated as mixed chimeras with detectable syngeneic and allogeneic lymphoid elements. When analyzed separately, these animals exhibited excellent survival (93% at 150 days). Of the remaining animals, 60% (31/52) repopulated as totally syngeneic and 3% (2/52) died at a time similar to radiation controls (Fig. 1). Survival of mixed chimeras was compared to that of a group of animals transplanted with TCD (LEW → LEW) in which 100% of the transplanted animals survived more than 150 days (Fig. 1).

In an attempt to achieve more reliable engraftment of allogeneic bone marrow, we performed titrations of increasing numbers of cells. When up to 200×10^6 TCD syngeneic + allogeneic bone marrow cells, mixed in the same 6:1 ACI to LEW ratio were infused per animal, no improvement in allogeneic bone marrow acceptance resulted.

Five out of 13 (38%) of the transplanted animals repopulated as true mixed chimeras. The remaining 8 repopulated as totally syngeneic when assessed 4 wk after BMT. All radiation controls expired within 16 days.

Assessment of Chimerism

Four weeks after BMT, animals were tested for the presence of host and donor type peripheral blood lymphoid elements using flow cytometry. Cell surface phenotype of peripheral blood lymphocytes (PBL) was demonstrated using rat-anti-rat monoclonal antibodies specific for anti-Class I staining (Fig. 2). Animals were subsequently reevaluated at 15 and 21 wk after reconstitution to assess potential fluctuations in the percentage of chimerism throughout the

study. In artificial titrations performed by mixing ACI and LEW lymphoid cells in varying ratios, chimerism could be detected to 0.5% donor type ACI cells by flow cytometry staining. As shown in Table 1, the percentage of donor type allogeneic cells varied from 0.5 to 82%. Animals with detectable mixed chimerism, as well as those that typed syngeneically were analyzed for the presence of donor-specific transplantation tolerance in vivo using skin or primarily vascularized cardiac allografts.

Skin Graft Survival In Syngeneically and Mixed Allogeneically Reconstituted Animals

Four weeks after BMT, each animal received three skin grafts: A syngeneic LEW (RT1^L), a donor type allogeneic ACI (RT1^a), and a MHC-disparate third party BN (RT1ⁿ) skin graft. All animals that had evidence for engraftment of allogeneic bone marrow when typed by flow cytometry also permanently accepted ACI (Fig. 3) and LEW skin (data not shown) grafts, yet rejected third party (BN) (data not shown) grafts in a similar time frame to negative controls. The level of allogeneic chimerism usually did not influence the outcome of skin graft survival as long as detectable chimerism was present. Even the 2 animals (animals 8 and 9) that had only a very low level of detectable chimerism accepted donor-specific skin allografts. All animals that repopulated totally syngeneic following mixed allogeneic reconstitution were analyzed separately, and rejected donor-specific ACI and BN skin grafts in the time frame similar to unirradiated controls (Fig. 3).

For virtually all recipients, donor-specific tolerance for skin graft acceptance occurred only in those animals with detectable allogeneic lymphoid chimerism, however low. One animal, animal #9, tested twice negative on 4 and 15 wk, then had a low percentage chimerism at 21 wk. However, tolerance to donor-specific skin allografts was present in spite of a very low level of allogeneic chimerism.

Histology of Donor-Specific Skin Grafts

Skin grafts surviving for more than 150 days were examined histologically in a blinded fashion. The epidermal-dermal architecture in the ACI grafts was similar to those of LEW grafts performed as controls, with no histologic evidence suggestive of chronic rejection. No obvious cellular infiltrates or architectural changes were identified.

Survival of Cardiac Allografts in Mixed Allogeneically Reconstituted Animals: Response to a Primarily Vascularized Graft

Four weeks after BMT, six mixed allogeneically reconstituted animals received a heterotopic ACI cardiac graft. Again, recipients of the mixed bone marrow inoculum, that had detectable allogeneic chimerism were analyzed separately from those animals that repopulated as totally syngeneic. All mixed chimeric animals accepted the ACI donor heart graft permanently, with all grafts beating now for more than 150 days (Fig. 4). Syngeneically reconstituted LEW animals ($n = 6$) prepared as controls that received an ACI cardiac graft rejected the graft with a time course similar to unmanipulated controls.

Donor-Specific Tolerance as Assessed In Vitro by Mixed Lymphocyte Culture Proliferative (MLR) and Cellular Cytotoxicity (CML) Assay

Animals that repopulated as true mixed lymphopoietic chimeras were tested at 100 days post-BMT for their response to donor and third party alloantigens in MLR assays. As seen in a representative one way MLR, lymph node cells obtained from mixed chimeric animals (LEW + ACI → Lew) were specifically non-reactive to allogeneic donor (ACI) as well as syngeneic (LEW) stimulator cells, yet were competent to respond to MHC-disparate third party cells (Table 2), suggesting the presence of systemic donor-specific transplantation tolerance.

DISCUSSION

We report here a model to achieve stable mixed allogeneic chimerism in the rat using transplantation of a mixture of TCD syngeneic plus TCD allogeneic bone marrow. Because of the lack of complement-fixing anti-rat monoclonal antibodies for T-cell depletion, immunomagnetic beads were utilized. Despite manipulation of allogeneic cell numbers administered, only approximately 40% of animals were true mixed chimeras. Both bone marrow inocula (syngeneic plus allogeneic) were TCD prior to transplantation using mAb OX-19 to specifically deplete CD-5-positive cells. This model was then utilized to evaluate the recipients for donor-specific transplantation tolerance to skin and cardiac allografts in vivo as well as to donor lymphoid elements in vitro.

Increasing the number of transplanted cells/animal did not overcome the limitation of failure engraftment of allogeneic bone marrow. Infusion up to 200×10^6 BM cells per animals mixed in the same 6:1 ACI:LEW ratio did not improve either the proportion of mixed chimeric animals or the level of engraftment of allogeneic donor type cells when engraftment was achieved. It is well recognized that syngeneic bone marrow engrafts more readily with fewer numbers of cells, when compared with allogeneic BM (5,23). Experiments in mice have shown that T-cell depletion of the syngeneic component of the mixed bone marrow inoculum is necessary in order to allow engraftment of the allogeneic bone marrow cells when mixed allogeneic reconstitution is carried out (10). It is possible that the bead-depletion using OX-19 did not remove all syngeneic T cells that prevent allogeneic stem cell engraftment. However, we feel that this is unlikely, because flow cytometric analysis showed complete removal of OX-19 positive T cells. The T-cell depletion protocol we applied was also not likely to remove stem cells, because engraftment of syngeneic BM was successful in 97% of the transplanted animals and bone marrow stem cells, although Thy-1.1-positive, express few other markers of differentiation (20). It is possible that the rat, as a recipient, exhibits a greater level of alloresistance to engraftment, because in fully xenogeneic bone marrow transplantation we have found that the mouse more readily accepts rat bone marrow than the converse (mouse $\rightarrow$ rat).

The presence of donor-specific transplantation tolerance was independent of the percentage of donor type cells present in the BM recipient if allogeneic chimerism was at all present. This corresponds to findings reported in mice (9), and is underscored by the low level of chimerism observed in animals 8 and 9, who exhibited donor-specific skin graft survival. Previous work has already indicated that long-term skin allograft survival was observed only in animals exhibiting hematolymphopoietic chimerism.

Experiments using mixed chimerism to achieve long-term tolerance to skin grafts were previously conducted in congenic mice strains where donor and recipient were matched for non-MHC antigens (9). Chronic skin graft rejection was reported when noncongenic mice strains were utilized with donor and recipient differing in MHC plus nonMHC antigens (9, 12). Skin-specific antigens were suggested to mediate this rejection, because animals remained chimeric even after donor-specific skin graft rejection occurred. The existence of tissue-specific antigens expressed selectively on skin, but not the bone marrow cells to which the recipient was rendered tolerant were first reported by Boyse and Olds (3) and Steinmuller (21,22). Mixed allogeneic reconstitution using noncongenic strain combinations (B10 + C3H $\rightarrow$ B 10) extended these observations (9,12). A recent report from Mayumi and Good (12) demonstrated that rejection of skin grafts by noncongenic mouse chimeras could be prevented if spleen cells were administered with the BMT. In this model, long-term tolerance to skin grafts was established using a combined treatment with mAb, bone marrow and spleen cell transplantation, and a single dose of cyclophosphamide. The administration of spleen cells was not necessary to induce in vitro tolerance to lymphoid cells, but was required to prolong the

median survival time of the donor type skin grafts from 53 days to 118 days. Differential survival of skin and heart allografts was reported using TLI followed by allogeneic BM infusion (19). The presence of donor-type lymphoid cells was necessary for skin, but not for heart allograft survival. If this phenomenon is related to the presence of skin-specific antigens, the development of suppressor cells, or to undetectable levels of chimerism in the heart recipients remains unclear.

Interestingly, in our model for mixed chimerism in the rat, there was no evidence for chronic skin or cardiac graft rejection up to 150 days post BMT. All skin grafts (10/10) and all cardiac grafts (6/6) survived despite transplantation over a MHC plus minor antigen disparity. When examined histologically, skin grafts had no evidence for chronic rejection. Different strain combinations are being evaluated to determine whether this is a strain-specific or general effect. Because the rat may be more homogeneous in minor histocompatibility antigens in comparison to the mouse (13,14), it is possible that minor disparities associated with skin-specific antigens in the mouse are not great enough in the rat to influence expression of these antigens on skin cells. Alternatively, skin-specific antigens may not be expressed in the rat.

Induction of tolerance to vascularized organ grafts in our rat model was not surprising because donor type skin grafts also survived in these chimeric animals. In contrast to skin, for the heart there has been no convincing evidence for the presence of tissue-specific antigens that could cause selective rejection of the heart in the presence of surviving skin grafts. Testing of different strain combinations will be necessary to evaluate survival of heart grafts in other genetic combinations.

Induction of specific tolerance in the rat supports the idea that chimerism might be a useful tool to achieve donor-specific transplantation tolerance in higher species and possibly in humans. Our present model of mixed chimerism in the rat allows testing of a variety of vascularized solid organs, such as liver and small bowel, which have been performed with limited success in the mouse. The existence of well characterized strain combinations in genetically inbred rats will allow the evaluation for potential organ-specific antigens in these specifically tolerant recipients.

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Fig. 1.
Survival of the rats reconstituted with mixed allogeneic (LEW + ACI → LEW) or syngeneic (LEW → LEW) bone marrow as calculated by the life table method (6). Irradiation controls received 1000 rad TBI without BM rescue.

Fig. 2.

Flow cytometric analysis of cell phenotype from LEW and ACI controls, two representative chimeric animals. Four wk after bone marrow transplantation, peripheral blood lymphocytes were tested for the presence of donor type ACI lymphoid cells using mAb 163 (anti-LEW) and mAb 211 (anti-ACI) (a generous gift from Dr. HW Kunz). Overlay of staining profiles with LEW and ACI mAb is shown for LEW and ACI controls. Straining of PBL with both mAb is presented separately for chimera controls. Calculation of chimerism showed 63% ACI cells for chimera 1 and 20% donor type ACI cells for chimera 2. These animals are the same as those represented in Table 1.

Fig. 3.

Life table survival of ACI skin grafts in mixed chimeras. Four wk after bone marrow transplantation, each animal received an ACI, LEW, and a BN full thickness skin graft. Animals which received mixed bone transplantation yet repopulated only syngeneically were analyzed separately. Grafts were followed for a minimum of 150 days. All animals accepted LEW skin grafts, but rejected third party BN grafts (data not shown).

Fig. 4.

Survival of ACI cardiac grafts in mixed chimeras, syngeneic bone marrow recipients and normal LEW rats. Four weeks after bone marrow transplantation, animals received a heterotopic abdominal cardiac graft. Mixed chimeras that failed to engraft the allogeneic BM component were analyzed separately (mixed chimera; syngeneic repopulation).

Table 1

Flow cytometry typing of mixed allogeneically reconstituted rat with detectable allogeneic chimerism

Animal (LEW = ACI → LEW)	% ACI lymphoid cells in PBL weeks after BMTx						Graft	
	4	15	21	64	64	64	Skin	Cardiac
Chimera	1	63	65	64	64	64	+	*
	2	20	22	N/A	†	+	+	
	3	50	32	36				+
	4	5	1	2				+
	5	73	82	N/A				+
	6	3	1	1				+
	7	36	49	49				+
	8	2	0.5	0			+	
	9	0	0	1.5			+	
	10	62	61	N/A				+
	11	55	40	N/A			+	
	12	48	48	48			+	
	13	62	36	N/A			+	
	14	77	78	N/A			+	
	15	0.5	1	3			+	
	16	19	21	N/A			+	

Flow cytometric analysis was performed on PBL using mAb against Lew (RT1^L) and ACI (RT1^A) Class I MHC antigens. A MAB against BN (RT1^{BN}) was used as an irrelevant control and subtracted from staining for calculation of percentage of chimerism. Values for each individual animal were normalized to 100%. Only animals with detectable allogeneic chimerism are presented.

* Graft viable.

† N/A = Not Available.

Table 2

Reactivity of mixed allogeneically reconstituted animals in a one way MLR

Animal	³ [H] Thymidine Uptake *			
	Anti-self	Anti-LEW	Anti-ACI	Anti-BN
Normal LEW	–	681 ± 122	25,264 ± 947	36,274 ± 3,735
Chim 1	1,362 ± 174	1,520 ± 58	535 ± 137	19,142 ± 976
Chim 2	1,050 ± 139	1,095 ± 172	741 ± 87	22,416 ± 2,171

* Mean ± SEM of triplicate cultures at a 2:1 stimulator responder ratio. Representative MLR (one of three) illustrating the responses of two individual chimeric animals. Animals are the same as those represented in Table 1 (Chimera 1, 63% ACI; Chimera 2, 20% ACI).