Cross-species Transplantation Tolerance: Rat Bone Marrow-derived Cells Can Contribute to the Ligand for Negative Selection of Mouse T Cell Receptor Vβ in Chimeras Tolerant to Xenogeneic Antigens (Mouse + Rat → Mouse)

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Summary

Mixed xenogeneic bone marrow reconstitution (mouse + rat → mouse) results in stable mixed lymphopoietic chimerism (1-48% rat), long-term survival, and the induction of stable functional donor-specific transplantation tolerance to xenoantigens in vivo. To examine the role of negative selection of potentially xenoreactive T lymphocytes during tolerance induction across a species barrier, mixed xenogeneic chimeras (mouse + rat → mouse) were prepared and analyzed using a mixture of mouse and rat bone marrow cells for relative T cell receptor (TCR)-Vβ expression on mouse T cells. In mixed xenogeneic chimeras (B10 mouse + rat → B10 mouse), T cell maturation proceeded normally in the presence of rat bone marrow-derived elements, and functional donor-specific tolerance to rat xenoantigens was present when assessed by mixed lymphocyte reactivity in vitro. Vβ5, which is expressed at high (undeleted) levels in normal B10 mice, was consistently deleted in B10 recipients of Wistar Furth (WF), but not F344 rat bone marrow, whereas the coadministration of either F344 rat or WF rat bone marrow with B10 mouse bone marrow cells resulted in a significant decrease in expression of TCR-Vβ11. Taken together, these data demonstrate for the first time that rat bone marrow-derived cells can contribute in a strain-specific manner to the ligand for negative selection of specific mouse TCR-Vβ during tolerance induction across a species barrier.

Progress in clinical organ transplantation has resulted in a critical shortage of solid organ allografts (1). Xenotransplantation has been suggested as a potential solution to this problem. However, traditional immunosuppressive agents that control rejection of allografts have not been successful in prevention of rejection of xenografts (2). The induction of tolerance to xenoantigens has been suggested as one method to overcome these limitations. We recently developed a model to induce stable donor-specific transplantation tolerance for a closely related species combination (mouse + rat → mouse) (3). Recipients engrafted with rat bone marrow stem cells exhibited excellent survival and specifically accepted donor-strain skin xenografts but rejected MHC-disparate third-party grafts (3, 4). Development of rat-derived T lymphocytes in the mouse recipients proceeded in a normal phenotypic fashion when analyzed by flow cytometry (3). We have now applied this model to examine whether negative selection of potentially xenoreactive T lymphocytes can be identified during tolerance induction across a species barrier.

The mature TCR repertoire has been shown to be the result of both positive and negative selection events that occur in the thymus during development. It is currently believed that a precursor T cell entering the thymus will undergo one of three developmental fates. If the developing T cell does not recognize any components of the thymic environment, it will die by programmed cell death (5, 6). If the TCR interacts with MHC molecules (or MHC plus peptide) on thymic epithelial cells, positive selection occurs with maturation into a functionally mature T cell (6-11). Interaction of the TCR with bone marrow-derived elements such as macrophages or dendritic cells may result in negative selection (clonal deletion) of potentially self-reactive lymphocytes (6, 12-16). These selection events with the thymus are believed to shape the final TCR repertoire, with the final result that self-antigens are not recognized by mature T cells and foreign antigenic peptides are recognized only in association with self-MHC (16, 17). In addition to this intrathymic pathway for T cell repertoire selection, it has recently been demon-

strated that clonal selection of T cells can occur extrathymically (18, 19).

The functional elimination of potentially self-reactive lymphoid cells through clonal deletion was first postulated by Burnet more than thirty years ago (17). mAbs specific for the β chain of individual TCR-α/β Vβ gene products, as well as molecular probes for gene expression, have recently been utilized to demonstrate the negative selection of TCR-Vβ reactive to endogenous ligands during T cell repertoire generation (12–29). These deletions appear to occur during T cell development at the double-positive (CD4$^+$CD8$^+$) stage of intermediate maturity (15, 30). TCR-Vβ-specific deletions have been identified that correspond to elimination of T cells reactive to endogenous mouse superantigens such as minor lymphocyte stimulatory (Mls)[1] antigens in association with self-MHC products (12, 14, 16, 23, 24, 26–29). Similar TCR-specific deletion was observed in transgenic mice expressing TCR-α/β specific for other endogenous antigens such as HY (31–34). However, Mls and HY are only very weak transplantation antigens (35–40) and how these mechanisms for deletion of potentially self-reactive T cells relate to the induction of donor-specific tolerance for class I and class II transplantation alloantigens has not yet been fully defined.

We recently reported the induction of stable mixed xenogeneic (mouse + rat → mouse) chimerism achieved when untreated rat bone marrow was transplanted into lethally irradiated B10 mouse recipients (3, 4). Recipients were specifically tolerant to donor-strain rat skin grafts, yet were competent to reject MHC-disparate third-party rat and mouse skin grafts. In the present studies we have investigated the role of negative selection in the induction and maintenance of donor-specific transplantation tolerance to xenoantigens. We have examined whether negative selection of potentially self-reactive TCR-Vβ could occur in the presence of xenogeneic bone marrow–derived cells by examining whether a detectable deletion in the mouse T cell repertoire occurs in the presence of rat xenoantigens. In mixed xenogeneic chimeras (mouse + rat → mouse), a selective deletion of Vβ5-positive mouse T cells was observed when a WF rat was the xenogeneic donor (B10 mouse + WF rat → B10 mouse), but not in B10 mouse + F344 rat → B10 mouse chimeras. Similarly, significant deletion of Vβ-11-positive TCR occurred when a WF rat, or F344 rat bone marrow, were coadministered with B10 mouse bone marrow into B10 mouse recipients. These data demonstrate that xenoantigens can contribute in a strain-specific manner to the ligand for mouse TCR-Vβ-specific deletion during tolerance induction across a species barrier.

Materials and Methods

Animals. 6–8-wk-old male C57BL/10SnJ (B10), B10.BR/SgSn (B10.BR), BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 4-8-wk-old male Fischer 344 (F344), ACI,

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[1] *Abbreviations used in this paper:* Mls, minor lymphocyte stimulatory; MST, median survival time.

and Wistar Furth (WF) male rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were housed in a specific pathogen-free facility at the Biomedical Science Tower at the University of Pittsburgh.

Mixed Xenogeneic Bone Marrow Reconstitution (Mouse + Rat → Mouse). Mixed xenogeneically reconstituted animals were prepared by a modification of the method previously described for preparation of mixed allogeneic chimeras (3, 4). Briefly, inbred C57BL/10Sn male recipients were lethally irradiated with a single dose of 950 rad from a Cesium source (Nordion, Ontario, Canada). Using sterile technique, bone marrow was flushed with Medium 199 (Gibco Laboratories, Grand Island, NY) containing 50 μl/ml gentamicin from the femurs and tibias of mice and rat donors with a 22-gauge needle. This medium mixture will hence be referred to as MEM. The marrow was mechanically resuspended in MEM by gentle aspiration through an 18-gauge needle and the suspension filtered through sterile nylon mesh. The cells were then pelleted at 1,000 rpm for 10 min, resuspended, and counted. B10 or B10.BR bone marrow cells were T cell depleted by treatment with a 1–40 dilution of rabbit anti–mouse brain serum in MEM (10^8 cells/ml) (4°C for 30 min) plus guinea pig complement (37°C for 30 min) (Gibco Laboratories) as previously described (3, 4). Cells were washed twice and resuspended in MEM at the appropriate concentration to allow injection of 1 ml per animal. Recipient animals were reconstituted within 4–6 h after irradiation via the lateral tail veins using a 27-gauge needle.

Mixed Xenogeneically Reconstituted Animals. These animals received 5 × 10^6 T cell-depleted bone marrow cells plus 4 × 10^7 untreated rat bone marrow cells unless otherwise specified. Under these conditions it has been demonstrated that the majority of T lymphocytes in these animals are derived from the rat bone marrow stem cell precursors and not contaminating T lymphocytes in the bone marrow inoculum, since T cell maturation proceeds in a developmentally regulated fashion in the thymus (3). Radiation controls were prepared to confirm adequacy of the radiation.

Characterization of Chimeras by Flow Cytometry. Recipients were characterized for engraftment with syngeneic and/or xenogeneic donor lymphoid elements using flow cytometry to determine the percentage of PBL bearing H-2^b or H-2^k and F344, WF, or ACI surface markers (3). Briefly, peripheral blood was collected into heparinized plastic serum vials. After thorough mixing, the suspension was layered over 1.5 ml of room temperature lymphocyte separation medium (LSM) (Organon Technica, Kensington, MD) and centrifuged at 23°C (1,700 rpm for 30 min). The lymphocyte layer was aspirated from the saline-LSM interface and washed with medium. Red blood cells were ACK lysed (ammonium chloride potassium carbonate lysing buffer; made in our laboratory) and the remaining cells stained with appropriate mAbs for 30 min at 4°C and counterstained with sandwich when required.

Analyses of splenic and thymic lymphoid cells were performed using a FACS II® (Becton Dickinson & Co., Mountain View, CA) as previously described (3). mAbs anti-WF and anti-F344-Biotin, which were generated in rats and generously supplied by Dr. Heinz W. Kunz (University of Pittsburgh), were utilized for class I staining of rat cells (3). Anti-H-2^b mAb 28-8-6S (IgG2a; HB31; American Type Culture Collection, Rockville, MD) (characterized in our laboratory) was utilized for anti-H2^b staining. Anti-CD4-PE mouse (Becton Dickinson & Co.), anti-Thy-1.2-PE (Becton Dickinson & Co.), anti-CD8-FITC mouse (Becton Dickinson & Co.), and anti-TCR-α/β FITC (Pharmangen, San Diego, CA) were utilized for cellular subset staining. Data were displayed as cell frequency histograms in which log fluorescence intensity was displayed on the horizontal axis and relative cell number of the vertical axis. The

percentage of cells considered positive after staining with the relevant mAb was calculated using a cut-off for positivity determined from the control fluorescence profiles of negative and positive control populations (B10 mouse and F344 rat).

Two-color Flow Cytometry Analysis Of Relative TCR-Vβ Expression. Spleen cells from normal or chimeric animals were enriched for T cells by passing over rabbit anti–mouse Ig-coated plates. 10^6 T-enriched spleen cells were stained as previously described (12). Antibodies used in the study were αVβ3, KJ25 (41), αVβ5, MR9.4 (42), αVβ6, RR4-7 (43), αVβ7, TR-310 (44), αVβ8, F23.1 (45), αVβ9, MR10.2 (46), αVβ11, RR3-15 (47), αVβ13, MR12.4 (48). The students t test was utilized to analyze significance estimates.

Mixed Lymphocyte Culture Cellular Proliferative Assay (MLR). Xenogeneic MLR were performed by a modification of that previously described by Hoffman et al. (50) for allogeneic MLR. Briefly, murine splenocytes and rat lymph nodes were made into single cell suspensions, ACK-lysed (prepared in our laboratory), washed, and reconstituted in DMEM (Gibco Laboratories) supplemented with a 0.75% normal mouse serum, 0.09 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05 mM 2-ME, and 1 mM N^G-monomethyl L-arginine (50). 4 × 10^5 responding lymphocytes were cocultured with 4 × 10^5 irradiated stimulator lymphocytes (2,000 rad) in a total of 200 μl of media. Cultures were incubated at 37°C, 10% CO_2 for 4 d, pulsed on the third day with 1 μCi [³H]thymidine (New England Nuclear, Boston, MA), and harvested on the fourth day with an automated harvester (PHD Cell Harvester; Technology, Inc., Cambridge, MA).

Results

Typing of Mixed Xenogeneically Reconstituted (Mouse + Rat → Mouse) Mice: Documentation of Xenogeneic Lymphoid Chimerism. To determine whether negative selection of potentially xenoreactive mouse T lymphocytes would occur as a result of tolerance induction across a species disparity, mixed xenogeneic chimeras were prepared and typed for rat and mouse lymphoid chimerism 28 d after reconstitution using anti-F344 (RtlA¹), or anti-WF (RtlA") class I and anti-H-2ᵇ mAbs. As in our previous experience, rat lymphoid chimerism was reliably present in all mixed xenogeneic chimeras and ranged from 1% to 46% (data not shown). To maximize the percentage of rat xenoantigens present, chimeras with a high percentage (>5%) of rat lymphoid chimerism were chosen for analysis of mouse Thy-1.2⁺ TCR-Vβ expression. B10 mice were reconstituted with a mixture of B10 mouse plus rat bone marrow using ACI, F344, or WF rat as the xenogeneic donors (i.e., B10 mouse + F344 rat → mouse). As previously described, B10 mice do not normally delete any of the characterized TCR-Vβ, while the congenic B10.BR strain reliably deletes Vβ5, -11, -12, and -16 (29). Chimeras prepared with B10 mouse as the syngeneic donor and recipient would therefore serve to detect any potential deletions in mouse TCR repertoire resulting from contribution of the rat bone marrow–derived cells to the ligand for TCR-Vβ recognition during T cell development and tolerance induction.

Maturation Of Mouse T Lymphocytes in Thymus Of Mixed Xenogeneic Chimeras. T cell maturation has been demonstrated to occur in the thymus through interaction with the thymic stroma and bone marrow–derived connective tissues

(6–17). We have now examined the staining profile of mouse-derived lymphoid cells in the thymus and spleen of mixed xenogeneic chimeras to determine whether the developmentally regulated expression of CD4, CD8, CD3, and TCR-α/β antigens would occur normally in the thymus and periphery. The expected populations of mouse TCR-α/β-negative, dull, and bright populations and CD4⁻CD8⁻, CD4⁺CD8⁺, and CD4⁺CD8⁻ or CD4⁻CD8⁺ cells were present in thymus of all chimeras analyzed (Fig. 1 *A*), while mature, single-positive TCR-α/βᵇʳⁱᵍʰᵗ, CD4⁻CD8⁺ or CD4⁺CD8⁻ cells were observed in splenic Thy-1.2⁺ lymphoid cells from chimeras (Fig. 1 *B*). The apparent increase in Thy-1.2-negative cells and in mouse CD4- and CD8-negative markers in thymus of the mixed xenogeneic chimeras is due to the presence of rat lymphocytes that were not stained by these species-specific reagents directed against mouse lymphocytes (3). The profile for T cell staining resembled that for normal B10 mouse thymus and spleen.

Evidence for In Vitro Functional Tolerance in Mixed Xenogeneic Chimeras. We have previously demonstrated that donor-specific rat skin xenografts were significantly prolonged (median survival time [MST] >180 d) while MHC-disparate third-party mouse and rat grafts were promptly rejected (MST = 10 and 20 d, respectively) by mixed xenogeneic (B10 + [F344 or WF] → B10) chimeras (3, 4). We have now examined splenic lymphoid cells from these recipients in vitro using mixed lymphocyte culture proliferative (MLR) assays for evidence of functional tolerance to donor and reactivity to third-party xeno and alloantigens. As seen in a representative MLR (Table 1), mixed xenogeneic chimeras exhibited functional tolerance to both host and donor xenoantigens, while their responses to MHC-disparate third-party rat or mouse stimulator cells were intact, demonstrating the presence of specific functional tolerance to donor xenoantigens.

Analysis of TCR-Vβ Expression in Mixed Xenogeneic Chimeras (B10 + Rat → B10). The Vβ-specific deletion of T cells specific for endogenous antigens (superantigens) has to date been demonstrated only in the mouse (49). To determine whether bone marrow–derived rat cells can contribute to the ligands that mediate Vβ-specific deletion, chimeras were prepared (B10 mouse + F344 rat → mouse, and B10 mouse + WF rat → B10 mouse) and typed for chimerism 28 d after reconstitution (Table 2). B10 mice have been shown to be permissive for expression of Vβ2, -3, -5, -6, -8, -9, -11, -13, and -14, and were therefore utilized as recipients to detect any potential deletions in repertoire that may have resulted due to the presence of additional ligands provided by rat bone marrow–derived cells. Those animals with the highest level of rat lymphoid chimerism (>5%) were again selected for analysis of mouse Thy-1.2⁺ TCR-Vβ expression. In B10 mouse + F344 rat → B10 mouse chimeras, no deletions were identified for TCR-Vβ2, -3, -5, -6, -8, -9, -13, or -14 expression (Table 2, and data not shown). In B10 mouse + WF rat → B10 mouse chimeras, no deletions were identified for TCR-Vβ3, -6, -8, -9, -13, or 14. In striking contrast, Vβ5-TCR expression was significantly reduced to levels similar to normal B10.BR mice (a Vβ5 deleting strain) in all mixed xenogeneic (B10 + WF → B10) chimeras ($p = 0.004$), but

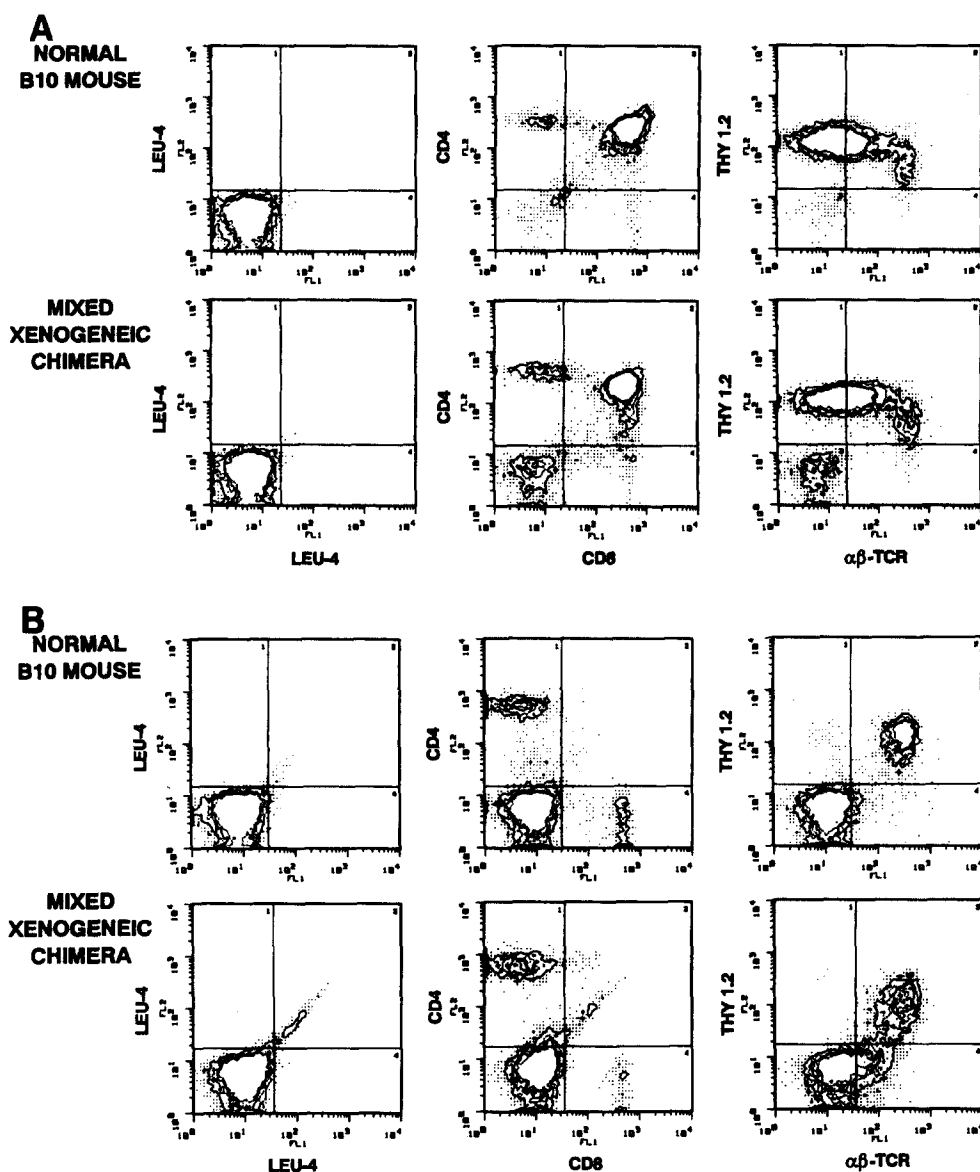Figure 1. (A) Flow cytometry analysis of mouse CD4, CD8, Thy-1.2, and TCR-α/β determinants on T lymphocytes in thymus of unmanipulated B10 mouse and mixed xenogeneic chimera (B10 mouse + ACI rat → B10 mouse) 8 wk after reconstitution. Total rat lymphoid chimerism in the chimera was 48%. Directly labeled antibody was utilized and unfractionated thymocytes were analyzed. Leu-4-FITC and Leu-4-PE were used as the fluorescent controls. All antibodies utilized were not crossreactive on rat lymphoid cells. (B) Flow cytometry analysis of mouse CD4, CD8, Thy-1.2, and TCR-α/β determinants on T lymphocytes in spleen of mixed xenogeneic chimera (B10 mouse + F344 → B10 mouse) 8 wk after reconstitution. Directly labeled antibody was utilized and unfractionated splenocytes were analyzed. The animal is the same as that for A.

not in B10 mouse + F344 rat → mouse chimeras ($p = 0.56$) (Table 2). A representative flow cytometry staining pattern is shown in Fig. 2. Decreases in mouse TCR-Vβ11 expression were also observed when either F344 rat ($p < 0.04$ compared with normal B10 mice) or WF rat ($p < 0.002$) were the xenogeneic donor, although the level of deletion was not as complete as that observed in syngeneically reconstituted B10.BR → B10.BR recipients (Fig. 3). These data suggest that rat bone marrow–derived cells can contribute in a strain-specific manner to the ligand for negative selection of selected mouse TCR-Vβ in mouse chimeras rendered tolerant to rat xenogeneic antigens.

Discussion

It has been demonstrated that during intrathymic T cell development, negative selection or clonal deletion occurs,

resulting in the elimination of those maturing T cells that express potentially self-reactive TCR. A similar process can occur in peripheral T cells (18, 19). In the mouse, a set of endogenous or self-antigens exists that has the property, when presented in association with a permissive MHC class II product, of interacting with all or nearly all T cells that express a given TCR-Vβ product. As a correlate of this Vβ specificity, mice expressing an endogenous antigen of this sort in association with a permissive class II product will delete most or all T cells expressing the corresponding Vβ product. These TCR-Vβ ligands have been termed superantigens and recent reports have indicated that the superantigen ligands characterized to date, including the ligands for Vβ3, -5, -6, -8.1, -9, and -11, are endogenous mouse mammary tumor virus products (48, 49). To date, Vβ-specific clonal deletion has not been demonstrated to occur in species other than the mouse. It thus remains to be determined whether the existence of Vβ-specific endogenous superantigens is

Table 1. *Reactivity of Xenogeneic (B10 Mouse + F344 Rat → B10 Mouse) Chimeras in One-way MLR*

Responding animal	[³H]Thymidine incorporation				
	Anti-B10	Anti-F344	Anti-BALB/c	Anti-ACI	Anti-self
			cpm ± SEM		
Normal B10	2,891 ± 177*	18,143 ± 5,225	23,416 ± 837	54,673 ± 8,215	
Normal F344	38,703 ± 7,278	1,319 ± 107	27,181 ± 4,925	38,899 ± 8,215	
B10 + F344→B10 chimera no. 1	5,503 ± 1,044	2,338 ± 194	36,842 ± 377	40,862 ± 4,210	5,423 ± 450
B10 + F344 → B10 chimera no. 2	4,682 ± 299	2,349 ± 56	63,307 ± 9,773	33,269 ± 8,781	4,569 ± 393

	Stimulation index			
	B10	F344	BALB/c	ACI
B10	1.0	6.3	8.0	18.9
F344	29.0	1.0	20.6	29.5
Chimera no. 1	1.0	0.4	6.8	7.5
Chimera no. 2	1.0	0.5	13.9	7.3

* Mean ± SEM of triplicate cultures in 1:1 responder-to-stimulator ratio. Animals were tested 5 mo after reconstitution. This is one representative of four experiments.

unique to the mouse, or is generalized to other species. In addition, superantigens such as Mls function poorly, if at all, as transplantation alloantigens (35, 36). In general, rejection of tissue grafts involving an isolated minor antigen disparity has been demonstrated to occur in a fashion distinct from responses to MHC-incompatible grafts in that they are recognized only in an MHC-restricted fashion and do not result in antibody production against the alloantigens (35–40). The role of specific clonal deletion in the induction of tolerance to transplantation alloantigens has only recently been addressed (51, 52).

There are obvious similarities between the model for tolerance to endogenous self-antigens and tolerance induced by allogeneic bone marrow transplantation in the adult recipient, in which precursor cells enter the thymus where they are educated. The role of clonal deletion in tolerance induction of new self for recipients of bone marrow allografts has been recently evaluated in allogeneic strain combinations using neonatal and adult bone marrow chimeras (51–53). In allogeneic radiation chimeras, the fate of potentially self-reactive T lymphocytes is largely determined by the derivation of bone marrow–derived stromal cells in the thymus (6, 13, 52–55). In fully allogeneic chimeras, Roberts et al. (14) and Ramsdell and Fowlkes (16) both demonstrated that clonal deletion of potentially autoreactive T lymphocytes in the thymus occurred if the appropriate donor bone marrow–derived stromal cells were present. In their absence, functional inactivation or anergy resulted. In addition, it has been reported that the

fate of Vβ6⁺ T cells was determined by the H-2 type of stem cells used for bone marrow reconstitution (39, 51). Speiser et al. (56) demonstrated that in radiation chimeras (Mlsᵃ recipients) reconstituted with bone marrow from IE⁻ donors, substantial numbers of mature Vβ6⁺ T cells that were functionally anergic to host Mls-1ᵃ in vitro were present in the peripheral lymphoid tissues despite host Mls-1ᵃ expression in vivo. In contrast, when an I-E⁺ strain was used as the donor for bone marrow transplanted into an I-E⁻ recipient, clonal deletion of potentially self-reactive T cells occurred (56).

We have now examined whether similar deletion of potentially self-reactive T cells could occur in chimeras tolerant across xenogeneic disparities to determine whether negative selection could proceed normally in a xenogeneic environment. We have also examined whether rat bone marrow–derived cells could contribute to ligands for deletion of mouse T cells during the induction of tolerance across a species barrier. The current report demonstrates for the first time that rat bone marrow–derived cells can contribute to the ligands for negative selection of mouse T cells expressing specific Vβs in chimeras rendered tolerant to rat xenoantigens (mouse + rat → mouse).

The Vβ-specific deletion of T cells specific for endogenous antigens (superantigens) has to date been demonstrated only in the mouse. To determine whether bone marrow–derived rat cells can contribute to ligands that mediate Vβ-specific deletion during the induction of tolerance across a species

Table 2. *PBL Typing and Relative Vβ-TCR Expression in Mixed Xenogeneic Chimeras (Mouse + Rat → Mouse)*

Reconstitution	Animal no.	PBL typing of chimeras		Relative TCR-Vβ antigen expression			
		Percent rat	Percent mouse	5	6	8	11
B10 + F344→B10	912	16.8	86.1	5.4	2.6	19.9	4.9
	916	31.0	78.0	5.3	7.3	6.6	5.0
	410	19.2	80.1	3.9	8.3	19.1	1.4
	992	20.3	78.8	0.4	9.0	18.2	0.5
	913	22.6	81.5	5.8	6.0	8.6	3.6
	668	21.0	80.0	6.8	6.0	21.6	4.4
	670	16.5	84.4	7.5	8.9	21.5	5.9
	409	11.2	89.8	2.5	7.0	20.4	1.0
B10 + WF→B10	935	29.0	69.5	0	9.5	24.8	2.1
	862	15.0	86.0	1.1	8.9	24.8	0.9
	710	12.2	88.0	1.2	7.8	27.1	2.9
	708	21.0	79.2	2.4	7.3	26.0	5.7
	936	45.0	64.0	0.8	8.9	12.4	3.3
	716	6.2	94.2	1.2	8.5	22.1	0.6
	717	5.7	95.3	1.7	7.4	27.0	0.6
	718	6.8	94.6	1.2	8.8	22.0	0.7
BR→BR	863	0.1	99.0	0.8	8.8	22.5	0.3
	864	0.2	99.6	0.3	10.1	20.3	0.2
	298	0	100.0	2.4	7.7	27.2	1.1
Normal B10	–			4.4	6.3	18.1	4.8

Thy-1.2 typing was performed on splenic lymphoid cells at the time of Vβ-TCR expression analysis. Animals were between 5 and 10 wk after reconstitution at the time of Vβ-TCR analysis.

barrier, mixed xenogeneic (mouse + rat → mouse) chimeras were prepared, and the fate of Thy-1.2$^+$ mouse T cells was examined. Maturation of mouse-derived T lymphocytes proceeded normally in the presence of rat-derived lymphoid cells in mixed xenogeneic chimeras (mouse + rat → mouse). The mouse T lymphocytes from chimeras were functionally tolerant to donor-strain rat xenoantigens in vitro when examined in MLR assays, yet responded to MHC-disparate third-party mouse and rat lymphoid cells, suggesting the presence of MHC-specific donor-specific transplantation tolerance for both mouse and rat antigens.

When mixed xenogeneic chimeras were prepared using F344 as the xenogeneic donor (B10 + F344 → B10), no significant differences in expression of TCR-Vβ2, -3, -6, -8, -9, -13, or -14 were detected when compared with B10 mouse controls. The same was true when WF rat was the donor in mixed xenogeneic reconstitutions involving B10 mouse recipients. In striking contrast, Vβ5-TCR expression was significantly decreased or absent in all chimeras examined when WF (RtlA^u) (n = 8; p = 0.004) was used as the xenogeneic donor, but not when F344 was used in the xenogeneic donor for this same recipient mouse strain (p = 0.56). Expression

of Vβ11 was significantly depressed when compared with normal B10 mice when F344 or WF rat bone marrow were coadministered with B10 mouse bone marrow. However, the level of deletion was not as complete as that observed for B10.BR → B10.BR syngeneically reconstituted mice. These data therefore suggest that for selected strain combinations, the exogenous rat xenoantigens contributed at least a portion of a ligand causing Vβ-specific deletions during the induction of tolerance to rat xenoantigens in mixed xenogeneic chimeras. In the mouse, the ligands for Vβ-specific T cell depletion have been shown to require both MHC-encoded and non-MHC-encoded gene products. Thus, there are a number of possible means by which rat gene products might contribute to the deletion of mouse T cells expressing specific Vβ products. The deletion of Vβ5-expressing T cells in (B10 mouse + WF rat → B10 mouse) chimeras may be mediated by ligand consisting entirely of WF rat gene products; that is, Vβ5-expressing T cells may recognize xenoantigens specific for the WF rat. Alternatively, Vβ5-specific deletion may involve an interaction of mouse and rat products. For example, MHC class II products expressed on rat bone marrow–derived cells may be capable of presenting a non-MHC-encoded
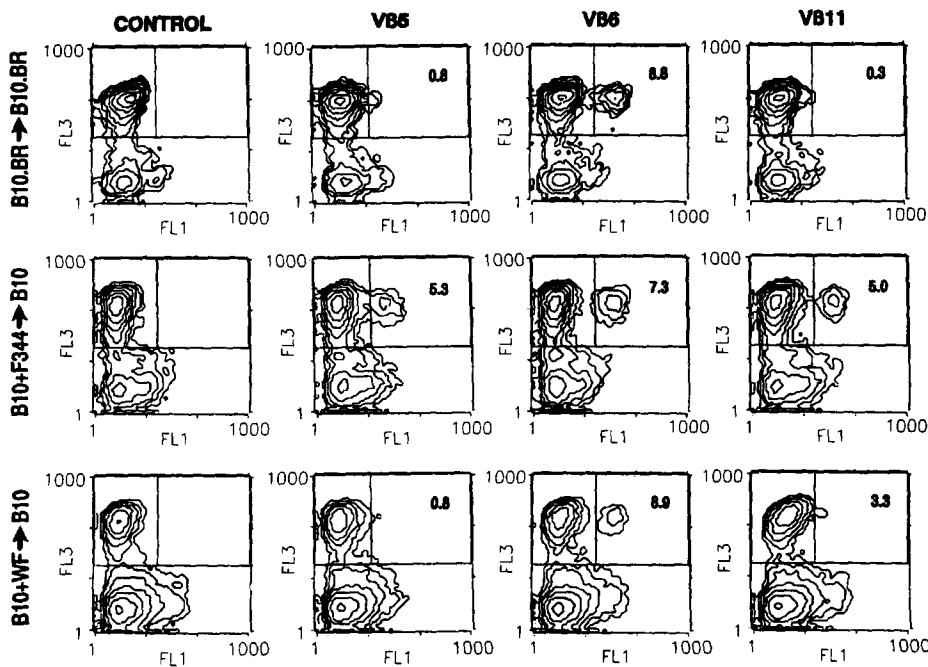
Figure 2. Analysis of Vβ expression in representative mixed xenogeneic chimeras (B10 mouse + F344 rat → B10 mouse or B10 mouse + WF rat → B10 mouse) by direct immunofluorescence. Animals were compared with syngeneically (B10.BR → B10.BR) reconstituted mice. 10^6 T-enriched spleen cells were stained with anti-Vβ culture supernatant and goat anti–rat FITC followed by Thy-1.2 biotin and texas-red avidin. Percentages indicate the number of Thy-1.2+ cells that express a specific Vβ. The percentage of cells staining with the control antibody has been subtracted.

superantigen product of the B10 mouse. The fact that B10 mice express the non-MHC component of the Vβ5 ligand, but fail to express a permissive class II MHC product, is consistent with this possibility (29). Attempts to identify the rat gene product that contributes to Vβ5 deletion are currently in progress using congenic strains of inbred rats.

Theoretically, if a specifically xeno-reactive subset of recipient T lymphocytes could be identified (e.g., Vβ5+ T cells), one might predict that in vivo depletion of this subset of T lymphocytes in B10 mouse recipients might allow a focused method to induce transplantation tolerance or hyporeactivity to xenoantigens utilizing a relatively benign but specific approach during tolerance induction across a species barrier. Studies are planned to evaluate in vivo depletion of Vβ5+ and/or Vβ11+ T cells in B10 mice before bone marrow

reconstitution to determine whether facilitated rat stem cell engraftment will occur using a nonlethal conditioning approach.

It has been suggested that at least one of the ligands for Vβ5 and Vβ11 deletion is the same, based upon similar strain distributions (25, 57, 58). Quantitative differences in Vβ5 and Vβ11 expression have been reported, however, in some mouse strains (25, 57, 58). Our present data in mixed xenogeneic chimeras are consistent with these findings, since Vβ5 was consistently deleted in all recipients, whereas Vβ11 deletion was less complete. Thus, qualitative or quantitative differences appear to exist in negative selection of Vβ5- and Vβ11-expressing T cells. Studies are now in progress to evaluate whether these potentially autoreactive Vβ11 TCR+ T cells in the periphery of mixed xenogeneic chimeras (mouse + rat → mouse) are functional or anergic. It is of note that there is no evidence for GVHD in the chimeras and survival is excellent (3), supporting the concept that although present, these potentially autoreactive TCR-Vβ11+ cells may not be functional.

In summary, we demonstrate for the first time that negative selection of selected mouse TCR-Vβ can occur across a closely related species barrier during the induction of tolerance to transplantation xenoantigens. Mixed xenogeneic chimeras exhibited functional tolerance in vitro to donor-specific rat xenoantigens yet were competent to respond to MHC-disparate third-party rat xenoantigens. We have identified that rat bone marrow–derived cells can contribute to the ligand for negative selection of specific mouse TCR-Vβ in mixed xenogeneic (mouse + rat → mouse) chimeras. Studies are in progress to examine the role of these specific TCR-Vβ in transplantation tolerance to xenoantigens.

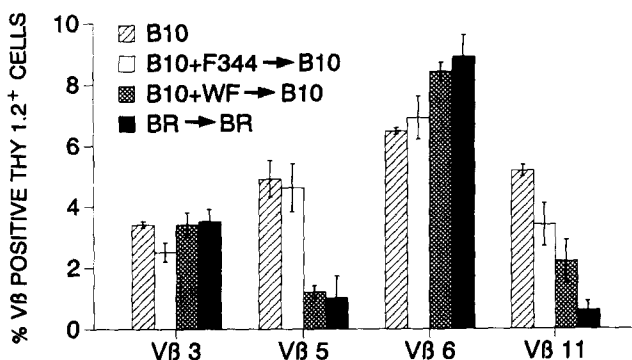**Figure 3.** TCR-Vβ expression in chimeric animals. Mean ± SEM specific TCR-Vβ3, -5, -6, and -11 expression in mixed xenogeneic chimeras, B10 mice, and B10.BR → B10.BR reconstituted mice.

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