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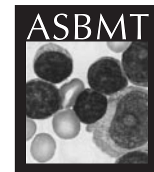

Induction of Stable Long-term Mixed Hematopoietic Chimerism Following Nonmyeloablative Conditioning With T Cell–Depleting Antibodies, Cyclophosphamide, and Thymic Irradiation Leads to Donor-Specific In Vitro and In Vivo Tolerance

Markus Y. Mapara, Michele Pelot, Guiling Zhao, Kirsten Swenson, Denise Pearson, Megan Sykes

Transplantation Biology Research Center, Bone Marrow Transplantation Section, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

Correspondence and reprint requests: Megan Sykes, MD, Transplantation Biology Research Center, Bone Marrow Transplantation Section, Massachusetts General Hospital-MGH-East Bldg 149-5102, Harvard Medical School, Boston, MA 02129 (e-mail: megan.sykes@tbrc.mgh.harvard.edu).

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ABSTRACT

Background: **Successful transplantation of solid organs relies on long-term immunosuppression for the prevention of graft rejection. Donor-specific tolerance without the need for continuous immunosuppression can be observed after allogeneic BMT. However, its routine use for tolerance induction has been precluded so far by the high conditioning-related toxicity of standard BMT regimens. Our laboratory has recently established a cyclophosphamide (CTX) plus thymic irradiation (TI)-based nonmyeloablative conditioning protocol for the treatment of hematologic malignancies. We have recently described the successful clinical application of this approach for the induction of donor-specific tolerance in a patient receiving a living-related kidney transplant, which resulted in graft acceptance without long-term immunosuppression. The aim of this study was to evaluate the induction and maintenance of host-versus-graft tolerance following this CTX-plus-TI–based regimen in a mouse model.**

Methods: **Induction of mixed hematopoietic chimerism and development of donor-specific tolerance following the CTX-based nonmyeloablative conditioning regimen (200 mg/kg CTX, in vivo T-cell depletion [anti-CD4 monoclonal antibody (MoAb) GK1.5 and anti-CD8 MoAb 2.43], and 7 Gy TI) was studied in the fully major histocompatibility complex (MHC)-mismatched B10.A ($H2^a$)→B6 ($H2^b$) strain combination.**

Results: **The conditioning regimen allowed allogeneic bone marrow engraftment and persistent (>30 weeks) mixed lymphohematopoietic chimerism in almost all recipients. TI was essential to allow engraftment and development of tolerance, which was evident in all lasting chimeras. Compared to animals receiving a similar TBI-based conditioning regimen, overall levels of chimerism were significantly lower in the CTX-plus-TI–conditioned animals. However, donor-specific tolerance in vitro and in vivo was evident in CTX-plus-TI–conditioned chimeras. Tolerance was associated with the presence of donor-type MHC class II^+ cells in the thymus and deletion of donor-reactive cells, as determined by Mtv-8 and Mtv-9 superantigen-mediated deletion of $V\beta11^+$ and $V\beta5/1.2^+$ T cells.**

Conclusion: **Engraftment, long-term chimerism, and induction of donor-specific tolerance can be achieved using a nonmyeloablative CTX-based conditioning regimen in fully MHC-mismatched BMT recipients without the induction of GVHD.**

KEY WORDS

Bone marrow transplantation • Cyclophosphamide • Clonal deletion • Mixed chimerism • Tolerance

M.Y.M. is now with Humboldt University, University Medical Center Charite Campus-Virchow Klinikum, Department of Hematology and Oncology, Berlin, Germany.

INTRODUCTION

Currently, pharmacological long-term immunosuppression is the only standard clinical treatment modality for the prevention of graft rejection in patients following solid organ transplantation. Although this approach is very successful and has enabled the widespread application of solid organ transplantation, there are considerable disadvantages to the lifelong use of immunosuppressive medication. Induction of donor-specific tolerance would obviate the need for nonspecific immunosuppressive therapy while retaining immunocompetence and is considered the ultimate goal in transplantation medicine. It has long been recognized that mixed hematopoietic chimerism is accompanied by the development of donor-specific tolerance without the need for long-term immunosuppression [1]. However, in humans, allogeneic bone marrow transplantation (BMT) in the context of myeloablative conditioning therapy is hampered by high treatment-related toxicity and the complication of graft-versus-host disease (GVHD), which is induced by alloreactive T cells in the graft. One pathogenic factor contributing to the development of GVHD is thought to be the inflammatory response to the preparative conditioning therapy usually needed for engraftment of allogeneic pluripotent hematopoietic stem cells in humans. Due to this high conditioning-related toxicity, patients older than 55 years or with organ dysfunction are usually not eligible for allogeneic BMT. Thus, it is a major goal to develop less toxic conditioning therapies for allogeneic BMT.

Recently, it has been demonstrated that mixed lymphohematopoietic chimerism can be established, even across major histocompatibility complex (MHC) barriers, by nonmyeloablative conditioning therapies in rodents [2] and large animals [3-6] and, recently, also in humans [7-9]. A total body irradiation (TBI)-based nonmyeloablative conditioning regimen consisting of 3 Gy TBI, in vivo T-cell depletion of the host, and 7 Gy thymic irradiation (TI) [2] has been established. Without inducing prolonged myelosuppression, this regimen led to multilineage hematopoietic chimerism and donor-specific tolerance, with preservation of immunocompetence as indicated by third-party alloreactivity in fully allogeneic MHC-mismatched murine strain combinations. Further decreases in conditioning-related toxicity were achieved by replacing TI with additional T-cell depletion or blockade of costimulatory molecules (CD40 or CD28) [10]. T-cell depletion and TI can both be avoided when 2 costimulatory blockers are given together [11]. Administration of high doses of stem cells has been shown to obviate the need for TBI in the conditioning regimen [12], and a regimen lacking any host preconditioning has been developed using costimulatory blockade and high-dose BMT [13]. These developments have paved the way to make allogeneic BMT applicable for the treatment of nonmalignant hematopoietic (eg, hemoglobinopathies, severe aplastic anemia) disorders and autoimmune diseases or for the induction of tolerance in the context of solid-organ transplantation.

In a modification of the TBI-based nonmyeloablative regimen described above [2] that was tailored for the treatment of hematologic malignancies, we recently showed that cyclophosphamide (CTX) could be used in place of low-dose TBI to achieve mixed chimerism in mice [14]. This approach was extended to a clinical trial involving patients with refractory hematologic malignancies [7,8]. The potential of this protocol to also induce allograft tolerance is underscored by a recent clinical observation, in which a patient with end-stage renal disease due to multiple myeloma received this nonmyeloablative treatment protocol with a simultaneous bone marrow and kidney transplant from a living HLA-identical sibling. Despite discontinuing the immunosuppressive therapy (cyclosporin A) by day 73, the patient accepted the kidney graft, demonstrating the successful induction of tolerance [15]. The aim of the current study was to assess the potential of this protocol to induce tolerance across full MHC barriers as well as the mechanisms underlying the induction of tolerance following this CTX-plus-TI–based conditioning regimen in the murine model.

MATERIALS AND METHODS

Animals

B10.A (H-2^a: K^k, A^k, E^k, D^d, L^d) female donor mice purchased from Frederick Cancer Research Facility, National Cancer Institute (NCI-Frederick) (Frederick, MD), were used at 8 to 12 weeks of age. Female C57BL/6 (B6: H-2^b, I-E$^-$) recipient mice were purchased from NCI-Frederick, and used after 8 weeks of age. A.SW (H-2^s) and B10.RIII (H2^r) mice were used as third-party donors for cell-mediated lympholysis (CML), mixed lymphocyte reaction (MLR) assays, and skin-grafting experiments. All mice were housed in autoclaved microisolator environments, and all manipulations were performed in a laminar flow hood.

Preparation of Chimeras

Two preparative regimens were used in this study. Control chimeras were prepared using our "standard" TBI-based nonmyeloablative regimen as previously described [2] and are referred to as TBI chimeras. Briefly, recipient B6 mice were treated on day –5 with depleting doses of anti-CD8 monoclonal antibody (MoAb) 2.43 (1.4 mg) and anti-CD4 MoAb GK1.5 (2.0 mg) intraperitoneally (IP) in sterile phosphate-buffered saline. On day 0, recipient mice were treated with 7 Gy TI from a ^{60}Co source and 3 Gy TBI from a ^{137}Cs source.

The second nonmyeloablative preparative regimen, in which CTX was substituted for TBI, has recently been developed in our laboratory [14]: instead of TBI on day 0, mice received 200 mg/kg CTX IP on day –1. To test the relevance of TI, we also prepared chimeras using the CTX regimen without TI. Bone marrow was harvested and single cell suspensions prepared as described [16]. Approximately 4 hours after the completion of TI, each recipient was injected intravenously through the lateral tail vein with 20×10^6 donor bone marrow cells.

Phenotyping of Chimeras

Chimerism in white blood cells (WBCs), spleen, bone marrow, and thymus was assessed by 2-color or 3-color flow cytometry (FCM) using a FACScan cytometer (Becton Dickinson, Mountain View, CA). Thymi and spleens were harvested and gently teased in bone marrow medium (Medium 199 [Mediatech, Herndon, VA], containing 2 μg/mL DNAse, 0.01 mol/L HEPES buffer [Biowhittaker, Walkersville, MD], and 4 μg/mL gentamycin) or in

ammonium chloride-potassium–lysing buffer (BioWhittaker), respectively. Single-cell suspensions were filtered through nylon mesh. Bone marrow was recovered from femora and tibiae by flushing the bone marrow cavity. Peripheral blood was collected into heparinized Eppendorf tubes and subjected to deionized water lysis.

Two-color staining was performed using MoAbs directly labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin (Bio), followed by subsequent development of Bio-labeled MoAbs with PE-conjugated streptavidin. To reduce nonspecific binding of MoAbs, 10 µL of 2.4G2 (anti–Fcγ-RII receptor, CDw32) hybridoma supernatant [17] was added to all tubes. The following antibodies were used for chimerism analyses: anti–CD4-FITC, anti–CD8β-FITC, anti–B220-FITC (all purchased from Pharmingen, San Diego, CA), anti–Mac-1-FITC (CalTag, San Francisco, CA), and 34-2-12-Bio (anti–H2-D^d). Nonreactive control MoAb HOPC-FITC or HOPC-Bio (immunoglobulin [Ig] G2a prepared in our laboratory) and rat IgG2b-PE (Pharmingen) were used as negative controls. The percentage of donor cells within each leukocyte population was determined as previously described [18]. Exclusion of dead cells was performed by propidium iodide (PI) staining and live gating on PI-negative cells. Ten thousand events were collected and analyzed. The different peripheral blood leukocyte populations were distinguished by their forward scatter (FSC) and side scatter (SSC) properties: FSC low and SSC low (lymphocytes), SSC high (granulocytes), and FSC high and SSC low (monocytes).

Flow Cytometric Analysis of T-Cell Receptor Vβ Subfamilies

Triple-color staining was performed for the analysis of thymic cell populations using FITC-conjugated (anti-Vβ5.1/2, anti-Vβ11, anti-Vβ8.1/2, HOPC), PE-labeled (anti-CD4, rat IgG2a), and Bio-labeled antibodies (HOPC, 34-2-12, anti-CD8β). Staining by Bio was developed using streptavidin-conjugated CyChrome (Pharmingen). Deletion of Vβ5.1/2^+ and Vβ11^+ thymocytes was assessed by live gating on $CD4^+CD8^-$ mature thymocytes. Five thousand gated cells were acquired. Nonspecific binding was determined by HOPC-FITC staining and was subtracted from the percentage of cells staining with each FITC-labeled Vβ MoAb. Expression of Vβ8.1/8.2^+, which are not deleted in I-E^+ animals, served as a control. Deletion of splenic Vβ5.1/2^+ and Vβ11^+ T cells was determined by 2-color staining with FITC-conjugated anti-Vβ5.1/2, anti-Vβ11, and anti-Vβ8 MoAbs versus CD4-PE and live gating on CD4-PE positive cells. Ten thousand gated events were analyzed. Nonspecific staining with HOPC-FITC was subtracted as described above.

Immunohistochemical Staining

Immunohistochemical analysis of thymic chimerism was performed as previously described [11,12,19]. Briefly, sections were prepared from frozen thymus tissue by using a Tissue Tek cryostat. After fixation and blocking, the specimens were incubated with MoAb, 25-9-17 (anti–I-A^b, IgG2a), ISCR-3 (anti-IE, IgG2b), HOPC (IgG2a control), and 74.11.10 (IgG2b control). After washing, slides were incubated with a biotinylated secondary MoAb followed by development with streptavidin-conjugated horseradish peroxidase (Vectastain; Vector Labs, Burlingame, CA) and AEC (20 mg of 3-amino-9-ethylcarbazole, dissolved in 2.5 mL of dimethyl formamide and 47.5 ml of 0.1 mol/L acetate buffer) for 2 minutes. The specimens were then immersed in 2% paraformaldehyde in 0.05 mol/L sodium phosphate buffer for 10 minutes. Counterstaining was performed using hematoxylin.

CML Assay

Spleen cells from normal control animals and BMT recipients were suspended in CML medium as previously described [12,13]. Responder and stimulator cells were diluted to a concentration of 8×10^6 cells/mL. Stimulator cells were irradiated with 3000 rad using a ^{137}Cs source. A total of 8×10^5 stimulator cells were cocultured with 8×10^5 responder cells in 96-well U-bottom plates. Cultures were set up in 2 rows of 3 replicates each. After 5 days of culture at 37°C, 2-fold serial dilutions were prepared from the second row of triplicates so that cytolytic activity could be determined at 5 different responder-to-target ratios. A total of 8×10^3 ^{51}Cr-labeled concanavalin A–stimulated lymphoblasts were added to each well and incubated for 4 hours at 37°C. Plates were harvested by using a Titertek supernatant collection system (Skatron, Sterling, VA), and ^{51}Cr release was determined with an automated gamma counter (Canberra-Packard, Meridien, CT). Percent specific lysis (PSL) was calculated using the formula: PSL = 100% × (experimental release – spontaneous release/maximum release – spontaneous release).

Mixed Lymphocyte Reaction

Spleen cells from normal control animals and BMT recipients were cultured in triplicate wells in serum-free MLR medium as previously described [12,13]. Stimulator cells were irradiated with 3000 rad using a ^{137}Cs source. Four hundred thousand stimulator cells were cocultured with 4×10^5 responder cells in 96-well flat-bottom plates for 3 to 4 days at 37°C before they were pulsed with 1 µCi of ^{3}H-thymidine per well for the last 16 to 18 hours of culture. Thereafter, cells were harvested on glass filter mats using a Tomtec harvesting device (Hamden, CT). ^{3}H-thymidine incorporation was measured using a betaplate beta counter. Stimulation index was determined by comparing antidonor response and anti–third-party response with anti-host response according to the formula: cpm antidonor (or anti–third party)/cpm antihost. The counts per minute obtained for antihost were generally similar to or lower than those obtained in the absence of stimulator cells.

Skin Grafting

Skin grafting with donor and third-party full-thickness tail skin was performed as previously described [19]. On day 7, bandages were removed and skin grafts were evaluated daily for signs of rejection or infection. Grafts were considered rejected when less than 10% of the graft bed contained viable grafted skin.

Statistical Analysis

Group means were compared using Student *t* test with Microsoft Excel software. A *P* value of less than .05 was considered to be significant. All experiments reported here were performed at least 3 times.

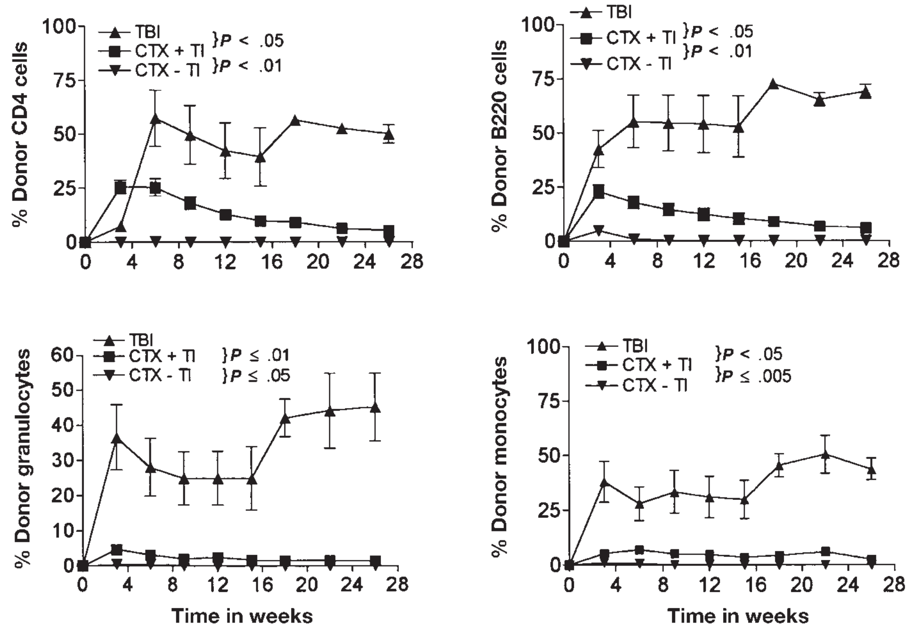

Figure 1. TI is required for sustained WBC chimerism in mice receiving BMT with the CTX-based nonmyeloablative conditioning regimen. Time course of WBC chimerism in mice treated with either the standard TBI-based conditioning regimen (TBI, n = 5), the CTX regimen without TI (CTX – TI, n = 8), or the full CTX regimen (CTX + TI, n = 8). Within the TBI group, 2 animals with low levels of donor chimerism died during the process of skin grafting, leading to an increase in the mean chimerism level in this group after week 15. The mean and the standard error of the mean are presented.

RESULTS

TI Is Necessary for the Development of Durable Mixed Lymphohematopoietic Chimerism

We have previously shown that the CTX-plus-TI–based nonmyeloablative conditioning regimen described in "Materials and Methods" allows engraftment and establishment of mixed hematopoietic chimerism when BMT is performed in fully MHC-mismatched strain combinations [14]. In the B10.A-to-B6 combination used here, lasting chimerism develops in about 90% of animals [14]. To evaluate the requirement for TI in this regimen and compare chimerism achieved with TBI versus CTX, 3 experimental groups were prepared: (1) the CTX-based regimen, including TI; (2) the above CTX-based regimen, without TI; and (3) our standard TBI regimen. Figure 1 shows the engraftment kinetics and the time course of chimerism in the peripheral blood leukocytes of the 3 groups. Animals that did not receive TI failed to engraft or showed only transient minimal levels of donor-type cells in the peripheral blood. Furthermore, animals receiving TBI conditioning had significantly higher levels of chimerism in all lineages compared to the group treated with CTX plus TI. Differences in levels of chimerism were statistically significant at each time point. Two animals from the TBI group with relatively low levels of chimerism died 15 weeks after BMT during the process of skin grafting, thus explaining the increase in the mean chimerism level in this group at 15 weeks.

Comparison of Chimerism in Hematopoietic Tissues of CTX Versus TBI Chimeras

We compared levels of chimerism in the lymphohematopoietic system in CTX- and TBI-conditioned mice. Mice were killed between days 219 and 232 post-BMT and studied for organ chimerism in the spleen, bone marrow, and thymus. Table 1 depicts chimerism results in marrow and spleen. Data on thymic chimerism are presented in Table 2. Mixed chimerism was observed in all tissues of 7/8 B6 recipients that had been treated with the full CTX regimen. One animal developed a skin infection after skin grafting and subsequently lost chimerism; it is presented separately in Tables 1 and 2. In mice receiving this conditioning regimen, donor-derived cells were found predominantly in the lymphoid lineages and, to a lesser extent, among the myeloid lineages. In contrast to these results, the standard TBI regimen led to a significantly higher ($P < .05$) level of donor cell engraftment in the lymphoid and myeloid lineages within bone marrow and spleen (Table 1). Although statistical significance was not achieved ($P = .08$), chimerism appeared to be higher in the Mac-1$^+$ population, and significantly higher chimerism was observed among B220$^+$ cells in the bone marrow of the TBI group. Chimerism was also detected among thymocytes in CTX chimeras (Table 2). However, a significantly higher level of donor thymocyte chimerism was detectable in the thymus of TBI animals (Table 2). Animals that received the CTX conditioning regimen without TI failed to show any detectable donor cells in any of the tissues studied.

Donor-Specific Tolerance in CTX Chimeras

Next, we studied the induction of donor-specific tolerance and its mechanism in B10.A→B6 chimeras prepared with the CTX-based regimen. We analyzed tolerance in vitro using CML and MLR assays, which were performed when animals were killed 31 to 33 weeks post-BMT. As is

Table 1. *Lymphohematopoietic Chimerism in Spleen and Bone Marrow*

	Normal B6 Controls,* Mean % H2D^{d+} Cells ± SD (n = 3)	Normal B10.A Controls, Mean % H2D^{d+} Cells ± SD (n = 3)	B10.A→B6 Chimeras TBI,† Mean % H2D^{d+} Cells ± SD (n = 3)	B10.A→B6 Chimeras CTX,† Mean % H2D^{d+} Cells ± SD (n = 7)‡	TBI Versus CTX, *P*
Bone Marrow					
B220	0.06 ± 0.06	99.84 ± 0.27	57.34 ± 16.40	4.17 ± 2.77	<.05
Mac-1	0.13 ± 0.20	97.77 ± 1.57	43.32 ± 22.06	2.3 ± 1.45	.08
Spleen					
CD4	0.13 ± .12	100 ± 0	50.08 ± 2.63	9.14 ± 5.76	<.05
CD8	0 ± 0	100 ± 0	55.97 ± 16.21	7.61 ± 6.88	<.05
B220	0.51 ± 0.08	100 ± 0	53.94 ± 6.34	5.72 ± 2.77	<.05

*Untreated B6 and B10.A mice were used as controls.

†Animals from experimental groups (TBI and CTX) were killed 219, 226, and 232 days after BMT.

‡One animal (#129/30) was excluded due to development of skin infection after skin grafting and loss of chimerism.

shown in Table 3, B10.A→B6 recipient mice treated with the CTX regimen revealed unresponsiveness to the B10.A donor in CML and MLR assays. For most animals, anti–third-party reactivity was evident in CML assays, demonstrating donor-specific CML tolerance. Nevertheless, anti–third-party CML responses in some experiments tended to be lower than those in untreated B6 control mice, which could be explained by the fact that the control animals were not age matched and thus were considerably younger than the experimental animals. Similar results were obtained for TBI chimeras. With both types of chimeras, anti–third-party MLR responses were frequently not measurable, probably reflecting the fact that A.SW is a particularly weak stimulator of MLR responses in mice that are tolerant of this donor and recipient strain, regardless of the regimen used to achieve tolerance (J. Kurtz, M.S., unpublished data, 1998). Nevertheless, the nontolerant (no TI) BMT recipients mounted significant responses to the donor (but also not to third party), consistent with the interpretation that TBI and CTX chimeras (treated with TI) were tolerant to their donors in the MLR assay.

One representative CML assay showing donor-specific tolerance in CTX-treated animals is depicted in Figure 2. As expected from the results presented above, animals receiving CTX without TI were not tolerant toward the marrow donor (Table 3 and Figure 2). The TBI-treated animal (#137/8) was specifically unresponsive toward donor and host cells, as previously described [2]. Third-party reactivity against A.SW (H2^{S}) stimulators was evident.

CTX Conditioning Therapy and BMT Lead to Acceptance of Fully MHC-Mismatched Donor Skin Grafts

Acceptance of donor-specific skin grafts is considered to be the most stringent test of tolerance. To measure donor-specific tolerance in vivo in chimeras prepared with the CTX regimen, skin transplantation was performed 16 weeks post-BMT. Animals received skin grafts from the bone

Table 2. *Thymic Deletion and Chimerism*

	Deletion of CD4^{+}CD8^{-} TCR Vβ Subfamilies*			Thymic Chimerism	
	%Vβ8^{+} Cells, Mean ± SD	%Vβ11^{+} Cells, Mean ± SD	%Vβ5^{+} Cells, Mean ± SD	%Donor Cells, MHC I^{+}†, Mean ± SD	Donor Cells MHC II^{+}‡
Normal B6 (n = 3)§	18.92 ± 0.48	4.74 ± 0.35	3.67 ± 0.71	0.27 ± 0.39‖	–
Normal B10.A (n = 3)	17.72 ± 0.73	0.24 ± 0.13	0.19 ± 0.03	60.33 ± 5.55	+
TBI (n = 3)	21.90 ± 2.16	0.28 ± 0.15	0.31 ± 0.19	18.39 ± 17.53	3/3
CTX without TI (n = 3)	18.67 ± 0.20	4.80 ± 0.15	3.81 ± 0.62	0.14 ± 0.13	–
CTX					
Stable chimeras (n = 7)	21.73 ± 3.06	0.95 ± 0.72	0.81 ± 0.63	0.91 ± 0.90	3/6¶
Unstable chimera#	20.63	4.42	2.28	0.03	–

*Deletion studies: CTX plus TI versus TBI: Vβ5.1/2, NS; Vβ11, $P < .05$; CTX plus TI versus CTX no TI: Vβ5.1/2, $P < .01$; Vβ11, $P < .0005$; CTX no TI versus TBI: Vβ5.1/2, $P < .01$; Vβ11, $P < .00001$.

†MHC class I H2D^{d+} thymocytes were detected by FCM.

‡MHC class II^{+} cells with dendritic cell morphology were detected by immunohistochemical staining of thymic tissue with a MoAb specific for donor MHC class II.

§Normal untreated B6 mice were used as controls.

‖One B6 animal was excluded due to high nonspecific staining.

¶Only 6 samples were available for histological analysis.

#Animal #129/30 developed a skin graft infection, lost its chimerism, and rejected its skin graft. Therefore, its results are presented separately.

Table 3. *Correlation of Splenic Deletion and Donor-Specific Tolerance**

	MLR, Stimulation Index		CML, Maximum PSL		Deletion of Splenic CD4⁺ Vβ Subfamilies		
	α-Donor	α-Third Party†	α-Donor	α-Third Party	Vβ8	Vβ11	Vβ5.1/2
B6 (n = 3)	11.1 ± 6	5.1 ± 1.9	44.9 ± 6.2	52.9 ± 10.4	17.4 ± 1.3	4.8 ± 0.3	2.5 ± 0.0
B10.A (n = 3)	1.0 ± 0	3.4 ± 1.0	7.3 ± 1	ND	17.4 ± 1.0	0.2 ± 0.1	0.1 ± 0.1
TBI (n = 3)	1.0 ± 0.1	1.9 ± 1	5.4‡	12.2	21.4 ± 1.5	0.4 ± 0.4	0.4 ± 0.5
CTX without TI (n = 3)	2.4 ± 0.8	1.6 ± 0.2	37.6 ± 12.5	26.4 ± 24.8	16.8 ± 1.9	3.8 ± 0.35	4.2 ± 3.5
CTX plus TI							
Stable chimeras (n = 7)	0.9 ± 0.2	1.4 ± 0.3	7.8 ± 3	19.2 ± 3.8	20.7 ± 1.0	0.6 ± 0.4	0.3 ± 0.3
Unstable chimera (#129/30)	1.7	2.7	NE	NE	17.41	3.83	1.5

**t* test: TBI vs. CTX Vβ8 Vβ5.1/2, Vβ11, NS; TBI vs. CTX no TI, Vβ11 $P < .05$; CTX plus TI vs. CTX no TI Vβ5.1/2, Vβ11 $P < .05$. ND indicates not determined; NE, not evaluable.

†A.SW were used as third-party stimulators.

‡Only 1 animal was evaluated.

marrow donor strain (B10.A) and from third-party donors (B10.RIII). In addition, animals that had been conditioned with the CTX regimen without TI received similar grafts. The survival curves for the skin grafts are presented in Figure 3. Whereas third-party B10.RIII grafts were rapidly rejected by day 11 in all groups, B10.A donor skin grafts were accepted for more than 100 days (ie, until the end of the observation period) in all CTX and TBI chimeras tested. The 1 animal prepared with the CTX/TI regimen that rejected its donor graft on day 29 had lost its chimerism. In contrast, animals that had received the CTX regimen without TI rejected their B10.A grafts by day 13.

Thymic Chimerism and Deletion of Donor-Reactive Cells Are Associated With the Presence of Donor-Type MHC Class II⁺ Cells in the Thymus

Donor-specific tolerance following BMT has been shown to be associated with intrathymic clonal deletion of donor-reactive cells [20,21]. To determine the mechanism underlying the maintenance of tolerance following our CTX regimen, we analyzed chimerism and deletion of donor-reactive thymocytes. Using FCM, donor-type cells (H2-D^d) were detectable among the thymocytes of most CTX chimeras killed at 31 to 33 weeks post-BMT (14 of 17 animals; Table 2 and data not shown). However, the level of thymocyte chimerism was much lower in these mice than in chimeras prepared with the TBI-based standard regimen (Table 2). A similar observation was made with regard to donor-type MHC II⁺ cells in the thymus, as demonstrated by immunohistochemical staining of thymic tissue. Whereas donor-type MHC class II⁺ cells were abundant in the medulla of the thymi of animals treated with the TBI regimen (3/3), rare MHC class II⁺ cells of donor origin were detectable in only 3/6 CTX chimeras. Figure 4 depicts 1 representative thymic section of a CTX chimera stained for donor and host MHC class II⁺ cells. In contrast, animals that had received the CTX regimen without TI were completely devoid of thymic chimerism and of donor MHC class II⁺ cells in the thymus (0/3) (Table 2).

To determine the fate of host-derived donor-reactive cells, we followed host-derived superantigen-reactive Vβ5.1/2⁺ and Vβ11⁺ T-cell receptor (TCR) subpopulations in mixed chimeras by FCM. Vβ5.1/2 and Vβ11 TCR subunits bind to superantigens derived from mammary tumor virus 8 and 9 endogenous retroviruses encoded in the B6/B10 background genome, which are presented by MHC class II I-E antigens. Thus, Vβ5.1/2- and Vβ11-expressing thymocytes are clonally deleted in the thymus of I-E⁺ (B10.A) animals, but not in I-E⁻ (B6) mice. We evaluated Vβ5.1./2⁺ and Vβ11⁺ T-cell populations in spleens and thymi of mixed chimeras.

The majority of CTX chimeras and all TBI chimeras showed complete or near-complete deletion of donor-reactive T cells, as determined by deletion of Vβ5/1.2⁺ and Vβ11⁺

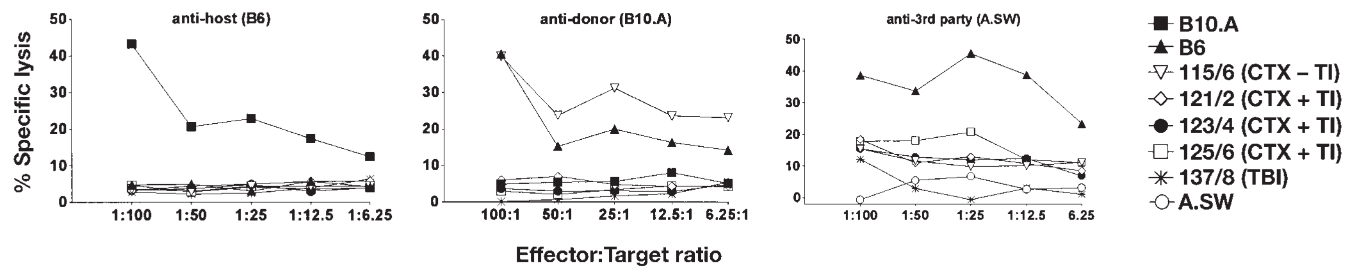

Figure 2. Tolerance to donor in CML assays. Spleen cells from representative animals conditioned with the complete cytoxan regimen CTX + TI (animals 121/2, 123/4, 125/6), CTX without TI (CTX-TI), and the TBI regimen (137/8) were studied with respect to antihost (B6), antidonor (B10.A), and anti–third-party cytotoxic responses. Normal B6, B10A, and A.SW splenocytes served as controls. All experimental animals were tolerant of host (anti-B6). Whereas all CTX + TI chimeras were tolerant of donor (B10.A) and demonstrated anti–third-party reactivity (anti-A.SW), the animal conditioned without TI (115/6, CTX-TI) showed a vigorous antidonor response.

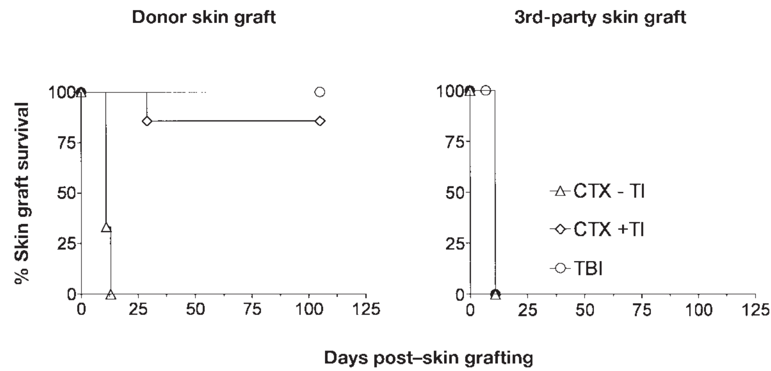

Figure 3. Donor-specific skin graft tolerance in CTX chimeras. Animals that had received the CTX-based regimen, with or without TI, or the standard TBI regimen were skin grafted as described in "Materials and Methods." All CTX- (n = 8) and TBI-treated animals (n = 3) rejected their third-party grafts (B10.RIII) by day 11, whereas donor-type (B10.A) skin grafts were accepted until the end of the observation period. Animals that received the CTX treatment without TI (n = 3) rejected their third-party grafts by day 11 and rejected donor grafts by day 13.

mature $CD4^+CD8^-$ cells in the thymus (Table 2) and of $CD4^+CD8^-$ cells in the spleen (Table 3). Because donor chimerism levels were generally low in CTX chimeras, the majority of $CD4^+$ cells analyzed for the presence of $V\beta5.1/2^+$ or $V\beta11^+$ subpopulations were of host origin, thus reflecting the deletion of donor-reactive host $CD4^+$ cells. Furthermore, in the thymus, deletion of superantigen-reactive $V\beta5.1/2^+$ or $V\beta11^+$ subpopulations appeared to be less complete in CTX versus TBI chimeras, with statistical significance achieved only for $V\beta11^+$ ($P < .05$). However, there was no statistically significant difference with regard to deletion of superantigen-reactive cells between CTX and TBI chimeras in the spleen. One animal (#129/30) had completely lost chimerism at the time of death, showed nontolerance in the MLR assay, and did not show deletion of these Vβ subpopulations (Tables 2 and 3). In all instances, donor-specific hyporesponsiveness in MLR was accompanied by complete or partial clonal deletion in the spleen and thymus of donor cells as determined by analysis of $V\beta5.1/2^+$ and $V\beta11^+$ TCR-bearing $CD4^+$ T cells. Animals that did not receive TI did not show deletion of superantigen-reactive cells and revealed significant antidonor MLR and CML responses (Tables 2 and 3, Figure 3).

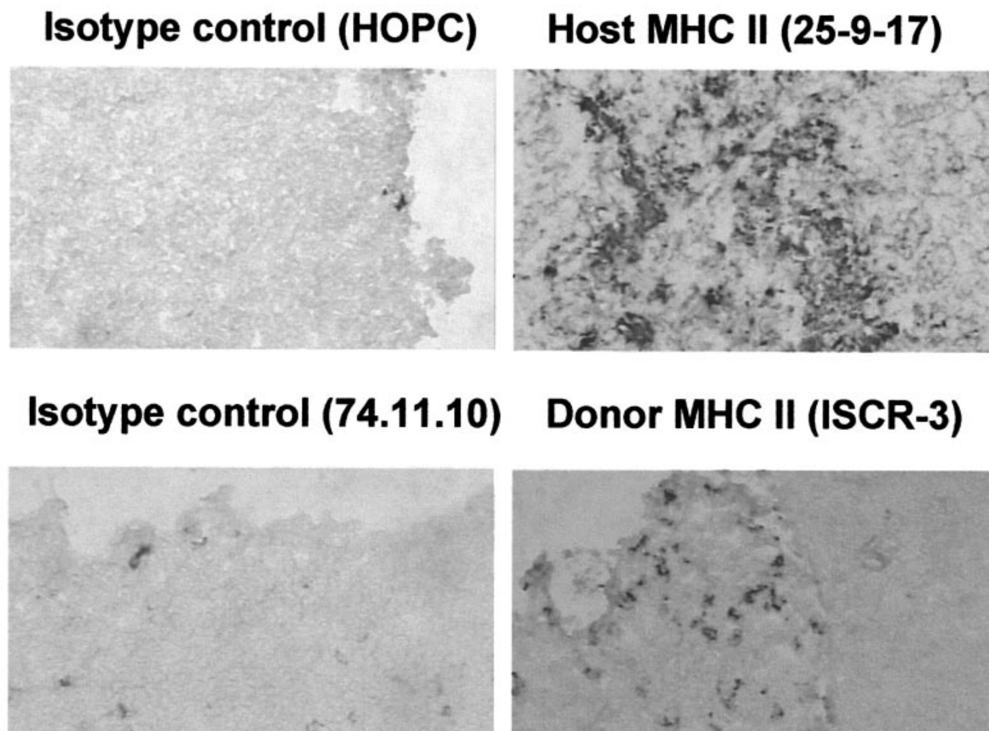

Figure 4. Immunohistochemistry of thymic tissues. Immunohistochemical staining of thymic sections of a CTX chimera killed 31 weeks post-BMT is shown. Donor and host MHC class II^+ cells are detectable in the thymus of CTX chimeras. Similar results were obtained in 3 of 6 chimeras prepared with this regimen.

DISCUSSION

The widespread application of allogeneic BMT for tolerance induction has been precluded thus far by 2 major limitations: first, the toxicity of the conditioning therapy required for engraftment of donor-derived hematopoietic stem cells; second, the development of GVHD. Accumulating data over the last decade have demonstrated that myeloablative conditioning is not necessary to induce mixed hematologic chimerism [2,12,14]. Recently, we were able to translate these findings into clinical application using a CTX-based nonmyeloablative conditioning regimen [15]. The current study was undertaken to investigate the tolerance induction and maintenance across full MHC barriers in murine mixed chimeras receiving BMT with the CTX conditioning regimen and to compare it to that achieved with our standard nonmyeloablative regimen, which is not designed for the treatment of hematological malignancies. Our data confirm that the CTX regimen is able to induce long-term hematopoietic chimerism in fully MHC-mismatched mouse strain combinations without the induction of GVHD. Chimerism was most pronounced in the B-cell lineage. Myeloid chimerism was also observable, but to a lesser extent compared to chimeras prepared with the TBI regimen. CTX is predominantly immunosuppressive and has only limited stem cell toxicity [22], consistent with results from several groups in which CTX is given on day 2 post-BMT to deplete alloantigen-activated T cells, and donor marrow engraftment is still achieved [23,24]. In contrast, low-grade TBI, although not myelotoxic, is highly stem cell toxic and leads to a high rate of stem cell engraftment [25].

TI is a crucial component of our CTX-based conditioning therapy, as animals that did not receive TI failed to engraft or develop donor-specific tolerance. These results confirm previous data from our laboratory showing that the administration of T cell–depleting MoAb as used in this protocol is not sufficient to deplete donor-reactive thymocytes in the standard TBI protocol [26]. However, TI could be eliminated in the standard protocol by administration of additional T cell–depleting MoAb [27,28] or using costimulatory blockade with anti-CD40 ligand or cytotoxic T lymphocyte–associated-4 antigen (CTLA4)-Ig [10]. Because the CTX regimen was primarily designed for the treatment of malignancies, prolonged in vivo T-cell depletion or suppression is not desirable, because it might mitigate graft-versus-tumor effects.

Chimeras prepared with the CTX regimen showed donor-specific tolerance in vitro and in vivo as assessed by CML/MLR assays and skin grafting. We evaluated superantigen-reactive Vβ5.1/2 and Vβ11 T cells as surrogate markers for the deletion of donor-reactive cells. Indeed, we were able to observe intrathymic clonal deletion of these T-cell subsets in mixed chimeras prepared with the CTX regimen. Deletion of thymic and splenic donor-reactive $CD4^+$ cells was evident in stable chimeras that showed tolerance to the donor in vitro and in vivo. Using 2C TCR transgenic mice as recipients, which carry a transgenic TCR specific for a donor MHC class I antigen L^d, we have recently confirmed that clonal deletion of such donor-reactive cells occurs in chimeras prepared using the TBI regimen, and these results concurred with results obtained by assessing the deletion of superantigen-reactive cells [19].

Induction and maintenance of tolerance following the TBI regimen has been shown to be associated with the presence of donor MHC II^+ cells in the thymus [21]. This association appears to be the only significant mechanism maintaining tolerance in such animals, as administration of anti-donor MoAb to euthymic mice resulted in loss of chimerism, deletion, and tolerance [29]. In contrast, when mice were thymectomized prior to elimination of donor cell chimerism, tolerance to donor skin grafts persisted despite the loss of peripheral chimerism. These results indicated that donor chimerism is required only in the thymus to maintain (deletional) tolerance. In the absence of a thymus, tolerance was maintained in the absence of donor antigen in the periphery, reflecting the fact that the T-cell repertoire had been fully deleted of donor-reactive cells. Anergy and suppression of donor-reactive cells did not play a role, as these phenomena depend on the continued presence of donor antigen. Further evidence against a role for suppression was the facility with which administration of nontolerant host-type T cells resulted in rapid rejection of donor grafts and chimerism [29].

Chimerism and the number of donor MHC II^+ cells were significantly lower in the thymi of CTX chimeras than in those of TBI recipients. This observation might also reflect a lower level of stem cell engraftment achieved with CTX compared to TBI (Table 1), resulting in a reduced number of donor bone marrow–derived antigen-presenting cells migrating to the recipient thymus. Nevertheless, deletion of donor-reactive thymocytes was quite extensive, although not as complete as that observed in TBI chimeras. In addition, some animals showed partial thymic and splenic deletion of donor-reactive $CD4^+$ cells but did not have donor MHC II^+ cells detectable in the thymus by immunohistochemistry at the time of death. This finding suggests that the number of MHC II^+ cells in the thymus of these mice might be very low and close to the limit of detection and that deletion might still be taking place even with very low numbers of donor-derived antigen-presenting cells in the thymus.

Another possible explanation for these findings could be that thymic chimerism disappears over time and that mechanisms other than deletion play a significant role in maintaining tolerance in the CTX chimeras. Indeed, deletion of donor-reactive T cells is incomplete in these animals, and we were able to detect donor MHC class II^+ cells in only half of the CTX-plus-TI–conditioned chimeras with stable chimerism and donor-specific tolerance. A role for suppression has been suggested in the long-term maintenance of tolerance in rat-mouse chimeras prepared with a nonmyeloablative regimen, which maintain donor-specific tolerance beyond the time when chimerism is no longer detectable [30]. Suppressive mechanisms might lead to a requirement for an intact thymus in the maintenance of tolerance in these chimeras, in contrast to results in TBI chimeras [29]. The induction of tolerance to MHC class I–disparate, MHC II–matched kidney grafts using a short course of cyclosporin A was critically dependent on the presence of a thymus in a miniature swine model not involving T-cell depletion or BMT [31,32], demonstrating that the thymus can play a role in maintaining peripheral tolerance. The role of the thymus in maintaining tolerance was not addressed in the present study.

In summary, data presented here show that the CTX regimen leads to induction of stable mixed hematopoietic chimerism with the concomitant induction of donor-specific tolerance, as indicated by the permanent acceptance of donor skin grafts and CML results, and that this tolerance develops in large part via a central deletion mechanism.

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