Mechanism for Cotolerance in Nonlethally Conditioned Mixed Chimeras: Negative Selection of the $V_{\beta}$ T-Cell Receptor Repertoire by Both Host and Donor Bone Marrow-Derived Cells

By Yolonda L. Colson, Jessica Lange, Kristin Fowler, and Suzanne T. Lidstad

Bone marrow (BM) chimeras prepared by complete recipient ablation (A→B) exhibit donor-specific tolerance, yet survival is often limited by graft-versus-host disease (GVHD). Negative selection of potentially donor-reactive T cells, as assessed by relative T-cell receptor (TCR-$V_{\beta}$) expression, is dependent on donor BM-derived deleting ligands. Mixed chimerism and tolerance for both donor and host antigens can be achieved using partial recipient myeloablation with 500 cGy total-body irradiation (TBI) before transplantation followed by cyclophosphamide (CyP) on day +2. To examine the influence of residual host elements on negative selection, the peripheral TCR-$V_{\beta}$ repertoire was analyzed in partially ablated C57BL/10SnJ (B10) recipients reconstituted with BM from major histocompatibility complex (MHC)- disparate B10.BR/SgSnJ or MHC, H-1 and Mls-disparate BALB/cByJ donors, which delete $V_{\beta}6^+$ and $V_{\beta}11^+$ or $V_{\beta}3^+$, $5^+$, and $11^+$ TCR subsets, respectively. As in myeloablated recipients, donor-reactive subfamilies were deleted in B10.BR→B10 and BALB/c→B10 chimeras, suggesting that donor I-E and minor lymphocyte-stimulating (Mls) antigens contribute to the deleting ligands in the nonmyeloablated host. In striking contrast to completely ablated B10→B10.BR chimeras, partially ablated recipients showed intramedullary I-E expression in the thymus and deleted host-reactive $V_{\beta}6^+$ and $V_{\beta}11^+$ subfamilies. These data demonstrate that efficient clonal deletion occurs after partial myeloablation and that both donor and host ligands contribute to TCR repertoire selection.

© 1996 by The American Society of Hematology.

CLONAL DELETION, or negative selection, of autoreactive T cells was postulated more than 35 years ago by Burnet. It is now considered to be the primary mechanism responsible for the acquisition of immunologic self-tolerance and the development of the T-cell repertoire. Monoclonal antibodies (MoAbs) specific for the variable region of the $\beta$ chain of the T-cell receptor (TCR-$V_{\beta}$), and transgene studies, have shown that negative selection of T lymphocytes primarily occurs within the thymus. Interaction of the TCR with specific endogenous retroviral integrant products, known as minor lymphocyte-stimulating (Mls) antigens, expressed on bone marrow (BM)-derived cells, results in the deletion of potentially autoreactive T cells during the double-positive TCR-CD4+CD8+ stage of T-cell development by programmed cell death, or apoptosis. Expression of the various Mls antigens in association with self-major histocompatibility complex (MHC) antigens, especially I-E, shape the T-cell repertoire through the deletion of potentially autoreactive $V_{\beta}$ T-cell populations. Although negative selection occurs most efficiently in developing T lymphocytes, mature activated T cells have also been shown to be deleted by apoptosis.

Roberts et al showed that deletion of potentially donor-reactive T cells is a major mechanism for achieving and maintaining tolerance in BM chimeras conditioned with lethal irradiation. However, it is clearly not the sole mechanism, because deletion occurred only if the donor marrow was from a deleting strain. SJL, as well as B10, mice are permissive for expression of $V_{\beta}6^+$ and $V_{\beta}17^+$ T cells, whereas CBA/J (Mls$^+$, I-E$^+$) mice delete these subfamilies. Transplantation of a mixture of SJL and B6 × CBA/J BM into B10 recipients resulted in the deletion of $V_{\beta}6^+$ and $V_{\beta}17^+$ T cells. However, $V_{\beta}6^+$ and $V_{\beta}17^+$ T-cell subfamilies were not deleted when the Mls$^+$ and I-E$^+$ determinants were expressed only by the host thymic stroma in SJL→B6 × CBA/J chimeras. Nonetheless, tolerance for these determinants was present in vitro due to the induction of clonalergy, as characterized by the survival of potentially self-reactive T cells and the lack of proliferation and interleukin-2 (IL-2) production in vitro in response to self-antigens.

These findings established that in fully ablated BM chimeras, clonal deletion is dependent on the expression of deleting ligands on donor-derived BM cells. In contrast, when Mls$^+$ and I-E$^+$ determinants were present only on radioresistant host elements, anergy and non deletion of autoreactive $V_{\beta}$-TCR subsets occurred. Unlike clonal deletion, anergy is reversible with the restoration of T-cell responsiveness, in the presence of IL-2.

Although BM chimeras reconstituted with donor BM after complete recipient ablation (A→B) exhibit donor-specific transplantation tolerance for subsequent tissue and organ grafts, survival is limited by graft-versus-host disease (GVHD) and an increased susceptibility to infection. In contrast, mixed allogeneic chimerism resulting from reconstitution with a mixture of syngeneic and allogeneic (A→B) BM confers tolerance to both donor and host alloantigens with a relative resistance to GVHD. The mechanism of tolerance induction by BM is still being characterized, but may be influenced by a number of variables, including the type of host conditioning used. It has now been established that mixed chimerism can be achieved across MHC disparities with incomplete recipient myeloablation. Although one could hypothesize that the fundamental mechanisms of tolerance induction are likely to be the same after

From the Department of Surgery, Division of Cellular Therapeutics, University of Pittsburgh, Pittsburgh, PA.

Submitted May 3, 1996; accepted August 2, 1996.

Supported in part by the American Heart Association Research Fellowship, Pennsylvania Affiliate, American College of Surgeons Resident Scholarship, National Institutes of Health Grants No. R01 AI30615 and R01 DK43901, and the American Heart Association.

Address reprint requests to Yolonda L. Colson, MD, PhD, Department of Surgery, Division of Cellular Therapeutics, University of Pittsburgh, Biomedical Science Tower E1656, Pittsburgh, PA 15261.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8812-0013$3.00/0

complete and incomplete host ablation, the kinetics of tolerance induction, the T-cell repertoire selected, and the dominant mechanism for maintaining long-term tolerance may be significantly different when the recipient is not totally ablated before transplantation. Cyclophosphamide (CyP)-induced tolerance to limited antigen disparities has been achieved through the generation of suppressor and/or anergic mechanisms with or without the requirement for donor chimerism. With more disparate donor and recipient combinations, a requirement for significant levels of donor chimerism and at least transient clonal deletion, has been observed. These differences suggest that the mechanisms for the induction and maintenance of tolerance are distinct, and are influenced by the degree of genetic disparity between the donor and host, as well as the strategy for host myeloablation.

We have recently established that mixed chimerism can be achieved without complete recipient ablation across multiple MHC, minor, and Hh-1 antigen disparities using a radiation-based conditioning approach (B10.BR → B10; B10 → B10.BR; BALB/c → B10). Recipients conditioned with 500 cGy total-body irradiation (TBI) and a single dose of intraperitoneal CyP on day +2, engraft and exhibit permanent donor-specific transplantation tolerance in vitro and in vivo for skin and primarily vascularized cardiac allografts. This approach is not completely ablative, because animals that are conditioned but not transplanted repopulate as syngeneic.

B10.BR mice delete several Vβ subfamilies, including Vβ5.1 and Vβ11* peripheral T cells, whereas B10 mice do not, BALB/c mice delete Vβ3*, Vβ5*, and Vβ11* T cells. In the current study, we have analyzed Vβ expression in BM chimeras after partial and complete recipient conditioning to determine the importance of Mls antigen expression by recipient and donor elements on the induction and maintenance of tolerance. All partially ablated B10 recipients engrafted as mixed chimeras (61.9% ± 7.3% donor), irrespective of the donor BM strain, and showed a selective deletion of donor-reactive Vβ T-cell subsets. Similarly, B10.BR recipients transplanted with B10 donor BM deleted potentially host-reactive Vβ T-cell subsets. However, the TCR-Vβ expression for each experimental group was compared with the expression seen in the nondeleting B10 strain using the unpaired Student’s t test. Analysis of variance (ANOVA) was used for statistical comparison of relative Vβ expression among the groups containing different levels of chimerism.

Immunohistochemical staining. Tissues for histologic analysis were immediately snap-frozen in pentane and kept at −70°C until use. Frozen sections (4 µm) of thymus were fixed in cold acetone, air-dried, and blocked for endogenous peroxidase and nonspecific protein interactions before incubation with 50 µL of biotinylated antimouse class II MoAbs (I-E
t, clone 17-3-3, mouse IgG2a, Pharmingen) for 1 hour at room temperature. This primary antibody was followed by incubation with the avidin-biotin complex conjugate (Vector Labs, Burlingame, CA) to detect the expression of nonspecific FCγR binding of labeled MoAbs. Staining for three-color flow cytometry was performed using anti-CD8-phycocerythrin (PE; clone 53-6.7; Pharmingen), anti-CD4-Tricolor (TC; CT-CD4; Caltag Laboratories, South San Francisco, CA), and the following FITC-conjugated anti-Vβ MoAbs for 45 minutes at 4°C: Vβ3 (KJ25), Vβ5 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8.1 (MR5-2), Vβ9 (MR10-2), Vβ10 (B2.15), Vβ11 (RR3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), Vβ14 (14-2), and Vβ17 (KJ23) (all from Pharmingen). A minimum of 50,000 gated events were collected within the region defined by forward- and side-scatter characteristic for the lymphoid gate. FITC- and PE-conjugated Leu-4 (antihuman CD3; Becton Dickinson) MoAbs were used as irrelevant controls for background staining.

Statistical analysis. To determine if the deletion of TCR-Vβ subsets was statistically significant, the relative Vβ expression for each experimental group was compared with the expression seen in the nondeleting B10 strain using the unpaired Student’s t test. Analysis of variance (ANOVA) was used for statistical comparison of relative Vβ expression among the groups containing different levels of chimerism.

Materials and Methods

Animals. Male, 6- to 8-week-old C57BL/10SnJ (B10; H-2b), B10.BR/5SnJ (B10.BR; H-2b), and BALB/cByJ (BALB/c; H-2b) mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Animals were housed in a specific pathogen-free facility at the Biomedical Science Tower.

Preparation of chimeras. B10 male recipients were irradiated with 950 cGy (complete ablation) or 500 cGy (partial ablation) TBI from a 137Cs source (Nordion, Ontario, Canada). Using sterile technique, BM was prepared from the femurs and tibias of donor B10.BR or BALB/c male mice as previously described for mixed allogeneic chimeras. Recipient animals were reconstituted with 15 × 106 allogeneic BM cells in 1 mL of Media 199 (GIBCO, Grand Island, NY) containing 0.25 µg/mL of gentamicin, 4 to 6 hours after irradiation, via lateral tail vein injection using a 27-gauge needle. Two days after transplantation, recipients initially treated with 500 cGy of TBI were administered a single intraperitoneal injection of 100 to 200 mg/kg CyP (Sigma, St Louis, MO) resuspended to 10 mg/mL in phosphate-buffered saline.

Peripheral blood lymphocyte (PBL) typing for chimerism. Recipients were characterized for engraftment using flow cytometry (FACS II; Becton Dickinson, Mountain View, CA) to determine the percentage of PBLs bearing H-2*, H-2k, and H-2d as previously described. Briefly, PB was collected into heparinized plastic serum vials, diluted 1:1 with Media 199, layered over 3 mL of Lymphocyte Separation Medium (LSM; Organon Teknik, Durham, NC), and centrifuged at 400 × g at 37°C for 20 minutes. The buffy-coat layer was aspirated from the saline-LSM interface and washed with medium. Lymphocytes were stained with fluorescein isothiocyanate (FITC)-labeled anti-class I MoAbs for 45 minutes at 4°C. Anti-H-2*, anti-H-2k, and anti-H-2d MoAbs (Pharmingen, San Diego, CA) were used for anti-class I staining.

TCR-Vβ flow cytometric analysis. Splenic lymphoid tissue from unmanipulated control mice and mixed allogeneic chimeras 1 to 17 months after reconstitution, was crushed using a sterile glass stopper, passed over a nylon wool column, and washed in Media 199, before the addition of 20 µL of a 1:10 dilution of mouse serum (prepared in our laboratory) to each sample to block nonspecific FCγR binding of labeled MoAbs. Staining for three-color flow cytometry was performed using anti-CD8-phycocerythrin (PE; clone 53-6.7; Pharmingen), anti-CD4-Tricolor (TC; CT-CD4; Caltag Laboratories, South San Francisco, CA), and the following FITC-conjugated anti-Vβ MoAbs for 45 minutes at 4°C: Vβ3 (KJ25), Vβ5 (MR9-4), Vβ6 (RR4-7), Vβ7 (TR310), Vβ8.1 (MR5-2), Vβ9 (MR10-2), Vβ10 (B2.15), Vβ11 (RR3-15), Vβ12 (MR11-1), Vβ13 (MR12-3), Vβ14 (14-2), and Vβ17 (KJ23) (all from Pharmingen). A minimum of 50,000 gated events were collected within the region defined by forward- and side-scatter characteristic for the lymphoid gate. FITC- and PE-conjugated Leu-4 (antihuman CD3; Becton Dickinson) MoAbs were used as irrelevant controls for background staining.

RESULTS

Donor-derived ligands contribute to Vβ deletion in chimeras conditioned with complete or partial myeloablation. To determine the influence of partial ablation on the selection
of the TCR repertoire, $V_{\beta}$ expression in fully ablated chimera was compared with expression in nonlethally conditioned B10.BR→B10 chimeras. B10 mice (H-2b; K$^\alpha$, A$^b$, E$^b$, D$^b$; Mls$^b$) were completely ablated with 950 cGy of TBI, or partially conditioned (500 cGy TBI + CyP; n = 9) conditioning. $V_{\beta}$-TCR expression in chimeras was compared with that in B10 mice using the unpaired Student’s t-test. Significant P values are indicated above the respective data bars (*P < .05 and **P < .0005). Comparison of $V_{\beta}$-TCR expression between B10.BR and either chimera group was not statistically significant for any given $V_{\beta}$.

Figure 1. Relative $V_{\beta}$-TCR expression in chimeras prepared with complete or partially ablative conditioning. Mean ± SEM of CD4$^+$ and CD8$^+$ splenic T-cell subsets for $V_{\beta}$3', $V_{\beta}$5', $V_{\beta}$6', $V_{\beta}$11', and $V_{\beta}$14' expression in host (B10; n = 8), donor (B10.BR; n = 8), and B10.BR→B10 chimeras. B10 mice (H-2b: K$^\alpha$, A$^b$, E$^b$, D$^b$; Mls$^b$) were completely ablated with 950 cGy of TBI, or partially conditioned (500 cGy TBI + CyP; n = 9) conditioning. $V_{\beta}$-TCR expression in chimeras was compared with that in B10 mice using the unpaired Student’s t-test. Significant P values are indicated above the respective data bars (*P < .05 and **P < .0005). Comparison of $V_{\beta}$-TCR expression between B10.BR and either chimera group was not statistically significant for any given $V_{\beta}$.

Table 1. Deletion of Donor-Reactive $V_{\beta}$ Subsets is Not Influenced by the Level of Donor Chimerism

<table>
<thead>
<tr>
<th>Reconstitution</th>
<th>% $V_{\beta}$5' T Cells</th>
<th>% $V_{\beta}$11' T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal B10</td>
<td>5.1 ± 0.9</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>Normal B10.BR</td>
<td>1.0 ± 0.3</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>&gt;30% donor chimerism</td>
<td>1.2 ± 0.3*</td>
<td>1.2 ± 0.04*</td>
</tr>
<tr>
<td>30-60% donor chimerism</td>
<td>0.5 ± 0.4*</td>
<td>0.5 ± 0.21</td>
</tr>
<tr>
<td>&gt;60% donor chimerism</td>
<td>0.6 ± 0.1*</td>
<td>0.8 ± 0.11</td>
</tr>
</tbody>
</table>

Relative $V_{\beta}$5 and $V_{\beta}$11 expression on T cells in control B10 (n = 8), B10.BR (n = 8), and partially ablated B10.BR→B10 chimeras (n = 9) with different levels of donor chimerism, as determined by PBL typing.

* ANOVA of relative $V_{\beta}$5 and $V_{\beta}$11 expression, respectively, as a function of increasing chimerism was not statistically significant for the different levels of donor chimerism.
donor TCR repertoire, but that the host-derived TCR repertoire remained permissive for expression of these subfamilies. Therefore, splenocytes from partially ablated B10.BR→B10 and BALB/c→B10 chimeras, previously shown to delete V8.5+ TCR subsets, were analyzed for coexpression of host (H-2<sup>b</sup>) class I<sup>+</sup> antigen and V8.5<sup>+</sup> or V6<sup>+</sup> TCR. Donor-reactive V8.5<sup>+</sup> T cells were deleted from the host-derived TCR repertoire (P = .003), whereas the level of expression of V6<sup>+</sup> TCR analyzed as a control was unaffected (Table 3).

Host-derived deletion ligands are present in partially ablated recipients. Previous studies using lethal recipient conditioning have shown that the pattern of V8 deletion is mediated, for the most part, by the deletion ligands of the BM donor, rather than those expressed by the recipient of the marrow. Therefore, it is not surprising that potentially host-reactive V8<sup>+</sup> T cells are not efficiently deleted when the donor is permissive for V8 expression, as in (I-E<sup>−</sup>−I-E<sup>+</sup>) chimeras.46 It has been previously shown that chimeras reconstituted with a mixture of host and donor hematopoietic stem cells are relatively resistant to GVHD as compared with lethally irradiated fully allogeneic chimeras.27,28 Therefore, we hypothesized that potentially host-reactive V8<sup>+</sup> T cells may be deleted in mixed chimeras conditioned with partial recipient myeloablation. The role of I-E and Mls expression on residual host cells in negative selection was determined by the comparison of V8 expression in myeloablated and nonlethally conditioned recipients. BM from nondeleting, I-E<sup>+</sup> B10 donors was transplanted into B10.BR recipients (100→100.BR) after complete (950cGy TBI) or partial (500cGy TBI + CyP) ablative conditioning. The B10.BR host expresses I-E<sup>+</sup> and Mls<sup>+</sup> gene products, and deletes V8<sup>+</sup> and V8<sup>+</sup> T cells. Therefore, Mls and I-E antigen expression within partially ablated B10→B10.BR chimeras is solely dependent on cells derived from host origin. All nonlethally prepared chimeras showed donor chimerism (9% to 86% donor; n = 4); however, the relative V8 expression for V8.3<sup>+</sup>, V8.5<sup>+</sup>, V8.6<sup>+</sup>, and V8<sup>+</sup> TCR subsets approximated that present in the host with the efficient deletion of V8.5 and V8.11 TCR subsets (Fig 3; P < .002). In striking contrast, V8<sup>+</sup> T cells were not deleted and V8<sup>+</sup> T cells were only decreased (P < .01), but not ablated, in B10.BR recipients conditioned with complete ablation.

Negative selection of V8<sup>+</sup> T cells occurs within the thymus.

### Table 2. V8 Deletion of Donor-Reactive T-Cell Subsets Directly Correlates With the Presence of Donor Chimerism

<table>
<thead>
<tr>
<th>Reconstitution</th>
<th>% V8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>% V8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>% V8&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal B10</td>
<td>2.7±0.6</td>
<td>9.4±1.2</td>
<td>4.4±0.4</td>
</tr>
<tr>
<td>Chimerism*</td>
<td>0.3±0.1</td>
<td>1.4±0.3</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>No chimerism</td>
<td>2.2±0.5</td>
<td>9.4±1.4</td>
<td>4.2±0.5</td>
</tr>
</tbody>
</table>

Relative V8<sup>+</sup> and V8<sup>+</sup> expression (mean ± SEM) of CD4<sup>+</sup> and CD8<sup>+</sup> splenic T-cell subsets in normal B10 mice (host strain; n = 16), conditioned (BALB/c→B10 and B10.BR→B10) chimeras, (TBI + CyP; n = 15) or recipients that failed to engraft (TBI alone; n = 5). Donor chimerism was determined by PBL typing 1 month following reconstitution.

* Comparison of relative V8 expression: chimerism versus no chimerism by Student’s t test, P < .0001 for all V8-TCR subsets.

### Table 3. Donor-Reactive V8 Subsets Are Deleted From the Host TCR Repertoire

<table>
<thead>
<tr>
<th>Reconstitution</th>
<th>V8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>V8&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal B10</td>
<td>3.7±0.5</td>
<td>4.6±0.4</td>
</tr>
<tr>
<td>Chimeras</td>
<td>0.9±0.3</td>
<td>6.5±0.8</td>
</tr>
</tbody>
</table>

Relative expression (mean ± SEM) of H-2<sup>+</sup> splenocytes for V8.5 and V8.6 subfamilies in normal B10 host mice (n = 3) and partially ablated BALB/c→B10 and B10.BR→B10 chimeras (n = 4).

Abbreviation: NS, not significant.

* Student’s t test comparison of relative V8 expression between B10 host and partially ablated B10 chimeras.
of nonmyeloablated recipients. The interaction of pre-T cells with stroma and BM-derived elements in the thymus results in the development of phenotypically and functionally mature T cells. Phenotypically immature (CD4+ CD8-), intermediate (CD4+CD8+), and mature (CD4+CD8- or CD4+CD8+) thymocyte subsets of donor and recipient origin were present within the thymus of partially ablated chimeras (n = 3) after reconstitution, suggesting that repopulation of the thymus occurs in the partially myeloablated host (Table 4).

Negative selection of Vα T-cell subsets has been shown to occur in fully ablated recipients before development of the mature single positive phenotype. To confirm that Vα deletion in a partially myeloablated host was due to intrathymic negative selection, expression of Vα3, Vα5, Vα6, and Vα11 on double-positive (CD4+CD8+) and mature, single-positive (CD4+CD8- or CD4+CD8+) thymocytes was examined in BALB/c→B10 nonlethal chimeras. As in the peripheral TCR repertoire, Vα3+, Vα5+, and Vα11+ bright thymocytes were absent in the mature CD4+CD8+ and CD4+CD8- thymocyte subsets in partially ablated chimeras (P < .05), but Vα6+ expression, analyzed as a control, was not affected (Table 5). In contrast, dim Vα3+, Vα5+, Vα11+ expression found on double-positive thymocytes was comparable between chimeras and controls, suggesting that negative selection of Vα subsets in partially ablated recipients occurs within the thymus, before the development of the mature single-positive thymocyte populations.

Host-derived I-E+ cells are present within the thymus of partially ablated recipients. The deletion of potentially autoreactive T cells in completely ablated chimeras is associated with expression of the I-E molecule on BM-derived dendritic cells within the medulla of the host thymus. We hypothesized that efficient deletion of host-reactive Vα-TCR subsets in partially ablated B10→B10BR chimeras, but not after complete myeloablation, may be due to the presence of host I-E+ cells within the thymus. Intense staining for I-Eβ antigen was present within the medullary regions of thymi obtained from B10→B10BR partially ablated chimeras, and resembled the immunohistology seen in unmanipulated B10BR controls (Fig 4). In contrast, I-Eβ expression was markedly decreased in the thymi of completely ablated chimeras, being present in small areas scattered primarily throughout the thymic cortex. Taken together, these results suggest that the BM-derived cells of the host thymus, which are necessary for deletion of potentially host-reactive T-cell populations, are present and functional in chimeras conditioned with partial myeloablation.

**DISCUSSION**

Negative selection of potentially autoreactive Vα T cells has been observed to occur at the CD4+CD8+ stage of intrathymic development. The relative contribution of radiosensitive epithelial and radiosensitive hematopoietic elements (macrophages and dendritic cells) to clonal deletion have been separated using radiation BM In conditioned chimeras, being present in small areas scattered primarily throughout the thymic cortex. Taken together, these results suggest that the BM-derived cells of the host thymus, which are necessary for deletion of potentially host-reactive T-cell populations, are present and functional in chimeras conditioned with partial myeloablation.

**Table 4. Presence of Thymocyte Subsets in Nonlethally Conditioned Chimeras**

<table>
<thead>
<tr>
<th>Reconstitution</th>
<th>CD4+CD8</th>
<th>CD4+CD8</th>
<th>CD4+CD8</th>
<th>CD4+CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10</td>
<td>4.0 ± 1.3</td>
<td>85.7 ± 0.6</td>
<td>4.4 ± 0.6</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>BALB/c</td>
<td>1.3 ± 0.1</td>
<td>85.7 ± 0.9</td>
<td>7.1 ± 0.6</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Chimeras</td>
<td>2.8 ± 0.9</td>
<td>86.9 ± 2.2</td>
<td>4.5 ± 1.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Immature (CD4+CD8+), intermediate (CD4+CD8+), or mature (CD4+CD8- or CD4+CD8+) thymocytes were analyzed by flow cytometry in control host (B10; n = 3), donor (BALB/c; n = 2), or partially ablated BALB/c→B10 chimeras (n = 3).
Table 5. TCR-Vβ Deletion in Partially Ablated Recipients Occurs Before the Single-Positive Stage of Thymocyte Maturation

<table>
<thead>
<tr>
<th>Reconstitution</th>
<th>% CD4+CD8-</th>
<th>% CD4-CD8+</th>
<th>% CD4-CD8-</th>
<th>% CD4+CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vβ3</td>
<td>Vβ5</td>
<td>Vβ6</td>
<td>Vβ11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>1.4 ± 0.6</td>
<td>1.4 ± 0.3</td>
<td>3.4 ± 0.3</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>BALB/c</td>
<td>1.5 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>5.1 ± 0.3</td>
<td>2.5 ± 0.0</td>
</tr>
<tr>
<td>Chimeras</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.5</td>
<td>3.5 ± 0.6</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2.3 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>4.2 ± 0.1</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>5.8 ± 0.7</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>7.5 ± 1.8</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2.7 ± 0.6</td>
<td>9.8 ± 2.4</td>
<td>7.2 ± 1.0</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>7.6 ± 1.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>0.3 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>7.8 ± 2.2</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

Relative Vβ expression (mean ± SEM) on immature and mature thymocyte subsets following reconstitution of partially ablated recipients. Vβ3, Vβ5, Vβ6, and Vβ11 expression is quantitated as the percentage of the thymocyte subpopulation expressing bright Vβ staining for mature thymocytes (CD4+CD8- or CD4-CD8+) or dim staining within the double-positive population (CD4+CD8+), as determined by flow cytometric analysis.

* Comparison of Vβ deletion between control B10 and chimeras by unpaired Student's t test.
tion of potentially donor- and host-reactive $V_\beta^+$ T cells. Recipients without evidence of donor chimerism, shown previously not to exhibit donor-specific tolerance, also do not delete donor-reactive $V_\beta^+$ subsets. Therefore, clonal deletion may be a critical requirement for the induction of tolerance in partially ablated recipients, with the prominent role of clonal anergy in tolerance induction being unique to MoAb-induced tolerance models. The mechanism(s) required for the induction and maintenance of tolerance may be important for the clinical application of chimerism, because T-cell unresponsiveness due to anergy, unlike deletional tolerance, can be bypassed by the exogenous or endogenous presence of T-cell–activating cytokines during periods of immune activation.\(^{19,21,22}\) One could hypothesize that the most robust, and therefore preferred, approach for achieving donor-specific transplantation tolerance would be deletional, if it could be achieved without significant morbidity.

In the current model of partial recipient ablation, donor chimerism across MHC and minor histocompatibility disparities is achieved in the context of clonal deletion and maintained for up to 17 months posttransplant. The deletion of $V_\beta^+$ and $V_{\beta\lambda}^{11^+}$ peripheral T cells occurred in partially ablated B10.BR→B10 chimeras. Furthermore, $V_\beta^+$-TCR subsets were analyzed in BALB/c→B10 nonlethal chimeras, which are disparate for MHC, Mls, and Hh-1 antigens. The TCR repertoire of these chimeras delete $V_\beta^3^+$, $V_\beta^5^+$, and $V_\beta^{11^+}$ TCR subsets as dictated by the BALB/c integrant products. Therefore, donor-derived elements can contribute the ligand for deletion, regardless of the degree of donor and recipient disparity and whether the recipient is conditioned with complete or partial myeloablation. Intrathymic clonal deletion requires that a permanent source of deletion ligand be present within the thymus, and therefore, $V_\beta^+$ deletion does not occur in this model, or in others, without the presence of donor chimerism.\(^{32}\) Thus, the permanent engraftment of donor hematopoietic stem cells across MHC, minor, and Hh-1 antigenic barriers with this nonlethal approach is central to the ability to maintain long-term clonal deletion and donor-specific tolerance.

Partially conditioned B10→B10.BR chimeras deleted host-reactive $V_\beta^5^+$ and $V_\beta^{11^+}$ T cells, whereas B10→B10.BR fully ablated recipients did not. The incomplete negative selection in completely ablated I-E$^+$→I-E$^+$ chimeras has previously been described for other donor and recipient combinations.\(^{36}\) Despite the presence of potentially autoreactive $V_\beta^+$ T cells in these recipients, numerous studies have estab-
lished that these T cells are tolerated in the lethally conditioned recipient via a mechanism of clonal anergy.\textsuperscript{3,15} Unlike clonal deletion, tolerance in these models can be overcome in vitro in the presence of exogenous IL-2. In the lethally ablated recipient, it was unclear whether deletion did not occur because the I-E\textsuperscript{+} donor was unable to delete host-reactive V\textsubscript{a} thymocytes or whether the I-E\textsuperscript{+} thymus was no longer able to support negative selection. The deletion of host-reactive V\textsubscript{a} subfamilies in partially ablated recipients suggests that developing donor lymphocytes can undergo negative selection in an I-E\textsuperscript{+} thymic environment, if the appropriate cellular components are present. This hypothesis is supported by the presence of host I-E\textsuperscript{+} cells in the thymus of B10→B10.BR chimeras after partial recipient ablation. In contrast, expression of I-E\textsuperscript{+} within the thymic medulla is no longer present in B10.BR recipients after completely ablative conditioning, and thus B10→B10.BR fully ablated chimeras do not delete host-reactive V\textsubscript{a} T cells. The immunohistologic presence of I-E\textsuperscript{+} cells after the current nonlethal conditioning approach confirms the nonablative nature of the host conditioning and suggests that BM-derived I-E\textsuperscript{+} cells, of either donor or host origin, may contribute the ligands for clonal deletion. Therefore, as in fully allogeneic chimeras, BM-derived elements contribute to the deletion ligands required for negative selection. The advantage in mixed chimeras, whether prepared with complete or partial host ablation, is that both donor and host-derived BM elements are present to result in clonal deletion instead of anergic tolerance.

Thymic selection is influenced by (1) the avidity of the interaction between the thymocyte TCR and the MHC/deleting ligand complex of the BM-derived thymic stroma, (2) the affinity of the thymocyte TCR for the stromal complex, and (3) the likelihood that maturing thymocytes encounter the deleting ligand.\textsuperscript{44-46} The level of donor chimerism and the conditioning of the host should not affect the avidity or intrinsic affinity of thymocytes for the deleting ligands. However, the deletion of V\textsubscript{a}5\textsuperscript{+} and V\textsubscript{a}11\textsuperscript{+} subfamilies in partially ablated recipients with donor chimerism ranging from 12\% to 70\% suggests that the number of MHC-bearing stromal cells, although important for negative selection, does not solely determine the likelihood of thymocyte-ligand interaction. Negative selection is thought to occur within the thymic medulla, most efficiently at the corticomedullary junction, where BM-derived I-E\textsuperscript{+} cells are normally concentrated.\textsuperscript{46,47} However, the thymic distribution of I-E\textsuperscript{+} cells can be significantly different depending on the degree and approach of host conditioning. After partial myeloablative conditioning, I-E\textsuperscript{+} cells were present in a relatively normal pattern of intramedullary distribution. In contrast, complete myeloablation altered this distribution resulting in the presence of islands of I-E\textsuperscript{+} cells throughout the thymus. These findings suggest that the incomplete negative selection of host-reactive V\textsubscript{a} subfamilies seen in fully ablated recipients may result from the disruption of the medullary architecture necessary for efficient V\textsubscript{a} deletion to occur, rather than simply fewer I-E\textsuperscript{+} stromal cells.

Mixed chimeras are more resistant to GVHD than are fully allogeneic chimeras.\textsuperscript{27} Consistent with the presence of potentially host-reactive V\textsubscript{a}-TCR subsets of donor origin in B10→B10.BR fully allogeneic chimeras, chimeras prepared by complete ablation and transplanted with untreated allogeneic BM cells have been shown to exhibit GVHD in vivo.\textsuperscript{24,48-49} In striking contrast, lymphocytes from partially ablated mixed allogeneic chimeras do not exhibit anti-host or anti-donor reactivity in vitro or in vivo.\textsuperscript{25} We have shown that this state of reciprocal cotolerance is associated with the deletion of autoreactive V\textsubscript{a} subfamilies from both host and donor TCR repertoires, as evidenced by the absence of the V\textsubscript{a}5\textsuperscript{+} subfamily in BALB/c→B10 chimeras, even among the small percentage of host-derived (H-2\textsuperscript{b}+) lymphocytes. The unique ability of mixed chimeras to induce clonal deletion of potentially host- as well as donor-reactive V\textsubscript{a} populations, suggests that this reciprocal cotolerance may result from donor and host hematopoietic populations each contributing the ligands required for efficient deletion of autoreactive T cells. One could hypothesize that activated donor- or host-reactive T cells would reenter the thymus, encounter their deleting ligand, and be removed from the T-cell repertoire.\textsuperscript{50}

The present model differs from other approaches for partial conditioning in that CyP is administered 2 days after transplantation of the donor BM inoculum, theoretically providing an antiproliferative agent during the time of maximum donor antithost (and host antidonor) reactivity.\textsuperscript{32,54} Therefore, we hypothesize that the primary mechanism for inducing and maintaining tolerance in the TBI/CyP model is deletional, but involves at least three contributing factors: (1) cytoablation for donor stem-cell engraftment so that a permanent source of donor antigen is present; (2) CyP on day +2 contributes to the elimination of proliferating mature donor antithost T cells contaminating the narrow inoculum and host antithost T cells remaining after partial host myeloblation; and (3) the multilinage chimerism resulting from the coengraftment of donor and host hematopoietic stem cells includes I-E\textsuperscript{+} stromal (dendritic) cells, which efficiently present deleting ligands within the medulla of the thymus, therefore providing the optimal environment for deletional rather than anergic tolerance of developing T cells.

Analysis of V\textsubscript{a} expression in different strain combinations of nonlethal chimeras (B10.BR→B10; B10→B10.BR; and BALB/c→B10) has allowed us to evaluate the importance of Mls and I-E antigen expression by host and donor elements, on the induction and maintenance of tolerance in partially ablated host environment. We have found that the presence of stable donor chimerism is associated with the deletion of autoreactive T-cell subfamilies and that both host and donor BM-derived cells in partially ablated chimeras can contribute to the intrathymic clonal elimination of potentially host-, as well as donor-reactive T-cell populations. Clonal deletion of both host- and donor-reactive T cells across MHC and minor antigen barriers may, in fact, decrease the risk of GVHD and allograft rejection. As we better understand the mechanism(s) for tolerance induction and maintenance in partially ablated recipients, new strategies will emerge in which deletional tolerance is favored over anergy as a means to induce durable donor-specific tolerance with minimal recipient morbidity and mortality in the clinical setting.
ACKNOWLEDGMENT

The authors thank William Dibella and David Parrish for expert technical assistance with flow cytometric analysis, MaryLynn Hronakes-Yurko for assistance with sample preparation, Jennifer Ionelie for preparation of immunohistochemical specimens, and Deborah Roberts, Brian Lanz, Mary J. Oles, and Marissa Massocchetti for excellent animal care.

REFERENCES

18. Alters SE, Song HK, Fathman CG: Evidence that clonal anergy is induced in thymic migrant cells after anti-CD4-mediated transplantation tolerance. Transplantation 56:633, 1993
38. Beutner U, Frankel WN, Cote MS, Coffin JM, Huber BT: Mls-1 is encoded by the long terminal repeat open reading frame of the mouse mammary tumor provirus Mtv-7. Proc Natl Acad Sci USA 89:5432, 1992


Mechanism for cotolerance in nonlethally conditioned mixed chimeras: negative selection of the Vbeta T-cell receptor repertoire by both host and donor bone marrow-derived cells

YL Colson, J Lange, K Fowler and ST Ildstad