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Bone Marrow Transplantation Induces Either Clonal Deletion or Infectious Tolerance Depending on the Dose

Frederike Bemelman,² Karen Honey, Elizabeth Adams, Stephen Cobbold, and Herman Waldmann³

The concept of immunologic tolerance arose from bone marrow transplantation in neonatal or irradiated mice, in which the predominant mechanism is clonal deletion of donor-specific T cells by donor hemopoietic cells in the recipient thymus. A short term treatment with nonlytic CD4 and CD8 mAbs can induce tolerance to tissue allografts or reversal of spontaneous autoimmunity. Such tolerance to skin or heart allografts is dependent on “infectious” tolerance mediated by regulatory CD4⁺ T cells. We show here, for multiple minor Ag differences, that while a large inoculum of donor marrow produces significant deletion of Ag-reactive cells as expected, a low marrow dose generates tolerance with little evidence of clonal deletion. Only this low dose tolerance can be transferred to unmanipulated recipients via CD4⁺ T cells, can be passed onto naive T cells as if infectious, and can act to suppress rejection of third party Ags when “linked” on F₁ grafts. The Journal of Immunology, 1998, 160: 2645–2648.

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2 F.B. was supported by the Dutch Kidney Foundation (Grant C94.1396).
3 Address correspondence and reprint requests to Prof. Herman Waldmann, Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, United Kingdom.
**Table 1. Deletion of Vβ6+ CD4+ and Vβ6+ CD8+ T cells depends on the dose of AKR bone marrow cell inoculum and correlates inversely with linked suppression of (AKR×B10.BR) F1 grafts**

<table>
<thead>
<tr>
<th>Bone Marrow Dose</th>
<th>Thymus</th>
<th>Peripheral Blood</th>
<th>Donor Chimerism</th>
<th>AKR Skin Graft</th>
<th>(AKR×B10.BR) F1 Skin Graft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+CD8+ % Vβ6+</td>
<td>CD4+CD8+ % Vβ6+</td>
<td>% Thy1.1+</td>
<td>MST (days)</td>
<td>MST (days)</td>
</tr>
<tr>
<td>5×10⁶</td>
<td>0.4±0.1/3.2±2.8</td>
<td>0.4±0.22/1.4±0.7</td>
<td>28.9±15.5</td>
<td>&gt;100</td>
<td>27</td>
</tr>
<tr>
<td>1×10⁷</td>
<td>0.8±0.26/2.4±1.9</td>
<td>10.0±7.8</td>
<td>&gt;100</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>2×10⁶</td>
<td>1.3±1.4/3.9±2.0</td>
<td>3.1±1.1</td>
<td>&gt;100</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>4×10⁵</td>
<td>1.5±1.1/6.7±1.3</td>
<td>0.2±0.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>1×10⁵</td>
<td>8.1±1.2/11.9±0.9</td>
<td>10.1±7.4/14.2±7.5</td>
<td>0.3±0.4</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>1×10³</td>
<td>12.2±1.5/17±1.2</td>
<td>0.2±0.4</td>
<td>16</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>“100”</td>
<td>7.9±0.6/15.6±0.1</td>
<td>0.1/11.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Different doses of T cell-depleted AKR bone marrow cells were given i.v. to CBA/Ca mice (n = 10 in each group) together with a course of nondepleting CD4 and CD8 Abs. AKR tail skin was grafted 6 wk later. Control groups consisting of CBA/Ca infused with syngeneic or no bone marrow cells rejected AKR skin within 20 days. At day 70, the percentage of Vβ6+ cells among single CD4+ and CD8+ cells was determined in the 5×10⁶, 1×10⁷, and no bone marrow groups in the thymus (n = 4) and in the peripheral blood (n = 6). Results are depicted as mean ± SD.
- Chimerism was determined by staining the peripheral blood for donor Thy1.1+ among CD4+ cells.
- MST, median survival time.

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**Immunofluorescence analysis and cell sorting**

Cell preparations of thymi were washed and resuspended in PBS, 1% w/v BSA, 5% v/v heat-inactivated normal rabbit serum, and 0.1% w/v sodium azide. Cells were incubated for 30 min at 4°C with anti-Vβ-FITC (clone 44-22-1 (12)), CD4-phycocerythrin (Sigma Chemical Co., cat. no. P-294), and CD8-Quantum Red (Sigma, cat. no. R-3762). Then cells were washed, resuspended in PBS, 1% BSA, and fixed in 1% v/v formaldehyde solution. Tricolor FACScan analysis (Becton Dickinson, Oxford, U.K.) was performed. Staining of peripheral blood lymphocytes was performed in a similar manner but was preceded by a water lysis of the erythrocytes. Chimerism was detected by staining donor lymphocytes with anti-Thy1.1-FITC (Seropec, cat. no. MCA 47F).

To determine the phenotype of APCs required for tolerance induction, T cell-depleted AKR bone marrow was negatively selected using a FACSort (Becton Dickinson) after staining with either anti-CD11b (YMB 6.1.10-FITC (13)). Sorted cells were collected by centrifugation and counted, and 2000 cells were injected into Ab-treated CBA recipients.

**Results**

**High dose marrow infusion generates tolerance by deletion**

CBA/Ca (H2k, Mls-1b) mice were tolerized to AKR (H2k, Mls-1a) using different doses of T cell-depleted bone marrow cells and a short course of nondepleting CD4 and CD8 Abs. Transplantation tolerance was tested by grafting donor AKR skin 6 wk later. The percentage of Vβ6+ cells among CD4+ and CD8+ cells in the thymus, peripheral lymphoid organs, and blood was used to reflect the degree of deletion elicited through Mls-1b recognition (12). Six weeks after transplantation of the test AKR graft, mice were regrafted with skin from (AKR × B10.BR) F1 donors, and graft survival was again monitored (Table I). Recipients of high dose bone marrow (>4×10⁶ cells) showed evidence of central and peripheral deletion of the Vβ6+ cells, sustained donor chimerism, and acceptance of donor skin. Recipients of low dose marrow (≤4×10⁵ cells) accepted donor skin, yet had no evidence of donor chimerism nor of deletion of the Vβ6+ population.

**Low dose marrow infusion generates tolerance associated with suppression**

A second graft expressing the donor Ags and an additional new set of minors ((AKR × B10.BR) F1) was then used to search for any evidence of linked suppression (14). These grafts were rejected rapidly in the high dose marrow groups but accepted indefinitely in the low dose groups (Table I). Evidence of dominant tolerance was then sought by the transfer of tolerant splenocytes from low or high dose marrow groups into naive CBA/Ca mice simultaneously grafted with AKR skin. Graft rejection was marginally delayed only in the recipients of spleen cells from the low dose bone marrow group. However, when tolerant donor mice were boosted with T cell-depleted AKR bone marrow cells 3 days before splenocyte transfer, graft rejection was significantly delayed in all recipients of low dose-tolerant cells but not in recipients of the high dose group (Fig. 1).

To demonstrate that transferable (dominant) tolerance depended upon CD4+ T cells, we treated low dose-tolerant, boosted mice with either depleting anti-CD4 or anti-CD8 Abs...
Adoptive transfer of low dose-tolerized cells (Thy1.2<sup>+</sup>) into Thy1.1<sup>+</sup> CBA/Ca recipients resulted in suppression of graft rejection as before. Depletion of donor lymphocytes immediately after transfer, with a depleting Ab against Thy1.2, abolished suppression (Fig. 2). However, if depletion was delayed until donor cells had resided in the secondary host for 3 wk, this resulted in prolonged survival of a fresh AKR graft and subsequently full acceptance of a graft from (AKR × B10BR) F<sub>1</sub> donors (Fig. 2). This demonstrates for the first time that tolerance to marrow can give rise to infectious tolerance and that the second-generation tolerated T cells also exhibit suppressive properties (linked suppression).

B220<sup>+</sup> bone marrow cells are required for low dose tolerance induction

As the injection of as few as 10,000 donor marrow cells was sufficient to induce tolerance, and as few as 100 cells produced a significant delay in graft rejection (Table I), we hypothesized that tolerance might require specific expansion or homing of a viable APC subpopulation from the bone marrow. In favor of this interpretation, neither fixed (1% formaldehyde) nor irradiated (2000 rad) cells (at a dose of 10<sup>3</sup>) could induce tolerance, even if the latter were given repeatedly during a 2-wk period (not shown). In addition, removal of B220<sup>+</sup> cells (but not surface Ig<sup>+</sup> cells nor myelomonocytic CD11b<sup>+</sup> cells) significantly impaired the tolerizing potential of a low dose (2000 sorted cells) marrow inoculum, suggesting that immature B-lineage cells were the most effective population at inducing dominant tolerance in this system.

Discussion

It has generally been thought that bone marrow induces tolerance centrally by clonal deletion in the thymus, and that no peripheral regulation was involved (15). The data in this paper confirm this to be the case when relatively high doses of multiple minor Ag (including Mls-1<sup>-</sup>)-mismatched bone marrow are given under cover of CD4<sup>+</sup> plus CD8<sup>+</sup> Abs. The demonstration that chimerism, clonal deletion, and tolerance can be achieved without any ablation of CD4<sup>+</sup> T cells or myeloid cells and with as few as 2 × 10<sup>6</sup> marrow cells, is notable but probably a function of the minor Ag differences, as we and others have previously shown that more aggressive protocols are required to achieve the high levels of chimerism necessary to induce deletion across MHC mismatches (16, 17). More surprising is that the infusion of much lower numbers of bone marrow cells (as low as 1000 cells per mouse) also led to tolerance in this Mls-plus-minors Ag difference, as defined by the acceptance of donor-type skin grafts, without any overt deletion of CD4<sup>+</sup> T cells or myeloid cells in and with as few as 2 × 10<sup>6</sup> marrow cells, is notable but probably a function of the minor Ag differences, as we and others have previously shown that more aggressive protocols are required to achieve the high levels of chimerism necessary to induce deletion across MHC mismatches (16, 17).

or a combination of both 24 h before transfer into naive CBA/Ca mice (Fig. 1). While those donor populations deprived of CD8<sup>+</sup> T cells were still able to delay rejection of AKR grafts by naive host lymphocytes, those deprived of CD4<sup>+</sup> T cells failed to do so, confirming that CD4<sup>+</sup> T cells were indeed required for the transfer of tolerance.
Although there is a general association between deletion and the loss of suppression, this is not absolute, as seen in the group of mice that received an intermediate \((4 \times 10^5)\) dose of bone marrow cells, in which deletion of CD4\(^+\)V\(\beta\)6\(^+\) cells was observed in the peripheral blood and yet (AKR \(\times\) B10(BR)) F\(_1\) skin was still accepted (Table I). This may be because the Mls-1\(^a\) Ag is not thought to be a major target for skin graft rejection (19), and this dose of marrow, while deleting the Mls-1\(^a\)-reactive T cells, may have been insufficient to delete all the other T cells that recognize Ags on the donor skin that can still act as targets for suppression.

Spleen cells from tolerant mice could suppress the rejection of grafts in otherwise unmanipulated secondary recipients. This suppression was most effective after boosting the tolerant CBA/Ca donor with AKR Ags some 3 to 4 days before transfer and was dependent on CD4\(^+\) T cells. This suggests that tolerant CD4\(^+\) T cells are not only responsible for initiating suppression, but are also able to respond to fresh Ag challenge in a manner that reinforces tolerance. It has previously been shown by titration that suppression of graft rejection in a secondary recipient represents a quantitative measure of dominant tolerance that is related to the ratio of tolerant to naive CD4\(^+\) T cells (20), and it is likely that the Ag boost is increasing the numbers of tolerant, suppressive CD4\(^+\) T cells. It is interesting to note that roughly half of the secondary recipient mice rejected their first AKR skin graft soon after the second graft (Fig. 2A), even though the latter was not itself rejected until further challenge with a third (AKR \(\times\) B10(BR)) F\(_1\) graft that was accepted indefinitely. This suggests that even after boosting, insufficient numbers of CD4\(^+\)tolerant T cells had been transferred to completely tolerate the secondary recipient.

Although the mechanisms of peripheral tolerance remain unresolved, the APC clearly plays an important role, as evidenced by the suppression of third-party responses only if they are coexpressed on F\(_1\) grafts, both in the primary tolerant mouse as well as in the secondary recipient through infectious tolerance. It was found that B220\(^+\)slg\(^-\) cells were required when limiting numbers of bone marrow cells were used to induce tolerance, suggesting a prominent role for an immature B cell. This could be related to the proliferative or Ag-presenting properties of a particular B cell subset (21) or to the restricted expression of the Mls-1\(^a\) Ag to B-lineage cells (and CD8\(^+\) T cells) but not dendritic or macrophage cells (19). However, it is still possible that long term maintenance of tolerance in these mice became dependent on the AKR skin that was later grafted to test for tolerance.

These findings show that adult transplantation tolerance induced with marrow inocula, under cover of CD4 Abs, can occur either via clonal deletion, and hence passive mechanisms, or dominant tolerance through active suppression. The degree to which either is involved seems dependent on the dose of marrow. These findings may have significant implications in the therapeutic application of tolerance.

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