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Chimeric Donor Cells Play an Active Role in Both Induction and Maintenance Phases of Transplantation Tolerance Induced by Mixed Chimerism

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Donor hemopoietic cell engraftment is considered to be an indicator of allograft tolerance. We depleted chimerism with cells specifically presensitized to the bone marrow donor to investigate its role in mixed chimerism-induced tolerance. Three experimental models were used: model A, B10.A cells presensitized to B6 (a anti-b cells) were injected into (B6 × D2)F1 → B10.A mixed chimeras grafted with DBA/2 skin; model B, anti-B6 presensitized cells prepared in DBA/2 → B10.A mixed chimeras, thus unresponsive to DBA/2 (a anti-btol-d cells), were injected into (B6 × D2)F1 → B10.A mixed chimeras grafted with DBA/2 skin; and model C, (BALB/c × B6)F1 cells presensitized to CBA (d/b anti-k cells) were injected into (B6 × CBA)F1 → BALB/c mixed chimeras grafted with B6 skin. Skin was grafted on day 30. Injection of each cell type before skin grafting abolished hemopoietic cell engraftment and prevented allograft acceptance. Injection of presensitized cells after skin grafting resulted in different outcomes depending on the models. In model A, injection of a anti-b cells completely depleted chimerism and caused allograft rejection. In model B, injection of a anti-btol-d cells markedly reduced, but did not deplete, peripheral chimerism and maintained skin allograft survival. In model C, d/b anti-k cells reduced chimerism to the background levels but failed to cause graft rejection, probably due to persistence of injected cells which share MHC with skin grafts. Together, the results show that presence of chimeric donor cells is essential in both the induction and maintenance phases of tolerance induced by mixed chimerism.

Materials and Methods

Mice

All mice except C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 mice were purchased from Taconic Farms (Germantown, NY). Mice were used at 8–10 wk of age. All care and handling of animals was performed in accordance with guidelines provided in the Guide for the Care and Use of Laboratory Animals published by the U.S. Department of Health and Human Services.

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Preparation of mixed chimeras

Recipient mice were irradiated with 1000 rad from a 137Cs source (Nordion, Ottawa, Ontario, Canada). BM was harvested from the femurs and humeri. Irradiated mice were reconstituted with 5 × 10^6 host BM plus 10 × 10^6 donor BM. BM was depleted of T cells by anti-Thy 1.2 mAb and magnetic beads (Dynal, Lake Success, NY). We used two mixed chimerism models. In one model, irradiated B10.A mice (H-2b) were infused with T cell-depleted (C57BL/6 × DBA/2)F1, (B6D2F1, H-2^bd) BM plus B10.A BM (b/d → a mixed chimeras) followed by DBA/2 (D2, H-2^d) skin grafting. In the second model, irradiated BALB/c (H-2^c) mice were infused with a mixture of (B6 × CBA)F1, (H-2^bc) and BALB/c BM (b/k → d mixed chimeras) followed by B6 (H-2^b) skin grafting.

Skin grafting

Full-thickness skin was transplanted using standard techniques 30 days after BMT as described previously (8, 9). Rejection was defined as complete loss of viable donor epithelium.

Thymectomy

Under anesthesia, a partial sternotomy was made and the thymus was removed by suction. The absence of thymic tissue was confirmed when thymectomized animals were euthanized. Animals with residual thymic tissue were excluded from analysis.

Flow cytometry

An anti-CD16/32 mAb was used as a blocking Ab. PBLs were stained with the FITC-, PE-, Cy-chrome-conjugated mAb directed to H-2Kb, H-2 d, H-2 k, and Thy1.2 (BD PharMingen, San Diego, CA). Isotype Abs were used as controls. Stained cells were analyzed on a FACScan (BD Biosciences, Mountain View, CA). The degree of background staining in negative control cells was 1.2 ± 0.1% (range: 0.1–3.2, n = 47).

Presensitized cells for depletion of chimerism

Three types of presensitized cells were prepared. 1) Naive B10.A mice were sensitized to H-2^b by B6 skin grafting and injection of 100 × 10^6 B6 splenocytes after skin graft rejection. Splenocytes were prepared from sensitized mice 7–10 days after immunization and used as “a anti-b presensitized cells” in the b/d → a mixed chimera model. 2) After establishment of stable mixed chimeraism (40–50 days post-BMT), D2 plus B10.A → B10.A mixed chimeric mice were depleted of H-2^d as described above. Splenocytes from presensitized chimeric mice were depleted of chimeric D2 cells with the use of anti-H-2 Kd mAb and magnetic beads. H-2^d-positive cells in the splenocytes were reduced from 63.2 ± 1.7% (n = 23) to 1.7 ± 0.2% after treatment. After removal of H-2^d cells, the splenocytes were cytotoxic against H-2^b but unresponsive to H-2^d in 51Cr-release assays (data not shown). The splenocytes were used as anti-b presensitized cells that were tolerant to D2 (a anti-btol-d) presensitized cells in the b/d → a mixed chimera model. 3) (BALB/c × B6)F1, (H-2^bc) mice were sensitized to H-2^b by CBA (H-2^c) skin grafting and immunization with CBA splenocytes. Splenocytes from sensitized mice were used as d^b anti-k presensitized cells. When injected into b/k → d^a mixed chimeras bearing b skin grafts, these cells were reactive with b/k chimeric cells but unresponsive to b skin grafts. The hosts (d^a) were also unresponsive to injected d^b cells by virtue of b/k chimism.

Detection of male Ag by PCR

We prepared b/d → a mixed chimeras bearing d skin grafts using male B6D2F1 (BM donor), female B10.A (recipients and syngeneic BM donor), and female DBA/2 (skin donor). Presensitized cells were prepared with the use of female mice for hosts as well as skin and splenocyte donors. Thus, following injection of presensitized cells, only chimeric b/d cells expressed male Ags.

Genomic DNA was extracted from blood, spleen, liver, thymus, and BM using the QIAamp Tissue kit from Qiagen (Chatsworth, CA). The reactions were completed in a total volume of 50 μl of reaction buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 50 mM MgCl2, 30 ng of template DNA, 0.2 mM each of 10 mM dNTP mixture (Invitrogen Carlsbad, CA), 1.0 U Taq polymerase (Invitrogen Carlsbad, CA), and 0.2 mM each of Y chromosome-specific primers under the following conditions in a thermocycler (PerkinElmer, Branchburg, NJ): 10 min denaturation at 95°C followed by 38 cycles amplification, each containing 1 min denaturation at 95°C, 1 min annealing at 60°C, 1 min polymerization at 72°C, followed by a 10-min amplification step at 72°C (holding temperature 4°C). The sequence of the Y chromosome-specific primers was: 5’-CTAACCCTGTACCACCCT and 5’-CTCTCTTCATTAACCTTTTCT (10). For analysis, the probes were run in a 1.5% agarose gel and detected by ethidium bromide staining. The Y chromosome-specific primers amplified the positive signal of male donors with a minimal detection level of 1:10^3 (male to female cells).

Results

Injection of a anti-b presensitized cells depletes chimerism and causes graft rejection in b/d → a mixed chimeras bearing d skin

In B6D2 plus B10.A → B10.A (b/d → a) mixed chimeras (n = 6), the levels of peripheral chimerism gradually increased from 0.1% to 0.4% during the first 40 days after injection of presensitized cells (Fig. 1A, Ia, and Ia). Flow cytometric analyses. Ib and Iib, PCR analyses of the PBLs. P, Positive control (male naive B10.A); N, negative control (female naive B10.A); 1–4 and 5–6, individual mice given a anti-b or a anti-btol-d cells, respectively. The same results were obtained with PCR analyses of other tissues.

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**Table I. Chimerism and skin graft survival after injection of a anti-b presensitized cells into b/d → mixed chimeras bearing d skin grafts**

<table>
<thead>
<tr>
<th>Days of Cell Injection (relative to day of skin grafting)</th>
<th>Number of Animals</th>
<th>Chimerism (%) (1 day before cell injection)</th>
<th>Time Interval to Achieve ≥1% Chimerism (days)</th>
<th>Skin Graft Survival (days postinjection/days postgrafting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−9</td>
<td>4</td>
<td>74.9 ± 2.5 (69.7–79.5)</td>
<td>21 (all)</td>
<td>25.8 ± 0.3/16.8 ± 0.3 (25–26/9–10)</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>82.2 ± 1.8 (75.4–85.4)</td>
<td>35 (all)</td>
<td>43.2 ± 1.5/73.2 ± 1.5 (41–49/71–79)</td>
</tr>
<tr>
<td>67</td>
<td>4</td>
<td>93.6 ± 1.0 (69.7–83.7)</td>
<td>36.8 ± 6.6 (28–56)</td>
<td>46.5 ± 6.5/113.5 ± 6.5 (33–59/100–126)</td>
</tr>
<tr>
<td>90</td>
<td>4</td>
<td>77.0 ± 4.2 (68.2–88.4)</td>
<td>36.8 ± 1.8 (35–42)</td>
<td>44.0 ± 1.8/134.0 ± 1.8 (41–49/131–139)</td>
</tr>
<tr>
<td>141</td>
<td>1</td>
<td>91.5</td>
<td>28</td>
<td>42/181</td>
</tr>
</tbody>
</table>

* a anti-b cells were resensitized in vitro against irradiated B6 (b) splenocytes.
To determine whether chimeric cells were completely depleted or remained at very low levels, we created b/d → a mixed chimeras in which only b/d chimeric cells expressed male Ags. After injection of a anti-b presensitized cells prepared in female mice caused marked reduction of peripheral chimerism (Fig. 1, Ia), mice were euthanized and various tissues were analyzed for the presence of a Y chromosome Ag. PCR analysis showed the absence of Y Ag, indicating that injection of the a anti-b presensitized cells into b/d → a chimeras resulted in complete elimination of chimeric cells (Fig. 1, Ib).

We also investigated the involvement of host thymus in skin graft rejection caused by injection of the presensitized cells. A group of b/d → a mixed chimeras bearing d skin grafts underwent thymectomy 60 days after BMT (30 days after skin grafting). We injected an anti-b presensitized cells into both thymectomized mice (21 days after thymectomy) and nonthymectomized mice (51 days after skin grafting). Injection of the presensitized cells abolished peripheral chimerism within 35 days in both thymectomized and nonthymectomized mice (Fig. 2A). However, skin allograft rejection in thymectomized mice was markedly delayed while the skin allograft was rejected within 30 days after cell injection in non-thymectomized mice ($p = 0.012$ by Mantel’s log-rank test) (Fig. 2B). Both chimerism and skin allografts remained intact after thymectomy without administration of the presensitized cells (not shown).

Injection of a anti-b presensitized cells that are unresponsive to skin donor Ag diminishes chimerism but fails to cause skin graft rejection in b/d → a mixed chimeras

To further eliminate the possibility that the injected presensitized cells directly damaged skin grafts, we injected a splenocytes that were sensitized to the b but tolerant to d (a anti-btol/d-sensitized cells) into b/d → a mixed chimeras. These a anti-btol/d-cells were highly cytotoxic to H-2b targets after 3 days of in vitro sensitization to H-2b stimulators, but failed to generate anti-H-2d cytotoxicity after in vitro sensitization with H-2d stimulators (not shown). We injected the cells into b/d → a mixed chimeric mice before or after d skin grafting. Injection of the cells 9 days before skin grafting induced rapid reduction of chimerism and skin graft rejection (Table II). However, injection of the cells 30 days after skin grafting failed to cause skin graft rejection despite the same degrees of reduction of the peripheral chimerism as those induced by a anti-b cells. In some mice, injection of $30 \times 10^6$ (n = 2) or $20 \times 10^6$ cells (n = 2) on day 30 had little effect on the degree of chimerism while second injection of $50 \times 10^6$ or $30 \times 10^6$ cells, respectively, was necessary to significantly reduce chimerism. In these mice, skin grafts remained intact for >150 days. When the a

Table II. Chimerism and skin graft survival after injection of a anti-b cells that were tolerant to d (a anti-btol/d-cells) into b/d → a mixed chimeras bearing d skin grafts

<table>
<thead>
<tr>
<th>Days of Cell Injection (relative to skin grafting)</th>
<th>Cell Numbers</th>
<th>Days Post Cell Injection/Chimerism (%)</th>
<th>Skin Graft Survival (days postgrafting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−9 (n = 4)</td>
<td>$50 \times 10^6$</td>
<td>1 Day: $79.3 \pm 0.8^<em>$ ($77.5-81.4^</em>$)</td>
<td>$24.8 \pm 3.9$ (17-34)</td>
</tr>
<tr>
<td>30 (n = 4)</td>
<td>$50 \times 10^6$</td>
<td>21 Days: $71.9 \pm 4.1$</td>
<td>NT $^a$</td>
</tr>
<tr>
<td>30 + 72 (n = 4)</td>
<td>$30 \times 10^6 + 50 \times 10^6$</td>
<td>70 Days: $2.6 \pm 0.6$</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>or $20 \times 10^6 + 30 \times 10^6$</td>
<td></td>
<td>100 Days: $1.2 \pm 0.3$ ($0.5-1.8$)</td>
<td>0.5, 0.7</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SEM.

$^d$ Not tested.

$^d$ Range.

$^d$ One mouse rejected skin graft on 114 days while the remaining survived >150 days.
anti-b/tol-d cells prepared in female mice were injected into female b/d → a mixed chimera mice bearing male b/d chimera cells, peripheral chimerism determined by flow cytometric analyses was markedly reduced (Fig. 1, IIa). However, PCR analysis of various tissues revealed the presence of the Y chromosome-specific Ag, suggesting the persistence of low level chimerism (Fig. 1, IIb).

**Table III. Chimerism and graft survival after injection of d/b anti-k presensitized cells into b/k → d mixed chimeras bearing b skin grafts**

<table>
<thead>
<tr>
<th>Days of Cell</th>
<th>mAb*</th>
<th>Days Post-Cell</th>
<th>Chimerism (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection (relative to skin grafting)</td>
<td></td>
<td>–1 Day</td>
<td>21 Days</td>
</tr>
<tr>
<td>–9 (n = 3)</td>
<td>H-2Kk</td>
<td>NT</td>
<td>1.7 ± 0.4 (1.1–2.5)</td>
</tr>
<tr>
<td>H-2Kk</td>
<td>NT</td>
<td>57.6 ± 4.1 (51.2–65.2)</td>
<td>8.2 ± 0.9 (6.5–9.2)</td>
</tr>
<tr>
<td>30 (n = 4)</td>
<td>H-2Kk</td>
<td>NT</td>
<td>3.6 ± 0.8 (1.9–5.2)</td>
</tr>
<tr>
<td>H-2Kk</td>
<td>NT</td>
<td>63.7 ± 1.8 (60.2–68.6)</td>
<td>13.0 ± 3.0 (97.6–21.5)</td>
</tr>
<tr>
<td>70 (n = 3)</td>
<td>H-2Kk</td>
<td>NT</td>
<td>4.2 ± 0.8 (2.8–5.7)</td>
</tr>
<tr>
<td>H-2Kk</td>
<td>NT</td>
<td>49.4 ± 3.3 (43.0–54.0)</td>
<td>9.1 ± 1.4 (6.6–11.5)</td>
</tr>
</tbody>
</table>

*Either anti-H-2Kk or H-2Kk mAb was used to detect chimerism.

**Discussion**

Injection of cells that were cytolytic to chimeric cells but not to skin grafts into mixed chimeric hosts, i.e., injection of a anti-b cells or a anti-b/tol-d into b/d → a mixed chimera bearing d skin and injection of d/b anti-k presensitized cells into b/k → d mixed chimeras bearing b skin, before skin grafting abolished engraftment of BM and prevented acceptance of the BM-MHC matched skin allografts. These results are in agreement with previous reports by others (11) and us (8) that robust chimerism is essential for induction of tolerance in a mixed irradiation chimera or allogeneic radiation chimera model. For example, Taniguchi et al. (11) reported that mice with >30% chimerism could accept skin grafts whereas mice with <10% chimerism showed prolonged but not permanent graft survival. Incomplete depletion of residual host T cells associated with <10% chimerism was considered responsible for failure of allograft tolerance induction. A study by Sharabi et al. (4) also showed that depletion of chimerism before skin grafting led to failure of allograft tolerance induction. We recently observed that administration of anti-H-2KkDk mAb in B6 × D2 → B10.A mixed chimeras starting 16–9 days before skin grafting caused rapid reduction of chimerism and rejection of D2 skin grafts even in the presence of a significant degree of chimerism (~10%) which persisted up to 150 days (A. Kanamoto, unpublished data). Injection of the presensitized cells into mixed chimeras after establishment of tolerance to skin allografts led to different results depending on the type of presensitized cells used as summarized in Table IV. When a anti-b presensitized cells were injected into b/d → a mixed chimera bearing d skin grafts, peripheral chimeric cells were completely eliminated as confirmed by PCR analysis. Effective elimination of chimeric cells may be attributable to the continuous presence of injected cells and/or expansion of anti-b cells in vivo by their restimulation by the b/d chimera cells. It is unlikely that injected cells directly mediated rejection of tolerant grafts. Reduction of chimerism to the background levels (~1%) always preceded skin allograft rejection by several days. Injection of a anti-b presensitized cells into d → a mixed chimera bearing d skin grafts failed to abolish peripheral chimerism or cause graft rejection. Host thymectomy before cell injection had no effect on depletion of chimerism by presensitized cells, but prevented rejection of tolerant skin grafts, indicating that the host thymus, most likely host thymus-derived T cells, is required for rapid rejection of skin grafts. Khan et al. (12) have demonstrated that tolerance can be broken when nontolerant T cells emerge from the thymus after intentional depletion of donor Ag or after exogenous administration of nontolerant T cells.
When the a anti-b/tol-d cells were injected into b/d → a mixed chimeras bearing d skin grafts, peripheral chimerism determined by flow cytometry was reduced to the same background levels as induced by the a anti-b cells, but skin grafts remained intact. PCR analysis revealed that low level chimerism persisted in these mice for up to 200 days. The reasons why tolerant presensitized cells were unable to completely eliminate chimeric cells are not immediately known. As the a anti-b/tol-d cells were prepared in d → a mixed chimeras, it is possible that small numbers of chimeric D2 (d) cells were present in the injected cells despite their careful removal before injection. Once injected into b/d → a mixed chimeras, these residual d cells might survive and possibly function as immunoregulatory cells preventing complete elimination of b/d chimeric cells and rejection of tolerant d skin.

When the d*/b anti-k cells were injected into b/k → d* mixed chimeras bearing b skin grafts, b/k chimeric cells were reduced to the background levels but skin grafts remained intact. It is possible that the background levels of b/k chimerisms represented low but true chimerism as in the case in which a anti-b/tol-d cells were injected into b/d → a mixed chimeras. Alternatively, as injected d*/b cells persisted, albeit in a small number, it is conceivable that these cells served as protectors of b skin allografts in place of abolished b/k chimeric cells.

The present study demonstrates that chimerism plays an active role not only in the induction phase but also in the maintenance phase of allograft tolerance induced by mixed chimerism. In the induction phase, macrochimerism (≥10%) is required for the induction of allograft tolerance. Prevention of allogeneic BM engraftment was always associated with inability to accept BM-MHC bearing skin allografts. In the maintenance phase, complete depletion of chimeric donor cells led to rejection of existing tolerant grafts, probably due to loss of immunoregulation by chimeric cells and activation of host antigraft immunity. In contrast, persistence of peripheral chimerism, even at the low levels detectable only by PCR, or continuous survival of injected cells that expressed graft MHC, was sufficient to maintain allograft tolerance. Failure by Schlitt and colleagues (5) to elicit rejection of allografts by mAb administered in the maintenance phase of cyclosporine-induced tolerance was explained by lack of involvement by the graft-derived chimeric cells in the maintenance of tolerance, complicating the involvement of tolerance mechanism(s) other than that mediated by chimerism. Alternatively, in view of our present results, their results could suggest that persistent microchimerism which they detected at 200 days by PCR is responsible for maintenance of tolerance. Thus, in their model, chimerism was indeed essential in both the induction and maintenance phase of allograft tolerance.

References