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Cutting Edge: Induction of Antigen-Specific Hyporesponsiveness by Transplantation of Hemopoietic Cells Containing an MHC Class I Transgene Regulated by a Lymphocyte-Specific Promoter

Susan A. Hansal,* Diane I. Morris,* Joan M. G. Sechler,* Paul E. Love, † and Amy S. Rosenberg 1*

We explored a novel approach to tolerance induction by the transplantation of bone marrow (BM) cells (BMCs) that themselves do not express a foreign histocompatibility Ag, but which give rise to mature lymphocytes that do so. Lines of transgenic (FVB) mice were generated that contained an MHC class I $D^d$ cDNA regulated by a CD2 promoter. Because the CD2 promoter is lymphocyte-specific and activated relatively late in lymphocyte ontogeny, $D^d$ is expressed on most mature lymphocytes in the periphery but only on developing B cells in the BM of transgenic mice. Transgenic BMCs are tolerogenic and reproducibly engraft in nontransgenic mice using a conditioning regimen that is nonpermissive for the engraftment of conventional (MHC promoter) $D^d$-transgenic BMCs. Engrafted BMCs generate transgene-expressing lymphocytes and confer a state of Ag-specific hyporesponsiveness on the host that is primarily attributable to a peripheral mechanism. The strategies by which tolerance can be optimized in this system are discussed. The Journal of Immunology, 1998, 161: 1063–1068.

Tolerance arising from bone marrow (BM)2 chimerism is thought to depend principally on the intrathymic deletion of allospecific lymphocytes by donor-derived dendritic cells (DCs) (1–2). The primary obstacle to achieving a tolerant state by this means is graft rejection. Indeed, the immunogenicity of BM cells (BMCs) is such that engraftment across MHC barriers requires extensive conditioning (3–5) and large doses of progenitor cells (4–5).

Distinct from central (thymic) tolerance, peripheral tolerance may be generated by several mechanisms: the deletion of alloreactive cells by T cell-mediated veto activity (6–7); the induction of anergy by small, resting B and T cells (8–9); suppression (10); or immune deviation (10–12). It is generally accepted that small, resting lymphocytes induce T cell anergy by presenting Ag (signal 1) in the absence of productive costimulatory interactions principally mediated by B7-CD28 and CD40-CD40L interactions (signal 2) (13–17).

This work was undertaken to explore tolerance induction, generated by bone marrow transplantation (BMT), in which the only cells arising from the engrafted marrow that express a foreign histocompatibility Ag are lymphocytes, which do not express B7 costimulatory molecules constitutively (14) and whose expression of CD40 fails to provide naive T cells with a productive costimulatory interaction (15).

Materials and Methods

Genetic constructs

$CD2-D^d$. The $D^d$ cDNA coding sequence in pDdSELFIX.34 (a kind gift of Dr. Randy Ribaudo, National Institutes of Health, Bethesda, MD) was ligated into the human CD2 promoter and enhancer expression cassette p29$\Delta$2(Sal−) (18–19).

$MHC-D^d$. The genomic $D^d$ gene, including the MHC class I promoter, was isolated from pDd1 (a kind gift of Dr. Gilbert Jay Origene Technologies, Rockville, MD) (20) and was used directly for the generation of transgenic mice.

Generation of transgenic mice

The CD2-D$^d$ and MHC-D$^d$ constructs were digested with restriction enzymes to remove vector sequences, and fragments were purified by gel electrophoresis and GeneClean (BIO 101, Vista, CA). DNA was resuspended in 10 mM Tris (pH 7.4) and 0.1 mM EDTA at a concentration of 10 ng/ml, and pronuclear injections were performed on fertilized FVB/N oocytes as described previously (21).

Mice

FVB/N mice, an inbred H-2b strain, were bred in-house or purchased from Taconic Farms (Germantown, NY).
Bone marrow transplantation. BM was harvested from posterior limbs and cervical vertebrae and washed three times in HBSS containing 5% FCS and 1% HEPES buffer. A total of $2 \times 10^6$ BMCs were infused into hosts that had been irradiated with a Cs$^{137}$ source (Gammacell 40 irradiator, Nordion, Ontario, Canada).

Chimerism following BMT. At 4 wk posttransplantation, PBLs were collected by tail vein incision, lysed with RBC lysing buffer (Sigma), and dually stained with 34-2-12 anti-Dd FITC (06134D; PharMingen, San Diego, CA) and B220 (01125A; PharMingen) or CD3 (01085A; PharMingen) phycoerythrin (PE). Cells were run on a FACScan (Becton Dickinson, Mountain View, CA), and data were analyzed using the CellQuest software program (Becton Dickinson).

Class I MHC expression on BM of CD2-Dd mice. BM was dually stained for Dd (PE (06135A) or biotin (06132D) with streptavidin-quantum red conjugate, both from PharMingen) and class I MHC (FITC) (06214D; PharMingen), c-kit (FITC) (01904D; PharMingen), Sca (01581D; PharMingen), Gr-1 (FITC) (01214A; PharMingen), Mac-1 (FITC) (01714D; PharMingen), 6C3 (FITC) (01284D; PharMingen), or GL-1 (FITC) (anti-B7.2 mAb), a generous gift of Karen Hathcock. DCs were identified by the expression of both Ia$^b$ (biotin (06302D; PharMingen) with streptavidin quantum red conjugate, Sigma, St. Louis, MO) and N418 (with anti-hamster IgG-FITC (06134D; PharMingen)) (22), which was a generous gift of Dr. Chris Norbury. Dd expression was evaluated in three-color assays using anti-Dd-PE.

Thymectomy. Thymectomized (ATX) FVB/N mice were purchased from Taconic or were generated in-house. Mice were thymectomized by sternotomy and excision under direct visualization.

Skin grafting. Mice were engrafted on the flank with tail skin from donor mice according to published methods (23). Grafts were scored daily or every other day until rejection (>80% loss of graft tissue).

**Results and Discussion**

We generated transgenic mice that express MHC class I Dd under the control of a human CD2 promoter/enhancer (CD2-Dd mice) (18–19). Tolerance to Dd was assessed in three transgenic lines with different expression levels by skin graft rejection (Fig. 1). Compared with control FVB mice, all transgenics were hyporesponsive to Dd, but there was a distinct hierarchy: 4905 mice rejected skin grafts faster than 4911 mice, which rejected grafts faster than 4906 mice. As shown in Figure 1, the degree of hyporesponsiveness correlated precisely with the expression level of the transgene, suggesting that the extent of TCR cross-linking, and thus the strength of signal 1, in the absence of costimulation, is crucial in tolerance induction.

In addition to the expression level, the distribution of transgene expression on lymphocyte subsets may play an important role in tolerance induction. As B cells, but not T cells, can generate the self/class II plus allo/class I peptide ligand recognized by CD4$^+$ class I allospecific T cells (25–27), B cells should be able to tolerate both CD4$^+$ as well as CD8$^+$ T cells mediating rejection. The degree to which the transgene is expressed on B cells may be a factor in the enhanced hyporesponsiveness of 4911 compared with 4905.

**FIGURE 1.** CD2-Dd-transgenic lines: expression level and tolerance to Dd. A, Expression of the Dd transgene on the spleen cells of transgenic mice by flow cytometry. B, A representative study in which CD2-Dd transgenic mice were engrafted with tail skin from transgenic mice expressing genomic Dd (MHC-Dd) and followed for rejection until day 75. MST in days: FVB = 12 ($n = 17$), 4905 = 25 ($n = 15$), 4911 = 35 ($n = 19$), and 4906 = 63 ($n = 17$), with 7 of 17 mice surviving 75 days.
4905 mice, since expression of the transgene can be detected on the B cells of 4911 mice but not 4905 mice (Fig. 1A). Similarly, the more profound hyporesponsiveness of 4906 mice may depend upon the expression of the transgene on a substantial population of B cells, as well as on the relatively high level of expression on T cells (Figs. 1 and 2, upper panels). These studies suggest that lymphocyte chimerism can potentially induce tolerance if high levels of expression can be induced on all or most B cell as well as T cell populations.

Because the CD2 promoter commences activity late in B cell maturation (pre-B cells) (28) and in T cells undergoing thymic maturation (CD25<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>) (29–30), the expression of D<sup>d</sup> was assessed on BMCs, with the thought that immature cells with progenitor activity might not express the transgene. As shown in Figure 2 (lower panel), CD2-D<sup>d</sup> BMCs express little D<sup>d</sup> relative to MHC-D<sup>d</sup> BMCs and relative to mature lymphocyte populations contained in the spleen (upper panel). The expression of the transgene in CD2-D<sup>d</sup> BMCs was restricted to B220<sup>+</sup> pre-B and B cells (Fig. 3), which were B7.2 dull, indicating that they were not activated (data not shown). Expression was negligible on cells of granulocyte, macrophage, and dendritic lineages (Fig. 3). A lack of transgene expression on the BM-derived DCs of CD2-D<sup>d</sup> mice was further demonstrated by the failure of their skin to elicit a rejection response or to prime nontransgenic mice to D<sup>d</sup> (data not shown), and therefore are not targets for the Ag-specific effectors mediating rejection (31). Taken together, these data demonstrate that the tissue-specific expression of alloantigen, which is mediated by the CD2 promoter, confers privilege on CD2-D<sup>d</sup> BMCs with respect to transplantation.

The hyporesponsiveness induced by lymphocyte chimerism may result from central (thymic) (32–33) as well as peripheral mechanisms (9–13). The role of the thymus was assessed by
thymectomizing recipients before irradiation and BMT. Whereas survival of Dd skin was modestly prolonged in BMT euthymic mice over non-BMT euthymic mice, skin grafts were accepted in four of five transplanted ATX mice (Table I). Tolerance correlated with a high ratio of transgene-expressing B vs T cells and with a reduction of total T cells. As thymectomy and irradiation in the

**FIGURE 3.** Expression of Dd on various lineages of BMCs of transgenic mice. In the first three columns, BMCs were dually stained for lineage markers and Dd. A gate was placed on lineage-positive cells (Gr-1, which are expressed on granulocytes and macrophages; 6C3, which are expressed on B cell progenitors; or B220, which are expressed on pre-B and mature B cells), and Dd expression was assessed by flow cytometry. DCs (last column) were generated and identified as previously described. A gate was placed on CD11c-positive cells, and Dd expression was measured.

**FIGURE 4.** Assessment of chimerism and tolerance following BMT with CD2-Dd BMCs. Nontransgenic mice were irradiated (300 rad) and infused with $2 \times 10^6$ BMCs from CD2-Dd or MHC-Dd mice. Mice were bled at 4 wk posttransplant, and lymphocyte fractions were isolated and tested for Dd expression by flow cytometry (A). The results are representative of 9 CD2-Dd BMT mice (median chimerism 18%, range 14-20%) and 11 MHC-Dd mice. After 2 wk, mice were engrafted with tail skin from MHC-Dd mice and followed for rejection (B). MST in days: CD2-Dd = 24, MHC-Dd = 10, FVB = 13.5.
absence of BMT only modestly delayed skin graft rejection (Table I), these data indicate that lymphocyte presentation of allotype tolerizes mature T cells by a peripheral mechanism that can be overwhelmed by the activity of the thymus. This finding was surprising, since CD8⁺ class I allospecific T cells are deleted by T cell expression of class I allotriken in the thymus (32), and CD4⁺ class I allospecific T cells that escape thymic deletion (32–33) should be tolerized in the periphery by B cells.

In this regard, the thymus (or its progeny) impacts on peripheral tolerance both by replenishing the allospecific T cell pool depleted by irradiation and by altering the distribution of D⁰ expression such that fewer B cells express the transgene in euthymic mice, even though overall engraftment is similar in euthymic and ATX mice (Table I). These effects serve to diminish the number of transgenic cells that can tolerate CD4⁺ as well as CD8⁺ allospecific T cells and allow D⁰ allospecific T cells that escape central tolerance to also escape peripheral tolerance.

Interestingly, the thymus does not appear to interfere with peripheral tolerance by generating cells that reject engrafted BMCs: lymphocyte chimerism persists at the same level following skin graft rejection, although more subtle changes in chimeric cells following skin graft rejection have not been excluded. The persistence of chimerism did not alter memory responses in transplanted mice, as second D⁰ skin grafts were rejected in an accelerated fashion.

In summary, these studies describe a novel approach to the induction of tolerance by BMT, the biological basis of which pertains to the tissue-specific, maturationally dependent expression of foreign Ags regulated by a lymphocyte-specific CD2 promoter. We have demonstrated that the restricted expression of the class I transgene in hematopoietic cell populations permits engraftment with minimal conditioning, which in turn gives rise to lymphocyte chimerism and Ag-specific hypersensitivity. These experiments suggest that tolerance induced by lymphocyte chimerism could be optimized by the manipulation of both host and donor elements: enhancing the level of cell surface expression, increasing the percentage of B cells that express high levels of the MHC transgene, and inhibiting thymic function (34) or the activity of mature T cells at the time of transplant. Such studies are in progress.

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References


Table I. Graft rejection in ATX vs euthymic BMT mice: cellular profiles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>D⁰⁺B220⁺ CD3⁻</th>
<th>D⁰⁺CD3⁺ CD3⁻</th>
<th>Total CD3⁺ Day of Graft Rejection</th>
<th>MST (days)</th>
</tr>
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<tbody>
<tr>
<td>ATX 300 rad/BMT (6)</td>
<td>14</td>
<td>33</td>
<td>&gt;66</td>
<td>&gt;66</td>
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<tr>
<td>ATX 300 rad (4)</td>
<td>11</td>
<td>31</td>
<td>&gt;66</td>
<td>&gt;66</td>
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<tr>
<td>Euthymic 300 rad/BMT (4)</td>
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<td>75</td>
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<td>21</td>
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<tr>
<td>Euthymic 300 rad (5)</td>
<td>0.8</td>
<td>75</td>
<td>21</td>
<td>21</td>
</tr>
</tbody>
</table>

* FVB nontransgenic mice were thymectomized and rested for several weeks prior to further treatment. ATX mice and controls were irradiated (300 rad) and infused with 20 × 10⁶ CD2⁺D⁰⁺ (4906) BMCs. Mice were bleed at 1 mo following infusion, and lymphocyte populations were assessed for the expression of D⁰⁺ and B220⁺ cell markers. After 1 wk, mice were engrafted with tail skin from MHC-D²⁺ mice and monitored daily for rejection. The number of mice per treatment is given in parentheses. The percentage of total CD3⁺ cells for irradiated/nontransplanted mice is the median with the range provided in parentheses.

* Partially thymectomized.


