## *Brief Definitive Report*

# ALLORESISTANCE TO ENGRAFTMENT OF ALLOGENEIC DONOR BONE MARROW IS MEDIATED BY AN Lyt-2$^+$ T CELL IN MIXED ALLOGENEIC RECONSTITUTION (C57BL/10Sn + B10.D2/nSn → C57BL/10Sn)

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We recently reported a murine model for the study of specific transplantation tolerance induced by total body irradiation and reconstitution with T cell depleted syngeneic (B10) plus T cell–depleted allogeneic (B10.D2/nSn) bone marrow (B10 + B10.D2 → B10) (1). Such recipients repopulated as mixed lymphopoietic chimeras and were specifically tolerant to the B10.D2 allogeneic donor in vivo and in vitro, yet retained immunocompetence at a level similar to that of syngeneically reconstituted (B10 → B10) recipients (2). T cell depletion of the syngeneic component of the mixed allogeneic bone marrow inoculum was essential for engraftment of the allogeneic donor and for induction of this specific transplantation tolerance. If the syngeneic donor marrow was not T cell–depleted, animals repopulated as fully syngeneic chimeras were not tolerant to B10.D2 allogeneic donor skin grafts, and were reactive in vitro to B10.D2 lymphoid elements in MLR and cell mediated lympholysis (CML) assays (3).

To the extent that failure of allogeneic engraftment in this model may correlate with failure of allogeneic bone marrow engraftment in general, these results provide a means for identifying the critical syngeneic cell phenotype responsible for the alloresistance to engraftment observed. We report here the results of selective depletions of various T cell subsets (L3T4$^+$, Lyt-2$^+$, Thy-1$^+$) from the syngeneic component of the mixed bone marrow inoculum by the use of mAbs plus complement. Our results indicate that the critical syngeneic cell phenotype that prevents engraftment of allogeneic donor bone marrow and tolerance to the donor alloantigens in mixed allogeneic reconstitution resides among the Lyt-2$^+$ subset of T lymphocytes.

## Materials and Methods

*Animals.* 4–6-wk-old mice of strains C57BL/10Sn (B10), B10.D2/nSn (B10.D2), and B10.BR (The Jackson Laboratory, Bar Harbor, ME) were used.

*Preparation of Chimeras.* Preparation of mixed allogeneic chimeras (B10 + B10.D2 → B10) was carried out as previously described (1, 2). T cell depletions of bone marrow were performed using rabbit anti–mouse brain (RAMB) plus guinea pig complement (GPC) as previously described, as well as using mAbs GK1.5 (anti-L3T4$_a$), 83-12-5 (anti-

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Lyt-2.2), and 20-10-5 (anti-Thy-1) (4), plus low-cytotoxicity rabbit complement. The monoclonal mouse anti-Lyt-2.2 antibody (83-12-5) was generated from the fusion of the spleen from a C3H/HeJ mouse immunized with an Lyt-2.2 bearing cytotoxic T cell clone, BM10-5.6. The monoclonal rat anti-L3T4 antibody (GK1.5) was a gift of Dr. Frank Fitch (University of Chicago, Chicago, IL). Adequacy of cellular depletions was confirmed using flow microfluorometry (FMF).

*Characterization of Chimeras by FMF.* Recipients were characterized for engraftment with syngeneic and/or allogeneic donor lymphoid elements using FMF to determine the percentage of PBL bearing H-$2^b$ and H-$2^d$ surface markers (1, 2). Arbitrary levels on log scale were selected based on the inflection point where staining of the control negative population was minimized while retaining staining of maximal numbers of positive cells.

*Skin Grafting.* Skin grafting was performed 6 wk postreconstitution as previously described (1, 2). Graft survivals were calculated by the life-table method (5), and the Median Survival Time (MST) derived from the time point at which 50% of grafts were surviving.

*Assays of Cellular Immunity.* Mixed lymphocyte culture proliferative assays and CML assays were performed as previously described (2).

## Results

*Cell Surface Phenotype of Syngeneic Bone Marrow Cell Responsible for Alloresistance to Engraftment in Mixed Allogeneic Reconstitution.* Chimeras were prepared according to the following five protocols and were characterized for engraftment of allogeneic donor bone marrow cells: Group 1, $5 \times 10^6$ Lyt-$2^+$-depleted B10 bone marrow cells + $15 \times 10^6$ RAMB B10.D2; Group 2, $5 \times 10^6$ L3T$4^+$-depleted B10 bone marrow cells + $15 \times 10^6$ RAMB B10.D2; Group 3, $5 \times 10^6$ Thy-$1^+$-depleted B10 bone marrow cells + $15 \times 10^6$ RAMB B10.D2; Group 4, $5 \times 10^6$ RAMB B10 bone marrow cells + $15 \times 10^6$ RAMB B10.D2; Group 5, $2.5 \times 10^6$ L3T$4^+$-depleted B10 + $2.5 \times 10^6$ Lyt-$2^+$-depleted B10 bone marrow cells + $15 \times 10^6$ RAMB B10.D2. As seen in Table I, the pattern of lymphopoietic repopulation of the recipients depended upon the protocols used for syngeneic T cell subset depletion. Those mixed, allogeneically reconstituted mice that received syngeneic bone marrow from which the Lyt-$2^+$-bearing cells had not been eliminated repopulated exclusively with syngeneic (B10) cells as shown by FMF (Table I; groups *2* and *5*). In contrast, all groups in which the Lyt-$2^+$ T cell phenotypes had been depleted repopulated as mixed lymphopoietic chimeras with variable percentages of B10 and B10.D2 cells (Table I; groups *1, 3,* and *4*), although some individual animals had very low levels of chimerism detected. However, the low number of B10.D2 cells in such animals was nevertheless distinctly detectable as a peak separated from the background on cell sorter profiles (data not shown). These data indicate that elimination of Lyt-$2^+$ cells from the syngeneic component of the mixed allogeneic bone marrow inoculum is necessary to permit engraftment of allogeneic donor bone marrow cells.

*Effect of T Cell Subset Depletions from Syngeneic Component of Bone Marrow in Mixed Allogeneic Reconstitution on Specific Acceptance of Donor Skin Allografts.* To assess the effect of selective depletions of various T cell subsets from the syngeneic component of the mixed marrow inoculum on the ability to induce specific transplantation tolerance to an allogeneic donor, animals from each of the treatment groups were grafted with B10.D2 (donor-type) and third-party B10.BR full-thickness tail skin grafts 6 wk after bone marrow transplantation. Those mice that had received bone marrow depleted of Lyt-$2^+$ cells (Lyt-$2^+$-depleted,

TABLE I

*Effect of Depletion of Lyt-2$^+$, L3T4$^+$, and Thy-1$^+$ Cell Subsets from Syngeneic Component of Bone Marrow Inocula on Engraftment of Allogeneic Donor*

| Group | Syngeneic (B10) bone marrow T cell depletions for reconstitution | Number of mice | Typing of chimeras by FMF | |
|---|---|---|---|---|
| | | | Mean percent B10 cells* (range) | Mean percent B10.D2 cells* (range) |
| 1 | Lyt-2$^+$ cells | 8 | 88.8 (74.8–99.2) | 14.4 (1.09–33.1) |
| 2 | L3T4$^+$ cells | 7 | 97.16 (95.7–98.4) | 1.63 (0.24–3.43) |
| 3 | Thy-1$^+$ cells | 5 | 74.14 (52.4–93.3) | 31.14 (13.87–52.5) |
| 4 | RAMB$^+$ cells | 4 | 78.25 (50.68–98.53) | 23.76 (0.9–51.93) |
| 5 | "Addback" of L3T4$^+$ plus Lyt-2$^+$ cells | 5 | 96.74 (95.1–98.3) | 1.24 (0.4–2.7) |
| | Normal B10 PBL | — | 98.4 | 1.3 |
| | Normal B10.D2 PBL | — | 3.71 | 95.5 |

* Percent of cells with fluorescence intensity ≥250 on log scale after staining with anti-H-2$^b$ or anti-H-2$^d$ Protein A–purified biotinylated mAb minus percent staining with FITC-Avidin alone on that respective animal. Correction was not made for low level of crossreactivity on normal B10 or normal B10.D2 peripheral blood lymphoid cells.

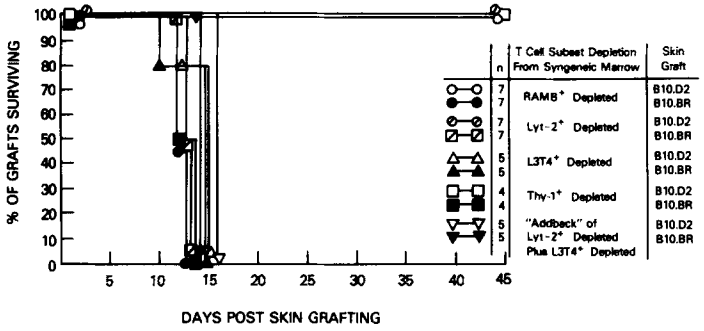


FIGURE 1. Survival of B10.D2 and third-party B10.BR full-thickness tail skin allografts placed on the lateral thoracic wall of mixed allogeneically reconstituted mice (B10 + B10.D2 ⇒ B10). B10 recipients were reconstituted with mixed marrow inocula of 15 × 10$^6$ RAMB B10.D2 bone marrow cells plus syngeneic bone marrow depleted of various T cell subsets, as indicated in the figure. Skin grafts were followed for a minimum of 45 d.

RAMB$^+$-depleted, or Thy-1$^+$-depleted groups) exhibited specific acceptance of B10.D2 allogeneic donor skin grafts (Fig. 1). In contrast, those animals that received either mixed marrow from which only L3T4-reactive cells had been depleted from the syngeneic component of the inoculum, or an "addback" mixture of L3T4-depleted plus Lyt-2-depleted syngeneic bone marrow, rejected B10.D2 grafts with a time course similar to that for rejection of third-party B10.BR skin grafts.

*Effect of Selective Syngeneic T Cell Subset Depletions on MLR and CML Reactivity.* To assess in vitro, specific transplantation tolerance to allogeneic donor and

TABLE II

*Mixed Lymphocyte Reactivity (MLR) in Mixed Allogeneic Reconstitution: The Effect of Selective Depletion of Syngeneic T cell Subsets on Reactivity to Allogeneic Donor*

| Responder | | Counts per minute ± SEM* ([$^3$H]thymidine incorporation) | | |
|---|---|---|---|---|
| Group† | Syngeneic bone marrow T cell depletions for mixed reconstitution (B10 + B10.D2 → B10) | Anti-B10 | Anti-B10.D2 | Anti-B10.BR |
| 1 | Lyt-2$^+$ cells | 8,631 ± 1,033 | 7,294 ± 636 | 55,814 ± 3,009 |
| 2 | L3T4$^+$ cells | 8,235 ± 568 | 36,911 ± 3,400 | 63,725 ± 4,208 |
| 3 | Thy-1$^+$ cells | 8,736 ± 298 | 6,904 ± 1,487 | 24,920 ± 724 |
| 4 | RAMB$^+$ cells | 5,830 ± 966 | 8,644 ± 385 | 12,024 ± 415 |
| 5 | "Addback" of L3T4$^+$ and Lyt-2$^+$ cells | 7,903 ± 1,154 | 21,550 ± 2,925 | 27,885 ± 2,763 |
| | Normal B10 | 7,946 ± 341 | 35,658 ± 373 | 40,786 ± 887 |
| | Normal B10.D2 | 29,373 ± 1,229 | 7,201 ± 267 | 36,665 ± 965 |

* Mean ± SEM of triplicate cultures at 1:1 responder/stimulator ratio.

† The animals represented above have typing data presented in Table I and are as follows: Group 1 (No. 6: 93.3% B10, 6.5% B10.D2); Group 2 (No. 4: 97.1% B10, 1.7% B10.D2); Group 4 (No. 2: 50.7% B10, 51.9% B10.D2); Group 5 (No. 2: 95.9% B10, 2.7% B10.D2).

immunocompetence to respond to third-party lymphoid elements, splenic lymphoid cells from animals reconstituted according to each of the five treatment protocol groups were tested in MLR assays. Table II illustrates the responses of individual animals in one representative experiment. Recipients reconstituted with mixed bone marrow inocula from which the Lyt-2$^+$ syngeneic T cells had been depleted before transplantation (Table II, groups *1*, *3*, and *4*) showed specific absence of reactivity to syngeneic (B10) and allogeneic (B10.D2) donor strains, yet were competent to respond to third party (B10.BR). In contrast, those animals that received mixed bone marrow inocula from which the Lyt-2-reactive cells had not been depleted were fully reactive to B10.D2 allogeneic donor, with a response similar in magnitude to that for third party (B10.BR) (Table II, groups *2* and *5*). These responses were observed consistently within groups irrespective of the degree of chimerism detected in individual animals. Similar results were obtained in an in vitro, cell-mediated lympholysis sensitization. Only lymphoid cells from mice that had received Lyt-2$^+$-depleted mixed bone marrow inocula were unreactive to the B10.D2 allogeneic donor, yet reactive to third party.

## Discussion

Alloresistance to engraftment of bone marrow has posed a longstanding challenge to the field of clinical transplantation. Murine, rat, and porcine models for this phenomenon have implicated both NK cells and cytotoxic T cells (6–8). However, the definitive cell phenotype has not been shown convincingly. To the extent that our model of mixed reconstitution simulates the general problem of resistance to bone marrow engraftment, our results appear to provide a means of determining this phenotype. Our data indicate that an Lyt-2$^+$, syngeneic T

cell in bone marrow is responsible for the failure of long-term engraftment of allogeneic donor lymphoid elements observed in mixed allogeneic reconstitution when the syngeneic component of the mixed bone marrow inoculum is not depleted of RAMB-reactive cells. These Lyt-$2^+$ T cells are perhaps cytotoxic T cells or their precursors. In general, Lyt-$2^+$ cells react with class I antigens, whereas L3T4$^+$ cells react with class II antigens. Therefore, the relevant target cell populations are likely to express class I but not class II antigens.

The level of engraftment at 6 wk posttransplantation varied from animal to animal. Some mice treated with either RAMB, anti-Thy-1, or anti-Lyt-2.2 repopulated with as little as 1% allogeneic cells. However, even this small percentage of cells was sufficient to induce tolerance in these mice, as determined by skin graft acceptance. Since we have not examined the short-term engraftment of these animals, it remains possible that mice reconstituted with marrow not depleted of Lyt-2 may engraft for a short time. However, the allogeneic bone marrow population must then be eliminated by the mature Lyt-$2^+$ syngeneic cells, since by 8 wk no evidence for allogeneic engraftment could be seen.

In our previous studies using RAMB for T cell depletions, we could not determine whether NK cells or T cells were responsible for the failure of engraftment of allogeneic donor bone marrow, because NK cells in culture have been reported to be crossreactive with RAMB (9–13). Since native NK cells do not express Lyt-2 (13, 14), the present data strongly suggest that the alloresistance to engraftment observed in mixed allogeneic reconstitution is mediated by an Lyt-$2^+$ T cell and not by NK cells.

This model of mixed allogeneic reconstitution should allow further discrimination of the characteristics of the syngeneic T cell component responsible for alloresistance. To the extent that a similar cell type mediates alloresistance in general, identification of such a cell phenotype may allow better targeting of treatment with specific mAbs rather than the aggressive, less specific approaches that are required for conditioning of bone marrow transplant recipients at this time.

## Summary

In the mixed allogeneic reconstitution (B10 + B10.D2 → B10) model, alloresistance to engraftment of allogeneic donor results if the syngeneic component of the mixed bone marrow inoculum is not depleted of Lyt-$2^+$ cells before transplantation. Resultant experimental animals repopulate as fully syngeneic, reject B10.D2 skin allografts, and are reactive to B10.D2 lymphoid cells in vitro, as assessed by mixed lymphocyte culture proliferative and cellular cytotoxicity assays. In contrast, depletion of Lyt-2-reactive cells from the syngeneic component of the mixed bone marrow inoculum results in mixed lymphopoietic chimerism and specific in vivo transplantation tolerance to B10.D2 allogeneic donor skin grafts and in vitro unreactivity to B10.D2 lymphoid elements. Full reactivity to third party is evident both in vitro and in vivo in these animals. This model may be helpful in further study of the syngeneic host-type cell phenotypes responsible for alloresistance to bone marrow engraftment.

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