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Cutting Edge: Administration of Anti-CD40 Ligand and Donor Bone Marrow Leads to Hemopoietic Chimerism and Donor-Specific Tolerance Without Cytoreductive Conditioning

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Transplantation tolerance, defined as allograft acceptance by an immunocompetent recipient in the absence of long-term immunosuppression, has remained an elusive goal in clinical transplantation. Robust experimental tolerance induction strategies have in common methods to induce mixed hemopoietic chimerism. To date, however, chimerism induction across allogeneic barriers has required recipient conditioning with irradiation or cytoreductive agents. In this paper we show that B6 recipients of fully allogeneic BALB/c skin grafts treated with repeated doses of donor bone marrow and anti-CD40 ligand (CD40L) develop durable (>300 days), readily detectable (6–12%) multilineage hemopoietic chimerism, indefinite allograft acceptance (>300 days), and donor-specific tolerance to secondary skin grafts. Analysis of the TCR repertoire of treated mice indicates that the underlying mechanisms of tolerance are in part mediated by deletion of donor-reactive T cells. These data demonstrate that durable hemopoietic chimerism and robust transplantation tolerance can be achieved without cytotoxic conditioning using a potentially clinically applicable regimen. The Journal of Immunology, 2000, 165: 1–4.

The induction of stable hemopoietic chimerism and deletional transplantation tolerance in MHC disparate recipients has, to date, required some form of recipient conditioning with either gamma irradiation or cytoreductive agents (1, 2). This strategy presumably allows for the creation of space within the marrow microenvironment and thus promotes pluripotent stem cell engraftment. In support of this concept, it has been observed that recipient conditioning greatly facilitates syngeneic stem cell engraftment (1, 3). However, there are data which suggest that a small number of niches within the marrow may be available for stem cell engraftment at any given time (4). For example, stable chimerism can be achieved without preconditioning when very high (2 × 10^6 cells), repeated doses of bone marrow are administered across an H-Y barrier (2, 5). In this paper we report that repeated administrations of allogeneic donor bone marrow under the cover of T cell costimulation blockade (anti-CD40 ligand [CD40L]) promotes the development of hemopoietic stem cell engraftment, durable hemopoietic chimerism, and robust donor-specific transplantation tolerance across a fully allogeneic barrier.

Materials and Methods

Mice

Adult male 6- to 8-wk-old C57BL/6 (H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions.

Bone marrow preparation and treatment regimens

Bone marrow was flushed from the tibiae, femurs, and humeri of BALB/c mice using sterile saline, needles, and syringes. Single cell suspensions of harvested bone marrow were made and lysed of red cells using a Trizma base ammonium chloride solution (Sigma, St. Louis, MO). The bone marrow cells were resuspended at 2 × 10^7 cells/500 μl sterile saline and injected i.v. on days 0, 2, 4, 6, 14, 28, 60, and 90. Hamster anti-mouse CD40L (MR1; Bioexpress, Lebanon, NH) was administered on days 0, 2, 4, 6, 14, 28, 60, and 90 (500 μg/dose i.p.).

Skin grafting

Full thickness skin grafts (~1 cm^2) were transplanted on the dorsal thorax of recipient mice and secured with a Band-Aid (Johnson & Johnson, Arlington, TX) for 7 days. Rejection was defined as the complete loss of viable epidermal graft tissue.

Abbreviation used in this paper: CD40L, CD40 ligand.

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Flow cytometric analysis

Peripheral blood was analyzed by staining with fluorochrome-conjugated Abs (anti-H-2Kd FITC, anti-IA d , FITC, anti-GR1 PE, anti-B220 PE, anti-CD11b APC, anti-Vb11 FITC, anti-Vb8.1/8.2 FITC, and anti-Vb5.1/5.2 FITC (PharMingen, San Diego, CA), or Ig isotype controls (Ms IgG2a, Ms IgG1, Rt IgG2b; PharMingen), followed by red blood cell lysis and washing with a whole blood lysis kit (R&D Systems, Minneapolis, MN). Stained cells were analyzed using CellQuest software on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Allogeneic mixed leukocyte reactions

T cell-enriched nylon wool nonadherent cells and dendritic cell-enriched transiently adherent splenocytes were used as responders and stimulators, respectively (6). A total of 10^6 irradiated (2000 rad, 137Cs) stimulator cells were added to 10^5 responder cells in a final volume of 0.20 ml in 96-well round-bottom plates. Proliferation in the wells was measured by adding 1 mCi of [3H]thymidine (Amersham, Arlington Heights, IL)/well between days 1 and 5. The cells were harvested 12–16 h later and counted on a beta-plate counter (LKB Instruments, Gaithersburg, MD). Results are the means of triplicate cultures.

Results and Discussion

B6 recipients of BALB/c skin grafts were treated with a regimen consisting of 2 × 10^7 donor bone marrow cells iv and 500 μg of anti-CD40L i.p. at the time of skin grafting (day 0) and on postoperative days 2, 4, 6, 14, 28, 60, and 90. Control groups included...
animals that received no treatment, bone marrow alone, or anti-CD40L alone. Recipient mice were monitored for evidence of peripheral hemopoietic chimerism by flow cytometry before each bone marrow dose beginning on day 14 and also on days 180, 205, 250, and 300. As expected, recipients in control groups showed no substantial hemopoietic chimerism at any time point. In contrast, all animals receiving both anti-CD40L and donor bone marrow developed readily detectable (6–12%) multilineage hemopoietic chimerism that persisted for >300 days (Fig. 1, A and B). Likewise, analysis of the recipient thymus at the end of the experiment.

FIGURE 3. Administration of anti-CD40L and repeated doses of donor bone marrow leads to donor-specific unresponsiveness and clonal deletion of donor-reactive T cells. Analysis of the T cells from the tolerant recipients was performed at ~300 days after transplantation. A, T cells from B6 mice treated with BALB/c bone marrow and anti-CD40L generated vigorous responses to C3H stimulators (■); in contrast, the response to BALB/c stimulators (●) was diminished to the level of the syngeneic response (■). B, In contrast to treated animals, T cells from control B6 mice generated vigorous responses to both BALB/c and C3H stimulators as compared with syngeneic stimulators. C and D, Peripheral blood CD4+ T cells in control and experimental mice was examined by flow cytometry at 25 wk. C, The utilization of Vβ5, Vβ11, and Vβ8 by CD4+ T cells in peripheral blood of mice that received no treatment, anti-CD40L alone, bone marrow alone, or anti-CD40L and bone marrow is shown (n = 5/group, error bars represent the SD). D, The CD4+ T cells of a representative animal within control groups (bone marrow or anti-CD40L alone) had Vβ11+ and Vβ5.1/2+ levels consistent with wild-type B6 levels (4–5% and 2–3%, respectively). Recipients of bone marrow and anti-CD40L therapy had levels of Vβ11+ cells and Vβ5.1/2+ similar to wild-type BALB/c levels. Vβ deletion is shown to be specific as Vβ8.1/2+ CD4+ T cells remain similar in all groups.
(>300 days) demonstrated similar levels of donor cells (data not shown). These results indicate that even across a fully allogeneic barrier, a regimen consisting of repeated doses of donor bone marrow and anti-CD40L can produce disseminated long-term hemopoietic chimerism in nonmyeloablated hosts.

Skin allograft survival among the groups was also strikingly different. Animals receiving either no treatment, bone marrow alone, or anti-CD40L alone all promptly rejected BALB/c skin grafts. In contrast, all animals receiving both anti-CD40L and donor bone marrow accepted their allografts for>300 days without evidence of rejection (Fig. 2A). To determine whether the combination of bone marrow and anti-CD40L is able to confer durable donor-specific tolerance, we rechallenged the animals ~180 days after the original transplant with donor (BALB/c) or third-party (C3H/HeJ) skin grafts. Animals in control groups promptly rejected both BALB/c and C3H/HeJ skin grafts (data not shown). In contrast, mice that received donor marrow and anti-CD40L uniformly accepted the donor-specific BALB/c skin grafts (median survival time [MST] >115 days) and promptly rejected C3H/HeJ grafts (MST 12 days) (Fig. 2B). Importantly, the original BALB/c skin grafts survived without evidence of rejection after donor or third-party rechallenge (all BALB/c skin grafts survived until the recipients were sacrificed for analysis, >300 days after the experiment’s initiation).

Next, to gain insight into the mechanisms by which bone marrow and anti-CD40L promote allograft acceptance and tolerance, we tested whether administration of donor bone marrow and anti-CD40L would lead to donor-specific unresponsiveness in mixed leukocyte reactions. At >300 days after transplantation, T cells were prepared from the spleens of mice from each experimental group. Although the T cells from mice treated with anti-CD40L and bone marrow generated vigorous responses to C3H stimulators, the response to BALB/c stimulators was diminished to the level of the syngeneic response (Fig. 3A). To determine whether or not the donor-specific unresponsive state was associated with selective deletion of donor-reactive T cells, we compared the utilization of V{beta}11, V{beta}5.1/2, and V{beta}8.1/2 by CD4{sup+} T cells from B6 recipients in the experimental group (accepted both hemopoietic and skin grafts) and from the control groups (rejected grafts). BALB/c mice delete V{beta}11- and V{beta}5-bearing T cells in the thymus due to their high affinity for endogenous retroviral superantigens (mouse mammary tumor virus [MMTV]) presented by I-E MHC class II molecules. B6 mice do not express I-E and utilize V{beta}11 on ~4–5% of CD4{sup+} T cells and V{beta}5.1/2 on ~2–3% of CD4{sup+} T cells (7–9). As anticipated, B6 mice that received either anti-CD40L or bone marrow alone failed to delete donor-reactive V{beta}11{sup+} or V{beta}5{sup+} CD4{sup+} T cells (Fig. 3, C and D). In contrast, recipients of BALB/c marrow and anti-CD40L developed near complete deletion of CD4{sup+} V{beta}11{sup+} and CD4{sup+} V{beta}5{sup+} T cells. For comparison, we also analyzed the percentage of V{beta}8-bearing CD4{sup+} T cells, which are expressed on ~15–20% of BALB/c and B6 CD4{sup+} T cells. B6 mice in the experimental group preserved their V{beta}8 CD4{sup+} T cells similar to control groups, indicating that the T cell deletion was donor specific in nature (Fig. 3, C and D). These results suggest that I-E-bearing bone marrow-derived cells populate the open recipient niches and are adequately protected from rejection with anti-CD40L in sufficient numbers to shape the selection of the T cell repertoire and confer robust donor-specific tolerance.

In previous experiments, transient donor-specific unresponsiveness has been achieved without preconditioning using donor splenocytes and anti-CD154, but despite a significant delay of skin allograft rejection, these grafts were all ultimately rejected (10). The prolonged graft survival in this model was not associated with hemopoietic chimerism. In addition, recipients of donor-specific transfusion and anti-CD154 rejected secondary donor grafts and their T cells were capable of rejecting donor skin when transferred into scid mice, indicating that graft acceptance was not mediated via a deletional mechanism (10). Our results are similar to those reported by Werkle et al. (9), who attained hemopoietic chimerism, T cell tolerance, and deletion using whole body irradiation (WBI) and costimulatory blockade. However, we report a strategy in the stringent BALB/c to B6 skin graft model that obviates the need for WBI or cytoablation using multiple doses of bone marrow transplantation and anti-CD40L. This approach provides many of the essential features for a successful clinical tolerance induction protocol including: 1) a means to control existing donor-reactive cells in the peripheral T cell compartment, 2) a means to shape the repertoire of developing T cells to effect inactivation and/or deletion of newly emerging donor-reactive cells, 3) allograft protection during tolerance induction, and 4) a clinically acceptable toxicity profile. Although the optimal dosing regimens for administration of both the bone marrow and costimulation blockade have yet to be defined, this strategy warrants prompt evaluation in preclinical primates.

References