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Overcoming T cell–mediated rejection of bone marrow allografts by T-regulatory cells: Synergism with veto cells and rapamycin

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Recently, we have shown that anti-third-party cytotoxic T lymphocytes (CTLs) depleted of alloreactivity against the host are endowed with marked veto activity and can facilitate bone marrow (BM) allografting without graft-versus-host disease. We also demonstrated synergism between rapamycin (RAPA) and the veto cells. $CD4^+CD25^+$ T-regulatory (Treg) cells are suppressor cells that can enhance alloengraftment. We investigated whether donor Tregs would be synergistic with veto CTLs and RAPA in augmenting alloengraftment or, conversely, would suppress veto CTL effects. Lethally irradiated C3H mice were transplanted at day 2 after irradiation with Balb-nude BM. Graft rejection was induced by purified host-type T cells infused 1 day prior to BMT. The addition of Tregs led to moderate enhancement of engraftment. RAPA at different doses was synergistic with Tregs. The addition of veto CTLs to Tregs enabled reducing the effective RAPA dose fourfold. Combining all three agents was necessary to overcome rejection at low-dose RAPA. Chimerism analysis at 5 to 9 months revealed a significant presence of host-type cells coexisting with the predominant donor T cells, suggesting that tolerance had been attained. The synergistic effects between Tregs, veto CTLs, and RAPA offer an attractive approach for facilitating alloengraftment. © **2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

Bone marrow transplantation (BMT) following supralethal radiochemotherapy is associated with dangerous infections due to the slow immune reconstitution during the first year after transplantation [1–6]. Thus, the use of reduced-intensity conditioning, associated with less severe immune ablation, could have remarkable potential in the treatment of a variety of nonmalignant diseases or for the induction of "mixed chimerism" as a prelude for cell therapy in cancer or in organ transplantation. However, the marked level of host hematopoietic and immune cells surviving mild preparatory regimens represents a difficult barrier for the engraftment of donor cells.

In patients with advanced hematologic malignancies who cannot withstand myeloablative conditioning because of age and/or performance status, recent attempts were made to develop low-toxicity conditioning protocols in conjunction with HLA-matched transplants [7–10]. Potent posttransplantation immunosuppression and the presence of large numbers of alloreactive T cells in the graft enabled a high rate of engraftment. However, graft-versus-host disease (GVHD), particularly lethal chronic GVHD, remains a major obstacle [9,11–13]. Although in high-risk leukemia such transplant-related mortality is acceptable, it would be totally intolerable if applied to patients with long life expectancy. Thus, the use of purified allogeneic stem cells, which do not pose any risk for GVHD and can continuously present donor-type antigens in the host thymus, thereby inducing durable tolerance to donor cells or tissues, represents one of the most desirable goals in transplantation biology.

One approach to overcoming immune rejection of incompatible stem cells rigorously depleted of T cells made use initially of increased doses of T cell–depleted bone marrow (BM) in mice [14–17] and rats [18]. Subsequently the cell-dose escalation concept was also shown with purified stem cells [19–22]. However, although this modality has become a clinical reality in the treatment of patients with leukemia conditioned by intensive chemotherapy, it has been

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suggested in studies in mice [21] and nonhuman primates (X. Yao, unpublished data, July 2001) that the number of hematopoietic precursors required to overcome the immune barrier in hosts pretreated with sublethal regimens cannot be attained with the state-of-the-art technology for stem cell mobilization. Rachamim et al. [23] demonstrated that when purified $CD34^+$ cells were added to bulk mixed-lymphocyte reaction, these cells suppressed cytotoxic T lymphocytes (CTLs) against matched stimulators but not against stimulators from a third party [23]. These results, which were further confirmed and extended by Gur et al. [24,25], strongly indicated that cells within the human $CD34^+$ population are endowed with potent veto activity.

Considering that the number of human CD34 cells that can be harvested is limited, the availability of other types of veto cells or immuneregulatory cells is crucial for further application of allogeneic stem cell transplantation under reduced intensity conditioning. We have demonstrated recently that anti-third-party CTLs exhibit potent veto activity in vitro and in vivo and are markedly depleted of graft-versus-host reactivity. Furthermore, these veto cells were shown to exhibit a synergistic effect with the immunosuppressive drug rapamycin (RAPA) when tested in a mouse model specifically designed to measure T cell–mediated BM allograft rejection without interference from stem cell competition, which might occur in mice exposed to reduced-intensity conditioning. In this study recipient mice were exposed to a lethal dose of total body irridation (TBI) and infused with host-type T cells prior to transplantation of mismatched T cell–depleted BM. Graft rejection mediated by the adoptively transferred host-type T cells could be effectively overcome only by the coadministration of RAPA and veto CTLs; each agent alone exhibited marginal enhancement of engraftment.

Recently, several studies demonstrated that donor- or host-type $CD4^+CD25^+$ T-regulatory (Treg) cells might control in vivo GVHD or graft rejection [26–35]. Because Treg cells are suppressive, we hypothesized that the addition of Treg cells to veto CTLs and RAPA might provide added benefits in engraftment promotion. Alternatively, it is possible that Treg cells may have impaired the engraftment facilitation by veto CTLs due to their global immune suppressive properties. Finally, it is possible that RAPA may interfere with Treg promotion of alloengraftment by precluding interleukin-2 (IL-2) responsiveness in Treg cells, thereby reducing Treg cell potency or survival. Thus, it was of interest to evaluate quantitatively, in the same graft rejection mouse model, the potential of Tregs to further affect engraftment of BM allografts in conjunction with RAPA and veto CTLs.

Materials and methods

Animals

Six- to 12-week-old female mice were used throughout the experiments. Balb/c, Balb/c-Nude, and FVB mice were obtained from the Weizmann Institute Animal Breeding Center (Rehovot, Israel). C3H/HeJ mice were obtained from the Roscoe B. Jackson Memorial Laboratory (Bar Harbor, ME, USA). All mice were kept in small cages (five animals in each cage) and fed sterile food and acid water containing 20 μg/mL cyprofloxacin (Bayer AG, Leverkusen, Germany).

T cell–mediated allograft rejection model

C3H/HeJ female mice were exposed to a single dose of 10 Gy (supralethal conditioning) TBI on day 0. The following day, the mice received intravenously 1.5×10^4 purified host T cells (HTCs). Transplantation of 2×10^6 allogeneic Balb/c-Nude BM cells was performed on day 2 in conjunction with the tolerizing cells to be evaluated. The survival of the mice was monitored daily.

Host T-cell preparation

Splenocytes of host C3H/HeJ mice were fractionated on Ficoll/Paque, and the isolated mononuclear cells were subjected to a positive selection of T cells (CD4 plus CD8) by magnetic cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Cytofluorimetric analysis of the fractionated cells was carried out by triple immunofluorescent staining using the following directly labeled antibodies (Pharmingen, San Diego, CA, USA): fluorescein isothiocyanate–CD4/L3T4 (clone H129.19), phycoerythrin (PE)-CD3e (clone 145-2C11), and Cy-Chrome–CD8a/Ly-2 (clone 53-6.7).

Immune suppression with RAPA

Synergism between RAPA (Wyeth Europa Ltd., Taplow, Maidenhead, Berks, UK) and different tolerizing cells was tested in the mouse model for BM allograft rejection. Rapamycin was given subcutaneously at different dosage of 1.25 to 5 μg/mouse/d for 5 consecutive days beginning on day 0.

Preparation of donor non-host-reactive CTLs

Anti-third-party veto CTLs were prepared as previously described by Bachar-Lustig et al. [36]. Briefly, spleen cells (Balb/c) 6 to 12 weeks of age were harvested, lysed in cold kalium buffered ammonium chloride to remove red blood cells, and suspended in RPMI-based medium. The single-cell suspension was then cocultured at a ratio of 1:2 with irradiated (2000 cGY), kalium buffered ammonium chloride-treated spleen cells from FVB mice (third-party origin) and cultured at 37°C in a humidified 5% CO_2/95% O_2 atmosphere. Six days later the cocultures were fractionated on Ficoll and the lymphoid fraction enriched for $CD8^+$ cells by positive selection using magnetically labeled anti-CD8 antibodies and a MACS (Miltenyi Biotec) system. The CD8-enriched fraction was then resuspended and cultured at a ratio of 1:4 with the same irradiated third-party stimulators, in the presence of 40 IU/mL recombinant human IL-2 (Eurocetus, Milan, Italy) added every 2 days for additional 2 weeks.

Purification of $CD4^+CD25^+$ T cells

Mouse auxiliary, mesenteric, and inguinal lymph nodes were harvested and single-cell suspensions passed through a wire mesh were prepared in phosphate-buffered saline containing 50% 199 medium and 2% fetal calf serum. Cells were first selected for $CD4^+$ cells, using negative selection by passage through a goat anti-mouse and goat anti-rat immunoglobulin immunocolumn (Cellect Cell Enrichment Immunocolumns, Cederlane, Hornby, Ontario, Canada). To enrich for $CD4^+CD25^+$ cells, purified