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Graft-Versus-Host Disease Prevention by Rapamycin: Cellular Mechanisms

Benny J. Chen, Randall E. Morris, Nelson J. Chao

Division of Bone Marrow Transplantation and Laboratory for Transplantation Immunology, Stanford University Medical Center, Stanford, California.

Correspondence and reprint requests: Nelson J Chao, MD, Bone Marrow Transplantation Program, Duke University Medical Center, 2400 Pratt St., Suite 1100, Durham, NC 27705

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ABSTRACT

Understanding the cellular mechanisms that lead to graft-versus-host disease (GVHD) may lead to alternative approaches in the prevention or therapy of this disease process. In this manuscript, we investigated the mechanisms of action of the immunosuppressive drug rapamycin for the prevention of GVHD. GVHD-free long-term survival was achieved in BALB/c ($H2^d$, Mls-2^a, Mls-3^a) recipients of B10.D2/nSnJ (H-2^d, Mls-2^a, Mls-3^a) bone marrow and spleen cells after a 30-day course of high-dose rapamycin (5 mg/kg per day). Low responses to recipient and third-party cells in a mixed lymphocyte reaction (MLR) were observed as well as decreased mature T-cell numbers in the spleen. This low response was not due to defective interleukin (IL)-2 production, because exogenous IL-2 did not improve the responses in the MLR. However, GVHD-free long-term survival was associated with a large number of infiltrating mononuclear cells in the target organs of GVHD. This observation suggested the possibility that these cells were responsible for suppressing the immune response. Regulatory cells, which could suppress both antirecipient and third-party responses in vitro, were demonstrated to be present in the spleens of these GVHD-free long-term survivors. These results suggest that in addition to impaired cellular immune function, the presence of nonspecific regulatory cells (ie, suppression) may contribute to maintenance of GVHD-free long-term survival induced by short-course rapamycin.

KEY WORDS

Graft-versus-host disease • Rapamycin • Tolerance • Suppressor cells

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) offers the hope of cure for certain lethal malignant and nonmalignant diseases, characterized by defective hematopoietic and immune function, through the generation of a new hematopoietic system [1]. The indications of allogeneic BMT are being investigated in other clinical situations such as autoimmune disease [2] and the induction of tolerance for organ transplantation [3]. The broad potential of allogeneic BMT has not been achieved mainly because of graft-versus-host disease (GVHD), a life-threatening complication associated with the procedure [4]. Although ideally the incidence of clinically significant acute GVHD could be controlled [5,6], in general, prophylaxis and therapy of GVHD remain unsatisfactory. The mortality rate can be as high as 50% of patients who develop GVHD [4]. Therefore, alternative approaches for prevention and treatment of GVHD are essential. Understanding the mechanisms by which GVHD prevention occurs may provide clues for new approaches to prevent or treat this disease.

Rapamycin is a natural macrolide produced by a filamentous bacterium *Streptomyces hygroscopicus* [7] and has been shown to be highly effective in prevention of GVHD, resulting in indefinite GVHD-free long-term survival after short-term treatment [8,9]. Although it is clear that rapamycin blocks signal transduction mediated by interleukin (IL)-2 and other cytokines [10-12] and the Ca^{2+}-independent CD28/CTLA-4–induced costimulatory pathway [13], these mechanisms of action do not explain why the GVHD-reactive T cells remain unresponsive after rapamycin withdrawal, because the half-life of rapamycin in the circulation is only 4 to 6 hours [14]. The mechanisms of rapamycin-induced long-term solid organ allograft survival have been carefully studied [15] and seem to be related to T-cell desensitization due to prolonged engagement of the T-cell receptor (TCR) by alloantigens. This mechanism is not necessarily the same in GVHD prevention.

In this study, the cellular mechanisms of GVHD-free long-term survival mediated by rapamycin were investigated in a murine model across a minor histocompatibility barrier.

A short-term treatment course with high-dose rapamycin (5 mg/kg per day) for 30 days following BMT protected all of the recipients from developing GVHD up to 160 days after transplantation. Of interest, mice surviving for the long term without GVHD demonstrated large numbers of infiltrating mononuclear cells in the usual target organs of GVHD. The presence of these mononuclear cells in the absence of histological evidence of acute GVHD suggests the possibility of regulatory cells. These regulatory cells, able to suppress both antirecipient and third-party responses in vitro, were demonstrated to be present in the spleens of these GVHD-free long-term survivors. These results suggest that in addition to impaired cellular immune reconstitution, the presence of nonspecific regulatory cells (ie, suppression) may contribute to maintenance of GVHD-free long-term survival induced by short-course rapamycin.

MATERIALS AND METHODS

Animals

B10.D2/nSnJ ($H2^d$, Mls-2^b, Mls-3^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). BALB/c ($H2^d$, Mls-2^a, Mls-3^a) and C57BL/6 ($H2^b$) mice were obtained from the specific pathogen–free colonies of Dr. Robert Kallman, Department of Radiology, Stanford University School of Medicine, Stanford, CA. Only female mice were used in this study.

GVHD Model

Via the tail vein, 10×10^6 bone marrow and 100×10^6 spleen cells in 0.5 mL plain RPMI 1640 medium from B10.D2 mice were injected into lethally irradiated (8.0 Gy) BALB/c recipients. It was previously shown that this protocol induced the most severe form of GVHD [16]. Donor mice were 8 to 14 weeks old and recipient mice were 12 to 13 weeks old at the time of transplantation. All experiments were performed according to federal and Stanford University guidelines. Recipients were monitored daily for clinical signs of GVHD by body weight and the extent of skin changes (hair loss and erythema) as scored on a cumulative severity scale from 0 (minimum) to 8 (maximum): head 1, neck 1, back (1/3, 2/3, 3/3) 1 to 3 and front (1/3, 2/3, 2/3) 1 to 3, as described [16-19]. Mortality was also recorded daily. Ear skin biopsies were routinely taken on days 30, 70, 100, and 150 after transplantation and when the mice were moribund. Histological changes were assessed as described [20].

Drugs and Treatment

Rapamycin was supplied as a pure compound in powder form from Wyeth-Ayerst Research (Princeton, NJ) and prepared fresh daily by suspending in 0.2% carboxymethylcellulose (CMC) and thoroughly homogenized before administration. Three doses of rapamycin were used to treat the animals: low dose, 0.5 mg/kg per day; medium dose, 1.5 mg/kg per day; and high dose, 5 mg/kg per day. cyclosporine A (Sandimmune injection: 50 mg/mL cyclosporine A, 33% ethanol, 65% cremophor) was obtained from Sandoz Pharmaceuticals (East Hanover, NJ) and diluted in phosphate-buffered saline (PBS). All drugs and CMC control were injected intraperitoneally once a day for 30 days beginning on the day of BMT. The volume of drug suspension given was 0.01 mL/g.

Histology and Immunohistochemistry

Specimens for regular histology were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin [21]. A total of 5 high-power (400×) fields per sample were studied to quantitate the numbers of infiltrating mononuclear cells. Portions for immunohistochemical analysis were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN). Frozen tissue blocks were kept at –70°C before sectioning. Sections were cut at 6 μm, picked up on glass slides, and fixed in absolute acetone at –20°C for 10 minutes. The sections were incubated at room temperature sequentially in primary antibody (anti-CD3ε: 145-2c11, anti-CD4: RM4-5, anti-CD8α: 53-6.7, anti-αβTCR: H57-597; PharMingen, San Diego, CA), in biotinylated secondary antibody (mouse anti-hamster immunoglobulin [Ig] G for CD3ε and αβTCR, goat anti-rat Ig for CD4 and CD8α; PharMingen) and in an optimal dilution of avidin-biotin-peroxidase complexes (Vector, Burlingame, CA). Sections were washed in PBS for 10 minutes after each step of the incubation. Positively stained cells were visualized with diaminobenzidine, 0.01% H_2O_2, and 0.3% sodium azide in 0.05 mol/L Tris buffer, pH 7.6. Slides were counterstained with Meyer hematoxylin and mounted. To quantitate the percentages of positive cells, 300 total cells were counted for each slide. Repeated analysis in a blinded fashion yielded results within 10% of initial scores.

Mixed Lymphocyte Reaction

A total of 5×10^5 responder spleen cells were plated in flat-bottom 96-well culture plates with 5×10^5 irradiated (10 Gy) spleen stimulator cells in a final volume of 200 μL. After 96 hours of incubation at 37°C and 5% CO_2, cultures were pulsed with 1 μCi [^{3}H]thymidine per well and harvested 16 hours later. Triplicate cultures were set up for every cell population tested. Culture medium was RPMI 1640 supplemented with 10% prescreened fetal calf serum, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. This medium was used for all the subsequent in vitro studies.

Cytotoxic T Lymphocyte Assay

The assay was designed to enhance the sensitivity to detect the cytotoxic T lymphocyte (CTL) against minor histocompatibility antigens as described [22]. Briefly, graded numbers of responder cells were plated with 5×10^5 irradiated (10 Gy) stimulator cells. Plates were cultured in a 37°C, 5% CO_2 incubator for 10 days. Recombinant human IL-2 (5 U/mL) (Cetus Immune, Emeryville, CA) was added on days 3 and 6 after initiation of the culture. The cells were tested in situ for lysis of ^{51}Cr-labeled day 2 concanavalin A blast cells. After 4 hours of culture, supernatant was taken out and counted in a γ counter. In each experiment, cultures were tested in parallel for lysis of irrelevant targets to ensure antirecipient specific killing. The percentage of specific release was calculated as follows.

$$\% \text{ specific release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

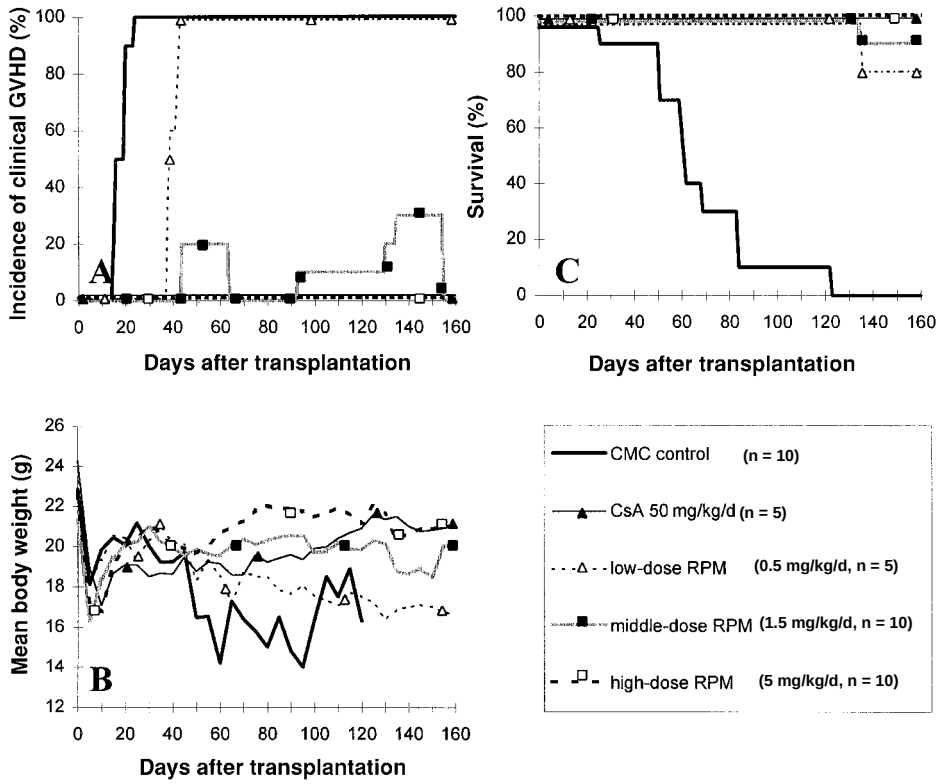


Figure 1. Short-term treatment with rapamycin (RPM) was highly effective in prophylaxis of murine graft-versus-host disease (GVHD), and GVHD-free long-term survival was achieved in high-dose RPM-treated mice. A total of 10×10^6 bone marrow cells and 100×10^6 spleen cells from B10.D2 mice were transplanted into lethally irradiated (8.0 Gy) BALB/c recipients. Recipient mice received treatment daily for 30 days starting on the day of transplantation. Data were pooled from 2 similar experiments. A, Incidence of clinical GVHD. Clinical GVHD was assessed daily by skin changes (hair loss and erythema) and loss of body weight. Of 10 mice treated with medium-dose RPM, 3 developed slight clinical GVHD and recovered from the GVHD process without any treatment. None of 10 mice treated with high-dose RPM developed clinical GVHD. B, Mean body weight curve. There was only 1 surviving mouse in the carboxymethylcellulose (CMC) control group after day 84. C, Actuarial survival from lethal GVHD. The median survival times in all 3 RPM-treated groups were more than 160 days and are superior ($P < .00001$) to the CMC control group and comparable with the cyclosporine A (CsA) (50 mg/kg per day) group ($P > .05$).

Spontaneous release was obtained by incubating target cells with stimulator cells only. Maximal release was obtained after treatment with 2% Nonidet P-40.

Coculture Assay

The assay was performed as described [23]. Briefly, spleen cells were added as cocultured cells to the 1-way mixed lymphocyte reaction (MLR) described in previous sections. Cocultured cells were irradiated (10 Gy) and added in a ratio of 1:1 to responder cells. Spleen cells from B10.D2 mice previously immunized to BALB/c (details in "Positive Control") and naive B10.D2 mice were used as responder cells in MLR against recipient and third-party cells, respectively. The percentage of suppression was calculated according to the following formula.

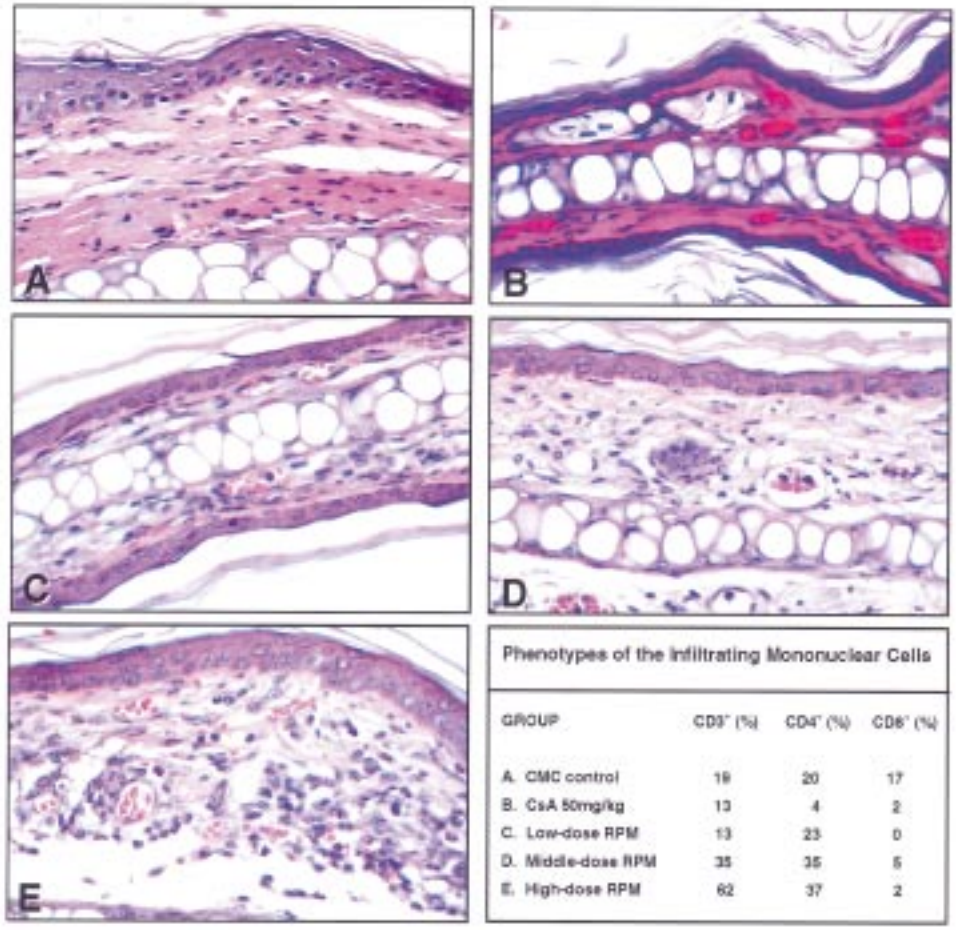
$$\% \text{ suppression} = \left(1 - \frac{\text{cpm with cocultured cells}}{\text{cpm without cocultured cells}}\right) \times 100$$

Flow Cytometric Analysis

A total of 1×10^6 spleen cells were stained with fluorescence–conjugated antibodies and incubated on ice for 30 minutes. Cells were washed and resuspended in 0.2 mL fluorescence-activated cell sorter (FACS) buffer (PBS with 0.1% bovine serum albumin and 0.01% sodium azide) for analysis. Phenotypic analysis was performed with a FACScan (Becton Dickinson, San Jose, CA), using monoclonal antibodies to CD3ε, CD4, CD8α, αβTCR, B220, and NK1.1 (PharMingen).

Positive Control

B10.D2 mice were injected intraperitoneally with 5×10^7 spleen cells from BALB/c mice in plain RPMI 1640 [16,24]. Spleen cells were harvested 21 days later as positive control (termed "immune B10.D2"). Because GVHD-free long-term survivors have been exposed to recipient antigens before, immune B10.D2 was thought to be a better control than naive B10.D2. All mice with GVHD had died and were not available to serve as a control group.



Phenotypes of the Infiltrating Mononuclear Cells

GROUP	CD3⁺ (%)	CD4⁺ (%)	CD8⁺ (%)
A. CMC control	19	20	17
B. CsA 50mg/kg	13	4	2
C. Low-dose RPM	13	23	0
D. Middle-dose RPM	35	35	5
E. High-dose RPM	62	37	2

Figure 2. More infiltrating cells were observed in the skin of rapamycin (RPM)-treated mice in a dose-dependent manner on day 30 after transplantation (hematoxylin and eosin staining, 400×). Representative skin changes from the animals of different groups are shown. A, Control mouse treated with carboxymethylcellulose (CMC). B, Control mouse treated with cyclosporine A (CsA) 50 mg/kg per day. C-E, Mice treated with 3 different doses of RPM: low dose, 0.5 mg/kg per day (C); medium dose, 1.5 mg/kg per day (D); and high dose, 5 mg/kg per day (E). Prominent infiltrating mononuclear cells and angiogenesis were noted in the skin of RPM-treated mice in a dose-dependent manner, without any evidence of histological graft-versus-host disease (vacuolated cells and dyskeratosis). Data in the table are the results from immunohistochemistry study of these infiltrating mononuclear cells. A total of 300 cells were counted for each slide. Results represent means from 3 animals per group.

Statistical Analysis

Group-wise comparison was made by Student *t* test. Survival data were analyzed by log-rank test.

RESULTS

Rapamycin Highly Effective in Prophylaxis of Murine GVHD

Daily administration of rapamycin for the first 30 days after allogeneic transplantation protected BALB/c recipients of B10.D2 bone marrow and spleen cells from lethal GVHD. Mice treated with low-dose rapamycin alone (0.5 mg/kg per day) had a delayed onset of clinical GVHD compared with CMC control mice. Of 10 mice treated with medium-dose rapamycin (1.5 mg/kg per day), 3 developed slight clinical GVHD. The other 7 mice in this group did not develop any signs of clinical GVHD with follow-up of 160 days after transplantation. Nevertheless, skin biopsy (ear) on day 150 after transplantation demonstrated that all of the mice in the medium-dose rapamycin group developed histological GVHD documented by the presence of vacuolated cells in the epidermis. None of the 10 mice treated with high-dose rapamycin (5 mg/kg per day) developed either clinical or histological evidence of GVHD during the observation period of 160 days after transplantation (Figure 1A). Treatment with all 3 doses of rapamycin decreased the overall disease severity as measured by the disease severity scores (data not shown) and by the weight curve of animals that underwent transplantation (Figure 1B), resulting in significantly higher survival rates compared with CMC control ($P < .0001$). The survival of the rapamycin groups was comparable with that of mice treated with cyclosporine A, 50 mg/kg per day (Figure 1C). However, GVHD-free long-term survival was achieved only in the high-dose group. Rapamycin syndrome, described by Blazar et al. [9], was observed in 1 of 10 recipients in the high-dose rapamycin group but none of the recipients in the lower-dose groups. Subsequent attempts to study the mechanisms of rapamycin-induced GVHD-free long-term survival

Table 1. *Decreased Antirecipient and Third-Party Responses of High-Dose Rapamycin-Treated GVHD-Free Long-Term Survivors in Mixed Lymphocyte Reaction**

Group	Δ cpm		
	Recipient	Third party	Background, cpm
Naive B10.D2	**0.4 ± 0.2**	**21.1 ± 8.6**	**2.2 ± 0.4**
Immune B10.D2	**7.5 ± 3.4**	**29.3 ± 4.6‖**	**2.1 ± 0.2**
High-dose rapamycin	**2.1 ± 0.4†‡**	**4.7 ± 2.0‖§**	**0.7 ± 0.1**

*A total 5×10^5 spleen cells were plated with 5×10^5 irradiated (10 Gy) stimulating spleen cells from normal mice in a 96-well flat-bottom plate. After 96 hours of incubation, cultures were pulsed with 1 μCi/well [^{3}H]thymidine for an additional 16 hours before harvesting. Data are proliferation (cpm $\times 10^3$) subtracted from syngeneic control, representing mean ± SD of cpm from 3 to 5 mice of each group. SDs were <15% of the mean from triplicate cultures. GVHD indicates graft-versus-host disease; Δ cpm, change in cell count per minute.

†$P > .05$ versus naive B10.D2.

‡$P < .05$ versus immune B10.D2.

§$P < .01$ versus immune B10.D2.

‖$P < .05$ versus naive B10.D2.

were carried out in the mice without rapamycin syndrome in the high-dose rapamycin-treated group (they were termed "GVHD-free long-term survivors"). All animals were full donor chimeras (data not shown).

Infiltrating Mononuclear Cells Observed in Skin of Rapamycin-Treated Mice

Histological analysis of skin (ear) from B10.D2 → BALB/c recipients on day 30 after transplantation demonstrated prominent infiltrating mononuclear cells (CMC control: 54.6 ± 7.8/field; rapamycin, 5 mg/kg: 206.9 ± 76.6/field; 5 animals per group; $P < .0001$) and angiogenesis in the skin of rapamycin-treated recipients in a dose-dependent manner (Figure 2A-E). The numbers of mononuclear cells increased in a dose-dependent manner: the higher the rapamycin dose, the more infiltrating cells were apparent (low dose: 72.1 ± 18.6; medium dose: 122.8 ± 29.4; high dose: 206.9 ± 76.6; 5 animals per group; $P < .05$ between groups). The infiltrating mononuclear cells decreased gradually after rapamycin withdrawal. Immunohistochemical studies on the infiltrating mononuclear cells of the high-dose rapamycin-treated mice demonstrated that 62% of them were $CD3^+$, 37% were $CD4^+$, and <2% were $CD8^+$, suggesting that about half of the $CD3^+$ infiltrating cells belonged to the cells with $CD3^+CD4^-CD8^-$ phenotype.

Decreased In Vitro Antirecipient and Third-Party Responses in GVHD-Free Long-Term Survivors

Treatment with high-dose rapamycin resulted in the long-term survival of B10.D2 → BALB/c recipients in the absence of GVHD, indicating that GVHD-reactive T cells were silent to the recipient antigens. The low antirecipient responsiveness was documented by the results from both MLR (Table 1) and CTL assay (Figure 3) compared with immune B10.D2. Spleen cells from these GVHD-free long-term survivors also had a low response against third-party cells (Table 1). Naive B10.D2 did not respond to recipient (BALB/c) in MLR as previously reported [25]. These results indicate that the low response in GVHD-free long-term survivors induced by high-dose rapamycin was not antigen-specific. Moreover, the addition of recombinant human IL-2 did not change the response, suggesting that this defect was not due to clonal anergy induced by a defect of IL-2 production (data not shown).

Nonspecific unresponsiveness in these GVHD-free long-term survivors induced by high-dose rapamycin could be due simply to impaired immune reconstitution. Flow cytometric analysis was performed to determine whether this was the case. As shown in Table 2, long-term survivors treated with high-dose rapamycin had fewer $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and $\alpha\beta TCR^+$ cells than did immune B10.D2 and naive controls, which could contribute to the low antirecipient and third-party responses. The percentages of T cells and T-cell subsets in the medium-dose rapamycin group were higher than in the high-dose rapamycin group but were lower than in both naive and immune B10.D2. There was no unique increase in the phenotypes tested ($CD3^+$, $CD3^+4^+$, $CD3^+CD8^+$, $\alpha\beta TCR^+$, $B220^+$, $NK1.1^+$) except for a slight increase in $\alpha\beta TCR^+ CD4^- CD8^-$ cell population in the spleens from high-dose rapamycin-treated, GVHD-free, long-term BMT survivors compared with all other groups.

Suppression of Antirecipient and Third-Party MLR in GVHD-Free Long-Term Survivors

The 5×10^5 irradiated (10 Gy) spleen cells from the high-dose rapamycin-treated long-term BMT survivors suppressed the MLR against recipient and third-party cells (immune B10.D2 versus BALB/c or naive B10.D2 versus C57BL/6 with 5×10^5 responder cells and 5×10^5 irradiated [10 Gy] stimulator) by 83.4% and 92.5%, respectively

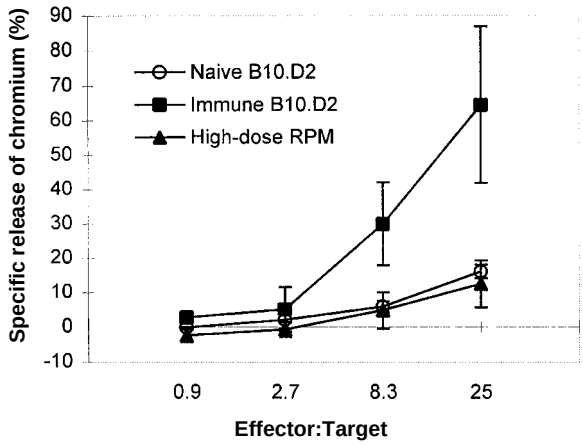


Figure 3. Decreased antirecipient cytotoxic T-lymphocyte activity. A total of 5×10^5 responder cells were plated with 5×10^5 irradiated stimulators from recipient mice. Interleukin-2, 5 U/mL, was added on day 3 and day 6 after initiation of the culture. On day 10, cultures were assayed for cytotoxicity on recipient strain day 2 concanavalin A blast cells in a 4-hour chromium release assay. Nonspecific release against irrelevant antigens was performed to ensure the specificity at each time. Spontaneous release was always <15% of maximum release. Results are means ± SD of specific releases from 3 to 5 mice in each group. RPM indicates rapamycin.

Table 2. *Flow Cytometric Analysis of Spleen Cells From Long-Term Bone Marrow Transplantation Survivors**

	Naive B10.D2	Immune B10.D2	CsA	Mid-Dose RPM	High-Dose RPM
$CD3^+$	30.1 ± 1.1	36.9 ± 1.9	13.9 ± 1.9	16.6 ± 1.2	11.5 ± 1.4†‡
$CD3^+CD4^+$	20.0 ± 0.7	22.2 ± 1.6	7.9 ± 1.0	8.8 ± 0.1	4.6 ± 0.4†
$CD3^+CD8^+$	10.1 ± 0.7	14.7 ± 0.7	6.0 ± 1.9	7.8 ± 1.0	5.1 ± 0.9†‡
$\alpha\beta TCR^+$	38.6 ± 2.4	43.0 ± 2.3	17.0 ± 2.2	18.0 ± 0.6	18.0 ± 1.6†‡¶
$B220^+$	59.9 ± 3.5	53.7 ± 1.8	77.8 ± 3.1	75.2 ± 0.3	60.5 ± 3.5†§
$NK1.1^+$	13.8 ± 1.5	18.1 ± 1.3	8.1 ± 3.3	15.4 ± 1.3	16.7 ± 1.4†§‖¶
$\alpha\beta TcR^+CD4^-CD8^-$	2.6 ± 0.5	5.5 ± 0.7	4.2 ± 1.3	4.8 ± 0.7	6.4 ± 0.4†‖¶

*Spleen cells from the long-term bone marrow transplantation survivors shown in Figure 1 were analyzed by flow cytometer. Results are expressed as mean percentages ± SD of spleen cells from 2 to 5 mice of each group. CsA indicates cyclosporine A; RPM, rapamycin.

†$P < .05$ versus other groups except those indicated in following footnotes.

‡$P > .05$ versus CsA group.

§$P > .05$ versus naive B10.D2.

‖$P > .05$ versus immune B10.D2.

¶$P > .05$ versus medium-dose RPM.

(Figure 4). Spleen cells from the recipients of medium-dose rapamycin, recipients of cyclosporine A 50 mg/kg per day, naive B10.D2, and immune B10.D2 controls had no suppressive effects, but rather enhanced the response in the MLR against recipient cells. Spleen cells from naive B10.D2, immune B10.D2, the recipients of cyclosporine A 50 mg/kg per day and medium-dose rapamycin all demonstrated some suppression of the MLR against third-party cells. However, the degree of suppression was much less compared with that of recipients of high-dose rapamycin ($P < .05$, $P < .01$, $P < .001$, and $P < .01$, respectively).

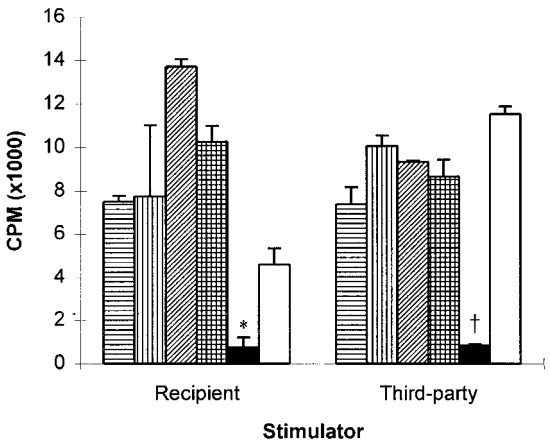


Figure 4. Suppression of antirecipient and third-party mixed lymphocyte reaction (MLR) by spleen cells from graft-versus-host disease (GVHD)-free long-term survivors. A total of 5×10^5 irradiated (10 Gy) spleen cells from different groups were added to either immune B10.D2 versus BALB/c MLR or naive B10.D2 versus C57BL/6 MLR with 5×10^5 responder and 5×10^5 irradiated (10 Gy) stimulator cells per well. Cultures were pulsed with [^{3}H]thymidine 1 μCi per well after 96 hours of incubation and harvested 16 hours later. Results are given as means ± SD of triplicate cultures and represent 1 of 3 separate experiments with comparable results. The 10 Gy–irradiated putative suppressor cells did not respond to concanavalin A (stimulation index <2). *$P < .01$, †$P < 0.001$ versus medium control. The background cpm for immune B10.D2 and naive B10.D2 were 2630 and 1799, respectively. ▤, naive B10.D2; ▥, immune B10.D2; ▨, cyclosporine A 50 mg/kg; ▦, medium-dose rapamycin (RPM); ■, high-dose RPM; □, medium control. CPM indicates counts per minute.

DISCUSSION

Absence of antigen-specific T cells, or clonal deletion, has been shown to be an important mechanism for the induction of tolerance to self-antigens and bacterial superantigens [26-28]. Clonal anergy has been shown to be responsible for tolerance in many models [29,30]. Exogenous IL-2 was reported to restore proliferation of anergic T-cell clones [31,32] and some in vivo–generated anergic T cells [29,33]. In our model, exogenous recombinant IL-2 failed to rescue the responses of GVHD-free long-term survivors against both recipient and third-party cells. T-cell desensitization due to prolonged engagement of TCR by persistent alloantigen could also be responsible for clonal anergy [15,34]. This mechanism is less likely to explain the low responses induced by rapamycin in our model, because GVHD developed late in the low-dose or medium-dose rapamycin-treated mice.

Active immune suppression mediated by regulatory cells has been proposed to be involved in maintenance of tolerance [35,36]. Cellular approaches using syngeneic or autologous bone marrow have been used successfully to reverse significant GVHD in allogeneic BMT [37,38]. Potential mechanisms by which such cells can modulate immune responses include natural suppressor [39-42] and veto activity [39,40]. Natural suppressor cells are potent nonspecific inhibitors of immune responses. Veto cells are capable of preventing the generation of CTL in a major histocompatibility complex–restricted fashion. Several cell phenotypes associated with suppressive activity have already been identified in mice, including null cells [40], $\alpha\beta TCR^+CD4^-CD8^-$ [41], Thy-1^+ NK1 asialo GM-1^+ $CD3^+$ [39], $\alpha\beta TCR^+$ $NK1.1^+$ [42], and $CD4^+$ [43]. Successful cloning of antigen-specific [43] and nonspecific [42,44] regulatory cells furnishes direct evidence of the existence of regulatory cells (ie, suppression).

Dose-dependent emergence of the infiltrating mononuclear cells in the skin of the rapamycin-treated mice that did not develop GVHD suggests that these infiltrating

mononuclear cells belong to a suppressive cell population rather than to a population of effector cells of GVHD. Blazar et al. [9] also observed the increase of a double negative T-cell population, which has been previously reported to belong to a nonspecific regulatory cell population [44,45]. To determine the mechanisms of prevention of GVHD with rapamycin, we performed a coculture assay and found that cells with suppressive activity to both antirecipient and third-party response exist in high-dose rapamycin-treated long-term survivors but not in cyclosporine A–treated mice (Figure 4). These cells did not appear to destroy skin tissue. These regulatory cells appear to be real, because we excluded the following possible nonspecific suppression: (1) cocultured cells were irradiated before culture and therefore could not proliferate, so the suppression was unlikely to be due to rapid depletion of essential nutrients, acceleration of responses by adding helper cells, and destruction of stimulator cells by adding CTL [46]; (2) the suppression of antirecipient responses was not due to the interference of responder-stimulator cell-to-cell contact because the addition of cocultured cells from all the control groups increased rather than decreased the MLR response; (3) it is unlikely that the suppression was due to the excess IL-2 receptor in the cocultured cells that could absorb IL-2 because additional IL-2 did not restore responsiveness [47]; and (4) the suppression was unlikely to be due to residual rapamycin because the half-life of rapamycin in the circulation in the rat is only 4 to 6 hours [14]. Our data suggest that rapamycin is able to regulate immune responses through the generation of a population of cells that suppress the immune response.

Our model suggests that rapamycin prevented GVHD not only because the responsiveness of GVHD-reactive T cells was directly inhibited by rapamycin, but also because antigen nonspecific regulatory cells (ie, suppression) were generated by rapamycin in a dose-dependent manner. These regulatory cells might interfere with the regeneration of mature T lymphocytes. Animals treated with the high-dose rapamycin induced enough regulatory cells to allow the mice in this cohort to survive GVHD-free in the period we monitored. Persistent presence of regulatory cells might also contribute to other mechanisms leading to the maintenance of more stable unresponsiveness, like clonal deletion in the current study (data not shown). Further analysis of the specific cell types involved in this suppressive activity is warranted. Because prevention and treatment of GVHD remains problematic, the potential to control GVHD by regulatory cells induced by rapamycin may be useful in developing alternative approaches to GVHD. However, it needs to be pointed out that nonspecific suppressor cells might also increase the incidence of opportunistic infection, as previously suggested by others [48].

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