Ex Vivo Rapamycin Generates Donor Th2 Cells That Potently Inhibit Graft-versus-Host Disease and Graft-versus-Tumor Effects via an IL-4-Dependent Mechanism

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Rapamycin (sirolimus) inhibits clinical allogeneic transplantation responses, including graft rejection (1) and graft-vs-host disease (GVHD) (2, 3). However, rapamycin therapy is limited by systemic toxicity and lack of single-agent efficacy (4). This relatively narrow therapeutic index indicates that optimization of rapamycin-based immune modulation may require alternative approaches. The molecular mechanism of action of rapamycin involves mammalian target of rapamycin (mTOR) inhibition (5), which in turn reduces mRNA translation, protein phosphorylation, and cell cycle progression (6, 7). Because signaling through mTOR is critical for homeostasis in immune and nonimmune cells (8–10), mTOR inhibition in vivo represents a relatively nonspecific immune modulation approach. Therefore, we reasoned that an ex vivo strategy that restricts rapamycin exposure to allogeneic T cells before transplantation may provide immune modulation while obviating systemic drug toxicity.

The cellular targets accounting for rapamycin immune suppression have not been fully characterized but involve both T cells and dendritic cells (DCs). Immune cells can be subdivided into cross-regulatory type I cells (CD4+Th1, CD8+Tc1, and DC1-type DC subsets) that promote cell-mediated immunity and type II cells (CD4+Th2, CD8+Tc2, and DC2-type DC subsets) that promote humoral immunity (11–13). Calcineurin signaling is essential for both Th1 and Th2 mRNA transcription (14), and as such, cyclosporine A or FK506 (tacrolimus) therapy does not clearly influence T cell cytokine polarity. In contrast, rapamycin can shift immunity toward Th2 cytokine production. As an initial example, rapamycin prevented rat cardiac allograft rejection increased IL-4 and IL-10 mRNA expression posttransplant (15). Similarly, rapamycin inhibition of GVHD after murine bone marrow transplantation (BMT) decreased T cell IFN-γ mRNA and increased IL-10 mRNA (16). In this latter study, rapamycin potently reduced CD8-mediated GVHD yet only modestly reduced CD4-mediated GVHD, thereby establishing CD8 cells as a more favorable target for rapamycin modulation. More recently, rapamycin was found to generate DC2-type cells that secreted reduced IL-12 and promoted T cell allostimulation toward IL-4 and IL-10 production (17). Although these in vivo studies indicate that rapamycin generally promotes type II immunity, it is currently not understood whether this effect occurs exclusively through an indirect mechanism (DC2 cell promotion) or also via a direct effect upon Th2 cell differentiation. To determine whether a direct mechanism might exist, we used an APC-free system for donor Th2 cell generation and adoptively transferred such rapamycin-generated Th2 cells after murine allogeneic BMT.

Allogeneic T cells transferred with the marrow graft primarily determine transplantation outcome, as donor T cells initiate both beneficial graft-vs-tumor (GVT) effects and detrimental GVHD. In an effort to define T cell subsets that might mediate GVT effects
with reduced GVHD, we and others have evaluated in vitro-generated, cytokine-polarized donor T cells. Unfortunately, these studies indicate that both GVHD and allogeneic antitumor effects are primarily type I cytokine processes that are cross-regulated by type II cells (18–22). That is, donor CD4+ Th1 and CD8+ Tc1 cells mediate potent graft-vs-leukemia (GVL) and GV T effects, with lethality from GVHD primarily limiting this T cell therapy. Conversely, donor CD4+ Th2 cells and CD8+ Tc2 cells mediate greatly reduced GVHD, with relatively weak GVL and GV T effects limiting therapeutic efficacy.

Thus, administration of highly purified Th1/Tc1 (type I) or Th2/Tc2 (type II) populations has an inherent limitation as an allogeneic BMT strategy. Therefore, we are currently evaluating approaches that provide some balance of type I and type II immunity post-BMT, with the hypothesis that a component of GV T effect can be realized (from type I immunity) with concomitant GVHD regulation (from type II immunity). In this study, we have evaluated whether augmentation of allografts with both unmanipulated donor CD4+ and CD8+ T cells and in vitro-generated donor Th2 cells might achieve such a type I/type II cytokine balance. In this context, we have tested whether ex vivo rapamycin might generate Th2 cells with an enhanced capacity to modulate immunity.

Materials and Methods

Animals

C57BL/6 × BALB/c F1 (CB6F1, H-2b/d), C57BL/6 × C3H/HeN (B6C3F1, H-2b), C57BL/6 (B6; H-2b), and congenic C57BL/6 (H-2b; Ly5.1 or Thy1.1) mice were obtained from Frederick Cancer Research Facility. B6 mice genetically deficient in IL-4 production were purchased from The Jackson Laboratory. All mice were 6–12 wk old, maintained in a specific-pathogen-free facility at the National Institutes of Health, and treated according to an approved animal protocol.

Generation of Th1 and Th2 subsets using CD3 and CD28 stimulation

B6 spleen cells were harvested, red cells were lysed (ACK buffer; Quality Biologicals), B cells were depleted (goat anti-mouse magnetic bioparticles; Polysciences), and CD8 cells were depleted (CD8 enrichment kit; StemCell Technologies). Anti-CD3 and anti-CD28-coated beads (CD3/CD28 beads) were produced (23) and used at 3:1 (bead:cell). CD4 cells were stimulated with CD3/CD28 beads in complete medium (CM) consisting of RPMI 1640 (Mediatech), 10% FCS (Gemi Bio-Products), pen-strep-glut (In-vitrogen Life Technologies), nonessential amino acids (Invitrogen Life Technologies), 2-ME (5 × 10−3 M; Invitrogen Life Technologies), and N-acetyl-cysteine (3.3 mM; Bristol-Myers Squibb). To generate control Th1 cells, CM was supplemented with recombinant murine (rm) IL-12 (2.5 ng/ml; R&D Systems), recombinant human (rh)IL-2 (20 IU/ml; National Cancer Institute (NCI)-Biologic Resource Branch (BRB) Repository), rhIL-7 (20 ng/ml; PeproTech), and anti-murine IL-4 (clone 11B.11 (10 µg/ml); NCBI-BR). To generate Th2 cells, CM was supplemented with rhIL-2 (20 IU/ml), rhIL-7 (20 ng/ml), and rmIL-4 (1000 IU/ml; PeproTech). In some cases, Th2 medium was supplemented with 0.1 or 10 µM rapamycin (sirolimus; Sigma-Aldrich). Cytokine- and rapamycin-containing medium was added on days 2–6 to maintain 0.2–1.0 × 10^6 cells/ml. However, rmIL-12 was only added on day 0 of Th1 culture.

Cytokine secretion assays

Th1, Th2, and rapamycin-generated Th2 cells (Th2.rapa) were harvested (day 6) and adjusted to 0.5 × 10^6 cells/ml in 24-well plates with or without CD3/CD28 beads. Twenty-four-hour culture supernatants were collected; cytokine content was evaluated by two-site ELISA (IL-2 and IL-5; R&D Systems; IL-4, IL-10, IL-13, and IFN-γ; BioSource International) or by Bio-Plex multiplex sandwich immunoassay (Bio-Rad).

BMT and tumor cell inoculation

Host CB6F1 mice were lethally irradiated (1050 centigray (cGy)). 125I gamma radiation source, gamma Cell 40; Atomic Energy of Canada) and reconstituted (i.v.) with B6 marrow (5 × 10^6 cells) containing host-type (H-2b) TS/A breast cancer cells (0.1 × 10^6 cells). The TS/A cell line (provided by Dr. R. Gress, NCI, Bethesda, MD) was derived from a spontaneously occurring mammary carcinoma of BALB/c origin, is TGF-β secreting, and is negative for mouse mammary tumor virus expression (24). Other treatment cohorts received additional donor cells, including T cell-containing spleen cells (7 × 10^6 cells) with or without Th1, Th2, or Th2.rapa cells (1 × 10^6 cells); Th2 cells were administered either on day 0 of BMT or in a delayed manner at day 4, 7, or 14 post-BMT. Mice were observed daily for survival.

Histology

Five mice from each cohort were evaluated on day 14 post-BMT. Liver, lung, small and large intestine, and skin were fixed in formaldehyde and stained with H&E. As detailed (21), semiquantitative GVHD scoring ranged from 0 to 4 (0 = normal; 4 = maximal GVHD); for GV T effect scoring, pulmonary tumor nodules were enumerated by light microscopy. Tumor and GVHD scoring was performed by a pathologist (M. Eckhaus) in a blinded manner.

Flow cytometry for surface markers

On day 6 of culture, Th1, Th2, and Th2.rapa cells were harvested for FACS analysis. Anti-murine CD3-PE, CD4-FITC, and CD8-allophycocyanin mAbs (BD Pharmingen) were used; three-color flow cytometry was performed (FACSCalibur instrument and CellQuest software; BD Biosciences). Live events (5,000–10,000) were acquired, with propidium iodide exclusion of dead cells. For cell tracking, Th1 and Th2 cells were generated from Thy1.1 congenic mice, and unmanipulated spleen cells were isolated from Ly5.1 congenic mice. Spleens were harvested post-BMT, and single-cell suspensions were labeled with Abs against murine CD4, CD8, CD19, Ly5.1, or CD90.1 (anti-Thy1.1) conjugated with FITC, PE, or allophycocyanin (BD Pharmingen).

Determination of in vivo allosensitization

Spleen cells collected post-BMT were subjected to: no stimulation, CD3/CD28 stimulation, B6 syngeneic DC stimulation, CB6F1 allogeneic DC stimulation, or third-party allogeneic DC stimulation (B6C3F1). For CD3/CD28 stimulation, spleen cells were adjusted to a concentration of 0.5 × 10^6 cells/ml and incubated with CD3/CD28 beads. DCs were obtained by culturing marrow cells for 4 days in rmGM-CSF and rmIL-4 (each at 1000 IU/ml; PeproTech); bacterial LPS (1 µg/ml; Calbiochem) was added to final 24 h of DC culture. Expanded DC were washed and used at 10:1 (spleen cell:DC). After 24 h, supernatants were collected for cytokine analysis, and cells were evaluated by FACS. For cytokine FACS, cells were labeled with IFN-γ or IL-4 catch reagent (Miltenyi Biotech), followed by 45 min of warm medium incubation (RPMI 1640 with 10% PBS) at 37°C (slow rotation). Cells were washed with cold buffer (PBS with 0.5% BSA and 2 mM EDTA), labeled with IFN-γ or IL-4 detection Ab (PE) and other surface Abs (anti-CD4 and anti-CD8 APC; anti-Ly-5.1 and anti-CD90.1 FITC), washed, and analyzed by FACS. FACS frequency information was multiplied by splenic cell yield to obtain number of cytokine secreting cells per spleen.

Statistics

Survival analysis was performed using the nonparametric, two-sided, matched log-rank test. For survival analyses involving day 7 or 14 post-BMT Th2 cell infusion, early deaths in the GVHD control group were censored out of the analysis. For small sample size comparison, Student’s t test was used. Comparison values of p < 0.05 were considered statistically significant.

Results

Donor Th2 cell generation in high-dose rapamycin (10 µM)

Using a method of APC-free T cell expansion and polarization that we have previously developed (22, 25), we evaluated whether in vitro rapamycin might enhance Th2 cell polarization. A total of 0.1 µM rapamycin was initially selected for evaluation, as this concentration abrogated primary in vitro allosensitization (B6 anti-CB6F1 MLC; data not shown). However, because Th2 cell expansion after costimulation was relatively preserved at 0.1 µM rapamycin, concentrations up to 10 µM were subsequently evaluated. In the representative experiment shown in Fig. 1a, relative to the standard Th2 condition, Th2 cell yield was reduced by 59% in 0.1 µM rapamycin and 88% in 10 µM rapamycin.

Addition of 10 µM rapamycin to the Th2 cell expansion condition dramatically altered the resultant T cell cytokine phenotype

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Each Th2 culture (Th2, Th2.rapa (0.1 μM rapamycin) and Th2.rapa (10 μM rapamycin)) secreted greatly reduced IFN-γ relative to control Th1 cells; although each Th2 culture secreted greatly reduced IL-2 relative to Th1 cells, Th2.rapa (10 μM) cells had increased IL-2 secretion. Each Th2 culture secreted increased IL-4 relative to Th1 cells (Fig. 1c); however, the magnitude of IL-4 secretion was reduced in Th2.rapa (10 μM) cells. Remarkably, Th2.rapa (10 μM) cells secreted low or undetectable levels of IL-5, IL-10, and IL-13.

Th2.rapa cells enhance Th2 skewing post-BMT in an IL-4-dependent manner

Allogeneic BMT and adoptive T cell transfers were performed to evaluate whether ex vivo rapamycin enhanced Th2 cell capacity for type II cytokine polarization post-BMT and to determine whether post-BMT cytokine skewing required Th2 cell secretion of IL-4. Because IL-4 is primarily responsible for initiation of type II immunity (26), we hypothesized that Th2 cell polarization of cytokine profiles post-BMT would require IL-4. In the context of this hypothesis, it was essential to determine the capacity of Th2.rapa cells, which secreted reduced IL-4 and other Th2 cytokines in vitro before BMT, to promote type II cytokine skewing post-BMT.

To address these hypotheses, control Th2 or Th2.rapa (10 μM) cells were generated from wild-type (WT) or IL-4-deficient (knockout (KO)) donors. Th2 and Th2.rapa cells generated from IL-4 KO donors were indeed deficient in IL-4 secretion (Fig. 2a). Results of this experiment were similar to data shown in Fig. 1c: Th2.rapa cells from WT donors secreted IL-4 but with reduced magnitude relative to control Th2 cells. Both WT and IL-4 KO CD4 cells express an intact IL-4R; each population would thus predictably proceed with Th2 differentiation via IL-4 added to culture. Indeed, both WT and KO Th2 cells secreted IL-5, IL-10, and IL-13; remarkably, secretion of these cytokines from WT or IL-4 KO donors was greatly reduced by rapamycin. Results shown in Fig. 2a demonstrate that each Th2 population secreted a similar quantity of IFN-γ. The magnitude of Th2 cell IFN-γ secretion was reduced relative to values typically observed in control Th1 cultures (see Th1 results in Fig. 1c). Therefore, the profile of polarized cells used in the experiment shown in Fig. 2 is most consistent with extensive Th2-type polarization, with some contamination with Th1-type polarization. Finally, rapamycin again resulted in Th2 cells with increased IL-2 secretion (both WT and IL-4 KO conditions).
We next evaluated Th2 (WT), Th2 (IL-4 KO), Th2.rapa (WT), and Th2.rapa (IL-4 KO) populations for their capacity to promote a Th2 shift post-BMT (Fig. 2b). Allograft augmentation with Th2 (WT) cells increased IL-4 secretion capacity post-BMT; as predicted, promotion of IL-4 secretion was not observed in Th2 (IL-4 KO) recipients. Surprisingly, recipients of Th2.rapa (WT) cells had the highest capacity for IL-4 secretion; this effect was completely dependent upon Th2.rapa cell IL-4 production. Recipients of Th2.rapa (WT) cells also had increased post-BMT capacity for IL-5, IL-10, and IL-13 secretion relative to Th2 (WT) recipients; induction of these cytokines in Th2 and Th2.rapa cell recipients generally occurred in an IL-4-dependent manner. However, increased IL-5 secretion was observed in recipients of IL-4 KO Th2.rapa cells.

In this experiment, recipients of Th2 (WT) cells had reduced post-BMT capacity for IFN-γ secretion; this effect was entirely dependent upon Th2 cell IL-4 secretion. Th2.rapa (WT) recipients had a more profound reduction in IFN-γ secretion capacity that was similarly IL-4 dependent. As such, relative to control Th2 recipients, Th2.rapa (WT) recipients developed enhanced post-BMT cytokine polarization, as evidenced by increased IL-4, IL-5, IL-10, and IL-13 secretion concomitant with reduced IFN-γ secretion. In light of the increased IL-2 secretion observed in Th2.rapa cells pre-BMT, it is interesting to note that recipients of Th2.rapa cells had increased post-BMT IL-2 secretion; this effect was fully dependent upon Th2.rapa cell production of IL-4.

Th2.rapa cells potently abrogate graft-vs-host reaction (GVHR) in an IL-4-dependent manner

GVHD is initiated by a T cell-mediated GVHR that can be quantified by post-BMT identification of donor anti-host allospecific T cells that secrete IFN-γ (27, 28). Thus, we evaluated whether Th2 cells inhibit GVHR, as determined by reduction in post-BMT allospecific IFN-γ secretion; in addition, we evaluated whether Th2 cell inhibition of GVHR occurred through an IL-4 mechanism and whether Th2.rapa (10 μM) cells might intensify GVHR inhibition.

As anticipated, Th2 or Th2.rapa cell recipients had greatly reduced allospecific CD8+ T cell numbers post-BMT (Fig. 3a, number of allospecific CD8+ T cells: (BMT+T) cohort, 2.5 ± 0.6 × 106; (BMT+T+Th2) cohort, 2 ± 1 × 106; (BMT+T+Th2.rapa) cohort, 1 ± 1 × 105 cells/spleen). Th2 and Th2.rapa cell inhibition of CD8-mediated GVHR was dependent upon IL-4 production; in an initial BMT experiment (data not shown), Th2 cells, but not control Th1 cells, reduced CD8-mediated GVHR. Furthermore, Th2 and Th2.rapa cells modestly reduced total CD8 numbers post-BMT in an IL-4-dependent manner.
**FIGURE 3.** Th2.rapa cell inhibition of CD8-mediated GVHR (a) and CD4-mediated GVHR (b) is IL-4 dependent. B6-into-CB6F1 BMT was performed (1050 cGy host irradiation). Cohorts received marrow and TSA tumor cells (“BMT”) and spleen cells (“T”) with or without Th2 cells. Th2 cells were generated from wild-type (“WT”) or IL-4 KO CD4 cells (“KO”) either without rapamycin (“Th2”) or with 10 μM rapamycin (“Th2.rapa”). On day 7 post-BMT, spleen cells were isolated; donor CD4 and CD8 cell number per spleen was calculated from flow data (cells per spleen, ×10^5). Day 7 post-BMT spleen cells were stimulated for 24 h with syngeneic or allogeneic DC (allogeneic DC results shown, left panels; syngeneic DC results, not shown). Cells were harvested, stained for CD4, CD8, and bispecific anti-CD45 × anti-IFN-γ; percentage of cells secreting IFN-γ was determined by flow cytometry. Number of cells per spleen secreting IFN-γ after in vitro allostimulation was calculated using flow data. Data shown are mean ± SEM (n = 5 cohort). * denotes that value for WT Th2 or WT Th2.rapa recipients is statistically different relative to cohort not receiving Th2 cells; ** indicates that value for KO Th2 or KO Th2.rapa recipients is statistically different relative to corresponding WT Th2 or WT Th2.rapa cohort, respectively.

Fig. 3b demonstrates that Th2 cells, and in particular, Th2.rapa cells, inhibited CD4-mediated GVHR. The absolute number of allospecific CD4^+ IFN-γ^+ cells per spleen in the (BMT+T) cohort was 1.5 ± 0.3 × 10^6, compared with the (BMT+T+Th2) cohort value of 9 ± 2 × 10^5 and the (BMT+T+Th2.rapa) cohort value of 4 ± 1 × 10^5. Th2 or Th2.rapa cell inhibition of CD4-mediated GVHR was fully dependent on IL-4 production. Concomitant with this reduction in allospecific CD4^+ cells, total CD4 cells post-BMT were actually significantly increased in Th2 recipients, particularly Th2.rapa cell recipients. The absolute number of total CD4^+ T cells in the (BMT+T) cohort was 5.2 ± 0.5 × 10^6, compared with the (BMT+T+Th2) cohort value of 1.5 ± 0.2 × 10^7 and the (BMT+T+Th2.rapa) cohort value of 3.5 ± 0.2 × 10^7 cells/spleen.

**Th2.rapa cells potently inhibit GVHD**

As Table I shows, recipients of Th2 cells, in particular Th2.rapa (10 μM) cells, had reduced histologically defined GVHD. BMT subjects receiving control Th2 cells (cohort D) had reduced GVHR relative to recipients administered Th1 cells (cohort C) (average GVHR score, 2.2 ± 0.6 vs 5.4 ± 0.5, p = 0.005). The difference between these groups was primarily attributable to reduced intestinal GVHD in Th2 recipients. BMT subjects administered Th2 cells expanded in low-dose rapamycin (0.1 μM; cohort E) had a similar GVHD target tissue spectrum and total GVHD score as control Th2 cell recipients. Most notably, cohort F, which received Th2 cells expanded in high-dose rapamycin (10 μM), had near-complete abrogation of GVHD (average GVHD score, 0.6 ± 0.3; reduced relative to cohorts B and C, p < 0.05). Strikingly, each recipient of Th2.rapa (10 μM) cells did not develop small or large intestinal GVHD; the sole evidence for GVHD in this cohort was stage 1 skin lesions in three of five recipients.

In the TS/A tumor model we used, allogeneic GVT effects correlate with post-BMT allospecific T cell IFN-γ secretion (22). These data predict that the significant reduction in IFN-γ-secreting allospecific T cells in Th2 recipients and, in particular, Th2.rapa recipients would reduce the anti-TS/A GVT effect. As Fig. 4a shows, mice receiving BMT not supplemented with T cells had extensive pulmonary infiltration with poorly differentiated carcinoma cells (day 14 post-BMT; 90.2 ± 25.0 tumor nodules/recipient). Transplant inocula supplemented with donor T cells alone or in combination with Th1 or control Th2 cells generated a significant GVT effect, as no pulmonary tumor cells were observed microscopically in these cohorts. In marked contrast, transplant inocula containing T cells and additional Th2.rapa (0.1 μM) or Th2.rapa (10 μM) cells mediated a blunted GVT effect, as early signs of tumor growth were observed in two of five and four of five of these recipients, respectively (mean number of pulmonary tumor nodules: 4.2 ± 2.6 and 1.4 ± 0.7).

As Fig. 4b illustrates, the early finding of microscopic tumor relapse in Th2.rapa recipients was a harbinger for later tumor-related death, which was confirmed by the presence of respiratory compromise, pulmonary nodules at necropsy, and clinical absence of ongoing GVHD (no skin rash, hunched posturing, or diarrhea). Median survival time in recipients of Th2.rapa (0.1 μM) cells and Th2.rapa (10 μM) cells was 40.5 ± 1.4 and 36.5 ± 4.9 days post-BMT; each of these values was increased relative to the tumor control group (cohort B: median survival time of 27.0 ± 1.4 days; cohort F > cohort B, p = 0.0001; cohort G > cohort B, p = 0.045). Recipients of transplant inocula supplemented with donor T cells alone or in combination with Th1 or control Th2 cells had a significant rate of lethality from GVHD, as evidenced by skin ulceration, diarrhea, and progressive weight loss (>20% loss of
initial body weight) in the absence of pulmonary compromise or pulmonary tumors at necropsy.

**Th2 IL-4 production influences lethality post-BMT**

An additional BMT experiment was performed to further evaluate the role of Th2 and Th2.rapa cells in the modulation of GVHD and GVT effects. One objective of this experiment was to determine the role of Th2 and Th2.rapa cell IL-4 production on post-BMT survival. In this particular experiment, a high rate of early lethality was observed in the GVHD control group (BMT/H11001 T cohort) (Fig. 5a, group C, 12 of 18 recipients died within 2 wk post-BMT). Of note, 6 of 18 recipients in this GVHD control cohort survived beyond the last subject in the tumor control group before eventual death due to ongoing GVHD, as evidenced by progressive weight loss in the absence of pulmonary compromise. Relative to the tumor control group (Fig. 5a, group B), the GVHD control group did not have increased overall survival ($p/H110050.76$).

By comparison, recipients of Th2.rapa cells (BMT/H11001 T/H11001 Th2.rapa cohort; Fig. 5a, left panel, group D) were protected from early death due to acute GVHD and had increased survival relative to the tumor control group ($p = 0.002$). To confirm that tumor progression was the cause of death in Th2.rapa cell recipients, the remaining 5 of 10 recipients in this cohort were euthanized at day 35 post-BMT for histology evaluation: each of the 5 recipients had numerous macroscopic tumor nodules at necropsy and an absence of small intestinal, large intestinal, liver, and skin GVHD. Taken together, these findings confirm that Th2.rapa cells prevented GVHD and abrogated GVT effects.

Further studies were performed to evaluate post-BMT immune function in the euthanized Th2.rapa cell recipients. As Fig. 5a, middle panel, illustrates, Th2.rapa cell recipients had persistent polarization toward Th2-type cytokine production at day 35 post-BMT; in fact, the magnitude of Th1- and Th2-type cytokine production in this cohort was very similar to that observed at day 5 post-BMT (shown in Fig. 2b). Th2.rapa cell recipients did not appear to be globally immunosuppressed, as day 35 post-BMT T cells had increased IFN-γ secretion in response to relevant host alloantigen and to third-party alloantigen (Fig. 5a, right panel).

In marked contrast to these results using Th2.rapa cells, allograft augmentation with Th2 cells not generated in rapamycin did not prevent lethality from acute GVHD (cohort F; Fig. 5b). That is, 6 of 10 recipients in this Th2 cell cohort developed early lethality in parallel to the GVHD control cohort; the remaining 4 of 10 cohort recipients survived longer than each tumor control recipient but

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<th>Table I. <strong>Th2 cell reduction in histologically defined GVHD: influence of ex vivo rapamycin</strong></th>
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* CB6F1 mice were lethally irradiated (1050 cGy) and received B6 marrow and host-type TS/A carcinoma cells. Cohorts B–F also received B6 splenic T cells; cohorts C–F also received B6 CD4 T cells generated under Th1 or Th2 conditions or the Th2 condition with low-dose rapamycin (0.1 μM) or high-dose rapamycin (10 μM).

On day 14 post-BMT, $n = 5$ recipients from each cohort were evaluated for histologic signs of GVHD, with each organ semiquantitatively scored (scale: 0–4).

* indicates statistically significant reduction in GVHD relative to cohort B ($p < 0.05$).
eventually died due to apparent ongoing GVHD (progressive weight loss and absence of pulmonary compromise). Relative to the tumor control group, this Th2 cell cohort did not have an overall survival advantage ($p < 0.65$). Most strikingly and unexpectedly, recipients of IL-4 KO Th2 cells (cohort G; Fig. 5b) were protected from early GVHD lethality and had increased survival relative to tumor controls ($p < 0.0002$); long-term survival in such recipients was limited by ongoing GVHD, as evidenced by progressive weight loss in the absence of pulmonary compromise.

Delayed administration of Th2.rapa cells improves balance of GVHD and GVT effects

These experiments indicated that Th2.rapa cells, when administered on day 0 of BMT, potently abrogated GVHD but also significantly reduced the GVT effect in this model. In previous studies (29), it was demonstrated that delay in administration of murine CD4+CD25+ T regulatory ($T_{reg}$) cells until day 2 after class I and class II disparate BMT maintained an anti-GVHD effect while permitting a GVL effect against a myeloid leukemia. Given these results, we hypothesized that a delay in Th2.rapa cell administration might allow for a beneficial GVT effect to develop with subsequent regulation of ongoing GVHD, thereby yielding a survival advantage post-BMT. To address this hypothesis, additional treatment cohorts in the BMT experiment shown in Fig. 5 received T cell replete inocula on day 0 of BMT, with additional donor Th2 or Th2.rapa cells administered on either days 4, 7, or 14 post-BMT.

Indeed, in our model, delay in Th2.rapa cell administration until day 4 post-BMT effectively prevented acute GVHD lethality and appeared to modestly enhance the GVT effect relative to day 0 Th2.rapa cell infusion (Fig. 6a, left panel); however, such day 4 Th2.rapa cell recipients eventually succumbed to tumor relapse, as evidenced by pulmonary compromise and presence of macroscopic lung tumors at the time of death. Delay in Th2.rapa cell administration until day 7 post-BMT appeared to reduce GVHD and provide an incremental increase in GVT effect; death in such recipients was again attributed to tumor progression. Remarkably, delay in Th2.rapa cell infusion until day 14 post-BMT, which was a time point of severe GVHD by histology parameters, reduced lethal GVHD; such recipients benefited from a potent GVT effect, as each of three healthy appearing mice in this cohort that were electively euthanized at day 74 post-BMT were free of tumor.

Delayed Th2 cell infusion was also evaluated for the Th2 cell product not generated in rapamycin. This donor T cell population, which was not capable of reducing early lethality from GVHD when administered on day 0 of BMT, effectively prevented lethal GVHD when administered at day 4 post-BMT. However, such day 4 Th2 cell recipients developed ongoing clinical GVHD in the absence of pulmonary compromise or visible tumor at the time of death. Post-BMT survival was similarly increased by Th2 cell administration on day 7 or 14 posttransplant, although such recipients also succumbed to ongoing GVHD.
respectively. On day 35 post-BMT, WT Th2.rapa cell recipients were euthanized, and splenic T cells were evaluated for cytokine phenotype (mean/H11006 WT Th2.rapa cells; “E”, IL-4 KO Th2.rapa cells). D–G received BMT, TSA cells, donor T cells, and donor Th2 cells (cipients of T cell replete transplantation followed by delayed re- shift toward Th2-type cytokines in such recipients was nominal, with only small increases in IL-5 and IL-13 detected. In sum, re-

FIGURE 5. Th2 cell prevention of GVHD: influence of ex vivo rapamycin and Th2 cell IL-4 secretion. Cohorts of B6-into-CB6F1 BMT included: BMT alone (“A”; n = 10), tumor control (“B”; BMT+TS/A carcinoma cells; n = 10), and GVHD control (“C”; BMT+TS/A + donor T cells; n = 18). Cohorts D–G received BMT, TSA cells, donor T cells, and donor Th2 cells (n = 10/cohort). a, left panel, Survival results with rapamycin generated Th2 cells (“D”, WT Th2.rapa cells; “E”, IL-4 KO Th2.rapa cells). * and ** indicate survival advantage relative to tumor control cohort “B” and GVHD control cohort “C”, respectively. On day 35 post-BMT, WT Th2.rapa cell recipients were euthanized, and splenic T cells were evaluated for cytokine phenotype (middle panel; mean ± SEM of n = 5; cytokine secretion after CD3 and CD28 costimulation; Bio-plex assay) and for DC-induced IFN-γ secretion (right panel; average ± SEM of n = 5; syngeneic DC (B6), relevant host allogeneic DC (CB6F1), or third-party allogeneic DC (B6C3F1)); * indicates increased IFN-γ release relative to B6 DC stimulation. b, Survival results of T cell replete BMT performed with Th2 cells generated without rapamycin (“F”, WT Th2 cells; “G”, IL-4 KO Th2 cells).

Long-term survivors receiving Th2.rapa cells: evaluation of immune function

Taken together, our experiments demonstrate that Th2.rapa cells represent a potent population for GVHD prevention and that Th2.rapa cell inhibition of GVT effects can be minimized through delayed cell administration. Delay in Th2.rapa cell infusion after the onset of significant clinical GVHD is a particularly attractive strategy, as this approach can yield a curative GVT effect in this model involving disseminated, refractory breast carcinoma cells. At the most basic level, success of GVHD treatment strategies can be measured by prevention in lethality; however, because the immune system is a sensitive target of GVHD (30), quantitation of immune function post-BMT is a more reliable indicator of the extent of GVHD protection.

To address this issue, long-term survivors in the day 14 Th2.rapa cell cohort were evaluated for immune function at day 74 post-BMT. Relative to BMT controls, recipients of T cell replete BMT followed by Th2.rapa cells at day 14 post-BMT had significantly reduced immune reconstitution, as indicated by reduced numbers of CD4+ and CD8+ T cells and CD19+ B cells (Fig. 7, left panel). In addition to this numerical evidence of immune deficiency, recipients of delayed Th2.rapa cells had functional defects, as post-BMT IFN-γ secretion in response to host alloantigen was preserved while response to third-party alloantigen was reduced (Fig. 7, middle panel). Furthermore, long-term survivors in the delayed Th2.rapa cell cohort had reduced IL-2, IFN-γ, and IL-10 secretion in response to CD3 and CD28 costimulation; a shift toward Th2-type cytokines in such recipients was nominal, with only small increases in IL-5 and IL-13 detected. In sum, recipients of T cell replete transplantation followed by delayed Th2.rapa cells had significant immune deficiency, which is consistent with subclinical GVHD.

Discussion

The immunosuppressive effect of rapamycin has been recognized for decades, yet an understanding of its molecular and cellular mechanism of action is only now emerging. Similarly, proof of its clinical utility has been demonstrated only recently for prevention of renal allograft rejection (1), with recent clinical trials showing promise for GVHD prevention (2, 3) or treatment (31). As knowledge of the mTOR pathway and cellular targets of mTOR inhibitors is identified, new therapeutic strategies not involving systemic drug administration might be harnessed to modulate this pathway with re-
duced host toxicity. As an initial example of such a strategy, rapa-
mycin eluting angioplasty stents can provide a high local concentra-
tion of drug, thereby inhibiting local endothelial cell proliferation and preventing coronary artery reoclusion without systemic immune sup-
pression (32). In this article, we have determined that usage of high-
dose rapamycin exclusively ex vivo generates a CD4+ Th2 cell product that potently inhibits alloreactivity after BMT, thereby il-
ustrating that a rapamycin-based immune modulation effect can be harnessed without systemic drug toxicity.

Allograft engineering through in vitro T cell expansion represents a particularly suitable setting to develop rapamycin-based therapies. In this study, we used an APC-free method of donor Th1 and Th2 cell expansion similar to the one we recently used for generation of mixed Th1/Tc1 and Th2/Tc2 subsets (22). In this method, anti-CD3 and anti-CD28 costimulation and cytokine polarization signals are provided independent of APCs. Therefore, T cell differentiation and survival signals are delivered independent of APC variables (33) such as
class of DC, quality of Ag presentation, strength of TCR and costimulatory pathway ligation, and without potential APC-mediated downregulatory interactions through B7:CTLA-4 interaction (34). Because rapamycin significantly modulates APC function (17, 35), our co-stimulation method provides a new opportunity to directly assess rapamycin effects on CD4 cell expansion and Th2 differentiation. In addition, our findings using this system have potential clinical relevance, as pilot trials using CD3 and CD28 costimulated T cells have been initiated for HIV therapy (36), lymphoma therapy (37), and GVHD modulation (38).

FIGURE 6. Delayed administration of Th2 and Th2.rapa cells: influence on post-BMT survival. a, left panel, Survival of cohorts receiving T cell replete B6-into-CB6F1 BMT (BMT + TS/A + T cells) and additional donor Th2.rapa cells administered on day 0 (“D”; n = 10) or day 4 post-BMT (“H”; n = 10); BMT (“A”), tumor (“B”), and GVHD control (“C”) cohorts are same groups shown in Fig. 5. * and ** indicate survival advantage relative to tumor control cohort B or GVHD control cohort C or CC, respectively. a, middle and right panels, Survival of cohorts receiving T cell replete BMT+Th2.rapa cells administered on day 7 (“I”; n = 10) or day 14 post-BMT (“J”; n = 6), respectively; for these comparisons, early deaths in the GVHD control group were censored (cohort CC; n = 10 for day 7 Th2.rapa comparison, n = 6 for day 14 Th2.rapa comparison). b, left panel, Results of additional donor Th2 cells not generated in rapamycin when administered on day 0 of BMT (“P”) or day 4 post-BMT (“Q”), b, middle and right panels, Survival of cohorts receiving T cell replete BMT+Th2 cells on day 7 (cohort “L”; n = 9) or day 14 post-BMT (cohort “M”; n = 7), respectively.

FIGURE 7. Delayed Th2.rapa cell infusion (day 14 post-BMT): evaluation of immune function at day 74 post-BMT. Recipients in cohort “A” (BMT control group; n = 5) and cohort “F” (day 14 Th2.rapa cell group; n = 3) were euthanized at day 74 post-BMT. For data in the left panel, the absolute number of CD4⁺ and CD8⁺ T cells and CD19⁺ B cells was calculated by total spleen cell counts and flow cytometry. For data in the middle panel, spleen cells were stimulated with syngeneic DC (B6), relevant host DC (CB6F1), or third-party allogeneic DC (B6C3F1); resultant 24-h supernatants were evaluated for IFN-γ content. ** indicates that the difference between BMT cohort and Th2.rapa cell cohort for third-party alloreactivity approached statistical significance (p = 0.08). For data in the right panel, spleen cells were stimulated with anti-CD3 and anti-CD28; resultant 24-h supernatants were tested for IL-2, IFN-γ, IL-4, IL-5, IL-10, and IL-13 content. Cytokine analysis was by multiplex assay, with results expressed as pg/ml/0.5 × 10⁶ cells/24 h. Results shown are mean values ± SEM; * indicates that the difference between value in Th2.rapa cell cohort (“F”) and BMT control cohort (“A”) is statistically significant.
We have demonstrated that this costimulation method provides an opportunity to evaluate and harness the direct T cell modulation effects of rapamycin at concentrations not achievable in vivo (10 \( \mu \text{M} \)). At this high drug concentration, Th2 polarization occurred with partial attenuation of Th2 cell expansion. This demonstration that Th2 cells can expand in high-dose rapamycin suggests that a form of “rapamycin resistance” may have developed. Such a finding may be analogous to rapamycin resistance described in human CD8\(^+\) T cells (39), which is induced through increased strength of CD3 and CD28 signaling. Further studies will be required to evaluate potential mechanisms that permit Th2 cell expansion in high-dose rapamycin.

High-dose rapamycin exposure generated a “precursor” Th2 cell secreting IL-4 in the virtual absence of other Th2-type cytokines. Rapamycin may have selected for or preferentially supported the expansion of naive CD4 cells or allowed Th2 cells to expand without significant differentiation; ongoing experiments seek to better understand this biology. Consistent with the concept that rapamycin can facilitate naive Th2 cell generation, we have found that Th2.rapa (10 \( \mu \text{M} \)) cells express increased L-selectin relative to control Th2 cells (U. Jung, unpublished observations). Of note, others have previously characterized a naive Th2 cell relatively restricted to IL-4 secretion (40). Similar to this previously described naive Th2 subset, IL-4-secreting Th2 cells generated through rapamycin exposure effectively promoted in vivo secretion of distally acting Th2 cytokines IL-5, IL-10, and IL-13 (41). Taken together, the ex vivo T cell expansion method we developed bypasses rapamycin APC inhibition and systemic toxicity to allow generation of a novel Th2 cell that likely does not occur to a comparable extent upon in vivo drug exposure.

Our experiments support the conclusion that Th1 and Th2 cells play differential roles in GVHD pathogenesis and have identified Th2.rapa cells as a particularly potent GVHD inhibitor. Using cytokine capture flow cytometry, we evaluated CD4 and CD8 cells for their post-BMT acquisition of allospecific IFN-\(\gamma\) secretion. Through this effort, we observed that Th2 and Th2.rapa cells (but not Th1 cells) prevented CD8-mediated GVHR. Of note, we have observed previously that Th2 cell administration can reduce donor CD8\(^+\) T cell engraftment; the mechanism of this immune regulation has not been characterized but may involve reduction in activation of host APC through Th2 cytokines such as IL-10 (42), consumption of necessary CD8 cell growth factors such as IL-15 (43), or conversion of CD8 cells to a Tc2 phenotype that have reduced capacity for expansion post-BMT (22). Perhaps most significantly, we found that CD4-mediated GVHR, which was modestly reduced by control Th2 cells, was most sensitive to inhibition by Th2.rapa cells. In previous studies, in vivo rapamycin effectively prevented CD8-mediated GVHD but was relatively ineffective at preventing CD4-mediated GVHD (16). The ability of Th2.rapa cells to more completely inhibit CD4-mediated GVHR may relate to a high-dose rapamycin effect, which is not likely attainable through in vivo administration.

Our findings using IL-4-deficient donors identify the importance of this cytokine to the observed Th2 and Th2.rapa cell promotion of Th2 immunity post-BMT. Strikingly, Th2.rapa cells, which secreted low levels of Th2 cytokines in vitro, yielded the most marked Th2 shift in vivo post-BMT. Further studies are ongoing to determine whether this paradoxical effect is due to enhanced Th2.rapa cell expansion or survival post-BMT or to increased Th2 cytokine production on a per cell basis. Our results also clearly demonstrate that IL-4 is required for Th2 and Th2.rapa cell inhibition of CD4- and CD8-mediated GVHR. This result is consistent with murine (44) and human (45) data indicating a protective role of donor IL-4 secretion in GVHD. Of course, IL-4 induces the immunosuppressive Th2 cytokines IL-10 and IL-13; further studies seek to determine whether the observed IL-4-dependent effect operates through these more distal Th2 cytokines. Positive results in such an effort would add to the body of work implicating IL-10 as an anti-GVHD cytokine (46, 47). The potential role of IL-13 as an anti-GVHD cytokine has not been defined; in fact, in the setting of unrelated human transplantation, donor IL-13 secretion was associated with increased GVHD (48). It should be noted that Th2.rapa cells likely prevent GVHD by a mechanism distinct from GVHD prevention by Treg cells (49, 50), as Treg cells typically mediate their effects through nonsoluble mediators (reviewed in Ref. 51); in cases where Treg cells mediate inhibition through cytokine secretion, such cytokines have not included IL-4.

Combined inhibition of CD4- and CD8-mediated GVHR through in vitro rapamycin exposure translated into reduced GVHD by standard histology criteria. Strikingly, recipients of allografts augmented with Th1 cells uniformly developed gut GVHD, whereas such lesions were absent in each recipient of allografts augmented with Th2 cells generated in high-dose rapamycin. These new data provide further support for application of the Th1/Th2 immune regulation paradigm to GVHD pathogenesis. The fact that other studies have not supported a strict Th1/Th2 view of GVHD may relate to their use of unmanipulated T cells (52–54), which does not result in the marked state of Th2 polarization that is achieved by an ex vivo polarized T cell therapy approach.

Th2 and Th2.rapa cells, in addition to reducing graft-vs-host alloreactivity and histologically defined GVHD, also inhibited lethality from GVHD. Only the Th2.rapa cells prevented lethal GVHD when cells were administered on the day of BMT; this increased efficacy of the Th2.rapa cells relative to the control Th2 cells is predictable, given this population’s increased capacity to polarize toward Th2-type cytokines post-BMT, to reduce alloreactivity, and to reduce histologically defined GVHD. Importantly, IL-4-deficient Th2.rapa cells, which weakly polarized toward Th2-type cytokines and nominally reduced alloreactivity, only partially reduced lethal GVHD.

By comparison, day 0 administration of control Th2 cells not generated in rapamycin did not prevent lethal GVHD. However, unexpectedly, control Th2 cells deficient in IL-4 production effectively reduced GVHD. Of note, a prior study using IL-4-deficient unmanipulated donor T cells determined a role for IL-4 production in GVHD (54). We propose that control Th2 cells, which secrete high levels of IL-4 at the time of infusion, exert a bifurmal effect on GVHD, with initial promotion of GVHD through IL-4 production and later inhibition of GVHD through IL-4 and alternative pathways. Consistent with this model, we found that a simple delay in Th2 cell administration until day 4 post-BMT reduced lethal GVHD.

In addition, for the first time, we have demonstrated that Th2 and Th2.rapa cell therapy can modulate established GVHD. Importantly, this anti-GVHD effect was realized even if Th2 cells were administered at the height of histologically defined GVHD and cohort lethality. Our analysis of day 74 post-BMT long-term survivors in recipients of Th2.rapa cells at day 14 post-BMT determined that this delayed cell administration approach was associated with immune compromise characteristic of ongoing, subclinical GVHD, including reduced IL-2 secretion capacity and reduced reactivity to third-party alloantigen. Future studies in our lab will seek to further understand the role of Th2.rapa cells in the treatment of established GVHD and to develop enhanced therapeutic strategies, such as multiple injections of Th2.rapa cells or combination of Th2.rapa cells with in vivo rapamycin therapy.
Potent inhibition of GVHD through day 0 Th2.rapa cell administration was also associated with attenuation of the GV'T effect, which occurred in an IL-4-dependent manner. In the TSI/A tumor model, we have previously found that the GV'T effect was mediated through an allospecific mechanism associated with IFN-γ secretion (22). Given the close association of GVHD and GV'T effects in this model, the attenuated GV'T effect observed in Th2.rapa cell recipients provides further evidence that this population potentially inhibited alloreactivity. It should be noted that the TSI/A model used is particularly refractory to allogeneic GV'T effects, in part through tumor cell secretion of TGF-β (24). Furthermore, the mix of T1/T2 immunity attained post-BMT through Th2.rapa cell augmentation of T cell replete allografts provided an improved GV'T effect relative to our historical control cohort that received only in vitro-expanded Th2/Th1 cells (22). Most importantly, we found that delay in Th2.rapa cell administration allowed GV'T effects to occur, with subsequent regulation of ongoing GVHD. As such, differential timing of Th2.rapa cell infusion can be used as an approach to modulate the balance of GVHD and GV'T effects.

In conclusion, rapamycin can be used exclusively ex vivo for modulation of transplantation responses through a Th2 cell adoptive transfer strategy. Although in vivo administration of rapamycin can modulate alloreactivity by several potential mechanisms, rapamycin as a single agent does not appear efficacious when both CD4⁺ and CD8⁺ immune pathways are operational. In the approach we have developed, high-dose ex vivo rapamycin generates a Th2 cell product that regulates both CD4⁺ and CD8 arms of immunity post-BMT through an IL-4-dependent mechanism. As such, we have demonstrated that it is possible to dissociate a beneficial immune suppression property of rapamycin from systemic drug toxicity. Further studies involving this in vitro pharmacologic approach to T cell therapy will seek to optimize Th1/Th2 cellular therapy for the goal of modulating graft rejection, GVL and GVT effects and GVHD.

Disclosures

The authors have no financial conflict of interest.

References


