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Ex Vivo Rapamycin Generates Apoptosis-Resistant Donor Th2 Cells That Persist In Vivo and Prevent Hemopoietic Stem Cell Graft Rejection1

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Because ex vivo rapamycin generates murine Th2 cells that prevent Graft-versus-host disease more potently than control Th2 cells, we hypothesized that rapamycin would generate Th2/Tc2 cells (Th2/Tc2.R cells) that abrogate fully MHC-disparate hemopoietic stem cell rejection more effectively than control Th2/Tc2 cells. In a B6-into-BALB/c graft rejection model, donor Th2/Tc2.R cells were indeed enriched in their capacity to prevent rejection; importantly, highly purified CD4+ Th2.R cells were also highly efficacious for preventing rejection. Rapamycin-generated Th2/Tc2 cells were less likely to die after adoptive transfer, accumulated in vivo at advanced proliferative cycles, and were present in 10-fold higher numbers than control Th2/Tc2 cells. Th2.R cells had a multifaceted, apoptosis-resistant phenotype, including: 1) reduced apoptosis after staurosporine addition, serum starvation, or CD3/CD28 costimulation; 2) reduced activation of caspases 3 and 9; and 3) increased anti-apoptotic Bcl-xL expression and reduced proapoptotic Bim and Bid expression. Using host-versus-graft reactivity as an immune correlate of graft rejection, we found that the in vivo efficacy of Th2/Tc2.R cells 1) did not require Th2/Tc2.R cell expression of IL-4, IL-10, perforin, or Fas ligand; 2) could not be reversed by IL-2, IL-7, or IL-15 posttransplant therapy; and 3) was intact after therapy with Th2.R cells relatively devoid of Foxp3 expression. We conclude that ex vivo rapamycin generates Th2 cells that are resistant to apoptosis, persist in vivo, and effectively prevent rejection by a mechanism that may be distinct from previously described graft-facilitating T cells. The Journal of Immunology, 2008, 180: 89–105.

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that allogeneic Th1/Tc1 cells generated in rapamycin mediated increased GVHD relative to control Th1/Tc1 cells (17). As such, the first goal of our studies was to evaluate whether rapamycin-generated Th2/Tc2 cells (Th2/Tc2.R cells) might more effectively prevent graft rejection relative to control Th2/Tc2 cells and to determine whether alloengraftment could be accomplished without lethal GVHD. To increase the stringency of our evaluation, we reduced the donor Th2/Tc2 cell to host T cell ratio from 100:1 (used in our previous studies) to 10:1. In addition, given the recent emergence of CD4+ Treg cells as a population capable of abrogating rejection (12–14), we also explored the effect of purified CD4+ Th2.R cells in this rejection model.

In immunotherapy models, the efficacy of adoptive T cell transfer appears to be determined in large part by the capacity of the transferred T cells to persist in vivo (18, 19). As such, we hypothesized that an enhanced efficacy of rapamycin-generated T cell therapy may be associated with an increase in T cell survival post-transplant. An ability of rapamycin to confer a T cell survival advantage seems somewhat paradoxical because receptor signaling for the prosurvival cytokines IL-7 (20) and the prosurvival co-stimulatory signal CD28 (21, 22) used in our ex vivo T cell expansion method are dependent in part upon mammalian target of rapamycin (mTOR) function. In contrast, mTOR inhibition can mediate antiapoptotic effects in several experimental models (reviewed in Ref. 23). Furthermore, rapamycin-resistant human CD8+ T cells have increased in vitro survival that may be mediated through increased expression of antiapoptotic Bcl-xL (24). Given this information, the second goal of our project was to perform serial T cell tracking studies to compare the in vivo persistence of Th2/Tc2.R cells to control Th2/Tc2 cells in the graft rejection model and to further characterize any observed antiapoptotic property of rapamycin-generated T cells.

Finally, the third goal of our project was to develop a method to monitor host-versus-graft reactivity (HVGR) as an immune correlate of graft rejection and to use this method to identify candidate mechanisms of Th2/Tc2 cell abrogation of rejection. Because the number of donor T cells capable of IFN-γ secretion in response to alloantigen is a suitable measure of graft-vs-host reactivity (GVHR) (25, 26), we reasoned that quantification of host T cells capable of IFN-γ secretion in response to donor alloantigen might represent a marker of HVGR. To quantify HVGR, posttransplant T cells were harvested, stimulated with either syngeneic host APC or CD4+ T cells modulated immunologically defined HVGR in vivo and to further characterize any observed antiapoptotic property of rapamycin-generated T cells.

HSC and T cell characterization by flow cytometry

Cells from HSC culture were harvested, resuspended in HBSS with FCS (2%) and HEPES (10 mM), and incubated with Hoechst 33342 (5 μg/ml; Sigma-Aldrich) for 90 min at 37°C without or with fumitromorgin C (10 μM; obtained from the Developmental Therapeutics Program, National Cancer Institute). Cells were stained for 10 min on ice with c-Kit, MHC class I, lineage markers, CD4 (H11001), CD8 (H11003), CD11b (H11002), and the side population (SP) was identified (28). Foxp3 expression within ex vivo-expanded T cells was determined by an intracellular analysis, Th2/Tc2 cells and Th2/Tc2.R cells were labeled with 1 × 106 or 1.0 × 106 cells). Mice were observed daily for survival. In some experiments, mice were injected (i.p. days 0 – 4 post-transplant) either with rhIL-2 (75,000 IU twice per day), rhIL-7 (10 μg/ day), or rhIL-15 10 μg/day (each cytokine obtained from Peprotech). T cells were expanded without or with 10 μM rapamycin (sirolimus; Sigma-Aldrich). Media were added to maintain T cells at 0.2–1.0 × 106 cells/ml. In some experiments, CD4+ or CD8+ cells were enriched before culture (CD4/CD8 selection kits; Stem Cell Technologies); cell purity before and after expansion was >90%. When CD4+CD25+ and CD4+CD25− cells were enriched before culture (Treg isolation kit; Miltenyi Biotec), purity was >92% and >98%, respectively.

Materials and Methods

**Animals**

C57BL/6 (B6, H-2Kb), BALB/c (H-2Kb), and C57BL/6 (B6, H-2Kb) Ly5.1-congenic (H-2Kb) mice were obtained from the Frederick Cancer Research Facility. B6 mice genetically deficient in IL-4, IL-10, IL-15R, perforin, and FasL were obtained from The Jackson Laboratory. Mice were maintained in a specific pathogen-free facility and treated according to an approved animal protocol.

**Hemopoietic stem cell (HSC) product**

B6 mice were injected with G-CSF (Amgen; 5 μg/day, i.p. 5 days). Spleen cells were harvested and mononuclear cells were separated (Histopaque-1077; Sigma-Aldrich) and cultured for 7 days in Stem-Span Media (Stem Cell Technologies) supplemented with penicillin-streptomycin-glutamine (Invitrogen Life Technologies), recombinant human (rh) thrombopoietin (TPO; 20 ng/ml), recombinant mouse (rm) stem cell-derived factor (SCF; 40 ng/ml), and rmFlt3L (20 ng/ml); cytokines from PeproTech. When T cell content of the HSC product was >1% by flow cytometry, T cell depletion was performed (anti-CD4/anti-CD8 beads; Miltenyi Biotec).

**Generation of cytokine-polarized T cells in rapamycin**

Spleens from Ly5.1 mice were harvested, RBC were lysed (ACK [ammonium chloride, potassium bicarbonate]; Quality Biologicals), and B cells were depleted (goat anti-mouse bioparticles; Polysciences). Anti-CD3- and anti-CD28-coated beads (CD3/CD28 beads) were produced as previously detailed (26); T cells were stimulated with CD3/CD28 beads (CD3/CD28 cultures; 20 IU/ml for purified CD4+ Th2 cultures, rhIL-7 (20 ng/ml), and rmIL-4 (1000 IU/ml); for Th1 cultures, CD4+ T cells were expanded in CM supplemented with rhIL-2 (20 IU/ml), rhIL-7 (20 ng/ml), 11B11 (10 μg/ml) and rmIL-12 (10 μg/ml; cytokines obtained from PeproTech). T cells were expanded without or with 10 μM rapamycin (sirolimus; Sigma-Aldrich) for 90 min on ice with c-Kit, MHC class I, lineage markers, CD4 (H11001), CD8 (H11003), CD11b (H11002), and the side population (SP) was identified (28). Foxp3 expression within ex vivo-expanded T cells was determined by an intracellular flow cytometry staining kit (eBioscience). To measure intracellular rapamycin, Th2/Tc2 cultures; 20 IU/ml for Th2/Tc2 cultures; 20 IU/ml for purified CD4+ Th2 cultures, rhIL-7 (20 ng/ml), and rmIL-4 (1000 IU/ml); for Th1 cultures, CD4+ T cells were expanded in CM supplemented with rhIL-2 (20 IU/ml), rhIL-7 (20 ng/ml), 11B11 (10 μg/ml) and rmIL-12 (10 μg/ml; cytokines obtained from PeproTech). T cells were expanded without or with 10 μM rapamycin (sirolimus; Sigma-Aldrich). Media were added to maintain T cells at 0.2–1.0 × 106 cells/ml. In some experiments, CD4+ or CD8+ cells were enriched before culture (CD4/CD8 selection kits; Stem Cell Technologies); cell purity before and after expansion was >90%. When CD4+CD25+ and CD4+CD25− cells were enriched before culture (Treg isolation kit; Miltenyi Biotec), purity was >92% and >98%, respectively.

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**Graft rejection model**

BALB/c mice were lethally irradiated (day −2; 1050 cGy; 137Cs gamma radiation, gamma Cell 40; Atomic Energy of Canada). Host T cells (1.0 × 106 or 1.0 × 106 cells) were administered i.v. on day −1. Other treatment cohorts received day 0 i.v. injection of the donor HSC product (10 × 106 cells) without or with separate i.v. injection of donor Th2/Tc2 cells or Th2/Tc2.R cells (10 × 106 or 1.0 × 106 cells). Mice were observed daily for survival. In some experiments, mice were injected (i.p. days 0–4 post-transplant) either with rhIL-2 (75,000 IU twice per day), rhIL-7 (10 μg/day), or rhIL-15 10 μg/day (each cytokine obtained from PeproTech).

**Chimerism, proliferation, and apoptosis**

For chimerism analysis, spleen, lymph node, and bone marrow were harvested, and single cells were labeled with Abs against H-2Kb, H-2Kb, CD45.1, CD4, and CD8 conjugated with FITC, PE, allophycocyanin, PE-Cy5, and allophycocyanin-Cy7 (BD Pharmingen). For in vivo proliferation analysis, Th2/Tc2 cells and Th2/Tc2.R cells were labeled with 1 μM CFSE.
FIGURE 1. Donor HSC-enriched population: characterization and host radioprotection.

**a**, Functional characterization. Donor B6 mice were injected i.p. with G-CSF for 5 days, single splenic cells were isolated, and the SP was identified by Hoechst dye efflux assay before ex vivo culture days 0 and after culture with rhTPO, rmSCF, and rmFLTL3 (day 7). Specificity of the assay was confirmed by inclusion of the efflux pump inhibitor, fumitromorin C (+inhibitor). **b**, Cell composition. Percentages of SP and lineage ‘Sca-1−/kit+’ (KLS) cells on days 0 and 7 of culture are shown (left and middle panels). Expansion of total cells, SP cells, and KLS cells was determined after 7 days of culture (right panel). Results are mean ± SEM of nine experiments. *p < 0.0001. **c**, Host radioprotection. Fully MHC-mismatched BALB/c hosts were lethally irradiated (1050 cGy) and either not reconstituted (“Co-matched BALB/c hosts were lethally irradiated and day 7 of culture differ from day 0”) or stimulated with CD3/CD28 beads, B6 dendritic cells (DC), or BALB/c murine marrow cells (cohort E). Overall survival (left panel) and body weight change (right panel) were monitored until day 90 posttransplant.

(Molecular Probes). T cell death and T cell apoptosis in vivo were determined by identifying CD45.1+ cells positive for Hoechst 33258 or annexin V-allophycocyanin (BD Pharmingen), respectively. For the in vitro proliferation analysis to assess T cell resistance to rapamycin, Th2 and Th2R cells were harvested and washed on day 6 of culture, labeled with 1 μM 5(6)-CFDA SE (Carboxyfluorescein Diacetate Succinimidyl Ester) and restimulated with CD3/CD28-coated beads (1:3 ratio) in CM supplemented with N-acetyl-1-cysteine (3.3 mM), rhIL-2 (20 IU/ml), and rhIL-7 (20 ng/ml) without or with rapamycin (0.1–10 μM). For the in vitro apoptosis assay, Th2, Th2R, Th1, or Th1R cells were harvested on day 6 of culture, washed, and resuspended at 0.5 × 106 cells/ml. Cells were subsequently not stimulated in CM, restimulated in CM with CD3/CD28 beads (1:3 ratio), and kept in CM containing staurosporine (1 μM; Sigma-Aldrich), or 4 resuspended in HBSS containing phenol red and no protein source (Invitrogen Life Technologies). To evaluate whether donor T cell subsets were differentially sensitive to host T cell cytotoxicity, donor CD45.1+ Th2 and Th2R cells were mixed with alloreactive host T cells that were generated in vivo in the graft rejection model; such alloreactive host T cells were harvested from rejection controls at day 6 posttransplant and were >90% pure for host T cell markers by flow cytometry.

**Quantification of HVGR using cytokine capture flow cytometry**

Posttransplant spleen cells (0.5 × 106 cells/ml) were either not stimulated or stimulated with CD3/CD28 beads, B6 dendritic cells (DC), or BALB/c DC generated by culturing marrow cells for 6 days in rmGM-CSF and rmIL-4 (each at 1000 IU/ml; PeproTech). Bacterial LPS (1 μg/ml) without or with rapamycin (0.1–10 μg/ml) or 10 μg/ml without or with rapamycin (0.1–10 μM). For the in vitro apoptosis analysis, Th2, Th2R, Th1, or Th1R cells were harvested on day 6 of culture, washed, and resuspended at 0.5 × 106 cells/ml. Cells were subsequently not stimulated in CM, restimulated in CM with CD3/CD28 beads (1:3 ratio), and kept in CM containing staurosporine (1 μM; Sigma-Aldrich), or 4 resuspended in HBSS containing phenol red and no protein source (Invitrogen Life Technologies). To evaluate whether donor T cell subsets were differentially sensitive to host T cell cytotoxicity, donor CD45.1+ Th2 and Th2R cells were mixed with alloreactive host T cells that were generated in vivo in the graft rejection model; such alloreactive host T cells were harvested from rejection controls at day 6 posttransplant and were >90% pure for host T cell markers by flow cytometry.

**Characterization of posttransplant cytokine phenotype**

Recipient spleen cells were harvested (day 5 after HSCT), RBC were lysed (Accurat Tek), and cells were adjusted to 0.5 × 106 cells/ml in 24-well plates with or without CD3/CD28 bead restimulation. Culture supernatants (24 h) were collected and cytokine content was evaluated by multiplex bead array (Bio-Rad).

**Western blot analysis**

Protein isolated from whole cells or fraction lysates were run on 10–20% SDS-PAGE gels and electrophoretically transferred to nitrocellulose. Nitrocellulose blots were blocked with 5% milk in TBST buffer (20 mMmL Tris-HCl, 500 mMmL NaCl, and 0.01% Tween 20) and incubated (overnight, 4°C) with the appropriate primary Ab in TBST containing 5% milk. Immunoreactivity was detected by sequential incubation with HRP-conjugated secondary Abs and enzyme chemiluminescence (Cell Signaling Technology). Abs used included: anti-Bim, -Bid, -Bcl-xL, -Bcl-2, -Bad, -Bax, -phospho(Ser112)-Bad, -Bank3, -asp3, -cas3-8, -cas9-2, -cleaved poly(ADP-ribose) polymerase (PARP), -mTOR,-phospho(Ser244)-mTOR, -4EBP1,-phosphoSer334,Ser377)-S6 ribosomal protein, -S6 ribosomal protein, Akt, -phosphoSer473-Akt, p27(S19)-1, glycogen synthase kinase (GSK) β, -phospho (Ser9)-GSK-3β, -phospho (Ser235,Ser237)-GSK-3β (all from Cell Signaling Technology); anti-β-actin, Pim-1, and Pim-2 were purchased from Santa Cruz Biotechnology.

**Statistics**

Survival analysis was performed according to the Kaplan-Maier method and survival curves were compared using log-rank testing. Flow and cytokine data were analyzed using Student’s two-tailed t tests. Values of p < 0.05 were considered statistically significant.

**Results**

Donor HSC-enriched population: generation, characterization, and host radioprotection

Increasing donor HSC dose improves transplantation across genetic barriers by exploiting the tolerance-inducing activity of Lin− Sca-1− cells (9) and by accelerating immune reconstitution (29). We therefore used a model that incorporated transfer of donor populations enriched for stem cell characteristics; other investigators have studied graft rejection in models using defined HSC isolated by flow sorting (30). To generate enriched HSC, G-CSF mobilization was performed (31) and resultant cells were incubated with SCF, Flt3L, and TPO (32). The stem cell frequency, as defined by the SP assay (28), increased from 0.2% at culture initiation to 2.2% at the end of culture (representative example, Fig. 1a). Stem cell enrichment was consis-
Donor Th2/Tc2.R cells abrogate rejection without lethal GVHD

The donor:host T cell ratio was held constant at 10:1. In experiments involving 1 × 10^6 donor Th2/Tc2 cells and 0.1 × 10^6 host T cells (Fig. 2a), Th2/Tc2.R cells prevented rejection more effectively than control Th2/Tc2 cells. Recipients of allografts augmented with Th2/Tc2.R cells (cohort D) had long-term survival (10 of 10 cases; Fig. 2a) and full donor chimerism (9 of 10 cases; Fig. 2a). By comparison, recipients of control Th2/Tc2 cells (cohort E) had long-term survival in only 3 of 10 cases; death was attributed to graft rejection, as day 30 donor chimerism in evaluable recipients was 0, 0, and 27%. Prevention of rejection through Th2/Tc2.R cell therapy did not cause clinical GVHD (absence of hunched posture, diarrhea, ruffled hair) but was associated with 5–10% weight loss relative to recipients of HSC incula alone (Fig. 2a). Relative to recipients of HSC alone, Th2/Tc2.R cell recipients had modestly impaired CD4^+ and CD8^- T cell reconstitution (Fig. 2a).

While maintaining this ratio of donor:host T cells (10:1), we evaluated whether donor Th2/Tc2 cells might prevent graft rejection mediated by a 1-log increase in host T cell number. Graft augmentation with Th2/Tc2.R cells prevented rejection, as 6 of 10 recipients had long-term survival (Fig. 2b; cohort D) and full donor chimerism (5 of 10 cases; Fig. 2b). In contrast, 10 of 10 recipients of control Th2/Tc2 cells died in parallel with rejection controls. Death in...
Th2/Tc2.R cell recipients was attributed in part to GVHD, because full donor chimerism at day 30 posttransplant was observed in 8 of 9 evaluable recipients (data not shown). GVHD was increased relative to Th2/Tc2.R cell therapy at the 1 × 10^6 cell dose, as high-dose Th2/Tc2.R cell recipients had 15–20% weight loss and more severely impaired T cell reconstitution (Fig. 2b).

In vivo tracking of Th2/Tc2 vs Th2/Tc2.R cells

We investigated whether the enhanced capacity of Th2/Tc2.R cells to prevent graft rejection was associated with increased Th2/Tc2.R cell persistence in vivo relative to control Th2/Tc2 cells. In the rejection control cohort, graft rejection was apparent in the spleen at day 5 posttransplant and in the lymph node and marrow by day 8 posttransplant (Fig. 3b). In recipients of control donor Th2/Tc2 cells, Th2/Tc2 cells were present in the spleen, lymph node, and marrow at day 5 posttransplant (Fig. 3a); however, at days 8 and 14 posttransplant, there was a progressive and dramatic decline in the number of Th2/Tc2 cells at each site evaluated. This in vivo reduction in donor Th2/Tc2 cell number was associated with an absence of alloengraftment (Fig. 3b).

In marked contrast, recipients of donor Th2/Tc2.R cells had a higher number of Th2/Tc2 cells in the spleen, lymph node, and marrow, and marrow at day 5 posttransplant relative to control Th2/Tc2 cell recipients and persistence of high Th2/Tc2 cell numbers at days 8 and 14 posttransplant. This in vivo persistence of Th2/Tc2.R cells was associated with donor engraftment in spleen, lymph node, and bone marrow at days 8 and 14 posttransplant (Fig. 3b).

Finally, recipients of the purified CD4^+ Th2.R cell population had 1) a dramatic in vivo expansion of the administered Th2.R cells in the spleen, lymph node, and marrow; 2) in vivo persistence of Th2.R cells at days 8 and 14 posttransplant; and 3) comparable donor engraftment at each time point and each organ tested relative to recipients of Th2/Tc2.R cells. As such, donor Th2.R cells appeared to have a similar capacity as Th2/Tc2.R cells to prevent graft rejection. To confirm the ability of purified Th2.R cells to prevent rejection, an additional long-term experiment was performed: at day 90 posttransplant, five of five recipients of donor Th2.R cells survived posttransplant, did not have clinical evidence of GVHD (similar to results with Th2/Tc2.R cells, a 5–10% persistent weight loss was observed), and had complete donor elements (>95%) at both days 30 and 90 posttransplant.

Increased Th2/Tc2.R cell number: reduced cell death and increased proliferation

Additional experiments were performed to determine the contribution of reduced Th2/Tc2 cell death or increased Th2/Tc2 cell proliferation to the observed in vivo persistence of the rapamycin-generated T cells. Using CFSE-labeled control Th2/Tc2 cells and Th2/Tc2.R cells, we found that Th2/Tc2.R cells had reduced cell death in vivo (Fig. 4a; percentage of Th2/Tc2 cells dead at day 2 posttransplant, 65 ± 2 vs 33 ± 1, p < 0.0001). Furthermore, there was a numerically modest but statistically significant reduction of in vivo apoptosis for CD4^+ Th2.R cells relative to control Th2 cells (Fig. 4b, left panel, percent apoptotic cells, 28 ± 31 ± 1, p = 0.04) and for CD8^+ Tc2.R cells relative to control Tc2 cells (50 ± 1 vs 55 ± 2, p = 0.03). The absolute number of nonapoptotic Tc2 cells in vivo was greater for Th2.R cells compared with Th2 cells (Fig. 4b, middle panel, cell number, ×10^6/spleen; 100 ± 0.4 vs 0.4 ± 0.02, p < 0.0001) and for CD8^+ Tc2.R cells relative to control Tc2 cells (0.70 ± 0.07 vs 0.10 ± 0.01, p < 0.0001). In contrast, the absolute number of apoptotic T cells in vivo was not significantly different for Th2.R cells compared with Th2 cells (Fig. 4b, right panel; 0.24 ± 0.02 vs 0.18 ± 0.01, p = NS); Tc2.R cells had reduced antiapoptotic characteristics in vivo relative to Th2.R cells, as the absolute number of apoptotic Tc2.R cells was increased relative to control Tc2 cells (0.70 ± 0.06 vs 0.20 ± 0.01, p < 0.0001).

Cell cycle analysis identified further in vivo differences between the adoptively transferred Th2/Tc2.R cells and control Th2/Tc2 cells. Control Th2/Tc2 cells were most commonly observed in vivo to have progressed through a relatively low number of cell divisions (three cell divisions; Fig. 4c, left panel). In contrast, Th2/Tc2.R cells predominated in vivo at a higher number of cell divisions (four and five cell divisions). Because of the increase in total number of Th2/Tc2.R cells in vivo, the absolute number of Th2/Tc2.R cells in each cell cycle number (divisions one through six) was increased relative to the control Th2/Tc2 cell number (Fig. 4c, right panel).

It is also possible that the reduced in vivo cell death of rapamycin-generated T cells might in part be due to a reduced T cell susceptibility to the host-vs-graft rejection response. As a step toward evaluating this possibility, the following experiment was conducted: 1) in vivo allosensitization was performed using the graft rejection model; 2) on day 6 posttransplant, splenic host T cells (>90% purity) from rejection controls were harvested and used as effectors; and 3) donor Th2.R or control Th2 cells were generated and evaluated as targets in an in vitro apoptosis assay. Relative to control Th2 cells, Th2.R cells indeed had reduced apoptosis in response to the allosensitized host T cells (Fig. 4d). As such, the increased number of donor Th2.R cells observed posttransplant may relate to reduced apoptosis, increased proliferation, and reduced target cell susceptibility to host anti-donor alloreactivity.

Th2.R cells have reduced apoptotic tendency via the extrinsic pathway

Additional experiments were performed to characterize the reduced apoptotic tendency observed with rapamycin-generated Th2 cells. To evaluate whether rapamycin-generated Th2 cells had a reduced apoptotic tendency via the extrinsic pathway (33), apoptosis of Th2.R cells and control Th2 cells was determined after CD3 and CD28 costimulation. At 7 h after costimulation, 54.7 ± 1.8% of control Th2 cells were annexin V^+ (Fig. 5a, right panel; n = 16); in marked contrast, only 11.2 ± 2.4% of Th2.R cells were annexin V^+ (Fig. 5a, right panel; p < 0.0001, n = 16).

The mechanism of extrinsic pathway apoptosis after T cell costimulation is mediated in part through Fas receptor signaling and subsequent caspase activation (34); in addition, granzyme B has been identified as an effector molecule of Th2 cell apoptosis (35). Given this information, further studies were performed to determine whether modulation of these effector pathways might influence apoptosis of Th2 cells generated either with or without rapamycin. First, with respect to the Fas pathway, we observed a modest reduction of Fas expression in Th2.rapa cells (Fig. 5a, left panel; percent CD4^+ CD95^+ cells; 60.4 ± 1.2% (control Th2 cells) vs 47.1 ± 1.6% (Th2.rapa cells); p < 0.05, n = 5). Control Th2 cells generated from Fas-deficient lpr mice had greatly reduced apoptosis relative to wild-type (WT) control Th2 cells (Fig. 5b; percent CD4^+ annexin V^+ cells, reduced from 66.6 ± 1.8% to 28.8 ± 4.5%; p = 0.001, n = 3). However, such Fas-deficient control Th2 cells had a much greater apoptotic tendency relative to WT Th2.rapa cells (percent CD4^+ annexin V^+ cells, 7.0 ± 0.4%). Of particular note, generation of Th2.rapa cells from Fas-deficient CD4^+ T cells did not further reduce the apoptotic tendency (percent CD4^+ annexin V^+ cells, 8.1 ± 0.6%).

Second, with respect to the potential role of granzyme B, we found no difference in granzyme B expression between Th2 and Th2.rapa cells (Fig. 5a, middle panel; percent CD4^+ granzyme B^+...
FIGURE 3. Posttransplant persistence of donor Th2/Tc2 vs Th2/Tc2.R cells. B6-into-BALB/c transplantation consisted of 1050 cGy host irradiation followed by donor HSC infusion alone (SCT), host T cell infusion alone (Host T; HT); or donor HSC and 1 × 10^6 host T cells infusion alone (SCT,HT) or in combination with 10 × 10^6 of donor Th2/Tc2 cells (SCT,HT,TH2/Tc2), donor Th2/Tc2.R cells (SCT,HT,TH2/Tc2R), or donor Th2.R cells (SCT,HT,TH2R). Spleen, lymph node, and bone marrow cells were isolated and analyzed by flow cytometry on days 5, 8, and 14 posttransplant. Results shown are mean ± SEM of n = 3–5/cohort per time point (day 14 time point for the SCT,HT cohort is not displayed, because all mice were dead by day 8). a, Number of CD45.1+ donor T cells: ** indicates that values for the SCT control cohort and Th2/Tc2R and Th2R recipients are different relative to the SCT,HT cohort (p < 0.0001); * indicates that values in recipients of Th2/Tc2, Th2/Tc2R, or Th2R inocula are different relative to the SCT,HT cohort (p < 0.05); and *** indicate that values in recipients of SCT, Th2/Tc2R, or Th2R are different relative to recipients of Th2/Tc2 cells (p < 0.05).
FIGURE 4. Increased number of donor Th2/Tc2.R cells posttransplant: contribution of reduced cell death and increased proliferation. Donor Th2/Tc2 cells and Th2/Tc2.R cells were stained with CFSE dye before infusion. B6-into-BALB/c transplantation consisted of 1050 cGy host irradiation followed by infusion of host T cells, donor Th2/Tc2 cells, or donor Th2/Tc2.R cells. On day 2 posttransplant, spleen cells were isolated and CFSE+H-2K^b+ cells were detected by flow cytometry. Results shown in a–c are mean ± SEM of n = 15/cohort. a, Percentage of dead cells was evaluated by gating for Hoechst 33258-positive cells among CFSE+H-2K^b+ cells. b, Percentage and absolute number of apoptotic and nonapoptotic cells among alive CFSE+H-2K^b+ cells were evaluated by annexin V staining. * indicates values in Th2/Tc2.R cell recipients different relative to the Th2/Tc2 cell cohort (p < 0.05). c, Number of donor T cells was calculated by applying percentage of cells per cycle to spleen cell numbers. * indicates that values in Tc2.R cell recipients are different relative to T2 cell cohort (p < 0.05). ** indicates that values in recipients of T2 cells are different relative to T2R cell cohort (p < 0.05). d, B6-into-BALB/c transplantation consisted of the rejection control cohort (irradiated host on day −2, host T cell add-back on day −1, and donor hemopoietic cell inocula on day 0). On day 6 posttransplant, alloreactive host splenic T cells were isolated (as effector cells) and mixed with CD45.1+ donor Th2 or Th2R cells (as target cells) at target: effector ratios of 1:1, 5:1, and 10:1. The percentage of CD45.1+CD4+ annexin V− cells among live cells was determined on day 3 of coculture. Results shown are from n = 3 experiments; * indicates Th2.R cell apoptosis reduced relative to control Th2 cells (p < 0.05).

cells: 51.4 ± 14.2 (control Th2 cells) vs 46.6 ± 13.1% (Th2.rapa cells); p = NS, n = 6). Apoptosis of control Th2 cells was reduced by granzyme B inhibition in a dose-dependent manner (Fig. 5b, 100 μM concentration of inhibitor; percent CD4+ annexin V− cells reduced from 66.6 ± 1.8% to 36.5 ± 3.4%; p = 0.001, n = 3). Inhibition of granzyme B further reduced Th2.rapa cell apoptotic tendency (percent CD4+annexin V− cells, reduced from 7.0 ± 0.4% to 4.1 ± 0.2%).

Third, with respect to the potential role of caspases, we found that caspase 8 was similarly cleaved in Th2 and Th2.R cells (Fig. 5c). Control Th2 cells had activation of caspase 3 and cleavage of PARP; however, in marked contrast, Th2.rapa cells had reduced caspase 3 activation and PARP cleavage. Concomitantly, caspase 9 cleavage was dramatically reduced in Th2.R cells. Pan-caspase inhibition greatly reduced control Th2 cell apoptosis (Fig. 5c, right panel; percent CD4+ annexin V− cells reduced from 66.6 ± 1.8% to 32.5 ± 0.5% and to 34.6 ± 5% (Z-vad-fmk concentrations of 50 or 200 μM, respectively); p < 0.005). Caspase inhibition further reduced Th2.rapa cell apoptotic tendency (percent CD4+ annexin V− cells reduced from 7.0 ± 0.4% to 3.1 ± 0.1%). As such, Th2.rapa cells had a markedly reduced propensity to apoptosis through an extrinsic pathway that appeared to operate via both caspase-dependent and caspase-independent pathways.

Th2.R cells have differential expression of Bcl-2 family genes

Because the balance of pro- and antiapoptotic members of the Bcl-2 gene family help determine apoptotic tendency (36), we evaluated whether such genes were differentially expressed in Th2 and Th2.R cells (Fig. 5d). We observed three differences in Bcl-2...
family gene expression: reduced expression of the proapoptotic genes Bim (37) (five of five cases) and Bid (38) (five of five cases) and increased expression of the antiapoptotic gene Bcl-x<sub>L</sub> (39) (five of five cases). Th2 and Th2.R cells did not appear to differentially express other Bcl-2 family genes having antiapoptotic properties (Bcl-2) or proapoptotic properties (Bax, Bak, Bad).

Both Th2.R and Th1.R cells have reduced apoptotic tendency

In previous experiments (17), we found that adoptive transfer of Th1-type cells generated in rapamycin yielded increased Th1 cell number in vivo and increased GVHD relative to control Th1 cell adoptive transfer. It was therefore of interest to determine whether the antiapoptotic tendency of rapamycin-generated T cells was a general phenomenon applicable to both Th2- and Th1-polarized subsets. To test this possibility, the apoptotic tendency of Th1 vs Th1.R cells and Th2 vs Th2.R cells was determined in response to challenge by costimulation, staurosporine, or serum starvation. Indeed, relative to control Th1 cells, Th1.R cells had reduced apoptosis after costimulation (Fig. 5e, left panel; percent annexin<sup>+</sup> cells, 40 ± 5 vs 20 ± 1, p = 0.003); however, Th2.R cells had the lowest rate of apoptosis after costimulation. We further compared the influence of rapamycin on Th1 and Th2 cell apoptosis via the intrinsic pathway through staurosporine challenge (40) and serum deprivation challenge (41). Both Th1.R and Th2.R cells had reduced apoptosis after staurosporine challenge, with Th2.R cells having the lowest rate of apoptosis. Similarly, with serum deprivation, Th2.R cells had a statistically significant reduction in apoptosis whereas the observed reduction of apoptosis in Th1.R cells did not reach statistical significance. In sum, these data indicate that rapamycin reduces both Th1 and Th2 cell apoptosis through both extrinsic and intrinsic pathways, with the effect on Th2 cell biology being more pronounced.

Characterization of Th2 cell rapamycin resistance: T cell expansion and proliferation

Upon repeat costimulation, control Th2 cell expansion was reduced in a dose-dependent manner by rapamycin (Fig. 6a, fold expansion reduced from 5.2 ± 0.4 (no rapamycin) to 1.4 ± 0.1 (10 μM rapamycin), p = 0.01). In contrast, Th2 cells generated in rapamycin were nominally inhibited by rapamycin (fold expansion of 2.6 ± 0.5 (no rapamycin) vs 1.8 ± 0.2 (10 μM rapamycin), p = NS). To evaluate rapamycin resistance in terms of cell proliferation, CFSE-labeled Th2 and Th2.R cells were costimulated and expanded in the presence or absence of rapamycin. Control Th2 cells had a high proliferative rate in the absence of rapamycin, with the majority of Th2 cells residing in cycle 3 (Fig. 6b, percentage of cells, 33 ± 0.8) and cycle 4 (percent, 25 ± 1). Remarkably, at the lowest rapamycin concentration tested (0.1 μM), Th2 cell capacity to progress to cycle 4 was completely abrogated; Th2 cell progression to cycle 3 was also significantly reduced by rapamycin, with no Th2 cells detected in cycle 3 at the highest rapamycin concentration tested (10 μM). By comparison, Th2.R cells had reduced proliferation in the absence of rapamycin, with the majority of cells in cycle 2 (percent, 44 ± 4) and cycle 3 (percent, 21 ± 0.5) and no cells detectable in cycle 4. Progression of Th2.R cells to cycle 3 was only partially inhibited by lower concentrations of rapamycin (percentage of cells, reduced from 21 ± 0.5 (no rapamycin) to 16 ± 3 (0.1 μM) and to 8 ± 5 (1.0 μM)) and more significantly inhibited by 10 μM rapamycin (percentage of cells, reduced to 2 ± 2). Remarkably, there was no apparent inhibitory effect of rapamycin on Th2.R cell capacity to proliferate to cell cycle 2. As such, Th2.R cells had a reduced intrinsic proliferative rate in the absence of rapamycin and were relatively resistant to subsequent rapamycin exposure.

**FIGURE 5.** Ex vivo expansion in Th2-generated Th2.R and Th1.R cells with reduced apoptotic tendency via the extrinsic and intrinsic pathways. a. After 6 days of ex vivo expansion in the presence or absence of rapamycin, Th2 and Th2.R cells were evaluated for surface expression of CD95 and intracellular expression of granelyme B (left and middle panels). Subsequently, Th2 and Th2.R cells were harvested and tested for their apoptotic tendency by the extrinsic pathway via costimulation with CD3/CD28-coated beads (right panel). PI<sup>+</sup> cells were excluded and apoptosis was determined by percentage of CD4<sup>+</sup> annexin V<sup>+</sup> cells at 7 h after the apoptotic stimulus. b. Extrinsic pathway apoptotic tendency was evaluated at 7 h after costimulation with CD3/CD28-coated beads either in WT or Fas-deficient (KO) Th2 and Th2.R cells and in the presence of different concentrations of the granelyme B inhibitor Z-aad-cmk. Results shown are mean ± SEM of n = 3–16 experiments. c. Cell lysates from Th2 cells (Rapamycin) and Th2.R cells (Rapamycin +) were used for Western blot analysis of members of the caspase family (left panel). Blots shown are from different experiments. β-actin served as the loading control. Th2 and Th2.R cell extrinsic pathway apoptotic tendency was evaluated in the presence of the pan-caspase inhibitor Z-vad-fmk (right panel). Results shown are mean ± SEM of n = 3 experiments. a–c. * indicates that values for Th2.R cells are different relative to Th2 cells (p < 0.05), whereas ** indicates that values for WT Th2 cells stimulated in the presence of granelyme or caspase inhibitor are different relative to control Th2 cells (p < 0.05). d. Cell lysates from Th2 cells (Rapamycin −) and Th2.R cells (Rapamycin +) were used for Western blot analysis of pro- and antiapoptotic members of the Bcl-2 gene family. Blots shown are from five different experiments; β-actin served as the loading control. e. Donor Th1, Th1.R, Th2, and Th2.R cells were harvested and tested for their extrinsic pathway apoptotic tendency by stimulation with CD3/CD28-coated beads and intrinsic pathway apoptosis by culture in staurosporine (1 μM) or serum starvation. PI<sup>+</sup> cells were excluded and apoptosis was expressed by percentage of CD4<sup>+</sup> annexin V<sup>+</sup> cells at 24 h after the apoptotic stimulus. * indicates that values for Th2.R or Th1.R cells are different relative to Th2 or Th1 cells (p < 0.05), respectively. Results shown are mean ± SEM of n = 6–15 experiments.

**Th2 cell rapamycin resistance: intracellular rapamycin and mTOR inhibition**

Additional experiments were performed to confirm that Th2 cells generated in rapamycin were indeed inhibited at the level of mTOR and to evaluate potential mechanisms that might contribute to Th2 cell proliferation in the setting of mTOR blockade. Because rapamycin is a known substrate for the multidrug resistance efflux pump (42), we hypothesized that the generation of Th2 cells in high-dose rapamycin may either select or induce Th2 cells with increased multidrug resistance activity and a resultant reduced propensity to accumulate intracellular rapamycin. Consistent with this hypothesis, we found that Th2.R cells had a reduced capacity to accumulate BODIPY-labeled rapamycin (Fig. 6c, left panel; mean fluorescence intensity of labeled drug reduced from 183 ± 2.6 (control Th2 cells) to 58 ± 6.5 (Th2.R cells), p < 0.0001). However, this reduced capacity to accumulate intracellular rapamycin did not appear to be physiologically relevant because Th2.R cells had greatly reduced mTOR kinase activity, as reflected by inhibition of phosphorylation of molecules downstream to mTOR, including 4EPB1 and S6 (Fig. 6c, right panel).

**Th2 cell rapamycin resistance: evaluation of potential mechanisms**

These data indicate that Th2.R cells accumulate sufficient drug to inhibit mTOR yet maintain sufficient signaling for ongoing T cell proliferation. Constitutive phosphorylation of upstream Akt
represents one potential mechanism contributing to cell proliferation in the context of mTOR blockade (43). However, this known negative feedback mechanism was not operational in our system, because Th2.R cells actually had greatly reduced phosphorylation of Akt in six of six experiments (Fig. 6d). A second potential mechanism for Th2 cell rapamycin resistance may relate to up-regulation of Pim-1 and Pim-2 kinases that confer T cell resistance to rapamycin (44, 45). Relative to control Th2 cells, we found that Th2.R cells expressed similar levels of Pim-1 (six of six cases) and Pim-2 (six of six cases). As a third

**FIGURE 6.** Characterization of Th2.R cell rapamycin resistance. Th2 and Th2.R cells were generated (culture day 6) and characterized as follows. **a,** Expansion of Th2 and Th2.R cells after repeated costimulation and expansion over 5 days of culture in rapamycin concentrations of 0, 0.1, 1, and 10 µM (fold increase from days 6 to 11 of culture, n = 3 experiments; * indicates that expansion was reduced by rapamycin). **b,** Proliferation of CFSE-labeled Th2 and Th2.R cells was evaluated 48 h after repeated costimulation. Values indicate percentage of Th2 cells advancing to a given cell cycle number (mean ± SEM; n = 3 experiments). **c,** Th2 and Th2.R cells were stained with BODIPY-labeled rapamycin to evaluate intracellular drug accumulation by flow cytometry (left panel, expressed as mean fluorescence intensity (MFI); n = 3 experiments, (*) indicates that the mean fluorescence intensity differs between Th2 and Th2.R cells. Cell lysates from Th2 cells (Rapamycin −) and Th2.R cell cells (Rapamycin +) were used for Western blot analysis of mTOR and downstream targets (right panel). **d,** Western blot expression of proteins potentially contributing to rapamycin resistance were evaluated in Th2 and Th2.R cultures (sum of n = 6 experiments shown).
**FIGURE 7.** Th2/Tc2.R cells induce type II cytokine polarization posttransplant and inhibit allospecific IFN-γ production by host T cells.  

**a.** Posttransplant cytokine phenotype. Lethally irradiated BALB/c hosts received donor B6 HSC alone (SCT), host T cell add-back alone (HT), or donor HSC and host T cell add-back either alone (SCT,HT) or in combination with donor Th2/Tc2 cells (SCT,HT,Th2/Tc2) or Th2/Tc2.R cells (SCT,HT,Th2/Tc2.R). On day 5 after SCT, spleen cells were isolated and restimulated with CD3/CD28 beads for 24 h; resultant supernatants were tested for cytokine content, with values expressed as pg/ml per 24 h per 0.5 × 10⁶ cells. Results are mean ± SEM of n = 10/cohort. (*) indicates that values in the SCT,HT cohort are different relative to the HT cohort (p < 0.05); * indicates that values in recipients of Th2/Tc2 and Th2/Tc2.R inocula are different relative to the SCT,HT cohort (p < 0.05); ** indicates that values in recipients of Th2/Tc2.R are different relative to recipients of Th2/Tc2 cells (p < 0.05).

**b.** Monitoring HVGR by cytokine capture flow cytometry. On day 5 after SCT, spleen cells were isolated and cultured for 24 h with no stimulation (left panels), host-type syngeneic DC stimulation (middle panels), or donor-type allogeneic DC stimulation (right panels). After stimulation, three-color flow cytometry using surface staining and cytokine capture reagents was used to detect host-type cells (H-2k⁺) that coexpressed CD4 or CD8 cell surface markers and secreted IFN-γ. Data shown are representative examples of each treatment cohort; values shown are the percentage of host-type CD8⁺ T cells secreting IFN-γ.

 possibility, we evaluated Th2.R cell expression of the cell cycle inhibitor p27, which is down-regulated in rapamycin-resistant human CD8⁺ T cells (24). Consistent with this previous observation, we found that Th2.R cells had reduced expression of p27 in six of six cases. Finally, as a fourth possibility, we hypothesized that rapamycin resistance in Th2.R cells might be associated with inhibition of GSK3β, which induces T cell apoptosis or cell cycle arrest (46). Of note, it has recently been shown that the mTOR and GSK3β pathways are interrelated (47). We found that the inactive, phosphorylated form of GSK3β was overexpressed in Th2.R cells relative to control Th2 cells (six of six cases).

Rapamycin-generated T cells induce marked Th2-type cytokine polarization in vivo

Additional experiments were performed to characterize the in vivo cytokine phenotype induced by graft rejection and to study the modulation of this biology by donor Th2/Tc2.R cells or control Th2/Tc2 cells. In vivo host T cell reactivity toward MHC-mismatched allografts yielded host T cells with increased IFN-γ secretion (Fig. 7a; relative to host T cell recipients not receiving HSC, increase from <100 to 9120 ± 2080 pg/ml; p = 0.007) and IL-2 secretion (<100 to 1265 ± 279 pg/ml; p = 0.009). By comparison, rejection controls had modestly increased secretion of IL-4 (<100 to 423 ± 85 pg/ml; p = 0.005), IL-5 (<100 to 64 ± 14 pg/ml; p = 0.05) and IL-10 (<100 to 258 ± 37 pg/ml; p = 0.0003). As such, graft rejection in this model was associated with a predominant Th1-type immune response.

Posttransplant T cell IFN-γ secretion was reduced from 9120 ± 2080 to 3365 ± 650 pg/ml in Th2/Tc2 cell recipients (p = 0.01) and more profoundly reduced to 995 ± 92 pg/ml in Th2/Tc2.R recipients (p = 0.004). Concomitantly, Th2/Tc2 cells increased posttransplant T cell secretion of IL-4, IL-5, and IL-10; importantly, Th2/Tc2.R cells more effectively promoted such type II cytokine secretion compared with control Th2/Tc2 cells. In sum, these data indicate that rapamycin-generated donor T cells induced...
a marked shift toward Th2-type cytokines in vivo and more effectively inhibited the increase in posttransplant IFN-γ that occurred during graft rejection.

Host antidonor allospecific IFN-γ secretion: modulation by Th2/Tc2 or Th2/Tc2.R cells

Additional experiments were performed to evaluate the capacity of Th2/Tc2.R cells or control Th2/Tc2 cells to modulate host antidonor alloreactivity in vivo. Because graft rejection was detectable in the spleen of rejection controls as early as day 5 posttransplant (Fig. 3), allospecific IFN-γ secretion by host splenic CD4+ and CD8+ T cells was analyzed by cytokine capture flow cytometry at this time point to correlate HVGR with graft rejection. Indeed, a high frequency of host CD4+ and CD8+ T cells secreted IFN-γ in an allospecific manner at the time of graft rejection (Fig. 7b, upper panels; representative host T cell cytokine capture assay). This immune response was amenable to modulation, as recipients of Th2/Tc2 cells (Fig. 7b, middle panels) and, in particular, Th2/Tc2.R cells (Fig. 7b, lower panels) had reduced frequencies of allospecific host T cells.

These results were consistently observed and relevant when expressed in terms of the absolute number of allospecific T cells (Fig. 8). Allospecific host CD4+ T cells were negligible without HSC allografting and increased to 1.2 ± 0.2 × 10^6 cells/spleen in rejection controls; IFN-γ+CD4+ host cells were negligible in rejection controls...
after syngeneic DC stimulation, thereby demonstrating assay specificity. Allospecific host CD4⁺ T cells decreased to 0.5 ± 0.2 × 10⁶ cells/spleen in Th2/Tc2 cell recipients and 0.1 ± 0.01 × 10⁶ cells/spleen in Th2/Tc2.R cell recipients. Th2/Tc2.R cell abrogation of CD4-mediated HVGR was more potent than abrogation mediated by control Th2/Tc2 cells (Fig. 5a, p = 0.008). Similarly, allospecific host CD8⁺ T cells increased from negligible values without HSC allografting to 0.9 ± 0.1 × 10⁶ cells/spleen in rejection controls. CD8-mediated HVGR was reduced to 0.4 ± 0.1 × 10⁶ cells/spleen in Th2/Tc2 cell recipients; in contrast, little or no detectable allospecific response was observed in Th2/Tc2.R cell recipients. Th2/Tc2.R cells abrogated CD8-mediated HVGR more potently than Th2/Tc2 cells (p = 0.0006).

HVGR was also evaluated by testing cytokine content of culture supernatants generated after syngeneic or allogeneic DC stimulation (Fig. 8b). Graft rejection was associated with increased IFN-γ production after alloantigen restimulation (Fig. 8b, left panel; < 50 pg/ml without HSC allografting to 956 ± 53 pg/ml in rejection controls); the assay largely reflected allospecific cytokine secretion, because IFN-γ production after syngeneic DC restimulation was relatively modest (167 ± 23 pg/ml). Consistent with results in the cytokine capture assay, allospecific IFN-γ was more potently reduced in Th2/Tc2.R cell recipients compared with Th2/Tc2 cell recipients. Furthermore, allospecific IL-2 secretion was elevated in rejection controls (Fig. 8b), modestly reduced by donor Th2/Tc2 cells, and profoundly abrogated in recipients of Th2/Tc2.R cells.

Marked reduction in allospecific host T cells in Th2/Tc2.R cell recipients was accompanied by a commensurate reduction in host T cell numbers to values observed without HSC allografting (Fig. 8c). Inhibition of host T cell expansion was not observed with control Th2/Tc2 cell therapy and occurred concomitantly with markedly increased Th2/Tc2.R cell numbers in vivo (Fig. 8c, right panel). In rejection controls, the number of spleen cells derived from the HSC inocula was reduced from 17.4 ± 2.4 × 10⁶ to 5.1 ± 0.5 × 10⁶ cells/spleen (Fig. 8d, left panel). In contrast, HSC-derived cells were not reduced in Th2/Tc2 cell recipients and were actually increased to 38.1 ± 3.0 × 10⁶ cells/spleen in Th2/Tc2.R cell recipients. Relative to engraftment controls, rejection controls had reduced chimera, Th2/Tc2 cell recipients had similar chimera, and recipients of Th2/Tc2.R cells had increased donor chimera (Fig. 8d, right panel).

Abrogation of HVGR is mediated by CD4⁺ Th2.R or CD8⁺ Tc2.R cells and occurs independent of IL-4, IL-10, perforin, FasL, and CD4⁺ FasL⁺ cells

Because HVGR appeared to correlate with graft rejection, we used this in vivo end point to screen for potential mechanisms operating in the prevention of graft rejection by rapamycin-generated...
donor T cells (data for all experiments summarized in Table I). Across six experiments, donor Th2/Tc2.R cells abrogated CD4-mediated HVGR by a median of >99% (range, 97 to >99%) and abrogated CD8-mediated HVGR by a median of >99% (range, 86 to >99%). Such inhibition of HVGR was mediated by both CD4+ Th2.R cells and CD8+ Tc2.R cells (experiment 1), did not require T cell secretion of the type II cytokines IL-4 (experiment 2) or IL-10 (experiment 3), and did not require T cell cytolytic perforin or FasL (experiment 4). An additional experiment using perforin knockout (KO) Th2/Tc2.R cells was performed to verify that an inability to reverse HVGR inhibition correlated with an inability to reverse allograft facilitation. In this experiment, day 30 posttransplant survival was 80% (4 of 5) in WT Th2/Tc2.R cell recipients, 90% (9 of 10) in Th2/Tc2.R perforin KO recipients, and 0% (0 of 5) in rejection controls.

Because rapamycin has been shown to promote the expansion of CD4+ Treg cells (48), we further evaluated whether a T cell component might be operational in our model. First, we evaluated whether CD4+Foxp3+ cells were preferentially expanded in our rapamycin-based culture system. In these experiments, CD4+Foxp3+ cells actually decreased from 9.7 ± 0.7% at culture initiation to 3.0 ± 0.2% by culture day 6 (n = 9 experiments). Second, we compared culture expansion of purified CD4+CD25+ Treg cells and CD4+CD25− conventional T cells. Purified CD4+CD25+ cells did not survive the culture interval (no viable cells on culture day 6 using IL-2 at 20 or 100 U/ml; n = 2). In contrast, culture initiation with CD4+CD25− cells yielded greater expansion than cultures initiated from total CD4+ T cells (fold expansion, 4.9 ± 0.7 in Treg-depleted cultures vs 2.0 ± 0.8 in total CD4 cultures; n = 2). Third, an in vivo experiment was performed using Th2.R cells generated from the CD25+ fraction of CD4+ T cells; in this experiment, recipients of Th2.R cells generated from total CD4 cells or Treg-depleted CD4 cells were equally capable of abrogating HVGR (Table I, experiment 5). As such, the mechanism of rejection abrogation used by rapamycin-generated T2 cells did not appear to substantially involve a Treg cell component.

To evaluate whether Th2/Tc2.R cells might be operating through deprivation of host T cell access to cytokines required for T cell proliferation and maintenance, we first tested whether Th2/Tc2.R cells generated from IL-15R KO mice would be devoid of the capacity to inhibit HVGR. However, both WT and IL-15R KO Th2/Tc2.R cells similarly abrogated host antitumor CD4+ and CD8+ T cell-mediated HVGR (Table I, experiment 6). Because IL-2 and IL-7 were required for the ex vivo generation of Th2/Tc2.R cells, it was not possible to use T cells from mice deficient in the IL-2R or IL-7R. As such, to evaluate these pathways of cytokine consumption, we evaluated whether posttransplant administration IL-2 or IL-7 (or IL-15) might reverse Th2/Tc2.R cell inhibition of HVGR. Cytokine therapy showed no significant capacity to reverse the efficacy of Th2/Tc2.R cells (Table I, experiments 7 and 8).

**Discussion**

In this report, we demonstrate that rapamycin-generated, fully MHC-disparate donor Th2/Tc2 cells or purified CD4+ Th2 cells are enriched in their capacity to prevent HSC graft rejection and do so without inducing lethal GVHD. Therefore, although previous studies have emphasized an important role of CD8+ T cells in abrogating rejection, the current study expands upon recent data supporting a role of specific subsets of CD4+ T cells, such as the Treg cell subset (12–14), in the promotion of alloengraftment. We propose that the increased in vivo efficacy of rapamycin-generated T cells is primarily a function of a multifaceted, apoptosis-resistant phenotype that dictates marked T cell accumulation and persistence posttransplant.

Our finding that rapamycin-generated Th2 cells manifest a broad apoptosis-resistant phenotype may not have been predicted from the numerous studies that associate mTOR inhibition with increased T cell apoptosis (49–53), but is consistent with knowledge that rapamycin-resistant human CD8+ T cells have an increase of antiapoptotic Bcl-xL (24) and that rapamycin resistance in murine T cells is mediated through the Pim-1 and Pim-2 kinases that confer antiapoptotic, growth, and proliferative effects (44, 45). The Th2.R cells used in our study were rapamycin resistant, as they expanded in high-dose rapamycin in the setting of reduced phosphorylation of molecules dependent upon mTOR kinase activity, 4EBP1 and S6 ribosomal protein. The mechanism of resistance did not appear to involve a compensatory increase in Akt phosphorylation that has been observed in tumor cell resistance to mTOR blockade (43). In fact, we observed greatly reduced Akt phosphorylation in Th2.R cells, which is consistent with findings that continuous rapamycin exposure interferes with the assembly of otherwise rapamycin-insensitive mTORC2, with subsequent "re trograde" inhibition of Akt (54). Our observation that Pim-1 and Pim-2 were equally expressed in Th2 and Th2.R cells suggests that these kinases contribute to the capacity of Th2.R cells to expand in the continued presence of rapamycin. However, the mechanism of resistance in our system is likely multifactorial, because Th2.R cells also had: 1) reduced expression of the cell cycle inhibitor p27, which is a previously described pathway of rapamycin resistance in human CD8+ T cells (24) and 2) reduced expression of the active form of the T cell inhibitor GSK3β. Additional experiments will be required to determine the relative contributions of pim kinase function, cell cycle inhibitor down-regulation, and modulation of the GSK3 pathway in the observed Th2.R cell rapamycin resistance.

The resistance to apoptosis in Th2.R cells was multifaceted, because it applied to both intrinsic and extrinsic apoptotic pathways and operated in both a caspase-dependent and caspase-independent manner. The reduced susceptibility to apoptosis in Th2.R cells was profound, as Fas-deficient, granzyme B-inhibited, or pan-caspase-inhibited control Th2 cells had greatly increased apoptosis relative to Th2.R cells. Because the balance of pro- and antiapoptotic Bcl-2 family member genes modulates both intrinsic and extrinsic apoptosis, it is possible that the potentially favorable Bcl-2 family gene balance we observed (increased antiapoptotic Bcl-xL expression concomitant with reduced proapoptotic Bim and Bid expression) may have contributed to the reduced Th2.rapa cell apoptosis. This demonstration that rapamycin-resistant T cells expressed both increased antiapoptotic Bcl-2 family gene members and reduced proapoptotic Bcl-2 family gene members appears to extend the work of Slavik et al. (24), who associated increased Bcl-xL with rapamycin-resistant human CD8 cells. Because the balance of the pro- and antiapoptotic members of the Bcl-2 family gene controls apoptosis in part through regulation of caspase 9 activation (55), we speculate that in our system the favorable Bcl-2 family gene balance that developed in the setting of rapamycin may have accounted in part for the observed reduction in caspase 9 activation, downstream caspase 3 activation, and resistance to apoptosis. Additional studies using gene-deficient or transgenic T cells or RNA inhibitor strategies will be required to determine whether these specific molecular changes are causally related to the reduced Th2.R cell antiapoptotic phenotype. Of note, rapamycin induction of an antiapoptotic T cell phenotype appeared to be a relatively general phenomenon, because we observed that both Th1 and Th2 cells generated in rapamycin were relatively resistant to apoptosis.
Importantly, this apoptosis-resistant phenotype of rapamycin-generated T cells appeared to translate into a greatly increased T cell capacity to persist in vivo, polarize in vivo toward a Th2 cytokine phenotype, and prevent graft rejection. That is, administration of $1 \times 10^7$ control Th2/Tc2 cells resulted in Th2/Tc2 cell detection in multiple organs (spleen, lymph node, marrow) early posttransplant (day 5). However, such Th2/Tc2 cells were essentially undetectable by day 14 posttransplant (<1 $\times 10^6$ total cells in these organ sites) at a time when loss of alloengraftment was observed in this cohort; as such, it is possible that the inability of control Th2/Tc2 cells to abrogate rejection may relate to their inability to persist in vivo. In marked contrast, rapamycin-generated Th2/Tc2 cells and, in particular, purified CD4$^+$ Th2.rapa cells were present in vivo through day 14 posttransplant in each organ evaluated. Of note, the number of rapamycin-generated T cells detected in vivo at day 14 ($\sim 2 \times 10^7$ cells) exceeded the $1 \times 10^7$ T cell number administered, thereby demonstrated not only in vivo persistence but also sustained in vivo donor T cell expansion without the development of lethal GVHD. The increase in rapamycin-generated T cell number posttransplant was associated with reduced in vivo T cell apoptosis, reduced in vivo T cell death, and enhanced accumulation of T cells in vivo at more advanced cell cycle numbers. As such, analogous to recent data emerging from murine adoptive T cell therapy of cancer models (19), we propose that the increased efficacy of rapamycin-generated donor T cells for prevention of graft rejection likely relates in great part to their enhanced in vivo survival.

We previously found that costimulated Th2/Tc2 cells abrogated fully MHC-disparate rejection when present at a donor T cell:host T cell ratio of 100:1 (15); however, such therapy was associated with a substantial incidence of lethal GVHD. Our current results using rapamycin-generated T cells appear to represent an advance relative to this initial study, since Th2/Tc2.rapa cells or purified CD4$^+$ Th2.rapa cells prevented fully MHC-disparate rejection at a donor T cell:host T cell ratio of 10:1 without lethal GVHD. It should be noted that the current experiments were performed using enriched HSC generated by ex vivo culture, whereas our previous studies and most studies in the literature have been performed with noncultured bone marrow cells. In recent experiments, we have found that rapamycin-generated T cells prevent rejection of nonexpanded, T cell-depleted bone marrow cells in the current model (J. Mariotti, unpublished data). Recipients of rapamycin-generated T cells did have GVHD-related weight loss and immune suppression and, as such, prevention of graft rejection by these populations is distinct from previously described nonalloreactive veto-type T cells (7–9). Of note, the magnitude of weight loss and immune suppression was greater with the $1 \times 10^7$ cell dose of rapamycin-generated T cells than with the $1 \times 10^5$ cell dose (each dose effectively prevented rejection if the ratio of donor:host T cells was held constant at 10:1). These results suggest that an improved therapeutic index for preventing graft rejection might be obtained by controlling both donor and host T cell number. It is important to note that our model used a fully MHC-mismatched strain combination; additional experiments will need to be performed to evaluate whether Th2.R cells might prevent rejection directed against minor histocompatibility Ags. It will also be important to characterize the antiviral and antitumor immune competence of hosts that achieve alloengraftment through Th2.rapa cell therapy. With respect to this latter point, we have previously shown that graft augmentation with donor Th2.rapa cells represents an approach to mediate graft-vs-tumor effects with reduced GVHD (16). Our current experiments found that Th2.rapa cell recipients had modest signs of ongoing GVHD (as indicated by absence of lethality and modest weight loss at day 90 posttransplant) and had nominal reduction in CD4$^+$ and CD8$^+$ T cell immune reconstitution at day 90 posttransplant; these results suggest that at least some component of immune competence is preserved in this model.

By using multicolor and cytokine capture flow cytometry on posttransplant T cells, we have also increased an understanding of the host-vs-graft reaction, because we associated host CD4$^+$ and CD8$^+$ allospecific T cell secretion of IFN-γ with graft rejection. It is important to note that a determination of the molecular mechanism accounting for graft rejection has been elusive, since cytokine- and cytolytic-deficient host T cells have retained a capacity to cause rejection, including IFN-γ-deficient T cells (56). Furthermore, even host T cells deficient in multiple cytolytic molecules can mediate rejection (57). As such, it is likely that several molecules working in concert or an unidentified dominant molecule mediate graft rejection and that allospecific host IFN-γ secretion represents a biological marker of graft rejection.

Significantly, we found that prevention of graft rejection through administration of rapamycin-generated T cells was associated with complete abrogation of HVGR, since the number of allospecific IFN-γ-secreting host T cells was consistently reduced by >99% in recipients of donor Th2/Tc2.R cells or Th2.R cells. Administration of single-molecule-deficient rapamycin-generated T cells, including IL-4, IL-10, perforin, or FasL, did not even partially reverse this complete inhibition of HVGR. It can be argued that quantification of HVGR may not represent a suitable marker for ultimate engraftment. However, we did find that perforin-deficient rapamycin-generated T cells were fully capable of abrogating HVGR and also fully capable of preventing graft rejection, thereby indicating that monitoring HVGR may represent a useful tool in our attempt to identify potential donor T cell mechanisms of action. This perforin-independent mechanism of action stands in contrast to previous studies of nonpolarized donor T cells that abrogated rejection in a perforin-dependent manner (58) or in a manner that involved both perforin and FasL (59). Our finding that FasL-deficient donor T cells completely inhibited HVGR suggests that the rapamycin-generated T cells likely operate by a mechanism distinct from that observed in studies by Reich-Zeliger et al. (60). Further studies using rapamycin-generated donor T cells multiply deficient in cytokine and/or cytolytic molecules will be required to further investigate their mechanism of action.

Our current data also do not support the possibility that rapamycin-generated T cells operate through a Treg cell mechanism, since a low frequency of Foxp3$^+$ cells was present in the final T cell inocula and purified Treg cells did not survive in the culture system we used. Furthermore, the marked in vivo type II cytokine polarization (increased IL-4, IL-5, and IL-10) we observed is not consistent with a strong Treg cell response, because Tregs have been shown to inhibit Th2-type immunity in vivo (61, 62). Nonetheless, it is possible that Th2.R cells may be analogous to a Treg cell population that does not express Foxp3, such as the Tr1 population (63). Finally, our data do not support “cytokine consumption” as a mechanism of rejection abrogation, since posttransplant therapy with IL-2, IL-7, or IL-15 did not even partially reverse the complete abrogation of host-vs-graft alloreactivity.

In conclusion, ex vivo rapamycin imparts a profound and multifaceted apoptosis-resistant phenotype upon donor Th2 and Th2/Tc2 cells that likely accounts for their dramatically increased in vivo survival and in vivo efficacy in terms of preventing graft rejection. Generation of antiapoptotic T cells through ex vivo rapamycin exposure is a powerful tool for enhancing in vivo T cell efficacy that may have application for
adoptive T cell therapy applications distinct from the ones that we have identified, namely, the prevention of GVHD and the abrogation of graft rejection.

Disclosures

The authors have no financial conflict of interest.

References


