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Induced Regulatory T Cells Promote Tolerance When Stabilized by Rapamycin and IL-2 In Vivo

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Natural regulatory T cells (nTregs) play an important role in tolerance; however, the small numbers of cells obtainable potentially limit the feasibility of clinical adoptive transfer. Therefore, we studied the feasibility and efficacy of using murine-induced regulatory T cells (iTregs) for the induction of tolerance after bone marrow transplantation. iTregs could be induced in large numbers from conventional donor CD4 and CD8 T cells within 1 wk and were highly suppressive. During graft-versus-host disease (GVHD), CD4 and CD8 iTregs suppressed the proliferation of effector T cells and the production of proinflammatory cytokines. However, unlike nTregs, both iTreg populations lost Foxp3 expression within 3 wk in vivo, reverted to effector T cells, and exacerbated GVHD. The loss of Foxp3 in iTregs followed homeostatic and/or alloantigen-driven proliferation and was unrelated to GVHD. However, the concurrent administration of rapamycin, with or without IL-2/anti–IL-2 Ab complexes, to the transplant recipients significantly improved Foxp3 stability in CD4 iTregs (and, to a lesser extent, CD8 iTregs), such that they remained detectable 12 wk after transfer. Strikingly, CD4, but not CD8, iTregs could then suppress Teff proliferation and proinflammatory cytokine production and prevent GVHD in an equivalent fashion to nTregs. However, at high numbers and when used as GVHD prophylaxis, Tregs potently suppress graft-versus-leukemia effects and so may be most appropriate as a therapeutic modality to treat GVHD. These data demonstrate that CD4 iTregs can be produced rapidly in large, clinically relevant numbers and, when transferred in the presence of systemic rapamycin and IL-2, induce tolerance in transplant recipients. ***The Journal of Immunology*****, 2013, 191: 5291–5303.**

Graft-versus-host disease (GVHD) is the major cause of treatment-related mortality after allogeneic bone marrow transplantation (BMT) and constitutes a major barrier to further improvements in survival. GVHD is initiated by the presentation of recipient alloantigens, predominantly by host APCs to donor T cells (1, 2). The subsequent differentiation and expansion of effector CD4 and CD8 T cells result in a complex proinflammatory cascade in which apoptosis is induced in target organs by both cytokines (especially TNF, IFN-γ, and IL-6) and cell-mediated cytotoxicity (3–6). Foxp3-expressing regulatory T cells (Tregs) include two major groups: those generated at steady-state within the thymus ("so-called" natural Tregs [nTreg]) and those induced regulatory T cells (iTreg) that are generated in the periphery from Foxp3$^-$ T cells. Both play an important role in immune regulation, including that after BMT (7–10). The adoptive transfer of freshly isolated or in vitro–expanded nTregs was shown to be an effective means to suppress GVHD and restore immune tolerance (11–13). However, the relative scarcity of nTregs in the peripheral blood and the time and expense of expansion protocols in vitro may ultimately limit their clinical usefulness (14). In contrast, iTregs can be generated in vitro from conventional T cells in large numbers to high purity within very short time frames (15–17). However, iTregs generated in vitro tend to be unstable as a result of a high level of methylation within the Treg-specific demethylated region of the Foxp3 promoter (9, 18–20). Because the suppressive function of Tregs was shown to correlate with Foxp3, the instability of Foxp3 within iTregs represents a significant potential therapeutic limitation.

Current efforts to improve the stability of Foxp3 involve the addition of demethylating agents and/or histone deacetylase inhibitors to in vitro–expansion protocols or administration of the agent to recipients after BMT (18–22). However, the use of these agents in vitro has yet to translate into improved Foxp3 stability and regulatory efficacy in vivo. Previous reports demonstrated that the mTOR inhibitor rapamycin (RAPA) can suppress conventional T cell expansion and function while sparing Foxp3$^+$ Tregs (14, 22–24). IL-2, which is critical for the survival and expansion of

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Abbreviations used in this article: BLI, bioluminescence imaging; BM, bone marrow; BMT, BM transplantation; CML, chronic myeloid leukemia; DC, dendritic cell; GVHD, graft-versus-host disease; GVL, graft-versus-leukemia; iTreg, induced regulatory T cell; nTreg, natural regulatory T cell; RAPA, rapamycin; TCD-BM, T cell–depleted bone marrow; Teff, effector T cell; Treg, regulatory T cell; VPD, violet proliferation dye.

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Tregs, was shown to preferentially expand Tregs in vivo and improve the stability of in vitro–generated iTregs (21, 25, 26). We (7) and other investigators (27) showed synergy between RAPA and IL-2 to expand iTregs in vivo; importantly, both agents are readily available in clinical practice (25, 28, 29).

The majority of studies have focused on nTregs and iTregs of the CD4 lineage. We (7) and other investigators (30, 31) recently described the in vivo generation of highly suppressive $CD8^+Foxp3^+$ iTregs in response to alloantigen. These iTregs appear to be more potent at suppressing MHC class I–restricted immune responses than are their CD4 counterparts (7). The in vitro generation and function of these CD8 iTregs has received only limited study.

We used Foxp3-GFP-luciferase knockin mice to generate Treg populations to allow the identification and tracking of regulatory cells in vivo (32–34) to identify the parameters that are permissive of Foxp3 stability and regulation in vivo. We find that the administration of RAPA in vivo, with or without IL-2, rescues iTreg function in vivo, significantly improving the stability of Foxp3 expression and the suppressive properties of both iTreg populations. Our findings suggest that CD4 iTregs, in particular, can be generated in clinically relevant numbers for adoptive transfer and hold promise for the suppression of clinical GVHD in BMT patients.

Materials and Methods

Mice

Female C57BL/6 (B6.WT,H-2^b,CD45.2), B6.SJL-Ptprca (PTPrca,H-2^b, CD45.1), BALB/c (H-2^d, CD45.2), and B6D2F1 (H-$2^{b/d}$,CD45.2) mice were purchased from the Animal Resource Center (Perth, WA, Australia). B6.Foxp3-GFP and B6.Foxp3-GFP-luciferase-DTR (B6.Foxp3-luc^+) mice were supplied by the Queensland Institute of Medical Research animal facility. The mice were used between 8 and 12 wk of age. Mice were housed in microisolator cages and received acidified autoclaved water (pH 2.5) after BMT. All animal studies were performed in accordance with the Queensland Institute of Medical Research Animal Ethics Committee.

Abs

The following Abs were purchased from BioLegend: PE-conjugated anti-$H2D^b$ (KH95), CD45.1 (A20), CD45.2 (104), CD25 (3C7), IFN-γ (XM61.2), IL-10 (JES5-16E3), TNF-α (MP6-XT22), IgG2b isotype control, allophycocyanin-conjugated anti-CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD45.2 (104), CD11c (N418), TNF-α (MP6-XT22), PE-Cy7–conjugated anti-CD45.1 (A20), CD8α (53-6.7), CD4 (RM4-5), I-A/I-E (M5/114.15.2), CD3ε (145-2C11), Alexa Fluor 647–anti-Foxp3 (150D), Alexa Fluor 700–anti-IL-17A (TC11-18H10.1), Brilliant Violet 605–conjugated anti-CD90.2, and biotin-conjugated anti-$H2D^d$ (34-2-12). Pacific Blue–conjugated CD8α (53-6.7) was purchased from BD Biosciences. 7-Aminoactinomycin D was purchased from Sigma-Aldrich. Intracellular cytokine staining was performed using the Foxp3 Cytofix/Cytoperm Kit (eBioscience), per the manufacturer's protocols. Anti-CD3 (2C11), anti-CD28 (N3751), anti-CD25 (PC61), and anti–IL-2 (JES6-1A12) Abs were produced in-house. Briefly, hybridoma cell culture supernatants were harvested, and products were precipitated using ammonium sulfate fractionation. Ab was selected using GammaBind G Sepharose (Amersham Biosciences), dialyzed with PBS using Membra-cel MD25 membrane, and concentrated using Amicon Ultra centrifugal filters (Millipore).

Cell preparation

T cell depletion of bone marrow (BM) was performed as previously described (35), and the resulting cell suspensions contained <1% $CD3^+$ T cells. For Treg depletion, donor mice were injected i.p. with anti-CD25 mAb (PC61, 250 μg/mouse on day −3, 125 μg/mouse on day −1) before harvesting of responder T cells. CD3 T cells were purified using magnetic bead depletion, as previously described (35), and subsequent CD3 T cell purities were >80%. CD4 and CD8 T cells were purified by MACS beads (CD4, CD8) and positive selection columns, according to the manufacturer's protocols (Miltenyi Biotec), and splenic dendritic cells (DCs) were enriched by gradient centrifugation, followed by positive selection by MACS (CD11c); the resulting cell population purities were >80%. nTregs or iTregs were sorted by FACS, based on Foxp3-GFP, CD4, and 7-aminoactinomycin D staining, by MoFlo (DakoCytomation, Fort Collins, CO) to >95% purity. Leukemic cells (bcr-abl GFP^+, $H2^{d/b}$, $CD45.2^+$), a primary myeloid blast crisis chronic myeloid leukemia (CML), were generated as previously described (36).

Murine iTreg induction

Twenty-four–well plates were coated with anti-CD3 (2C11, 10 μg/ml) and anti-CD28 (N3751, 1 μg/ml). Magnetically purified splenic CD4 and CD8 T cells (with purity ranging from 80 to 95%) were seeded at 1×10^6 cells/well and resuspended in 1 ml IMDM with 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate, supplemented with 100 U/ml recombinant human IL-2 (Aldesleukin), 10 ng/ml TGF-β1 (BioLegend), and 100 ng/ml RAPA (Invitrogen). Medium was changed on day 2, and cells were harvested on day 4 for analysis or further studies.

Cell labeling

For cell-proliferation assays, purified T cells or Tregs were suspended at 3×10^7 cells/ml in serum-free media, and CFSE (Sigma-Aldrich) or CellTrace violet proliferation dye (VPD; Invitrogen) was added at 1 or 5 μM final concentration, respectively. After incubation at 37°C for 15 min, cells were washed using serum-free media and resuspended in complete media.

In vitro T cell–suppression assays

For in vitro–suppression assays, CFSE-labeled, BioMag-purified total T cells or MACS-selected CD4 or CD8 T cells (B6, $CD45.1^+$) were seeded at 5×10^4/well in 96-well round-bottom plates, with DCs (B6, $CD45.2^+$) at 5×10^3/well, in the absence or presence of Tregs (B6.Foxp3-GFP, $CD45.2^+$) at 5×10^4/well, and supplemented with anti-CD3 (2C11, 1 μg/ml). Cells were harvested for CFSE dilution analysis at 72 h of culture for BioMag T responders and between 72 and 96 h for purified CD4 and CD8 responders.

Intracellular cytokine staining

Cells were stimulated by anti-CD3 (2C11, 2.5 μg/ml) for 4 h at 37°C and in the presence of brefeldin A (1 μg/ml) for the last 3 h. Then cells were harvested, fixed and permeabilized using an eBioscience Foxp3 intracellular staining kit, followed by staining of Foxp3, IFN-γ, and TNF-α. For IL-17A, and for IL-10 with or without IFN-γ and TNF-α staining, cells were stimulated by PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h at 37°C, and brefeldin A (1 μg/ml) was added for the last 3 h.

Bone marrow transplantation

On day −1, recipient mice received 1100 cGy (B6D2F1), 1000 cGy (B6), or 900 cGy (BALB/c) total body irradiation ([^{137}Cs] source at 108 cGy/min), split into two doses separated by 3 h. On day 0, recipients were transplanted with 5×10^6 (B6, B6D2F1) or 10^7 (BALB/c) BM cells from C57BL/6 or B6.SJL-Ptprca mice, where indicated, with or without 0.5–1×10^6 Tregs from B6.Foxp3-GFP or B6.Foxp3-GFP-luciferase-DTR donors. On day 2, recipients were transplanted with purified T cells (same doses with Tregs) from B6.SJL-Ptprca or C57BL/6 donors (34). Intraperitoneal injections of RAPA (1.5 mg/kg; Wyeth) were administered daily starting on day 0, as previously described (7). Recombinant murine IL-2 (1.5 μg; eBioscience) was incubated for 30 min with the Ab JES6-1A12 (50 μg) to form IL-2/anti–IL-2 Ab complexes, which were injected i.p. on days 0 and 4 post-BMT.

GVHD clinical scoring

The scores of systemic GVHD were based on the following five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity (maximum index = 10) (37). Mice were monitored regularly, those with clinical scores ≥ 6 were sacrificed, and the date of death was recorded as the next day, in accordance with institutional animal ethics guidelines.

In vivo and organ luminescence imaging

Recipients were injected s.c. with luciferin (0.5 mg) and then anesthetized with isoflurane 5 min before imaging with the Xenogen imaging system (Xenogen IVIS 100; Caliper Life Sciences). For organ imaging, mice were injected s.c. with luciferin (0.5 mg), and organs were taken 5 min later and imaged with the same system. When whole-body and organ imaging were conducted on the same day, they were separated by 4–5 h to allow for complete excretion of luciferin. Data were analyzed with Living Image Version 4 software (Xenogen).

Histology

Organs were taken, preserved in 10% formalin, embedded in paraffin, and processed to generate 5-μm-thick sections. H&E-stained sections of skin, small intestine, and liver were examined in a blinded fashion (by A.D.C.) using a semiquantitative scoring system for GVHD, as previously published (35, 38). Images were acquired using an Olympus BX51 microscope, Evolution MPVersion 5.0 Camera, and QCapture software (QImaging).

Cytokine analysis

Serum levels of IFN-γ, TNF-α, IL-4, IL-6, IL-10, and IL-17A were detected using the BD Cytometric Bead Array system (BD Biosciences Pharmingen, San Diego, CA), according to the manufacturer's protocols.

Statistical analysis

Data are presented as mean ± SEM. The Mann–Whitney *U* test was used for group comparisons. Survival was estimated and plotted using Kaplan–Meier methods, and the difference between subgroups was estimated using log-rank methods. Mortality due to leukemia or GVHD was treated as competing risks, and the incidences were plotted and compared using competing risk regression. A two-sided p value < 0.05 was considered statistically significant. Statistical analyses were performed using Prism Version 6 software (GraphPad) or R2.15.3 (39).

Results

Tregs can be induced with high efficiency from both conventional CD4 and CD8 T cells

We used B6.Foxp3-GFP and B6.Foxp3-GFP-luciferase-DTR mice as a source of donor T cells and iTregs from conventional CD4 and CD8 T cell populations in vitro. To simplify procedures and make as clinically transferable as possible, we magnetically selected initial cell populations. Purified T cells (10^6/well) were seeded in CD3/28-coated plates in the presence of IL-2, TGF-β1, and RAPA. After 4 d of culture, the frequency of Foxp3 was 78.5 ± 3.1% in CD4 T cells and 70.7 ± 3.9% in CD8 T cells (Fig. 1A, 1B). The numbers of CD4 and CD8 Foxp3$^+$ cells recovered per million seeded T cells were 1.81 ± 0.11 and 2.34 ± 0.11 × 10^6, respectively (Fig. 1B). Cytokine production (IFN-γ, TNF, IL-10, IL-17A) was analyzed by intracellular staining. Both iTreg populations produced IFN-γ and particularly TNF, which was seen most dramatically in the CD8 iTregs. Neither population produced significant amounts of IL-10 or IL-17A (Fig. 1C).

We then verified the suppressive function of the CD4 and CD8 iTregs relative to sort-purified (CD4$^+$Foxp3$^+$) naive nTregs that were >95% Foxp3$^+$ (Fig. 1A). Purified CFSE-labeled syngeneic (B6.CD45.1$^+$) CD3$^+$ or individual CD4$^+$ and CD8$^+$ T cells (data not shown) were used as responder cells in conjunction with B6. CD45.2$^+$ DCs, together with CD3 mAb in the presence or absence of Treg populations. CFSE dilution in CD45.1$^+$ responder cells was determined 3 d later. All Treg populations were suppressive in vitro, but CD4 iTregs were consistently the most potent suppressors of proliferation in both CD4 and CD8 responder cells (Fig. 1D–F).

CD4 iTregs are suppressive in vivo

We used a previously reported transplant model to test the suppression of CD4 and CD8 iTregs in vivo. We transferred 1 × 10^6 CD4 iTregs, CD8 iTregs, or sort-purified naive nTregs from B6. Foxp3-GFP donors along with B6.WT (CD45.2$^+$) T cell–depleted BM (TCD-BM) into lethally irradiated B6D2F1 recipients on day 0. One million VPD-labeled conventional T cells (CD45.1$^+$), which were depleted of Tregs (by in vivo depletion with CD25 mAb PC61), were transferred on day 2 (Fig. 2A). Because the iTregs were only 80% Foxp3$^+$, we initially compared Foxp3 expression 5 d after transfer of sort-purified Foxp3-GFP$^+$ iTreg populations relative to the unfractionated parental iTreg populations. As shown in Supplemental Fig. 1A, 80% of nTregs maintained their expression of Foxp3 in vivo but only 25% of the CD4 and CD8 iTreg populations maintained Foxp3 after BMT, and this was associated with a large outgrowth of Foxp3$^-$ cells. Importantly, sort-purification of Foxp3$^+$ starting populations based on GFP did not influence the stability or recovery of Foxp3$^+$ populations after BMT. To examine effector function in the Treg populations after BMT, we quantified IFN-γ and TNF expression. CD4, and particularly CD8, iTregs secreted significant amounts of these inflammatory cytokines after BMT, predominantly in the Foxp3$^-$ fraction; again, sort-purification of the initial Foxp3-GFP$^+$ cell fraction did not influence outcome (Supplemental Fig. 1B). Thus, unlike naive nTregs, iTregs were highly unstable after BMT and rapidly reverted to effector T cells (Teffs); this was not due to preferential expansion of Foxp3$^-$ cells in the starting iTreg population.

The administration of RAPA in vivo enhances Foxp3 stability and suppressive properties of iTregs

Given the ability of RAPA to inhibit Teffs, but not Tregs, we also analyzed the expansion and Foxp3 stability in unseparated iTreg populations in the presence or absence of RAPA in vivo.

By gating on the adoptively transferred Treg population (CD45.2$^+$ H2D^{d-}Thy1.2$^+$), we analyzed the expansion of and Foxp3 expression in the adoptively transferred Tregs posttransplant, which consist of the remaining Foxp3$^+$ Tregs and Foxp3$^-$ Teffs that had reverted from the original Treg population. When RAPA was administered in vivo, although the overall Foxp3$^+$ Treg number was marginally reduced (CD4 iTregs: 0.57 ± 0.07 × 10^6 versus 0.40 ± 0.05 × 10^6, $p = 0.09$; CD8 iTregs: 0.89 ± 0.10 × 10^6 versus 0.32 ± 0.09 × 10^6, $p = 0.005$; Fig. 2B, 2C), the reversion to Foxp3$^-$ cells was prevented to a much greater extent (CD4 iTregs: 2.76 ± 0.82 × 10^6 versus 0.95 ± 0.23 × 10^6, $p = 0.01$; CD8 iTregs: 5.90 ± 0.78 × 10^6 versus 0.56 ± 0.23 × 10^6, $p < 0.0001$, Fig. 2B, 2C). We then investigated the function of the recovered Tregs by analyzing the production of proinflammatory cytokines. Importantly, RAPA administration in vivo significantly suppressed the production of IFN-γ and TNF (data not shown) in Foxp3$^+$ and Foxp3$^-$ Tregs (Fig. 2D).

The in vivo migratory dynamics of Tregs determine their immunomodulatory role during GVHD. To study the migration of Tregs in vivo, we used Tregs from B6.Foxp3-luciferase-DTR mice in which luciferase is driven off the Foxp3 promoter. Whole-body and organ imaging indicated that, like nTregs, iTregs migrated and expanded predominantly in the gastrointestinal tract and spleen (Fig. 2E). The treatment of recipients with RAPA attenuated Treg expansion, but the same pattern of migration remained.

We next investigated the suppressive function of the adoptively transferred iTreg populations with and without RAPA in vivo. CD4 iTregs demonstrated similar potency to nTregs in suppressing CD4 and CD8 Teff proliferation (Fig. 3A, 3B) and expansion (Fig. 3A, 3C) and were more potent at suppressing IFN-γ secretion (Fig. 3A, 3D). In contrast, CD8 iTregs were less potent, and suppression was predominantly confined to a partial inhibition of proliferation (Fig. 3A, 3B) and expansion (Fig. 3A, 3C) in CD8 T cell responders, whereas IFN-γ secretion was partially inhibited in both CD4 and CD8 T cell responders (Fig. 3A, 3D). As expected, the administration of RAPA alone (without Treg transfer) to BMT recipients attenuated proliferation and IFN-γ secretion by both CD4 and CD8 responder T cells. In the presence of RAPA, nTregs and CD4 iTregs demonstrated an enhanced but, again, similar capacity to suppress proliferation of both CD4 and CD8 responder T cells; however, the addition of RAPA resulted in a marked enhancement of CD8 iTregs' capacity to suppress the proliferation

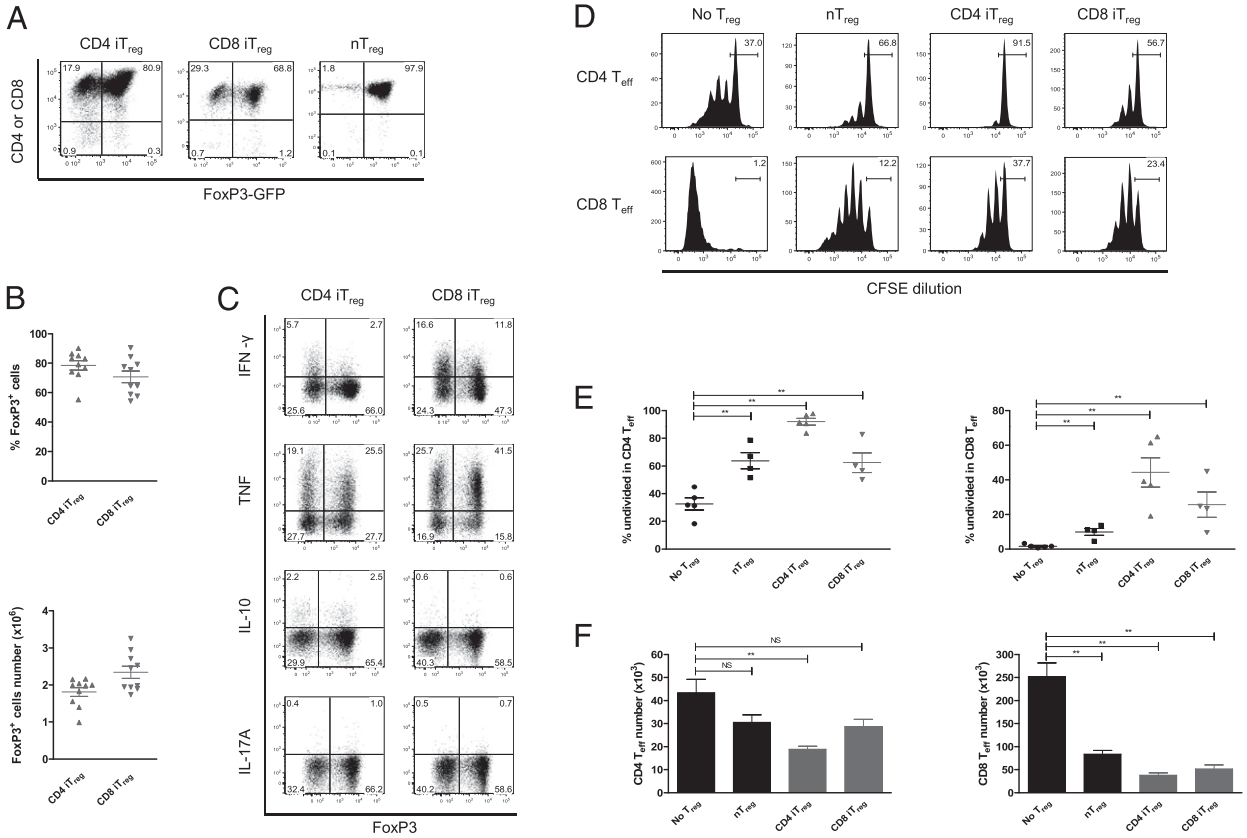

FIGURE 1. Tregs can be induced with high efficiency from both conventional CD4 and CD8 T cells. Splenic CD4 and CD8 T cells from B6.Foxp3-GFP or B6.Foxp3-GFP-luciferase-DTR mice were magnetically purified and seeded at 1×10^6/well in CD3/28-coated 24-well plates in the presence of recombinant human IL-2 (100 U/ml), TGF-β1 (10 ng/ml), and RAPA (100 ng/ml). (**A**) Representative plots of Foxp3 expression in CD4 iTregs, CD8 iTregs, and sort-purified ($CD4^+Foxp3\text{-}GFP^+$) naive nTregs. (**B**) On day 4 of culture, $Foxp3^+$ cells frequencies were determined by expression of GFP, and $Foxp3^+$ cell numbers recovered per well were calculated. Data are from 10 independent experiments. (**C**) Representative plots of iTreg intracellular cytokine production generated after 4 d of culture and stimulated with PMA/ionomycin. (**D**) CFSE-labeled syngeneic (B6, $CD45.1^+$) $CD3^+$ or individual $CD4^+$ and $CD8^+$ T cells (data not shown) were used as responder cells (5×10^4/well) in conjunction with DCs (B6, $CD45.2^+$) (5×10^3/well), together with CD3 mAb (2C11, 1 μg/ml), in the presence or absence of Tregs ($CD45.2^+$, 5×10^4/well). CFSE dilution was determined 72 h later in $CD45.1^+$ responder populations. Suppression of CD4 (*left panel*) and CD8 (*right panel*) responder proliferation was determined by the proportion of undivided cells (**E**) and the numbers of responder cells recovered per well (**F**). Data are from 5 independent experiments. $^{**}p < 0.01$.

and expansion of CD8 Teffs (Fig. 3B, 3C). In the presence of RAPA, effector function in both CD4 and CD8 responders was largely eliminated by nTregs and CD4 iTregs and remained partially inhibited by CD8 iTregs (Fig. 3D).

Consistent with the ex vivo data, both nTregs and CD4 iTregs significantly suppressed IFN-γ, TNF, and IL-6 levels in sera 6 d after BMT, whereas higher IFN-γ levels were detected in recipients of CD8 iTregs (Fig. 3E). When RAPA was coadministered to recipients, the production of IFN-γ and TNF was almost completely suppressed in all groups, whereas the production of IL-6 was not (Fig. 3E).

CD4 iTregs transferred after BMT in the presence of RAPA potently suppress GVHD

We adopted the aforementioned transplant model to investigate the ability of iTregs to regulate lethal GVHD. As described in Fig. 4A, 0.5×10^6 nTregs or iTregs from B6.Foxp3-luciferase-DTR donors were transplanted into BALB/c mice on day 0 in conjunction with TCD-BM. Splenic conventional T cells (0.5×10^6) depleted of Tregs were transferred on day 2. Systemic GVHD was monitored thereafter, together with in vivo quantification of Tregs by weekly whole-body bioluminescence imaging (BLI). CD4 and CD8 iTregs only survived short-term; hence BLI signals peaked on day 6 but declined quickly. In contrast, nTregs were more stable and remained detectable 12 wk posttransplant (Fig. 4B). In line with their in vivo stability, nTregs reduced GVHD clinical scores and protected the recipients from GVHD lethality. In contrast, GVHD mortality was exacerbated in recipients of both CD4 and CD8 iTregs, and clinical scores were not attenuated (Fig. 4B).

To define the optimal conditions by which RAPA regulates iTreg stability, we repeated the transplants and administered RAPA from days 0 to 6 posttransplant. After RAPA administration alone, the stability of nTregs and iTregs was improved, with BLI signals detectable long-term in recipients of CD4 iTregs (Fig. 4C). Strikingly, CD4 iTregs conferred complete protection from lethal GVHD when coadministered with RAPA and markedly suppressed GVHD clinical scores, as did nTregs. CD8 iTregs partially reduced GVHD clinical scores and marginally, but significantly, prolonged overall survival (43.0 versus 30.5 d, $p = 0.009$), although they failed to prevent GVHD mortality.

Given that the CD4 iTregs and naive nTregs demonstrated potent suppressive function in vivo, we assessed their impact on graft-versus-leukemia (GVL) effects using primary myeloid blast crisis CML cells. In these studies, 0.5×10^6 CD4 iTregs or naive nTregs from B6.Foxp3-GFP-luciferase-DTR ($CD3^+GFP^+$) donors were transplanted with 10^6 leukemic cells ($CD3^-GFP^+$) and TCD-

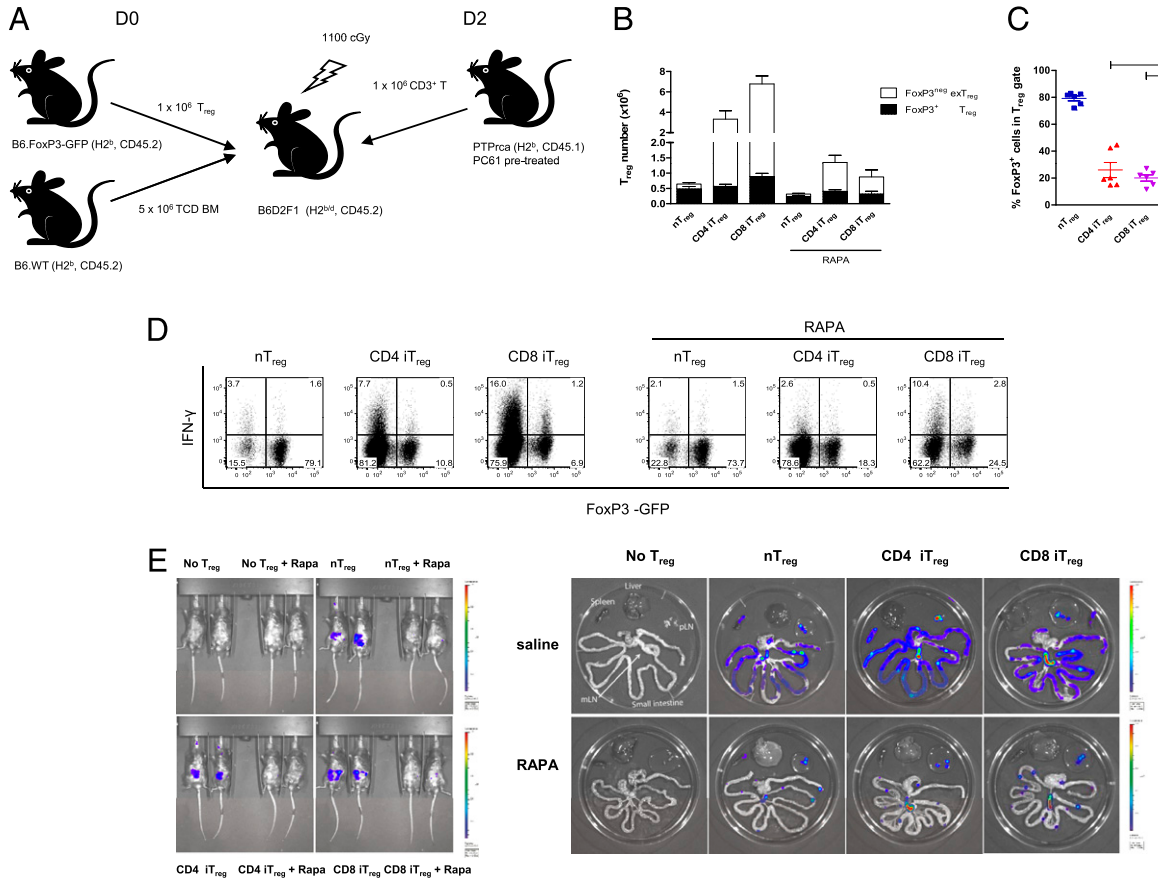

FIGURE 2. RAPA administration in vivo inhibits the expansion of Foxp3$^-$ cells from iTregs. (**A**) Experimental schema. A total of 1×10^6 CD4 iTregs, CD8 iTregs, or sort-purified naive nTregs from B6.Foxp3-GFP or B6.Foxp3-GFP-luciferase-DTR mice (CD45.2$^+$, H2D^b), together with TCD-BM (B6, CD45.2$^+$, H2D^b), were transplanted into lethally irradiated B6D2F1 (CD45.2$^+$, H2D$^{b/d}$) recipients on day 0, followed by the transfer of 1×10^6 VPD-labeled conventional T cells (B6, CD45.1$^+$, H2D^b) on day 2. Conventional T cells were depleted of Tregs (by treatment with CD25 mAb). Cohorts of animals received RAPA (1.5 mg/kg) from days 0 to 5. On day 6 after BMT, the absolute number (**B**) and frequency (**C**) of Foxp3$^+$ and Foxp3$^-$ cells within the adoptively transferred Tregs (CD45.2$^+$H2D^{d-}Thy1.2$^+$) were quantified in spleen. (**D**) Intracellular cytokine secretion (IFN-γ) was analyzed after restimulation with CD3 mAb (n = 6–8, data combined from four experiments). (**E**) For in vivo tracking, Tregs were generated from B6.Foxp3-GFP-luciferase-DTR donors, and imaging was conducted on day 6 after transplant. *$p < 0.05$, **$p < 0.01$.

BM (B6) into lethally irradiated B6D2F1 recipients on day 0. At day 2, 0.5×10^6 conventional T cells (B6, CD3$^+$), depleted of Tregs, were transferred. RAPA and IL-2/IL-2 Ab complexes (referred to as IL-2 complexes hereafter) were given, as previously described (7). Leukemia growth was monitored in the peripheral blood, and animals were killed if they became sick or when their total WBC was $>100 \times 10^9$/l. The cause of death was confirmed to be leukemia by subsequent analysis of BM. Surprisingly, both nTregs and CD4 iTregs resulted in a partial, but dramatic, suppression of the GVL effect, with animals succumbing to leukemia at similar times as the recipients of T cell–depleted grafts (Fig. 5A–C). In contrast, recipients of conventional T cells, but no Tregs, controlled leukemia long-term, albeit in the presence of GVHD (Fig. 5A–C). We next investigated the contribution of RAPA and IL-2 complexes to the suppression of the GVL effect by Tregs. The suppression of GVL by Tregs was not due to concurrent RAPA/IL-2 complex administration, because recipients of Tregs without RAPA and IL-2 complexes actually developed leukemia at an accelerated rate (Fig. 5D). This is consistent with the known ability of RAPA to inhibit leukemia growth and/or the ability of IL-2 to expand T and NK cell effector responses (40, 41).

Twelve weeks after BMT, the CD4 iTregs were still detectable by in vivo imaging and were located in the intestine, spleen, and thymus (Fig. 6A). We analyzed the contribution of the Tregs transferred on day 0 to Treg numbers 12 wk after BMT and found that the transferred nTregs constituted 20.0 ± 0.7% of the total Treg pool, whereas the transferred CD4 iTregs constituted 10.9 ± 1.3% of the Treg pool (Fig. 6B, 6C). Next, we administered IL-2 complexes that are known to preferentially expand Tregs when coadministered with RAPA after BMT (7). Again, the addition of IL-2 complexes to RAPA resulted in similar protection by nTregs and CD4 iTregs, and some long-term survivors were now seen in recipients of CD8 iTregs (Fig. 4D). The addition of IL-2 complexes did not appear to result in improved long-term Foxp3 expression and expansion, as determined by BLI, relative to RAPA alone (Fig. 4C, 4D). However, target organ (Fig. 6A) and phenotypic analysis of Tregs in animals that received RAPA and survived 12 wk after BMT demonstrated that a significant number (10–20%) of the Tregs at this time were derived from the Tregs initially transferred on day 0 (Fig. 6B, 6C). The addition of IL-2 complexes to RAPA resulted in an increase in this proportion in recipients receiving CD4 iTregs, whereas the recovery of CD8 iTregs remained limited (Fig. 6D–F).

Next, we studied whether iTregs (particularly CD4 iTregs) remained suppressive long-term after BMT. In these studies, lethally irradiated BALB/c recipients were transplanted with 1×10^6 nTregs or CD4 iTregs, and recipients were given RAPA

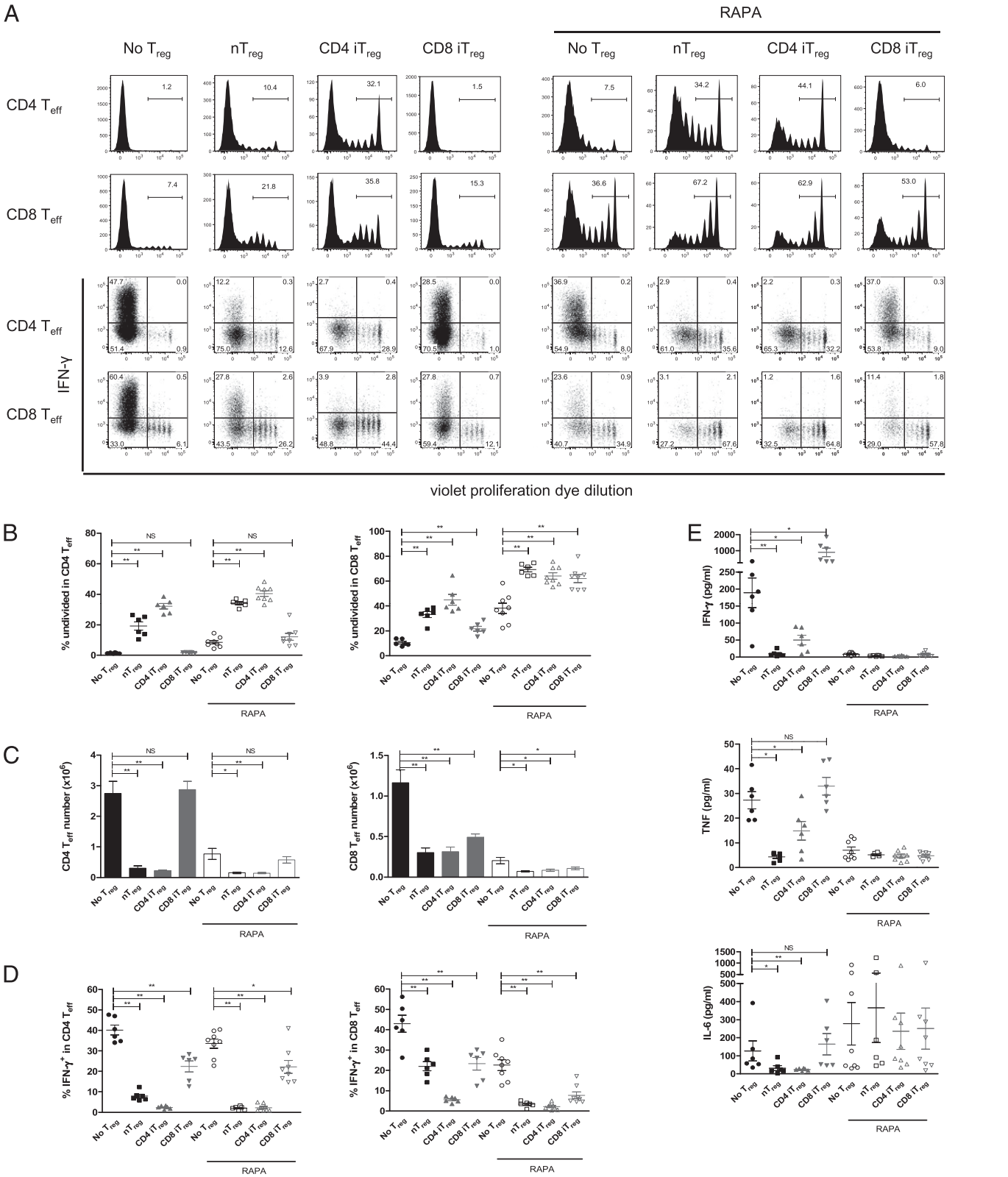

FIGURE 3. The effects of RAPA administration in vivo on Treg suppression. Mice were transplanted as in Fig. 2A, and analysis was performed 6 d after BMT. (**A**) Representative plots of CD4 and CD8 Teff proliferation (indicated by dilution of VPD) and cytokines in mice receiving the various Treg populations. (**B**) Suppression of Teff proliferation by Tregs, as determined by the proportion of undivided cells. (**C**) Teff expansion, as determined by the numbers recovered per spleen. (**D**) Intracellular IFN-γ production in CD4 and CD8 Teffs. (**E**) IFN-γ, TNF-α, and IL-6 in the sera of animals 6 d after BMT. All data (n = 6–8) are combined from four experiments. $^*p < 0.05$, $^{**}p < 0.01$.

and IL-2 complexes. GFP$^+$ Tregs were purified from spleens by FACS sort 28 d posttransplant. An in vitro–suppression assay using the previously described assays demonstrated that both nTregs and CD4 iTregs retained suppressive properties that were similar to those of the freshly sorted naive nTregs (Fig. 6G).

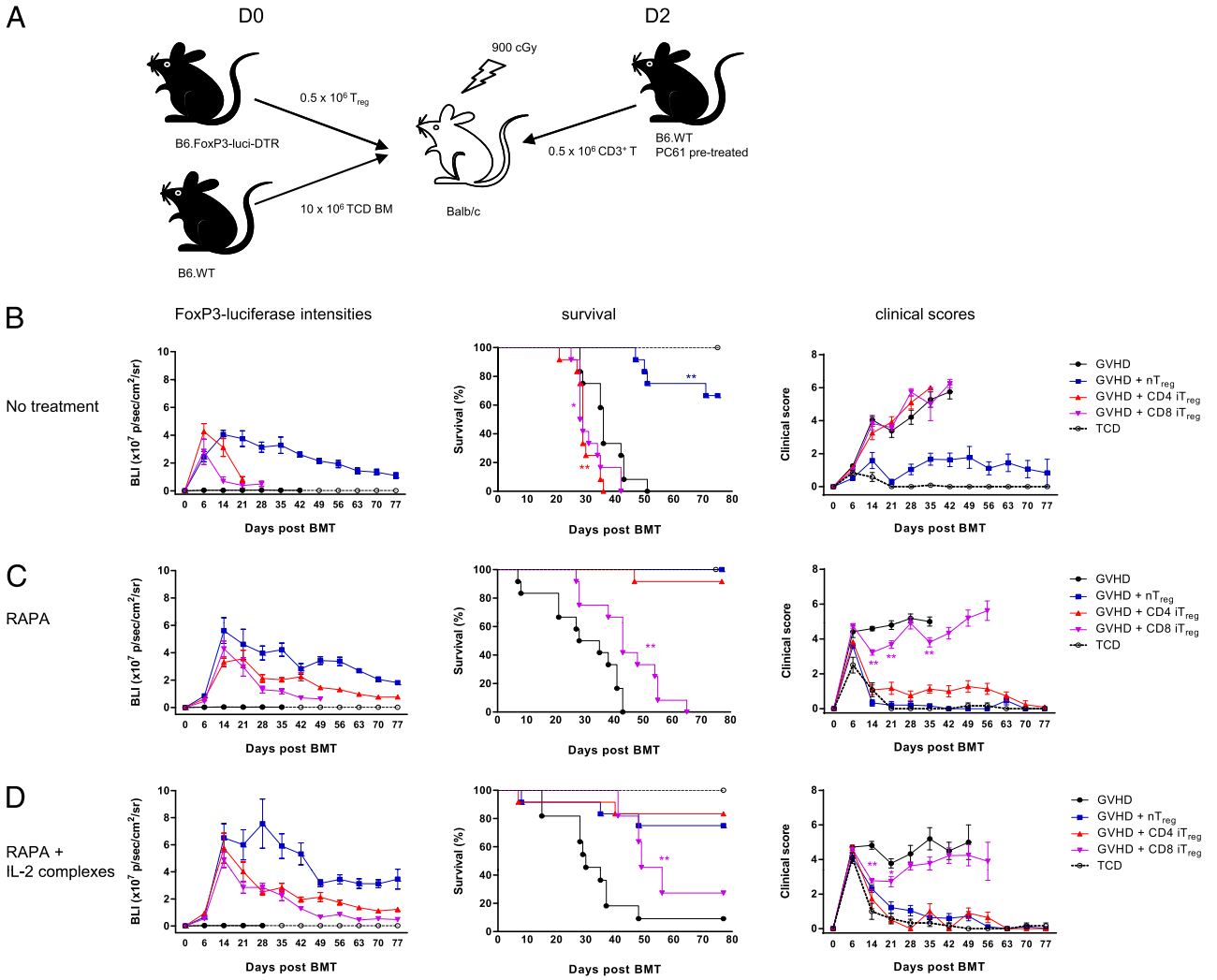

FIGURE 4. iTreg and RAPA administration inhibits GVHD. (**A**) Experimental schema. A total of 0.5 × 10^6 CD4 iTregs, CD8 iTregs, or sort-purified nTregs from B6.Foxp3-GFP-luciferase-DTR mice, together with wild-type B6 TCD-BM, were transplanted into lethally irradiated BALB/c recipients on day 0, followed by transfer of B6 Treg-depleted $CD3^+$ T cells on day 2. Treg expansion and survival were monitored by BLI, and GVHD was determined by survival and clinical score. (**B**) Recipients received no additional therapy. (**C**) Recipients received RAPA from days 0 to 6. (**D**) Recipients received RAPA from days 0 to 6 and IL-2 complexes on days 0 and 4. For BLI data, n = 6–12 in Treg groups, and n = 3–6 in each TCD group. For GVHD and clinical score data, n = 11–12 in Treg groups, and n = 6 in each TCD group. Data are combined from two replicate experiments. $^*p < 0.05$, $^{**}p < 0.01$.

CD4 and CD8 iTregs are unstable and pathogenic in the absence of RAPA in vivo

Given that $Foxp3^+$ iTregs rapidly reverted to $Foxp3^-$ effectors after BMT, we asked whether the adoptively transferred iTregs themselves had the capacity to induce GVHD in the longer term. Thus, we transplanted lethally irradiated BALB/c mice with 0.5 × 10^6 nTregs, CD4 iTregs, or CD8 iTregs from B6.Foxp3-GFP ($CD45.2^+$) donors and TCD-BM from B6.$CD45.1^+$ donors. Recipients of CD4 and CD8 iTregs demonstrated higher GVHD scores in comparison with those receiving TCD-BM in isolation and recipients of nTregs (Fig. 7A). Furthermore, significantly higher levels of IFN-γ and TNF were detected in the sera of recipients of CD4 and CD8 iTregs 12 d after BMT, whereas IL-6 was also elevated in recipients of CD4 iTregs (Fig. 7B). We next examined the effects of RAPA and IL-2 complexes (which expand both CD4 and CD8 Tregs) (7) on Treg function in vivo. Notably, the coadministration of RAPA and IL-2 complexes with iTregs significantly reduced clinical scores and eliminated cytokine production in transplant recipients compared with iTregs alone (Fig. 7A, 7B). Examination of GVHD target organs on day 21 after BMT demonstrated that iTregs could induce GVHD in the absence, but not the presence, of RAPA and IL-2 complexes (Fig. 7C, 7D). In particular, CD4 iTregs generated significant GVHD within the gastrointestinal tract and liver, whereas CD8 iTregs were pathogenic in the skin in the absence of RAPA and IL-2 complexes (Fig. 7C, 7D). The ability of CD4 iTregs to induce GVHD was prevented by short-term RAPA and IL-2 complex administration, whereas CD8 iTregs still resulted in GVHD within the gastrointestinal tract in some animals, even under these conditions (Fig. 7C, 7D), consistent with the only partial improvements seen in survival and clinical scores (Fig. 4). Examination of Foxp3 within nTregs revealed that this lineage was relatively stable over 3 wk (Foxp3 frequency, 57.4 ± 3.4%); however, Foxp3 in both CD4 and CD8 iTregs was lost over the same time period (corresponding frequencies, 7.0 ± 1.0% and 2.7 ± 0.5%, respectively). The administration of RAPA and IL-2 complexes significantly inhibited the conversion and expansion of $Foxp3^+$ cells into $Foxp3^-$ effectors but did not impair the recovery of $Foxp3^+$ CD4 iTregs or nTregs (Fig. 7E, 7F). Importantly, the recovery of $Foxp3^+$ CD8 iTregs was minimal at 3 wk after BMT, regardless of RAPA and IL-2 complex administration.

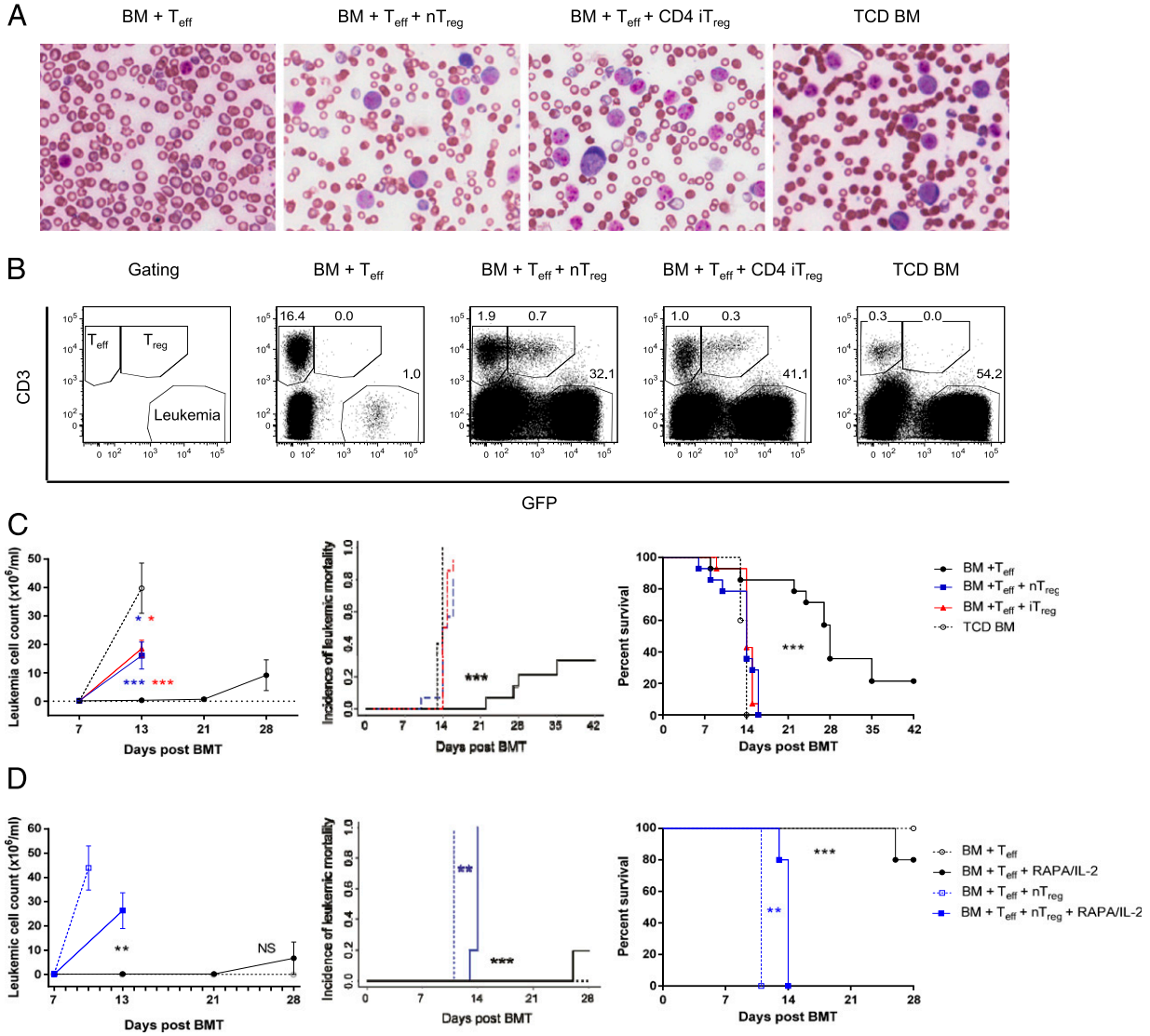

FIGURE 5. Tregs impair GVL effects. CD4 iTregs or naive nTregs (0.5×10^6) from B6.Foxp3-GFP-luciferase-DTR ($CD3^+GFP^+$) mice were transplanted with 1×10^6 leukemia cells ($CD3^-GFP^+$) and TCD-BM (B6) into lethally irradiated B6D2F1 recipients on day 0. At day 2, 0.5×10^6 conventional T cells (B6, $CD3^+$) depleted of Tregs were transferred. All animals in (**A**–**C**) received RAPA and IL-2 complexes, as previously described. (A) Representative images of blood smears across groups (Wright Giemsa stain, original magnification ×200). (B and C) Leukemia ($CD3^-GFP^+$) was quantified in the peripheral blood, as demonstrated. Representative plots are shown. (C) Incidence of leukemic mortality and overall survival of transplant recipients (n = 14 in T cell–replete groups; n = 10 in the TCD group). Data are combined from two replicate experiments. (**D**) Leukemic count in peripheral blood, incidence of leukemic mortality, and overall survival of recipients of nTregs, with or without RAPA and IL-2 complexes (n = 5/group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

We next determined the factors controlling the reversion of $Foxp3^+$ Tregs to $Foxp3^-$ Teffs in vivo in relation to alloantigen and GVHD. Sort-purified, VPD-labeled CD4 iTregs and CD8 iTregs from B6.Foxp3-GFP mice were transplanted with TCD-BM (B6, $CD45.1^+$) into lethally irradiated syngeneic (B6, $CD45.1^+$) or allogeneic (B6D2F1) recipients. Teffs (B6, $CD45.1^+$) were transferred 2 d later to the syngeneic and one allogeneic group to generate syngeneic (non-GVHD, no alloantigen) and allogeneic (both GVHD and alloantigen present) groups, respectively. A third allogeneic group did not receive any Teffs (non-GVHD but alloantigen present). As shown in Fig. 7G through 7I, Foxp3 within CD4 iTregs ($CD45.2^+H2D^{d-}Thy1.2^+$) was relatively stable 6 d after syngeneic BMT (Foxp3 frequency 65.2 ± 2.3%) but was lost quickly in the presence of alloantigen, regardless of the concurrent presence of GVHD (corresponding frequencies 20.1 ± 1.3% and 19.4 ± 1.1%, respectively). In contrast, CD8 iTregs proliferated rapidly into $Foxp3^-$ Teffs in vivo under all conditions, regardless of the presence of alloantigen or GVHD (Fig. 7G, 7H). The loss of Foxp3 in both populations correlated with alloantigen (CD4 iTreg) or homeostatic (CD8 iTreg) proliferation and the subsequent expansion of $Foxp3^-$ effector cells.

Suppression of systemic TNF and IL-6 by Tregs correlates with transplant outcome

Our in vivo T cell suppression studies indicated that nTregs and CD4 iTregs suppressed the proliferation and expansion of responder T cells and, correspondingly, reduced the serum levels of proinflammatory cytokines. Thus, we investigated the relationship between serum cytokine levels and recipient survival. In the absence of RAPA and IL-2 complexes, only nTregs suppressed the production of TNF, whereas the CD8 iTregs increased its production, consistent with the respective beneficial and pathogenic effects of each population on survival (Fig. 8A). When RAPA was given to recipients, TNF was suppressed significantly by nTregs

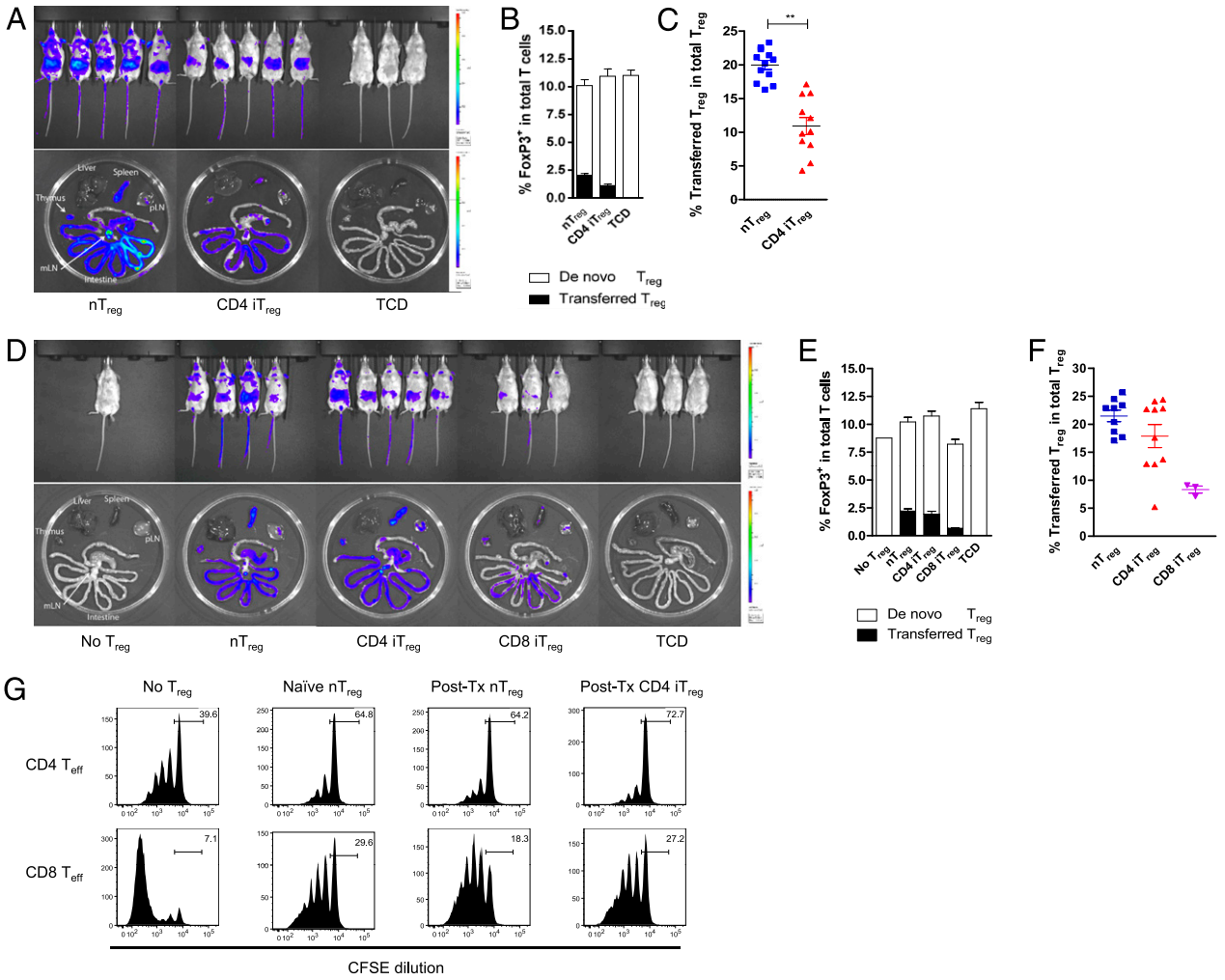

FIGURE 6. Contribution of the adoptively transferred Tregs to the long-term population. (**A**) Whole-body and organ luciferase imaging in BMT recipients receiving RAPA alone and surviving 12 wk after BMT. (**B**) The expression of GFP in Foxp3$^+$ Tregs as a proportion of splenic CD3 T cells. (**C**) The proportion of splenic Foxp3$^+$ Tregs expressing GFP (i.e., originating from the initial transferred Tregs). n = 11–12 in Treg groups; n = 6 in TCD group. (**D**) Whole-body and organ luciferase imaging in BMT recipients receiving RAPA and IL-2 complexes and surviving 12 wk after BMT. (**E**) The expression of GFP in Foxp3$^+$ Tregs as a proportion of splenic CD3 T cells. (**F**) The proportion of Foxp3$^+$ Tregs expressing GFP (i.e., originating from the initial transferred Treg). n = 9–10 in CD4 Treg groups, n = 3 in CD8 Treg group. Data are combined from two independent experiments. (**G**) Foxp3$^+$ cells were sort-purified (on the basis of Foxp3-GFP$^+$) from spleens 28 d after transplantation of adoptively transferred nTregs or CD4 iTregs. Suppression was analyzed, as previously described, using naive nTregs as a positive control. Representative plots from two replicate experiments are shown. $^{**}p < 0.01$.

and CD4 iTregs (Fig. 8B), and the addition of IL-2 complexes reduced TNF in all groups (Fig. 8C). IL-6 production was suppressed by nTregs and CD4 iTregs when RAPA was given (Fig. 8B) and also was suppressed by CD8 iTregs when IL-2 complexes were added (Fig. 8C). As previously noted, RAPA suppressed IFN-γ secretion in general, and this was further suppressed in the presence of nTregs and CD4 iTregs. Thus, suppression of TNF and IL-6 at this time point correlated with the prevention of GVHD mortality following Treg transfer and was maximal when recipients received RAPA and IL-2 complexes.

Discussion

Attempts to regulate GVHD by the expansion of nTregs either directly in vivo or by the adoptive transfer of in vitro–expanded nTreg has been studied extensively and demonstrates clear proof of principle (11, 12, 25, 26). Nevertheless, the ability to generate large, clinically relevant numbers of nTregs remains problematic. In contrast, iTregs can be generated rapidly in large numbers and constitute a promising alternative choice for adoptive transfer (15, 17). However, the instability of Foxp3 expression within iTregs and their potential to revert to pathogenic Teffs remain a concern and have represented major limitations (18, 42). Attempts to improve the stability of Foxp3 expression within iTregs have yielded only limited success. In the current study, we generated iTregs from both conventional CD4 and CD8 T cells, verified their suppressive properties both in vitro and in vivo, and identified the optimal conditions for their in vivo suppression of lethal GVHD. To our knowledge, this is the first study of CD4$^+$Foxp3$^+$ and CD8$^+$Foxp3$^+$ iTregs in parallel. We demonstrate that short-term RAPA with or without IL-2 administration to BMT recipients was permissive of highly effective suppression by CD4 iTregs. In contrast, suppression by CD8 iTregs remains suboptimal and, indeed, without further optimization and confirmation of efficacy in fully murine systems in vivo, the translation to clinic would seem premature.

Previous studies showed that CD4$^+$Foxp3$^+$ iTregs can be induced from conventional T cells in vitro in the presence of IL-2 and TGF-β, with similar suppressive properties to naive nTregs in vitro. Recently, Foxp3-expressing CD8 T cells were identified both in vitro and in vivo (7, 16, 30, 31, 43), although little remains

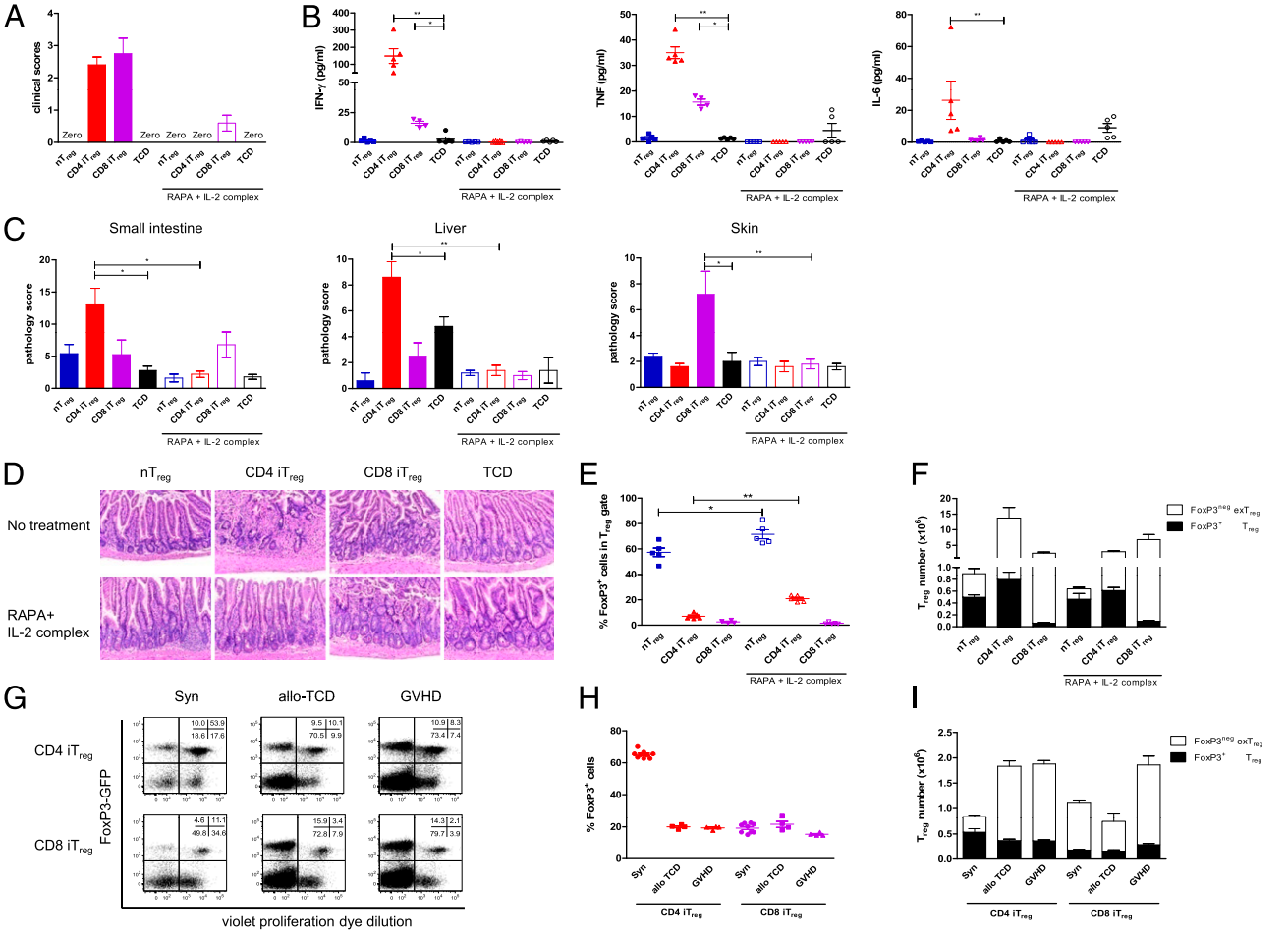

FIGURE 7. iTregs are pathogenic in vivo in the absence of RAPA. Lethally irradiated BALB/c recipients ($CD45.2^+$, $H2D^{d+}$) were transplanted with 0.5×10^6 nTregs, CD4 iTregs, or CD8 iTregs from B6.Foxp3-GFP ($CD45.2^+$) mice together with TCD-BM (B6, $CD45.1^+$), without conventional T cells. In a second cohort, recipients were given RAPA plus IL-2 complexes, as previously described. (**A**) GVHD clinical scores at day 21. (**B**) IFN-γ, TNF, and IL-6 levels in sera at 12 d after BMT. (**C**) Semiquantitative histopathology of GVHD target organs 21 d after BMT. (**D**) Representative images of small intestine (H&E stain, original magnification ×200). (**E**) The proportion of cells expressing Foxp3 within the transferred Tregs ($CD45.2^+H2D^{d-}Thy1.2^+$). (**F**) Quantification of transferred Tregs in spleen 21 d after BMT, divided into $Foxp3^+$ and $Foxp3^-$ populations. $n = 4$–5 per group in (A–F). (**G–I**) A total of 1×10^6 sort-purified, VPD-labeled CD4 iTregs and CD8 iTregs from B6.Foxp3-GFP mice, together with TCD-BM (B6, $CD45.1^+$), was transplanted into lethally irradiated syngeneic (B6, $CD45.1^+$) or allogeneic (B6D2F1, $CD45.2^+$, $H2D^{d/b}$) recipients. A total of 1×10^6 Treg-depleted $CD3^+$ T cells (B6, $CD45.1^+$) was transferred into syngeneic and allogeneic GVHD groups on day 2. An allo-TCD group did not receive conventional T cells. Foxp3 expression and VDP dilution were determined in iTreg ($CD45.2^+H2D^{d-}Thy1.2^+$) populations 6 d after BMT. (G) Representative plots of Treg proliferation, as gated on $CD45.2^+H2D^{d-}Thy1.2^+$ cells within spleen. (H) Foxp3 expression in iTreg populations after BMT. (I) Number of $Foxp3^+$ and $Foxp3^-$ cells within the adoptively transferred Tregs ($CD45.2^+H2D^{d-}Thy1.2^+$) quantified in spleen. $n = 9$ in syngeneic groups, and $n = 4$ in allo-TCD and GVHD groups. $^*p < 0.05$, $^{**}p < 0.01$.

known about their therapeutic potential. Importantly, we demonstrate that the outgrowth of $Foxp3^-$ cells from transferred CD4 or CD8 iTregs in vivo is a major limitation in both populations. Importantly, this loss of Foxp3 is driven by alloantigen- and homeostatic-driven proliferation and can be prevented by RAPA administration in vivo. Although RAPA administration in vivo would appear to have limited benefit in the scenario of nTreg transfer, it is relatively neutral in this setting; thus, RAPA still appears to be a significantly better option for Teff-targeted inhibition than are calcineurin inhibitors (22, 44). However, in the context of CD4 iTreg transfers, the coadministration of RAPA appears to be mandatory, rendering the combination cell therapy–immunosuppression approach a highly promising therapeutic strategy. In addition, the use of iTregs is not limited by the cell subset separation and low starting cell numbers that are characteristic of nTreg-based approaches.

With regard to the impact of Tregs on GVL, initial reports demonstrated that nTregs could impair the priming and expansion of CTLs but not the cytolytic capacity (13). In contrast, the same study demonstrated that nTregs could prevent GVHD lethality and that these animals did not die of leukemia, unlike the recipients of TCD-BM only (13). Thus, like most studies, this work demonstrates that some GVL effects remain in the presence of nTregs, but this usually is not quantified because the controls die of GVHD. It also should be noted that many of the tumor lines used for these studies (and in particular B cell lymphoma cell lines) are very sensitive to eradication by alloreactive T cells in vivo. Indeed, in our hands, as few as 50,000 donor B6 T cells will eradicate high numbers (1 million) of the A20 line in BALB/c recipients, a T cell dose that induces minimal or no GVHD (data not shown). Our current findings were based on experiments using a primary myeloid blast crisis CML cell, which is aggressive and more faithfully represents human acute myeloid leukemia. Importantly, the leukemia can relapse (like human acute myeloid leukemia), even in the presence of a GVL effect, albeit in a highly attenuated fashion, As such, these leukemias may be preferable to quantify the effects

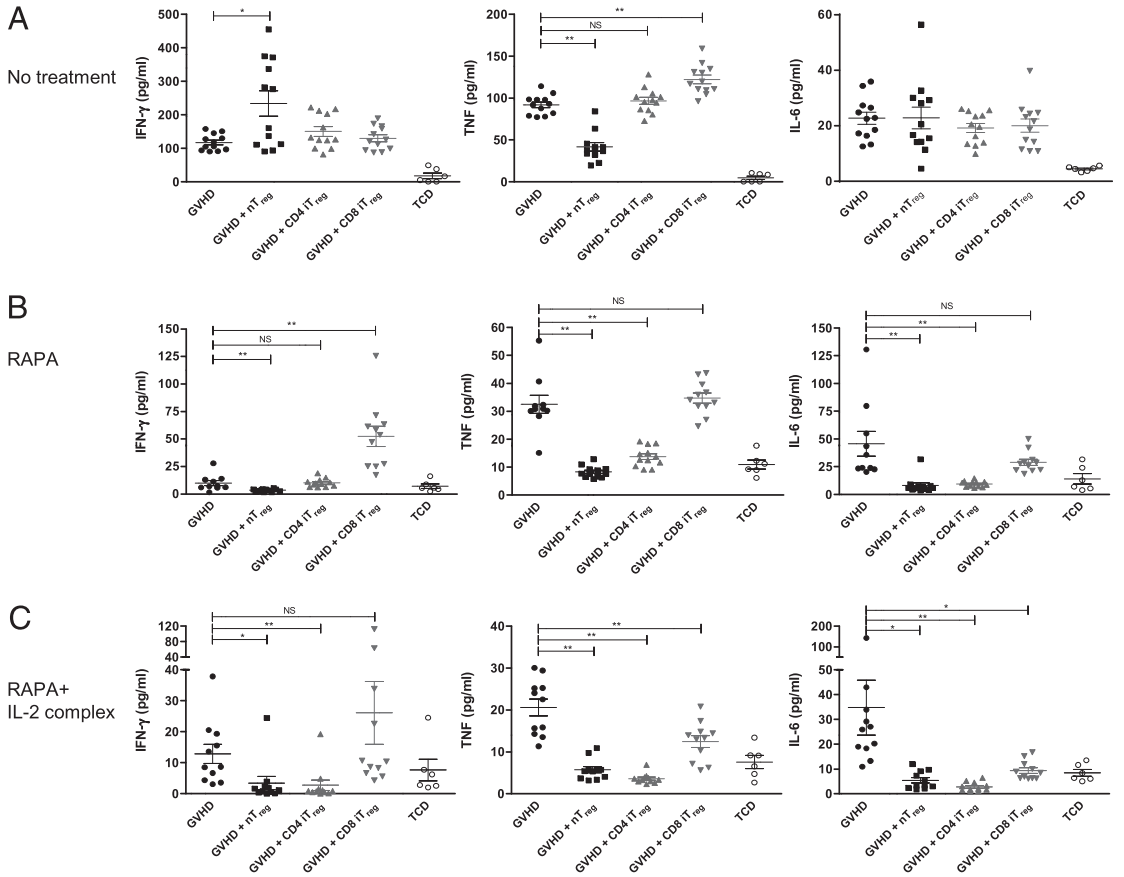

FIGURE 8. The suppression of TNF and IL-6 by Tregs correlates with survival. Animals were transplanted as in Fig. 4 and cytokine levels determined in the sera 12 days after BMT. Recipients receiving no RAPA or IL-2 in vivo (**A**), recipients receiving RAPA alone on days 0–6 (**B**), or recipients receiving RAPA and IL-2 complexes on days 0 and 4 (**C**). Data are combined from two independent experiments, and each data point represents an individual animal. $*p < 0.05$, $**p < 0.01$.

of Tregs on GVL. As would be expected given that Tregs potently suppress both CD4 and CD8 expansion and effector function in vivo, we demonstrate that, at a high Treg/Teff ratio, GVL is significantly abrogated. To our knowledge, this is the first time that this has been definitively demonstrated in vivo and has potential ramifications for strategies in which Tregs are to be used as GVHD prophylaxis. This is less of an issue if treating chronic GVHD when the leukemia has likely been eradicated and the goal is to rescue patients from otherwise fatal GVHD.

Previous studies used various pharmacologic agents in culture or administered them systemically after BMT in attempts to improve nTreg expansion and Foxp3 expression long-term (18–20, 45). In the studies by Zeiser et al. (22), adoptive transfer of freshly separated nTregs in conjunction with the administration of RAPA after BMT demonstrated significant synergy in suppressing acute GVHD. We (7) and other investigators (27) demonstrated that RAPA and IL-2 can be used to expand nTreg or iTreg populations in vivo, improving the control of GVHD. However, studies are lacking regarding the ability of iTregs generated in vitro to prevent GVHD after adoptive transfer. Murine studies demonstrated the inability of transferred CD4 iTregs alone to prevent GVHD in the presence or absence of IL-6 inhibition (18). In contrast, studies of human CD4 iTregs demonstrated partial (17) protection from GVHD in humanized mice. A more recent study suggested dramatic protection by CD8 Ag-specific iTregs in humanized mice (46), a result in contrast to that seen in this study in a fully murine system. It is worth considering the limitations of xenograft systems in studying immune responses, particularly GVHD. First, human T cells cannot recognize and be stimulated by resting murine APCs (47). By extension, the cognate TCR–MHC interaction between human T cells and murine GVHD target organs is ineffective. Thus, GVHD in these traditional xenograft systems is generated by human APCs, presenting murine-derived xenoantigens to (predominantly) human CD4 T cells, driving inflammatory cytokine-mediated GVHD that is independent of direct T cell–mediated cytotoxicity against target tissues. Indeed, CD8 T cells cannot induce GVHD in these systems (48). Although this pathway can effectively induce CD4-dependent cytokine-mediated GVHD (49), the ability of CD8 T cells to mediate GVHD is extremely limited in the absence of effective cognate TCR–MHC interactions (49, 50). A recent study of CD8 iTregs used a xenograft system in which human APCs were engrafted prior to secondary transplantation to allow the induction of GVHD by direct presentation of human alloantigens to human T cells (46). However, the absence of appropriate (human) MHC on GVHD target organs dictates that any alloreactive CD8 T cell would be unable to induce GVHD in these systems, regardless of origin. In contrast, they will suppress CD4 T cell responses via IL-2 consumption (51). Thus, we feel that caution needs to be exercised in the clinical use of CD8 iTregs, especially given their pathogenic propensity as confirmed in this study.

The protective effects of Tregs seen in this study correlated with suppression of TNF and IL-6, which are known to be important cellular mediators of GVHD (52–54). Although it is attractive to consider IL-6 the critical cytokine driving Th17 at the expense of Treg differentiation, any such role after BMT appears to be relatively small, and significant redundancy appears to exist (18, 52). In this regard, it was demonstrated that the ability to generate

Foxp3$^+$ iTregs after BMT is inhibited by STAT3 signaling (55). The cytokines signaling through this pathway include IL-6, IL-11, G-CSF, IL-10, IL-21, IL-23, and IL-27 (56); thus, it is likely that, in the context of the inflammatory milieu created during acute GVHD, significant redundancy exists within this family of cytokines.

Clarification of the mechanisms that drive the expansion of Foxp3$^-$ Teffs from iTregs is important to improve long-term outcomes with regard to the induction of tolerance. Previous studies demonstrated that the maintenance of Foxp3 expression in Tregs tends to be unstable within an inflammatory milieu, and IL-10 may reverse this process (42, 55, 57). In the current study, we demonstrate that the loss of Foxp3 in iTregs following adoptive transfer correlates with proliferation, and the concurrent administration of RAPA attenuates this process. The fact that this loss of Foxp3 occurs independently of GVHD suggests that the positive effects of RAPA do not occur indirectly via inhibition of GVHD.

Together, these data suggest that CD4 iTregs, in conjunction with RAPA, with or without exogenous IL-2, represent an attractive strategy to promote tolerance. In contrast, CD8 iTregs need to be approached more cautiously as a therapeutic strategy because of their propensity to revert rapidly to pathogenic Teffs. Initially, the CD4 iTregs could be explored in well-designed clinical studies focusing on the treatment of GVHD, but there clearly are wider potential applications in solid organ transplantation and autoimmunity.

Disclosures

The authors have no financial conflicts of interest.

References

1. Koyama, M., R. D. Kuns, S. D. Olver, N. C. Raffelt, Y. A. Wilson, A. L. Don, K. E. Lineburg, M. Cheong, R. J. Robb, K. A. Markey, et al. 2012. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. *Nat. Med.* 18: 135–142.
2. Shlomchik, W. D., M. S. Couzens, C. B. Tang, J. McNiff, M. E. Robert, J. Liu, M. J. Shlomchik, and S. G. Emerson. 1999. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science* 285: 412–415.
3. Morris, E. S., and G. R. Hill. 2007. Advances in the understanding of acute graft-versus-host disease. *Br. J. Haematol.* 137: 3–19.
4. Ferrara, J. L., J. E. Levine, P. Reddy, and E. Holler. 2009. Graft-versus-host disease. *Lancet* 373: 1550–1561.
5. Socié, G., and B. R. Blazar. 2009. Acute graft-versus-host disease: from the bench to the bedside. *Blood* 114: 4327–4336.
6. Blazar, B. R., W. J. Murphy, and M. Abedi. 2012. Advances in graft-versus-host disease biology and therapy. *Nat. Rev. Immunol.* 12: 443–458.
7. Robb, R. J., K. E. Lineburg, R. D. Kuns, Y. A. Wilson, N. C. Raffelt, S. D. Olver, A. Varelias, K. A. Alexander, B. E. Teal, T. Sparwasser, et al. 2012. Identification and expansion of highly suppressive CD8(+)FoxP3(+) regulatory T cells after experimental allogeneic bone marrow transplantation. *Blood* 119: 5898–5908.
8. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30: 626–635.
9. Bilate, A. M., and J. J. Lafaille. 2012. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu. Rev. Immunol.* 30: 733–758.
10. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345–352.
11. Brunstein, C. G., J. S. Miller, Q. Cao, D. H. McKenna, K. L. Hippen, J. Curtsinger, T. Defor, B. L. Levine, C. H. June, P. Rubinstein, et al. 2011. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 117: 1061–1070.
12. Di Ianni, M., F. Falzetti, A. Carotti, A. Terenzi, F. Castellino, E. Bonifacio, B. Del Papa, T. Zei, R. I. Ostini, D. Cecchini, et al. 2011. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* 117: 3921–3928.
13. Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9: 1144–1150.
14. Hippen, K. L., S. C. Merkel, D. K. Schirm, C. M. Sieben, D. Sumstad, D. M. Kadidlo, D. H. McKenna, J. S. Bromberg, B. L. Levine, J. L. Riley, et al. 2011. Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Sci. Transl. Med.* 3: 83ra41.
15. Selvaraj, R. K., and T. L. Geiger. 2007. A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta. *J. Immunol.* 179: 11 p following 1390.
16. Mayer, C. T., S. Floess, A. M. Baru, K. Lahl, J. Huehn, and T. Sparwasser. 2011. CD8+ Foxp3+ T cells share developmental and phenotypic features with classical CD4+ Foxp3+ regulatory T cells but lack potent suppressive activity. *Eur. J. Immunol.* 41: 716–725.
17. Hippen, K. L., S. C. Merkel, D. K. Schirm, C. Nelson, N. C. Tennis, J. L. Riley, C. H. June, J. S. Miller, J. E. Wagner, and B. R. Blazar. 2011. Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. *Am. J. Transplant.* 11: 1148–1157.
18. Beres, A., R. Komorowski, M. Mihara, and W. R. Drobyski. 2011. Instability of Foxp3 expression limits the ability of induced regulatory T cells to mitigate graft versus host disease. *Clin. Cancer Res.* 17: 3969–3983.
19. Lal, G., N. Zhang, W. van der Touw, Y. Ding, W. Ju, E. P. Bottinger, S. P. Reid, D. E. Levy, and J. S. Bromberg. 2009. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. *J. Immunol.* 182: 259–273.
20. Polansky, J. K., K. Kretschmer, J. Freyer, S. Floess, A. Garbe, U. Baron, S. Olek, A. Hamann, H. von Boehmer, and J. Huehn. 2008. DNA methylation controls Foxp3 gene expression. *Eur. J. Immunol.* 38: 1654–1663.
21. Chen, Q., Y. C. Kim, A. Laurence, G. A. Punkosdy, and E. M. Shevach. 2011. IL-2 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo. *J. Immunol.* 186: 6329–6337.
22. Zeiser, R., D. B. Leveson-Gower, E. A. Zambricki, N. Kambham, A. Beilhack, J. Loh, J. Z. Hou, and R. S. Negrin. 2008. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood* 111: 453–462.
23. Tresoldi, E., I. Dell'Albani, A. Stabilini, T. Jofra, A. Valle, N. Gagliani, A. Bondanza, M. G. Roncarolo, and M. Battaglia. 2011. Stability of human rapamycin-expanded CD4+CD25+ T regulatory cells. *Haematologica* 96: 1357–1365.
24. Waickman, A. T., and J. D. Powell. 2012. mTOR, metabolism, and the regulation of T-cell differentiation and function. *Immunol. Rev.* 249: 43–58.
25. Koreth, J., K. Matsuoka, H. T. Kim, S. M. McDonough, B. Bindra, E. P. Alyea, III, P. Armand, C. Cutler, V. T. Ho, N. S. Treister, et al. 2011. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N. Engl. J. Med.* 365: 2055–2066.
26. Matsuoka, K., J. Koreth, H. T. Kim, G. Bascug, S. McDonough, Y. Kawano, K. Murase, C. Cutler, V. T. Ho, E. P. Alyea, et al. 2013. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. *Sci. Transl. Med.* 5: 179ra43.
27. Shin, H. J., J. Baker, D. B. Leveson-Gower, A. T. Smith, E. I. Sega, and R. S. Negrin. 2011. Rapamycin and IL-2 reduce lethal acute graft-versus-host disease associated with increased expansion of donor type CD4+CD25+Foxp3+ regulatory T cells. *Blood* 118: 2342–2350.
28. Ahmadzadeh, M., and S. A. Rosenberg. 2006. IL-2 administration increases CD4+ CD25(hi) Foxp3+ regulatory T cells in cancer patients. *Blood* 107: 2409–2414.
29. Cutler, C., S. Li, V. T. Ho, J. Koreth, E. Alyea, R. J. Soiffer, and J. H. Antin. 2007. Extended follow-up of methotrexate-free immunosuppression using sirolimus and tacrolimus in related and unrelated donor peripheral blood stem cell transplantation. *Blood* 109: 3108–3114.
30. Beres, A. J., D. Haribhai, A. C. Chadwick, P. J. Gonyo, C. B. Williams, and W. R. Drobyski. 2012. CD8+ Foxp3+ regulatory T cells are induced during graft-versus-host disease and mitigate disease severity. *J. Immunol.* 189: 464–474.
31. Sawamukai, N., A. Satake, A. M. Schmidt, I. T. Lamborn, P. Ojha, Y. Tanaka, and T. Kambayashi. 2012. Cell-autonomous role of TGFβ and IL-2 receptors in CD4+ and CD8+ inducible regulatory T-cell generation during GVHD. *Blood* 119: 5575–5583.
32. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329–341.
33. Suffner, J., K. Hochweller, M. C. Kühnle, X. Li, R. A. Kroczek, N. Garbi, and G. J. Hämmerling. 2010. Dendritic cells support homeostatic expansion of Foxp3+ regulatory T cells in Foxp3.LuciDTR mice. *J. Immunol.* 184: 1810–1820.
34. Nguyen, V. H., R. Zeiser, D. L. Dasilva, D. S. Chang, A. Beilhack, C. H. Contag, and R. S. Negrin. 2007. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood* 109: 2649–2656.
35. Burman, A. C., T. Banovic, R. D. Kuns, A. D. Clouston, A. C. Stanley, E. S. Morris, V. Rowe, H. Bofinger, R. Skoczylas, N. Raffelt, et al. 2007. IFNgamma differentially controls the development of idiopathic pneumonia syndrome and GVHD of the gastrointestinal tract. *Blood* 110: 1064–1072.
36. Dash, A. B., I. R. Williams, J. L. Kutok, M. H. Tomasson, E. Anastasiadou, K. Lindahl, S. Li, R. A. Van Etten, J. Borrow, D. Housman, et al. 2002. A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9. *Proc. Natl. Acad. Sci. USA* 99: 7622–7627.
37. Cooke, K. R., L. Kobzik, T. R. Martin, J. Brewer, J. Delmonte, Jr., J. M. Crawford, and J. L. M. Ferrara. 1996. An experimental model of idiopathic pneumonia syndrome after bone marrow transplantation: I. The roles of minor H antigens and endotoxin. *Blood* 88: 3230–3239.
38. MacDonald, K. P., V. Rowe, C. Filippich, R. Thomas, A. D. Clouston, J. K. Welply, D. N. Hart, J. L. Ferrara, and G. R. Hill. 2003. Donor pretreatment with progenipoietin-1 is superior to granulocyte colony-stimulating factor in preventing graft-versus-host disease after allogeneic stem cell transplantation. *Blood* 101: 2033–2042.
39. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org/. Accessed September 30, 2013.

40. Boyman, O., M. Kovar, M. P. Rubinstein, C. D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science* 311: 1924–1927.
41. Maude, S. L., S. K. Tasian, T. Vincent, J. W. Hall, C. Sheen, K. G. Roberts, A. E. Seif, D. M. Barrett, I. M. Chen, J. R. Collins, et al. 2012. Targeting JAK1/2 and mTOR in murine xenograft models of Ph-like acute lymphoblastic leukemia. *Blood* 120: 3510–3518.
42. Schmitt, E. G., D. Haribhai, J. B. Williams, P. Aggarwal, S. Jia, L. M. Charbonnier, K. Yan, R. Lorier, A. Turner, J. Ziegelbauer, et al. 2012. IL-10 produced by induced regulatory T cells (iTregs) controls colitis and pathogenic ex-iTregs during immunotherapy. *J. Immunol.* 189: 5638–5648.
43. Mahic, M., K. Henjum, S. Yaqub, B. A. Bjørnbeth, K. M. Torgersen, K. Taskén, and E. M. Aandahl. 2008. Generation of highly suppressive adaptive CD8(+) CD25(+)FOXP3(+) regulatory T cells by continuous antigen stimulation. *Eur. J. Immunol.* 38: 640–646.
44. Zeiser, R., V. H. Nguyen, A. Beilhack, M. Buess, S. Schulz, J. Baker, C. H. Contag, and R. S. Negrin. 2006. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 108: 390–399.
45. Jeon, E. J., B. Y. Yoon, J. Y. Lim, H. J. Oh, H. S. Park, M. J. Park, M. A. Lim, M. K. Park, K. W. Kim, M. L. Cho, and S. G. Cho. 2012. Adoptive transfer of all-trans-retinal-induced regulatory T cells ameliorates experimental autoimmune arthritis in an interferon-gamma knockout model. *Autoimmunity* 45: 460–469.
46. Zheng, J., Y. Liu, M. Liu, Z. Xiang, K. T. Lam, D. B. Lewis, Y. L. Lau, and W. Tu. 2013. Human CD8+ regulatory T cells inhibit GVHD and preserve general immunity in humanized mice. *Sci. Transl. Med.* 5: 168ra9.
47. Lucas, P. J., C. V. Bare, and R. E. Gress. 1995. The human anti-murine xenogeneic cytotoxic response. II. Activated murine antigen-presenting cells directly stimulate human T helper cells. *J. Immunol.* 154: 3761–3770.
48. Wilson, J., H. Cullup, R. Lourie, Y. Sheng, A. Palkova, K. J. Radford, A. M. Dickinson, A. M. Rice, D. N. Hart, and D. J. Munster. 2009. Antibody to the dendritic cell surface activation antigen CD83 prevents acute graft-versus-host disease. *J. Exp. Med.* 206: 387–398.
49. Teshima, T., R. Ordemann, P. Reddy, S. Gagin, C. Liu, K. R. Cooke, and J. L. Ferrara. 2002. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat. Med.* 8: 575–581.
50. Matte-Martone, C., J. Liu, D. Jain, J. McNiff, and W. D. Shlomchik. 2008. CD8+ but not CD4+ T cells require cognate interactions with target tissues to mediate GVHD across only minor H antigens, whereas both CD4+ and CD8+ T cells require direct leukemic contact to mediate GVL. *Blood* 111: 3884–3892.
51. McNally, A., G. R. Hill, T. Sparwasser, R. Thomas, and R. J. Steptoe. 2011. CD4+ CD25+ regulatory T cells control CD8+ T-cell effector differentiation by modulating IL-2 homeostasis. *Proc. Natl. Acad. Sci. USA* 108: 7529–7534.
52. Tawara, I., M. Koyama, C. Liu, T. Toubai, D. Thomas, R. Evers, P. Chockley, E. Nieves, Y. Sun, K. P. Lowler, et al. 2011. Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation. *Clin. Cancer Res.* 17: 77–88.
53. Hill, G. R., J. M. Crawford, K. R. Cooke, Y. S. Brinson, L. Pan, and J. L. M. Ferrara. 1997. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. *Blood* 90: 3204–3213.
54. Chen, X., R. Das, R. Komorowski, A. Beres, M. J. Hessner, M. Mihara, and W. R. Drobyski. 2009. Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. *Blood* 114: 891–900.
55. Laurence, A., S. Amarnath, J. Mariotti, Y. C. Kim, J. Foley, M. Eckhaus, J. J. O'Shea, and D. H. Fowler. 2012. STAT3 transcription factor promotes instability of nTreg cells and limits generation of iTreg cells during acute murine graft-versus-host disease. *Immunity* 37: 209–222.
56. Hill, G. R., R. D. Kuns, N. C. Raffelt, A. L. Don, S. D. Olver, K. A. Markey, Y. A. Wilson, J. Tocker, W. S. Alexander, A. D. Clouston, et al. 2010. SOCS3 regulates graft-versus-host disease. *Blood* 116: 287–296.
57. Zhou, X., S. L. Bailey-Bucktrout, L. T. Jeker, C. Penaranda, M. Martínez-Llordella, M. Ashby, M. Nakayama, W. Rosenthal, and J. A. Bluestone. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* 10: 1000–1007.