

Escape from suppression: tumor-specific effector cells outcompete regulatory T cells following stem-cell transplantation

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Immune reconstitution of autologous hematopoietic stem-cell transplant recipients with the progeny of mature T cells in the graft leads to profound changes in the emerging functional T-cell repertoire. In the steady state, the host is frequently tolerant to tumor antigens, reflecting dominant suppression of naive and effector T cells by regulatory T cells (T_{regs}). We examined the relative frequency and function of these 3 components within the tumor-specific T-cell compartment during immune reconstitution. Grafts from tumor-

bearing donors exerted a significant anti-tumor effect in irradiated, syngeneic tumor-bearing recipients. This was associated with dramatic clonal expansion and interferon- γ (IFN γ) production by previously tolerant tumor-specific T cells. While donor-derived T_{regs} expanded in recipients, they did not inhibit the antigen-driven expansion of effector T cells in the early posttransplantation period. Indeed, the repopulation of tumor-specific effector T cells significantly exceeded that of T_{regs}, the expansion of

which was limited by IL-2 availability. Although the intrinsic suppressive capacity of T_{regs} remained intact, their diminished frequency was insufficient to suppress effector cell function. These findings provide an explanation for the reversal of tolerance leading to tumor rejection in transplant recipients and likely contribute to the efficacy of adoptive T-cell therapies in lymphopenic hosts. (Blood. 2008;111:2112-2121)

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Introduction

Hematopoietic stem cell transplantation (HSCT) is a well-established procedure for treating a variety of hematologic diseases. The dose-intensive “host conditioning” for HSCT is both myeloablative and lymphodepleting, requiring that the progeny of the infused cells reconstitute hematopoiesis, including a T- and B-cell repertoire capable of restoring adaptive immunity. In the case of allogeneic HSCT, mature lymphocytes contained within the graft not only initiate immune reconstitution, but are potent killers of cancer cells that survive chemo/radiation therapy, providing an immune-mediated graft-versus-tumor (GVT) effect. Unfortunately, this response against allo-antigens lacks tumor specificity, accounting for graft-versus-host disease (GVHD), a toxicity that limits the overall success of allogeneic HSCT. Efforts to reduce this immune-mediated toxicity have been associated with a reduction in the GVT effect as well, leading to increases in relapse rates.¹ It remains to be determined whether novel strategies for manipulating the graft, the host, and/or posttransplantation immune modulation can widen the window between GVT and GVHD.

Autologous HSCT, by contrast, provides a less toxic alternative to allogeneic transplantation. However, it is generally assumed that the autologous nature of the graft precludes any immune-mediated antitumor effect. Besides lacking the potency of the allo-response, the infused lymphocytes come from a donor in whom the very cancer being targeted has successfully evaded endogenous immune surveillance.² We and others have shown that one mechanism contributing to this immune evasion is the induction of T-cell tolerance to tumor antigens.^{3,4} Accordingly, immunotherapy in the setting of autologous HSCT must contend with targeting weakly

immunogenic tumor-associated antigens (as opposed to allo-antigens) with a T-cell repertoire that has been rendered functionally unresponsive to those antigens.

In spite of these considerations, there is ample experimental evidence that infusion of tumor antigen-sensitized lymphocytes into lymphopenic, tumor-bearing recipients can mediate significant tumor rejection.⁵⁻⁹ Indeed, we previously reported the paradoxical observation that mice with established B-cell lymphoma that underwent transplantation with marrow and lymphocytes from syngeneic tumor-bearing donors had superior progression-free survival to identical cohorts receiving grafts from non-tumor-bearing donors. Mature postthymic T cells from the tumor-bearing donors were an essential component of the graft in mediating this effect.¹⁰ Furthermore, this syngeneic GVT effect could be sustained with repeated immunizations in the posttransplantation period, using a granulocyte-macrophage colony-stimulating factor (GM-CSF)-producing tumor cell vaccine, a strategy that has since been taken into the clinic in patients with multiple myeloma and acute myelogenous leukemia undergoing autologous HSCT.^{11,12}

These findings suggest that the profound changes that accompany immune reconstitution of a lymphopenic host somehow lead to the unmasking and/or amplification of an endogenous antitumor immune response that was ineffective in the relative steady state of the lymphocyte-replete tumor-bearing host. Understanding the mechanisms by which such a state of tumor-specific unresponsiveness is altered during reconstitution is essential for fully exploiting the platform of autologous HSCT and adoptive T-cell therapy as effective modes of cancer treatment.²

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We have previously demonstrated that T-cell receptor (TCR) transgenic (tg) T cells specific for influenza hemagglutinin (HA) undergo partial activation followed by functional anergy in mice harboring HA-expressing A20 B-cell lymphoma (A20HA).³ This unresponsive state is manifest as an overall decreased capacity to proliferate, undergo clonal expansion, and produce interferon- γ (IFN γ) in response to HA antigen *in vitro* and *in vivo*. However, further analysis has revealed that the HA-specific CD4⁺ T cells from A20HA-bearing mice are actually functionally heterogeneous, consisting of naive, effector, and regulatory CD4⁺Foxp3⁺ T cells (T_{regs}). The appearance of anergy in the HA-specific CD4⁺ T-cell pool as a whole is the net result of the suppression exerted by an expanding pool of HA-specific T_{regs}, which mask the functional competence of naive and effector T cells (T_{effs}).¹³

We hypothesized that the superior relapse-free survival of transplant recipients receiving grafts from tumor-bearing donors relative to tumor-free donors involves an alteration in the function and/or the frequency of T_{regs} during immune reconstitution, thereby allowing effector cells (present at increased frequency in grafts from tumor-bearing donors) to launch a transient antitumor immune response. In the present study, we show that tumor-specific T_{effs} outcompete T_{regs} during the early posttransplantation period, resulting in a significant fall in the T_{reg}/T_{eff} ratio that is no longer sufficient to blunt effector function. These changes may in part account for the efficacy of adoptive T-cell therapies in lymphopenic hosts and provide an explanation for the recovery of host antitumor immunity and window of vaccine responsiveness observed in the posttransplantation period.

Methods

Mice

BALB/c mice (4-8 weeks old) were purchased from Harlan (Indianapolis, IN). BALB/c TCR tg ("6.5") mice expressing a TCR specific for amino acids 110 to 120 of influenza hemagglutinin (HA) were a generous gift from Dr H. von Boehmer (Dana-Farber Cancer Institute, Boston, MA).¹⁴ 6.5 mice on a Thy1.1^{+/+}, Thy1.1^{+/Thy1.2}+, or Rag2^{-/-} background were used in experiments where indicated. Experiments were conducted in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Tumor cells and T-cell isolation

Murine A20 lymphoma cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μ g/mL streptomycin, 2 mM L-glutamine, nonessential amino acids, 5 mM HEPES buffer, and 100 μ M 2-mercaptoethanol and grown at 37°C in 5% CO₂. A20HA cells were generated as described previously¹⁵ and were selected in complete medium supplemented with the neomycin analog G418 (400 μ g/mL). A20HA-luciferase (A20HA-Luci) was generated by electroporating A20HA cells with a luciferase-encoding plasmid. A20HA-Luci cells were grown in medium containing 400 μ g/mL G418 and 400 μ g/mL hygromycin. Tumor cells (10⁶) in a total volume of 0.2 mL Hanks balanced salt solution (HBSS) were injected into each mouse intravenously.

Single-cell suspensions were made from peripheral lymph nodes (LNs) and spleens. CD4⁺ T cells were enriched by removing CD8⁺ and B220⁺/MHCII⁺ cells, as previously described.¹⁶ The percentage of tg lymphocytes doubly positive for CD4 and the clonotypic TCR was determined by flow cytometry as described "Flow cytometric analysis." Cells were washed 3 times and injected into the tail vein of BALB/c recipients (2.5 \times 10⁶ 6.5/CD4⁺ T cells/recipient). For experiments involving CFSE^{low}/GITR⁺ or CFSE^{low}/GITR⁻ tg cells, pre-enriched TCR tg

CD4⁺ cells were labeled with CFSE (Molecular Probes, Eugene, OR) by incubating with 1 μ M CFSE at 37°C for 10 minutes in HBSS containing 0.1% bovine serum albumin (BSA). Cells were washed 3 times with ice-cold HBSS before injection.

Bioluminescent imaging

In vivo optical imaging was performed with a prototype IVIS 3D bioluminescence/fluorescence optical imaging system (Xenogen, Alameda, CA) at indicated time points. Each mouse received an intraperitoneal injection of luciferin (Promega, Madison, WI) at a dose of 125 mg/kg. General anesthesia was induced with ketamine (90 mg/kg) and xylazine (10 mg/kg). After acquiring photographic images of each mouse, luminescent images were acquired with 3-minute exposure times. Optical images were analyzed with Igor (WaveMetrics, Lake Oswego, OR) and IVIS Living Image (Xenogen) software packages.

Syngeneic HSCT

Donor mice (with or without tumor) were killed, and their T cells were isolated by negative depletion of MHC II⁺ and B220⁺ cells as described.¹⁶ The femurs and tibiae were obtained from wild-type (WT) BALB/c mice, and bone marrow (BM) was harvested by flushing the bones with medium. The marrow was T-cell depleted with magnetic selection using antibodies against CD4 and CD8. The graft consisted of 4 \times 10⁶ T cell-depleted BM cells along with 4 to 16 \times 10⁶ T cell-enriched lymphocytes (Figures 1-3), or a total of 300 000 sorted CD4⁺ T cells (T_{regs} + T_{effs}; Figures 4-6; Table 1). Recipients were 5- to 6-week-old BALB/c mice that had been irradiated with 850 cGy total body irradiation (TBI), followed by injection of the graft in a volume of 0.2 mL intravenously. The animals that underwent transplantation were maintained in sterile microisolator cages and received sterile food and water containing 1 mL trimethoprim/sulfamethoxazol (Alpharma, Baltimore, MD). Where indicated, recipients received intraperitoneal injections of IL-2 (10 μ g/mouse) every day following HCST.

Flow cytometric analysis

Antibodies were purchased from BD Biosciences (Mountain View, CA), except anti-GITR antibody, which was prepared from the DTA-1 hybridoma, kindly provided by Dr Ethan Shevach (National Institutes of Health, Bethesda, MD). 6.5 T cells were stained with biotinylated rat anticonotypic TCR antibody 6.5 followed by PE-conjugated streptavidin. Single-cell populations from LNs and spleens were stained with the indicated Abs for cell-surface markers. Events were collected on a FacsCalibur (Becton Dickinson, San Jose, CA) and analyzed using CellQuest Pro software (Becton Dickinson).

Antigen-specific proliferation and *in vitro* suppression assay

T cell-enriched (5 \times 10⁴ per well) lymphocytes from the experimental groups were mixed with fresh WT splenocytes (15-30 \times 10⁴ per well, serving as antigen-presenting cells [APCs]) from BALB/c mice. HA₁₁₀₋₁₂₀ peptide was added at indicated concentrations. CD4⁺-enriched T cells from 6.5/Rag2^{-/-} mice were used as responder cells. Responder cells (N) were incubated with or without sorted T_{regs} or T_{effs} (10⁴ per well) plus APCs and were pulsed with HA peptide (10 μ g/mL). At 48 hours after incubation, supernatant from each well was collected for enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Cells were then pulsed with [³H] thymidine (1 μ Ci/well [0.037 MBq/well]) and cultured for 16 hours before harvesting and measuring scintillation counts.

In vivo priming with Vacc-HA

A recombinant vaccinia virus encoding hemagglutinin from the 1934 PR8 strain of influenza (VaccHA) has been described previously.³ Mice were primed by intraperitoneal or subcutaneous injection with 10⁷ plaque-forming units (pfu) of VaccHA suspended in 0.1 mL HBSS. Mice were killed 5 to 6 days after vaccination.

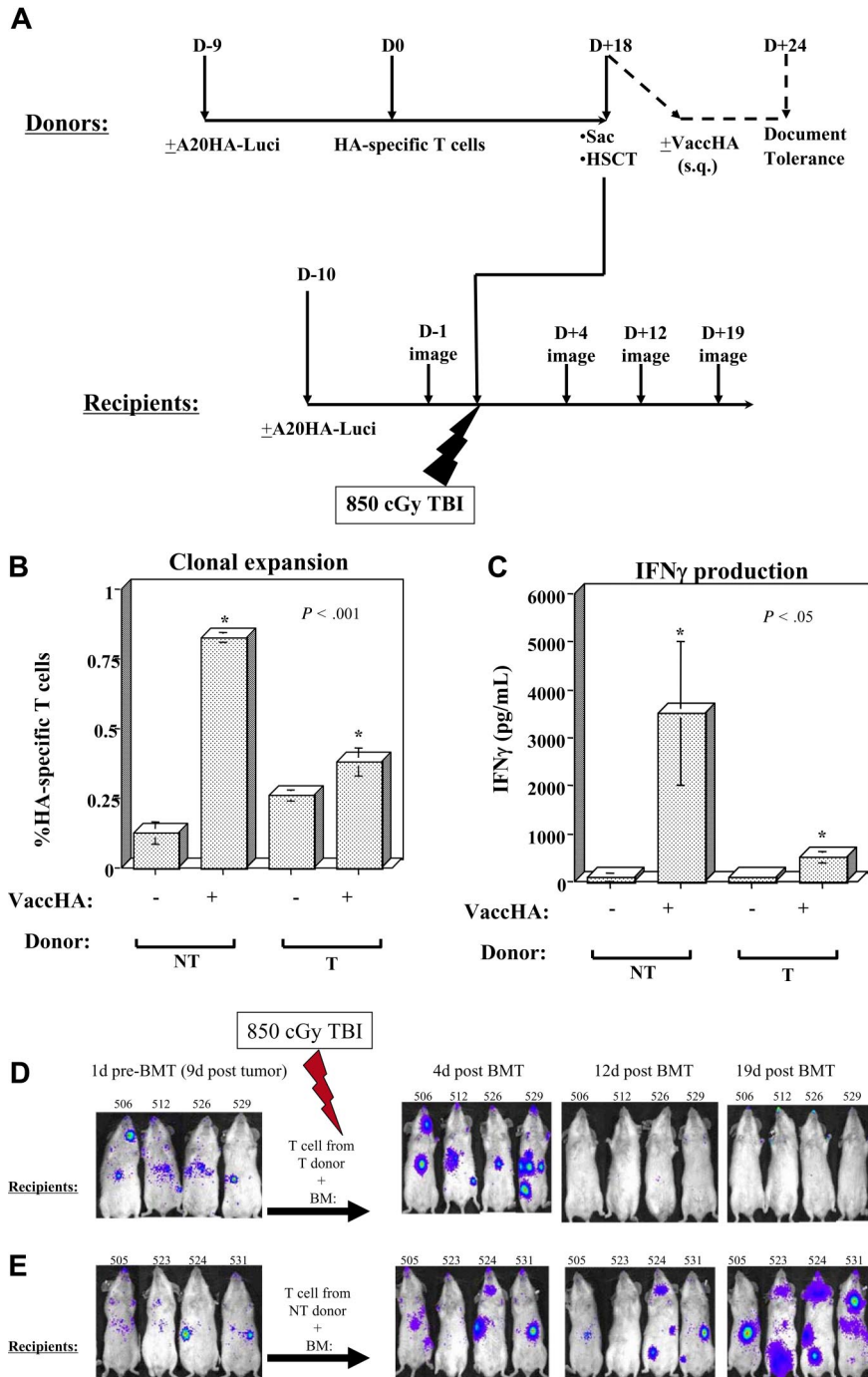


Figure 1. Antitumor immunity visualized by A20HA-Luci imaging. (A) Experimental outline. Donor mice received 2.5×10^6 CD4⁺-enriched HA-specific T cells with or without a tumor challenge (10^6 A20HA-Luci intravenously 9 days before T-cell transfer). At 18 days after T-cell transfer, donor mice were killed, and their spleens and LNs were harvested and T cell-enriched to be transferred into transplant recipients. Recipients were challenged with or without 10^6 A20HA-Luci intravenously 10 days prior to transplantation. Recipients underwent transplantation as described in "Methods." A total of 3 tumor-bearing and 3 non-tumor-bearing mice were randomly removed from the donor pool and received plus or minus 10^7 pfu VaccHA (subcutaneously). They were killed 6 days later to assess HA-specific T-cell function. (B) Percentage of HA-specific T-cell expansion in vivo and (C) IFN γ production in response to HA peptide in vitro in these non-tumor-bearing (NT) and tumor-bearing (T) mice are shown. Data represent mean plus or minus SE. (D,E) Photon emission was used as an indication of tumor size and dissemination. Images of 4 ear-tagged mice per group at the indicated time points are shown.

Statistical analysis

P values were calculated using the Student *t* test. *P* values less than or equal to .05 were considered to be statistically significant.

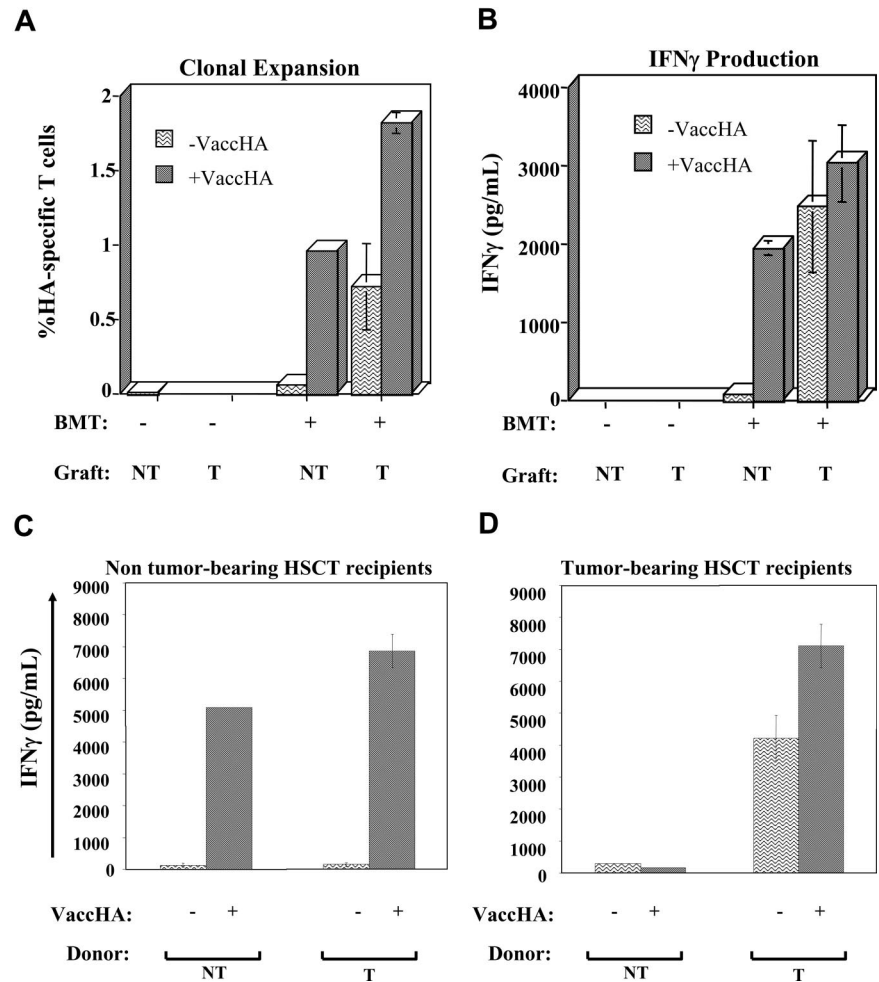
Results

Visualizing antitumor immunity in HSCT recipients

We previously established a murine HSCT model in which mice with disseminated A20 lymphoma are treated with lethal TBI followed by grafting with syngeneic marrow and mature T cells. Paradoxically, we found that relapse-free survival was superior in

recipients of grafts obtained from tumor-bearing donors compared with recipients that received grafts from non-tumor-bearing donors.¹⁰ To visualize the kinetics of tumor progression in this model in relation to changes in the frequency and function of tumor-specific T cells, we generated A20HA-Luci. Transplant donors with or without established lymphoma received 2.5×10^6 CD4⁺-enriched, HA-specific T cells (Figure 1A), enabling an assessment of the frequency and function of this reference population in the donors compared with tumor-bearing recipients during immune reconstitution. To document impaired HA-specific T-cell responsiveness in the tumor-bearing donors at the time of graft collection, 3 tumor-bearing and 3 non-tumor-bearing mice were randomly

Figure 2. Endogenous activation of tumor-specific T cells in HSCT setting. Recipient mice with (A,B,D) or without (C) established tumor underwent transplantation on day 0 (D0), then were inoculated with or without 10⁷ pfu of VaccHA (subcutaneously) 15 days (A,B) or 35 days (C,D) after HSCT/adoptive transfer, and killed 6 days later. Percentage of HA-specific T cells (A) and IFN γ production in response to HA peptide (B-D) in recipient mice was measured. Values are means plus or minus SE of triplicate cultures from 3 mice in each group.



removed from the donor cohort and immunized with VaccHA. Similar to previous findings,³ HA-specific T cells expanded modestly in mice with established A20HA-Luci relative to their frequency in non-tumor-bearing mice. However, they were impaired in their ability to undergo further clonal expansion or to produce IFN γ following vaccination with VaccHA in vivo when compared with the responses seen in vaccinated tumor free mice (Figure 1B,C). By these criteria, “tumor antigen-specific T-cell tolerance” was evident in cells obtained from the donors.

One day before HSCT, recipients were imaged, and photon emission was measured as an indication of tumor size and dissemination (Figure 1D,E). On the day of HSCT, mice received 850 cGy of TBI and grafts from either non- or A20HA-Luci-bearing donors. TBI alone was ineffective lymphoma therapy, as all mice showed an increased intensity and distribution of the bioluminescence signal 4 days after HSCT. This was in agreement with our earlier findings that the dose of irradiation given, while myeloablative and lymphodepleting, is not curative. Interestingly, however, by 12 days after HSCT, recipients of grafts from tumor-bearing donors all had a significant reduction in tumor signal, while recipients of grafts from non-tumor-bearing donors continued to show evidence of disseminated lymphoma. By 19 days after HSCT, there was no detectable signal in recipients of grafts from tumor-bearing donors, and these mice remained progression-free (data not shown). In contrast, recipients of grafts from non-tumor-bearing donors all clearly progressed by 19 days and succumbed to tumor about 3 weeks later. These results graphically illustrate the

“unmasking” of tumor-specific effector function of tumor antigen-experienced lymphocytes upon transfer into irradiated recipients.

Reversal of tumor-specific T-cell tolerance during immune reconstitution

Whereas the reference population of tumor-specific T cells clearly displayed blunted responses in tumor-bearing donors, the rejection of tumor in the recipients suggested that either this population was not representative of the cells responsible for the antitumor response, or that its capacity to respond to tumor antigen was significantly altered during immune reconstitution. Such a change in function of the reference population was indeed found in recipients evaluated 3 weeks after transplantation. HA-specific T cells from both tumor-bearing and non-tumor-bearing grafts expanded and produced IFN γ (Figure 2A,B). This response was particularly pronounced in grafts obtained from tumor-bearing donors and was associated with a restored capacity of these cells to be primed by a therapeutic vaccination. The clonal expansion and IFN γ responses were largely antigen-driven, as they were not seen in tumor-free transplant recipients in the absence of vaccination (data not shown). Furthermore, these changes were not observed by simply transferring the grafted lymphocytes into nonirradiated tumor-bearing recipients. Finally, at the time of this analysis, tumor was clearly evident in recipients of grafts from non-tumor-bearing donors (Figure 1E). In spite of this, however, HA-specific CD4⁺ T cells remained responsive in the early posttransplantation period

(Figure 2A,B). Overall, these findings reveal that lymphocyte repopulation in the early posttransplantation period favors activation rather than tolerance of naive T cells and leads to the restored responsiveness of a previously tolerant population of tumor antigen-specific T cells.

Interestingly, the endogenous activation of naive, HA-specific T cells contained in the grafts from non-tumor-bearing donors was short-lived in the recipients, as these T cells no longer were capable of producing IFN γ or responding to immunization 6 weeks after HSCT (Figure 2D). This indicated that, at later stages of immune reconstitution, the progeny of naive T cells in the recipients could be rendered tolerant in the face of progressing tumor, whereas they remained responsive to vaccination 6 weeks after being transplanted into tumor-free recipients (Figure 2C). In marked contrast, HA-specific T cells from recipients of grafts obtained from tumor-bearing donors maintained an effector/memory response 6 weeks out from transplantation (Figure 2D), corresponding to the successful eradication of tumor seen in this cohort.

Assessing the frequency and function of T_{regs} and T_{effs} isolated from tumor-bearing donors

Given that the appearance of anergy in the HA-specific CD4⁺ T cell pool of A20HA-bearing mice actually reflects suppression exerted by T_{regs} on both naive and T_{effs},¹³ we hypothesized that the effector function unmasked during immune reconstitution might be secondary to changes in the frequency and/or function of these subpopulations. Specifically, we wished to determine whether (1) the relative ratios of T_{regs}/T_{effs} had changed in tumor-bearing hosts during immune reconstitution; (2) T_{regs} themselves were driven to further differentiate into T_{effs}; and/or (3) T_{regs} lost the capacity to suppress during the period of expansion in irradiated recipients.

We first compared the overall frequency of HA-specific T_{regs} present in the donor grafts with that present at 1 and 2 weeks after HSCT. Whereas nearly 60% of the antigen-experienced HA-specific CD4⁺ T cells expressed Foxp3 when harvested from tumor-bearing donors, this frequency fell by more than half during the first 2 weeks of immune reconstitution (Figure 3). These results are consistent with the unmasked effector function and proliferative capacity of T_{effs} demonstrated in Figures 1 and 2.

Availability of IL-2 after HSCT limits T_{reg} repopulation

T_{regs} have been reported to undergo robust homeostatic proliferation in lymphopenic recipients without losing suppressive function.¹⁷ However, posttransplantation expansion of tumor-antigen specific T cells is an antigen-driven process that may be more dependent on cytokine availability than is homeostatic proliferation. Given the exquisite dependence of T_{regs} on IL-2, we hypothesized that their failure to compete with T_{effs} in repopulating the tumor-specific T-cell pool might result from the relative paucity of this cytokine (produced largely by the rare T cells present during early reconstitution). Indeed, as shown in Figure 3, the fall in T_{reg} frequency was partially abrogated when exogenous IL-2 was administered daily during the first week after HSCT. While this represents a fall relative to the starting frequency of tumor-specific T_{regs} in the graft, it is unlikely that the administration of exogenous IL-2 precisely mimics the IL-2-dependent signaling that arises from paracrine production by T cells in vivo.

The overall fall in frequency of T_{regs} detected here does not provide a direct measure of the relative repopulation rates of T_{regs} and T_{effs}, nor does it determine whether conversion of T_{regs} into T_{effs}

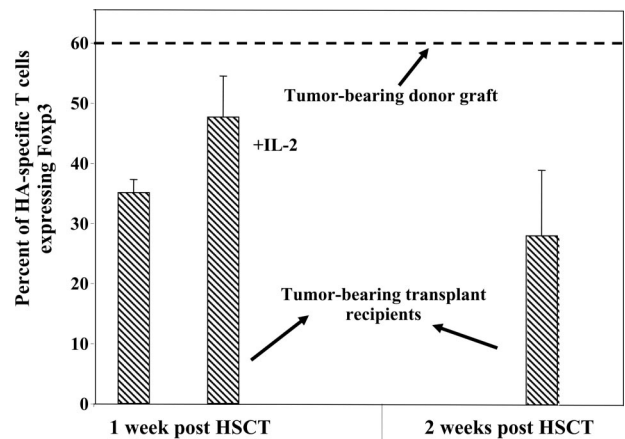


Figure 3. The frequency of antigen-specific T_{regs} in transplant recipients decreases immediately after HSCT. Donor mice (Thy1.2^{+/+}) with a 10-day established tumor burden received 2.5×10^6 CD4⁺-enriched HA-specific T cells (Thy1.1^{+/1.2}). At 19 days after T-cell transfer, donor mice were killed, and their spleens and LNs were harvested and T-cell-enriched to be transferred into transplant recipients. Recipients (Thy1.1^{+/+}) were challenged with 1×10^6 A20HA intravenously 10 days prior to HSCT and underwent transplantation as described in "Methods." Half of the transplant recipients received daily injections of 10 μ g/mouse IL-2 intraperitoneally. The frequency of HA-specific CD4⁺ T cells (Thy1.1^{+/1.2}) expressing Foxp3 was determined by flow cytometry in the graft and in recipients killed 1 and 2 weeks after transplantation. Data represent means plus or minus SE.

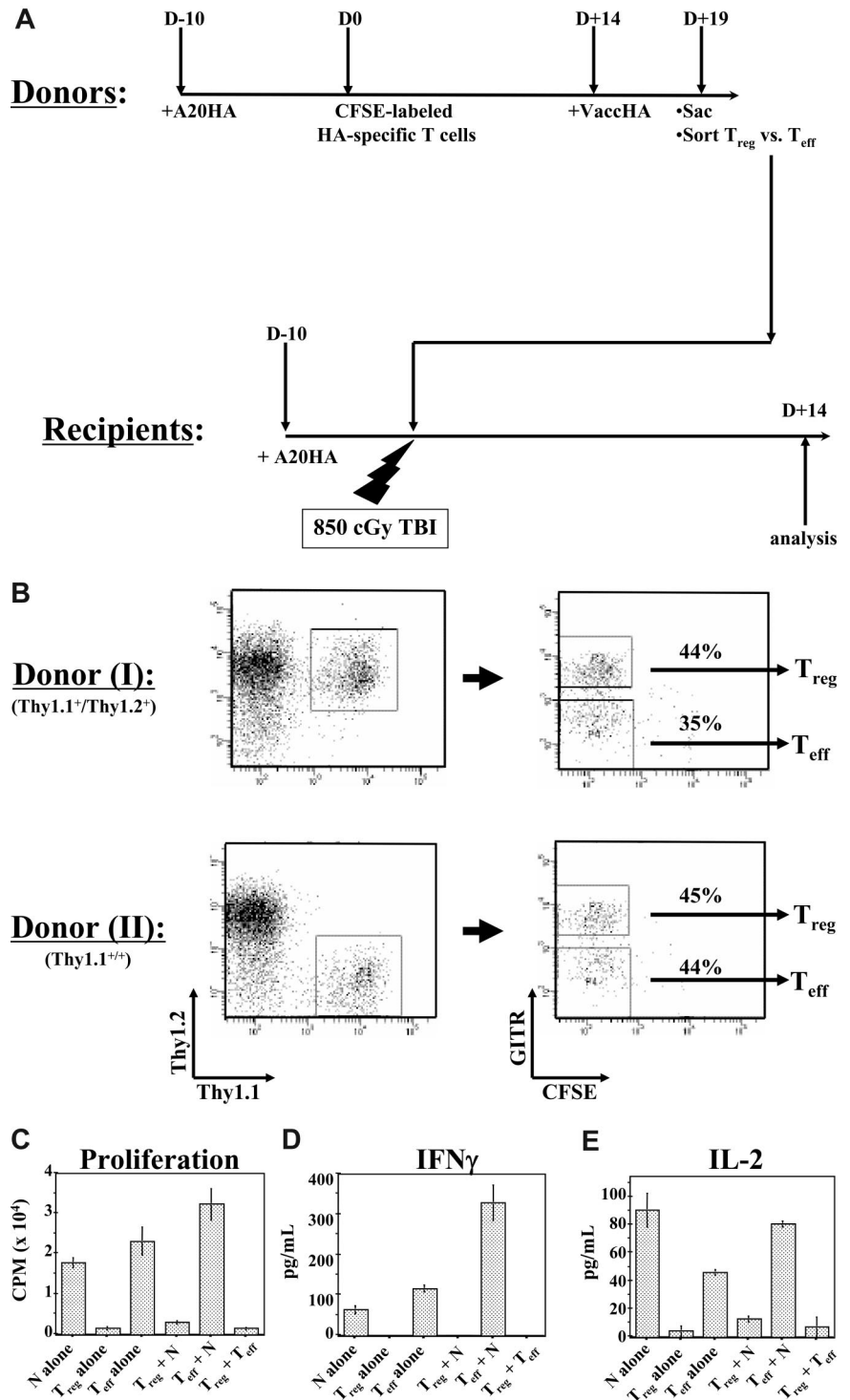
might contribute to these altered ratios that emerge after HSCT. Finally, changes in the intrinsic suppressive capacity of T_{regs} during reconstitution could not be directly examined in this system. To address these questions therefore, we examined the fate of congenically marked T_{regs} and T_{effs} obtained from the donors as they repopulated tumor-bearing transplant recipients. A20HA-bearing BALB/c donors (Thy1.2^{+/+}) received CFSE labeled HA-specific CD4⁺ T cells that were either homozygous (Thy1.1^{+/+}) or heterozygous (Thy1.1^{+/Thy1.2}) at the Thy1 locus (Figure 4A,B). Because HA-specific T cells are present at very low frequencies in tumor-bearing mice, donors were vaccinated with VacCHA 5 days before T-cell isolation. Divided (CFSE^{low}) CD4⁺Thy1.1⁺ cells were sorted into GITR⁺ (T_{regs}) or GITR⁻ (T_{effs}) based on our earlier work identifying that suppressive function (and Foxp3 expression) was largely confined to the GITR⁺ subset, whereas the divided GITR⁻ subset were T helper 1 (Th1) cells.^{13,18} This design enabled the collection of congenically marked T_{regs} and T_{effs} from donors, the progeny of which could then be distinguished in recipients based on the pattern of Thy1 expression.

To verify that the isolated cells from tumor-bearing donors exhibited their expected functions, a small aliquot of the sorted GITR⁺ T_{regs} and GITR⁻ T_{effs} was cultured either alone, together (1:1), or mixed with naive HA-specific CD4⁺ T cells freshly isolated from 6.5/Rag2^{-/-} mice (N), in the presence of fresh APCs and HA peptide. As previously reported,¹³ sorted GITR⁺CFSE^{low} T_{regs} were hypoproliferative in vitro and were unable to produce IFN γ or IL-2. Furthermore, they suppressed HA-specific responses by naive or T_{effs} in vitro, whereas GITR⁻CFSE^{low} cells exhibited effector function and did not suppress (Figure 4C-E).

The fate of tumor-specific T_{effs} and T_{regs} in the posttransplantation period

Analysis of multiple donor mice with established A20HA demonstrated that at the time of graft harvest, CD4⁺, HA-specific GITR⁺ T_{regs} and GITR⁻ T_{effs} existed at approximately a 1:1 ratio (Figure 4B). Accordingly, recipients with established A20HA were lethally irradiated and grafted with T cell-depleted

Figure 4. Validation of function of T_{regs} and T_{effs} isolated from donors. (A) Experimental outline. Donor mice received 10⁶ A20HA intravenously, followed 10 days later by 2.5 × 10⁶ CFSE-labeled, CD4⁺-enriched HA-specific T cells. At 14 days after T-cell transfer, mice were vaccinated with 10⁷ pfu VaccHA (intraperitoneally) and were killed 5 days later. (B) Spleens and LNs were harvested and analyzed by fluorescence-activated cell sorter (FACS). T_{regs} (CFSE^{low}GITR⁺) or T_{effs} (CFSE^{low}GITR⁻) were sorted. (C-E) A total of 10 000 sorted T_{regs} or T_{effs} were cultured in vitro either alone or with 10 000 naive CD4⁺ cells from a 6.5/Rag^{-/-} mouse (naive responder [N]), along with 200 000 splenocytes from a WT BALB/c mouse, and stimulated with 10 μg/mL HA peptide. Proliferation (C) and cytokine production (D,E) were measured as described in "Methods." Data represent mean (± SE) of triplicate cultures.



BM, along with a 1:1 mixture of the sorted Thy1.1^{+/+} and Thy1.1^{+/}Thy1.2⁺ HA-specific: (1) T_{regs} only; (2) T_{effs} only; or (3) T_{regs} and T_{effs} (for experimental design, see Table 1). At 2 weeks after transplantation, the frequency and function of the progeny of the infused cells were examined (Figure 5A,B). Whereas the progeny of donor T_{regs} were readily identified at this time point, their expansion was markedly less than that of the T_{effs} (T_{reg} = 77 500 ± 53 000 vs T_{eff} = 461 000 ± 177 000, P = .03). Furthermore, the clonal expansion of T_{effs} was not diminished by the cotransfer of an equal number of T_{regs} (T_{effs} in

Table 1. Makeup of T-cell grafts transferred into BM transplant recipients

BM transplant recipients	Thy1.1 ^{+/+}	Thy1.1 ^{+/} Thy1.2 ⁺	Phenotype
T _{regs} only	1.5 × 10 ⁵	1.5 × 10 ⁵	CFSE ^{low} GITR ⁺
T _{effs} only	1.5 × 10 ⁵	1.5 × 10 ⁵	CFSE ^{low} GITR ⁻
T _{regs} + T _{effs}	1.5 × 10 ⁵	0	CFSE ^{low} GITR ⁺
T _{regs} + T _{effs}	0	1.5 × 10 ⁵	CFSE ^{low} GITR ⁻

On the day of HSCT, recipients received 850 cGy TBI and received 4 × 10⁶ T cell-depleted BM cells along with 300 000 sorted T_{regs} and/or T_{effs} on different Thy1 congenic backgrounds.

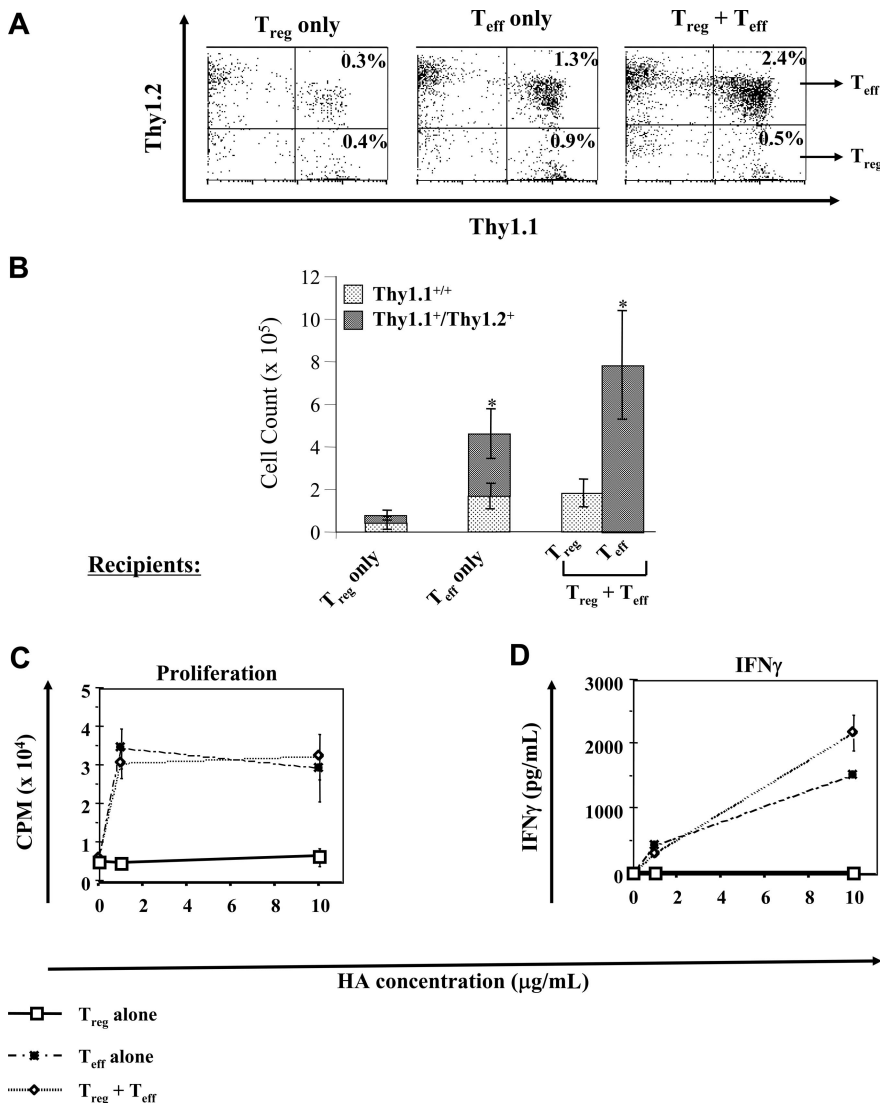


Figure 5. Tumor-specific T_{eff}s outcompete T_{regs} during early immune reconstitution. Mice with a 10D established tumor burden were lethally irradiated and received transplants with BM along with T_{regs} only, T_{eff}s only, or T_{regs} plus T_{eff}s. Mice were killed 2 weeks after HSCT, and spleens were harvested. CD4⁺ T cell frequency was measured by flow cytometry (A) and absolute CD4⁺ cell numbers were calculated (B). CD4⁺ T-cell frequency as measured by flow cytometry is shown in panel A. Data in panel B represent mean (± SE) of 2-4 mice per group. (*P* = .1). A total of 50 000 tumor-purged splenocytes were cultured with 200 000 WT splenocytes to serve as APCs. HA peptide was added at the indicated concentrations. Proliferation (C) and IFN γ production (D) were measured as described in "Methods." *P* values were calculated using the Student *t* test. Data in panels C and D represent mean (± SE) of triplicate cultures.

T_{eff}-only group = 461 000 ± 177 000 vs T_{eff}s in T_{reg} plus T_{eff} group = 784 000 ± 255 000; *P* = .1). This is in marked contrast to the potent capacity of HA-specific T_{regs} to blunt the burst size of an effector response to vaccination in A20HA-bearing mice in the nontransplantation setting.¹³ As a result of this differential repopulation, T_{eff}s outcompeted T_{regs} by 4- to 5-fold both in terms of cell frequency and cell numbers in groups receiving both T_{regs} and T_{eff}s.

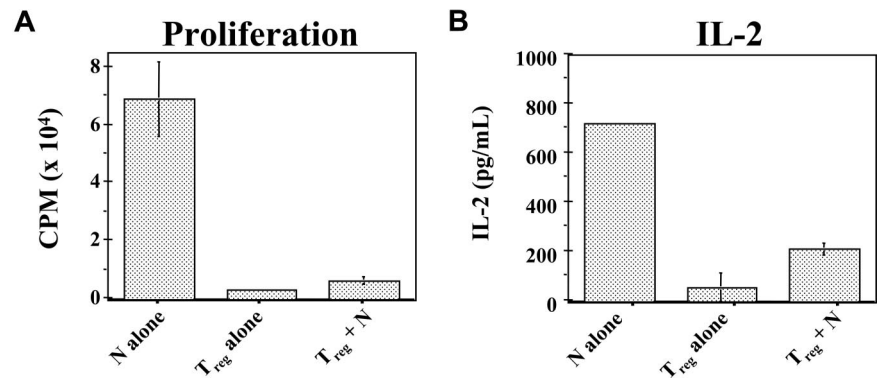
To assess the impact of this change in frequency on the overall function of HA-specific T cells repopulating the transplant recipients, unfractionated splenocytes isolated from each group were evaluated for their capacity to proliferate and produce IFN γ in response to HA peptide in vitro. Whereas HA-specific T cells in the splenocytes of mice that received transplants of T_{regs} only remained hyporesponsive in vitro, those containing T_{regs} and T_{eff}s proliferated and produced IFN γ to the same extent as splenocytes of recipients transplanted with T_{eff}s only (Figure 5C,D). Together, these data demonstrate that T_{regs} do not convert into T_{eff}s as a consequence of homeostatic and antigen-driven expansion during immune reconstitution. Significantly, however, T_{regs} have a negligible impact on the expansion of T_{eff}s in vivo, and as a result, their relative frequency is

inadequate to block effector function as measured in vitro, and potentially in vivo.

T_{regs} isolated during immune reconstitution maintain their intrinsic suppressive capacity in vitro

Although the relative frequency of tumor-specific T_{regs} falls significantly in the early posttransplantation period, those that persist have a negligible impact on T_{eff} expansion. However, it is unclear whether this results from an intrinsic change in the potency of these cells to suppress, or simply reflects their becoming outnumbered. To distinguish these possibilities, HA-specific GITR⁺CFSE^{low} T_{regs} were sorted from tumor-bearing donors and validated to express Foxp3 and suppress in vitro (data not shown). These cells were transplanted into irradiated A20HA-bearing recipients; 2 weeks later, their progeny were sorted based on congenic markers. The sorted cells remained hypoproliferative and were impaired in their ability to produce IL-2 in response to HA peptide in vitro (Figure 6). Furthermore, when assayed for suppression at a 1:1 ratio with a freshly isolated naive HA-specific reference population, they maintained their suppressive activity in vitro (Figure 6A,B). These data demonstrate that, on a per-cell basis, T_{regs} did not

Figure 6. T_{regs} isolated from transplant recipients maintain their suppressive activity in vitro. Donor mice were prepared as in Figure 4. Tumor-bearing transplant recipients were killed 2 weeks after HSCT, and their spleens were harvested. T_{regs} were then sorted out of recipients and cultured in vitro with or without CD4⁺-enriched T cells from 6.5/Rag2^{-/-} mice as responder cells, as described in Figure 4. Proliferation (A) and IL-2 production (B) were measured. Data represent mean (\pm SE) of triplicate cultures.



lose their intrinsic capacity to suppress as a consequence of the events associated with immune reconstitution of transplant recipients.

Discussion

During the course of tumor progression, tumor-specific T cells are rendered tolerant as measured by their lack of ability to proliferate and produce IFN γ in response to tumor antigen.³ The induction of tumor-specific T-cell tolerance is a major obstacle in the development of immune-based strategies.² Understanding the underlying mechanisms leading to this state is critical in achieving a measurable antitumor effect.

Using a syngeneic murine transplantation model, we previously reported that T-cell grafts from tumor-bearing donors impart a survival advantage over those from non-tumor-bearing donors when transferred into tumor-bearing syngeneic transplant recipients.¹⁰ This effect was seen with WT tumor (ie, not expressing a model antigen) and depended only upon the endogenous donor T-cell repertoire (ie, no TCR tg T cells). To elucidate the underlying mechanism at the level of antigen-specific T-cell function, the model was modified here to introduce a “reference population” of TCR tg T cells specific for tumor antigen. This system again demonstrated a measurable GVT effect of syngeneic T cells harvested from tumor-bearing donors (Figure 1D,E), enabling a direct comparison of tumor-specific T-cell function as measured: (1) in the donors; (2) in the recipients rejecting tumor; and (3) in the recipients of naive grafts in which tumor progresses.

This analysis reveals striking changes in the function of a tolerant tumor-specific T-cell population which, as a whole, undergoes a rapid transition to an activated, effector phenotype upon transplantation into irradiated, syngeneic tumor-bearing recipients. This endogenous activation is manifest as clonal expansion and IFN γ production, and is accompanied by a restored ability to respond to vaccination in the posttransplantation period. The increased responsiveness to tumor antigen in the hosts that received transplants is most robust and only maintained in recipients of grafts from tumor-bearing donors (Figure 2A,B). This presumably reflects an increase in the number of effector/memory cells generated during the endogenous response to tumor in these donors relative to that present in tumor antigen-naïve donors. Furthermore, this effector population, which is held in check by T_{regs} in the donors, effectively escapes this suppression in repopulating the transplant recipient, exerting unmasked effector function while increasing in frequency relative to T_{regs} (Figure 5).

More than 25 years ago, Brendt and North reported that it was possible to cause the regression of large, established tumors by

intravenous infusion of tumor-sensitized T cells from immune donors, but only if the tumors are growing in T cell-deficient recipients.¹⁹ Since that time, considerable attention has been given to the antitumor properties associated with infusion of T cells into lymphopenic hosts.⁵⁻⁹ In this setting, the increased availability of cytokines such as IL-7 and IL-15 that govern lymphocyte homeostasis, as well as access to APCs that provide low-level TCR stimulation, both contribute to proliferation and expansion.^{20,21} Several lines of evidence suggest that the mechanisms responsible for maintaining peripheral tolerance are altered during this “nonequilibrium” phase. Dummer et al reported that T cells undergoing homeostatic proliferation exhibited a memory/effector phenotype with evidence of the induction of an antitumor immune response.²² Similarly, in a model of organ allograft rejection, it was demonstrated that T cells that have undergone homeostatic expansion show resistance to tolerance, consistent with known properties of memory cells in vivo.²³

In addition to these intrinsic changes in the susceptibility of naive and T_{effs} to become tolerant, there is ample evidence that extrinsic influences exerted by T_{regs} play a crucial role in regulating T-cell repopulation of a lymphopenic host. Rag2^{-/-} mice receiving naive syngeneic CD4⁺ T cells were shown to develop inflammatory bowel disease that was prevented by cotransfer of T_{regs}, which controlled the size of the resulting activated/memory peripheral T-cell compartment.^{24,25} These findings are consistent with the lymphoproliferative disease and autoimmunity seen in mice that naturally lack T_{regs}, such as Foxp3 mutants, or T_{reg} function as in IL-2^{-/-} and IL-2R^{-/-} mice.²⁶⁻²⁹

CD4⁺Foxp3⁺ T_{regs} have been shown to play a key role in inhibiting a multitude of host immune responses.³⁰ These cells, although hypoproliferative in vitro, undergo both homeostatic¹⁷ and antigen-driven³¹ expansion in vivo, while maintaining their suppressive activities. However, this is the first report to examine the relative antigen-driven expansion of T_{regs} versus T_{effs} in the setting of lymphopenia, a parameter that is particularly relevant to HSCT. Indeed, in the absence of antigen (ie, in irradiated non-tumor-bearing recipients) we find that both T_{regs} and T_{effs} undergo modest homeostatic proliferation. However, the magnitude is far less than that seen in response to antigen, and the ratio of T_{reg}/T_{eff} is not significantly altered (data not shown). In sharp contrast, we find that antigen-driven proliferation in the setting of lymphopenia clearly favors T_{eff} expansion, enabling the execution of effector function.

This study intentionally focuses on the early phases of immune reconstitution because we have observed that there is a strong correlation between the extent of early T-cell engraftment and posttransplantation relapse-free survival (P.M. and H.I.L., manuscript in preparation). For most human cancers treated by HSCT,

tumor relapse precedes significant thymic recovery, placing the burden of any immune-mediated resistance to tumor recurrence on the progeny of mature T cells. Our data illustrate that while HA-specific T_{regs} cotransferred with T_{effs} can be found in tumor-bearing transplant recipients, during this early phase their frequency remains low relative to the progeny of tumor-specific T_{effs} (Figure 5).

Importantly, although T_{regs} had no measurable impact on the expansion of cotransferred T_{effs} in vivo, they did suppress naive HA-specific responder T cells when cocultured in vitro at the initial high frequencies found in the donors (Figure 6). Furthermore, they maintained Foxp3 expression (data not shown), a molecule known to be exclusively expressed by murine T_{regs} .³²⁻³⁴ We have also observed that transplant recipients receiving grafts containing T_{regs} generally have a larger tumor burden at the time of death than those receiving T_{effs} only (data not shown), providing additional evidence for their maintained suppressive capacity. While T_{regs} have little impact on the systemic expansion of T_{effs} in the early posttransplantation period, they may still act locally by inhibiting T_{eff} function at the site of tumor, a possibility we are now investigating.

The balance between T_{effs} and T_{regs} was recently shown to be strongly correlated with the effectiveness of antitumor immune responses.^{35,36} In the current study, we demonstrate that changes in this parameter during immune reconstitution also favor the productive antitumor responses seen in recipients of grafts from tumor-bearing donors. Whereas the focus of the current study is on CD4⁺ T_{effs} , the well-described inversion of the CD4/CD8 T-cell ratio during immune reconstitution suggests that similar principles may apply for T_{reg} influence on CD8 homeostasis.

T_{regs} have been shown to be exquisitely dependent upon IL-2.^{37,38} Given that the only source of IL-2 immediately after HSCT is the repopulating lymphocytes, T_{regs} may be at a competitive disadvantage, especially during antigen-driven proliferation. Indeed, consistent with related studies,³⁷⁻⁴¹ we find that the administration of systemic IL-2 during immune reconstitution increases the frequency of T_{regs} , suggesting that this cytokine may be limiting. The abundance of IL-7 and IL-15 in lymphopenic hosts,²⁰ which inhibit T_{regs} in vitro,⁴² may further abrogate T_{reg} -suppressive activities in vivo.

Many of the principles examined here likely have contributed to the early successes reported in the clinical translation of adoptive T-cell therapy into lymphopenic patients. Dudley et al demonstrated that the infusion of melanoma-specific T cells in fludarabine/

cyclophosphamide-treated patients led to sustained T-cell expansion, associated with clinically measurable tumor regressions.^{43,44} The frequency of responses was significantly greater than that previously reported in lymphocyte-replete patients treated with similar cellular products. Moreover, Rapoport et al have demonstrated that potent T-cell and antibody responses to an immunogen could be elicited in patients with myeloma undergoing autologous HSCT, but optimal responses required the infusion of vaccine-primed and ex vivo–costimulated autologous T cells after myeloablative conditioning, followed by a series of posttransplantation vaccines early during immune reconstitution.⁴⁵

The findings reported here provide insight into the complex interplay between T_{regs} and T_{effs} during the early posttransplantation period, a window of opportunity in which tumor-specific immunity can be restored and amplified. As the underlying elements responsible for these changes become defined, autologous HSCT has the potential to more fully evolve into a platform for effective immunotherapy of cancer.

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Authorship

Contribution: P.M., G.T., H.I.L., and I.B. designed the research and analyzed and interpreted the data. P.M., G.T., G.Z., K.N., and T.M. performed the research. P.M. and H.I.L. drafted the manuscript.

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