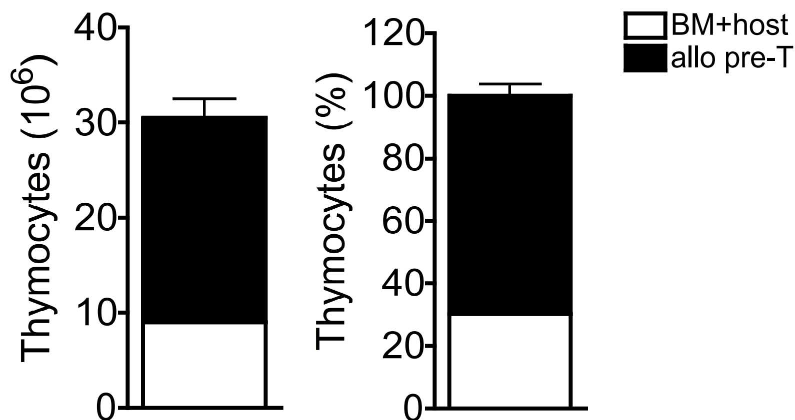
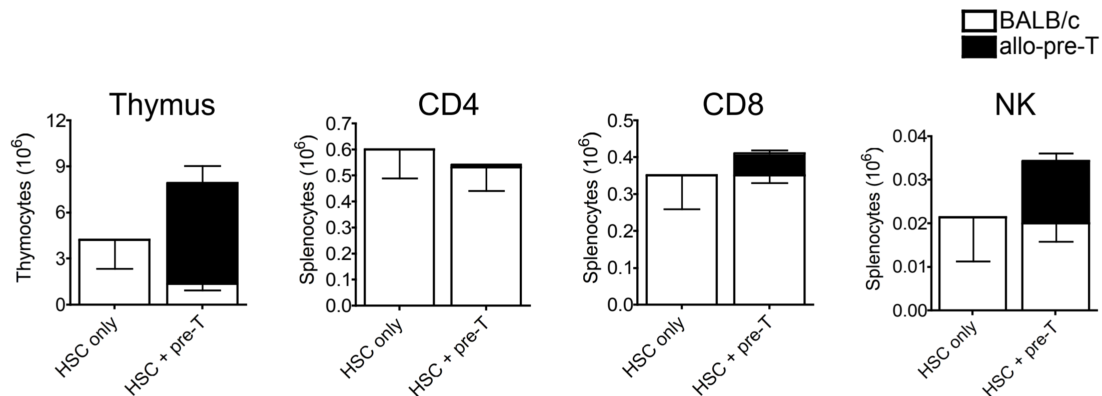
Supplementary Figure 1



Adoptively transferred allogeneic T-cell precursors enhance thymic reconstitution in syngeneic lin$^-$ BMT recipients

Lethally irradiated BALB/c recipients were transplanted with 10^5 BALB/c-derived lin$^-$ BM cells and received $6x10^6$ C57BL/6-derived T-cell precursors. Thymi were harvested on day 14 after transplantation and donor and host progenies were determined by total cellularity and multicolor flow cytometric analysis using CD45.1 and CD45-specific antibodies. Absolute numbers as well as percentages + SEM are presented (*n* = 5). The experiment was performed more than five times.

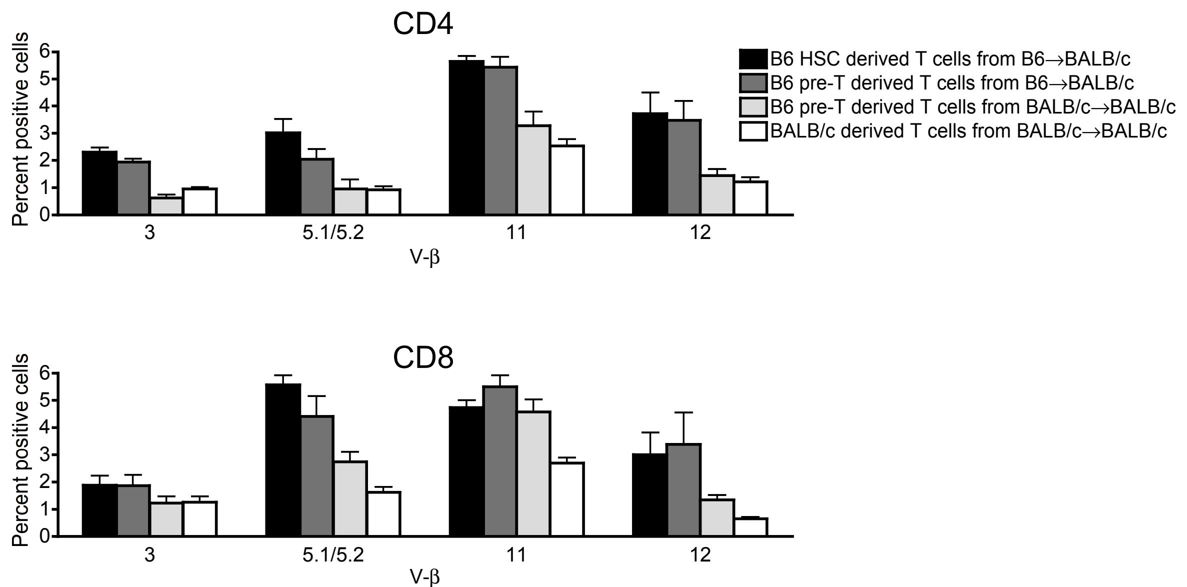
Supplementary Figure 2



Adoptively transferred allogeneic T-cell precursors enhance thymic reconstitution in aging HSCT recipients

Lethally irradiated 12 months old BALB/c recipients were transplanted with BALB/c-derived HSCs; control mice received HSCs only, the pre-T group received additional C57BL/6-derived T-cell precursors. Thymi and spleens were harvested on day 14 after transplantation. Donor and host progenies were determined by total cellularity and multicolor flow cytometric analysis using Ly9.1 and CD45-specific antibodies, T and NK cells were analyzed using CD4, CD8, CD3, and DX5-specific antibodies. Mean cell numbers + SEM are presented (n = 4-5).

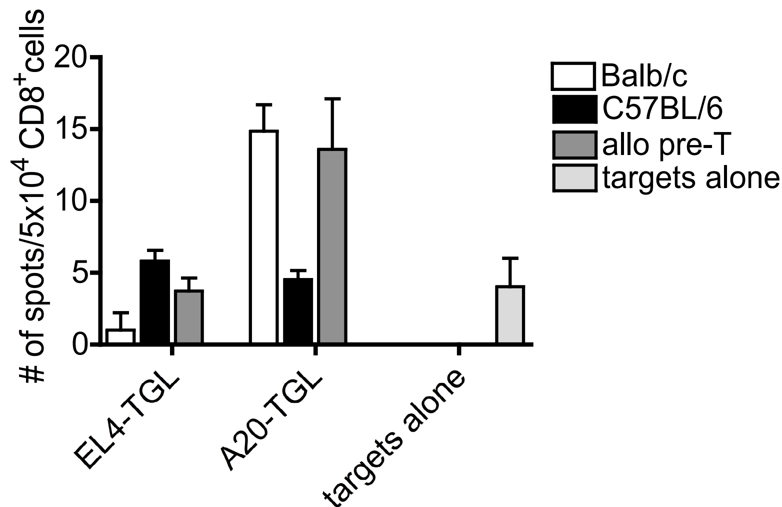
Supplementary Figure 3



Negative selection of adoptively transferred allogeneic T-cell precursors in HSCT recipients is determined by donor-derived hematopoietic cells

Lethally irradiated BALB/c recipients were transplanted with BALB/c HSCs or C57BL/6 HSCs and received additional C57CL/6(CD45.1$^+$)-derived *in vitro-generated* T-cell precursors. Animals were sacrificed on day 60 after HSCT and splenocytes were obtained for multicolor flow cytometric analysis of the presented TCR-Vβ families on CD4$^+$ and CD8$^+$ cells of C57BL/6 origin. Mean + SEM are presented (n = 7–8). Combined data from four independent experiments are presented.

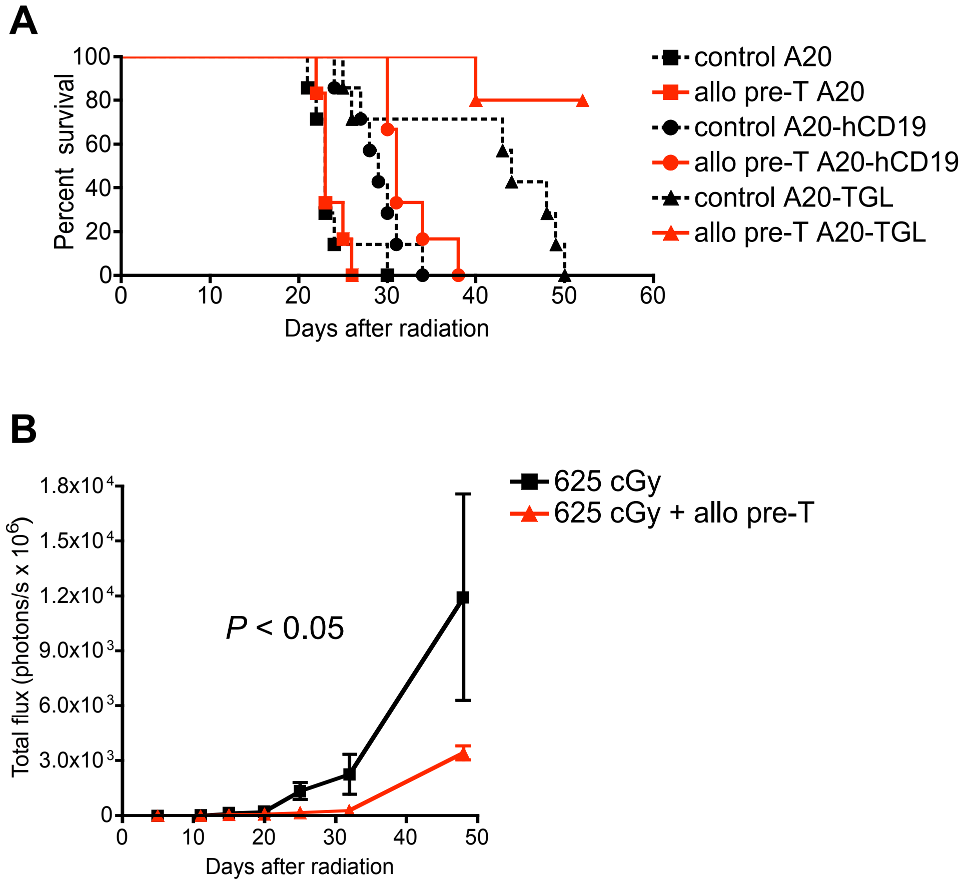
Supplementary Figure 4



CD8^{+} T-cells derived from adoptively transferred allogeneic T-cell precursors respond to host-MHC expressing stimulators

Lethally irradiated BALB/c (H-2^{d}) recipients were transplanted with BALB/c Lin^{-} BM and lethally irradiated C57BL/6 (H-2^{b}) recipients were transplanted with C57BL/6 Lin^{-} BM. All mice received *in vitro* generated C57BL/6(CD45.1^{+}) T-cell precursors (8 x 10^{6}) at day 0 and were immunized with irradiated GFP-expressing OP9-DL1 cells (H-2^{k}) at days 43 and 49 after HSCT. At day 55 after HSCT we isolated splenic CD8^{+} T-cells of BALB/c, C57BL/6 and allo pre-T (CD45.1^{+}) origin and stimulated them with irradiated GFP-expressing cells of either H-2^{d} background (A20-TGL) or H-2^{b} background (EL4-TGL), followed by quantification of INF-γ secretion by ELISPOT. Mean + SEM are presented (*n* = 4–7). Combined data from two independent experiments are presented.

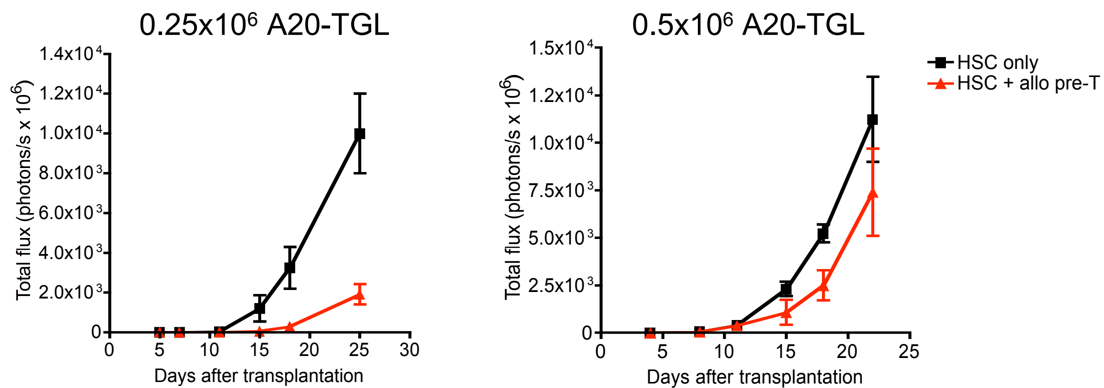
Supplementary Figure 5



Anti-tumor responses of adoptively transferred allogeneic T-cell precursors in sublethally irradiated recipients depend on the immunogenicity of the tumor

Sublethally irradiated (625 cGy) BALB/c recipients were challenged with 2.5 x 10^5 A20, A20-hCD19 or A20-TGL tumor cells i.v. on day 0 ± administration of $8x10^6$ allogeneic T-cell precursors (control groups versus allo pre-T groups). Survival was monitored daily (A) and in recipients of A20-TGL cells tumor growth was monitored by *in vivo* BLI (B) (*n* = 5-7).

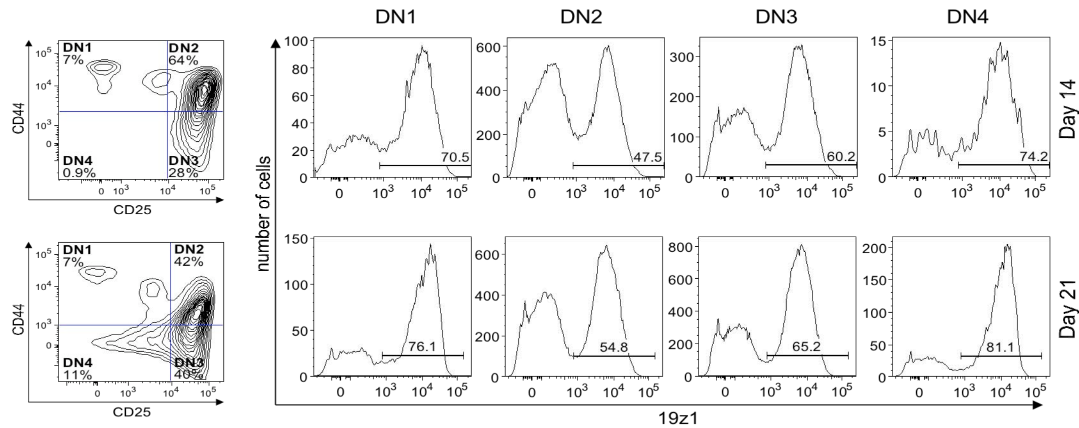
Supplementary Figure 6



Anti-tumor responses of adoptively transferred allogeneic T-cell precursors in are less effective in advanced tumors

Lethally irradiated BALB/c mice received 10^3 syngeneic HSCs and were challenged with 2.5 x 10^5 A20-TGL or 5 x 10^5 A20-TGL tumor cells i.v. on day 0 ± administration of $8x10^6$ allogeneic T-cell precursors. Tumor growth was monitored by *in vivo* BLI (*n* = 5-8).

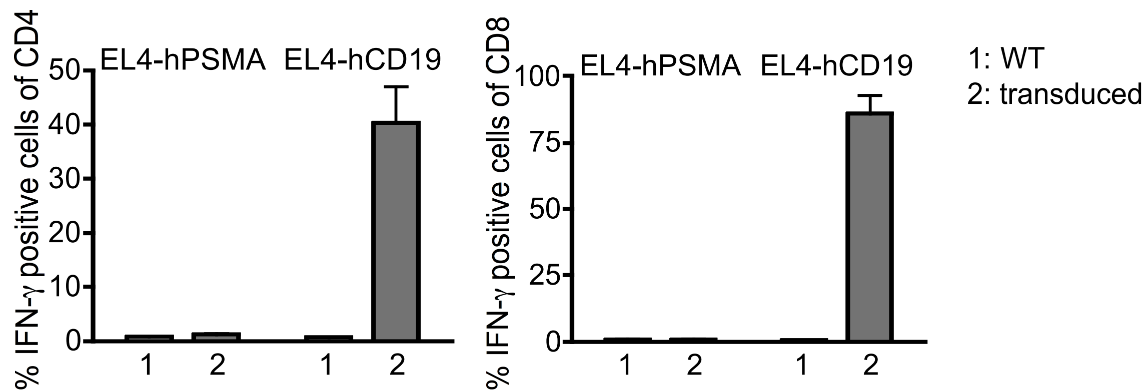
Supplementary Figure 7



T-cell precursors can be genetically engineered to be antigen-specific

OP9-DL1-derived C57BL/6 T-cell precursors on days 5 and 6 of coculture were retrovirally transduced with a 19z1–encoding lentiviral vector and cells from days 14 and 21 of coculture were analyzed by multicolor flow cytometry. Cells were gated on the CD4/CD8 DN population and further resolved into the DN1 to DN4 stages based on expression of CD44 and CD25. DN1–DN4 T-cell precursors were then analyzed for expression of 19z1. A representative example of six experiments is presented.

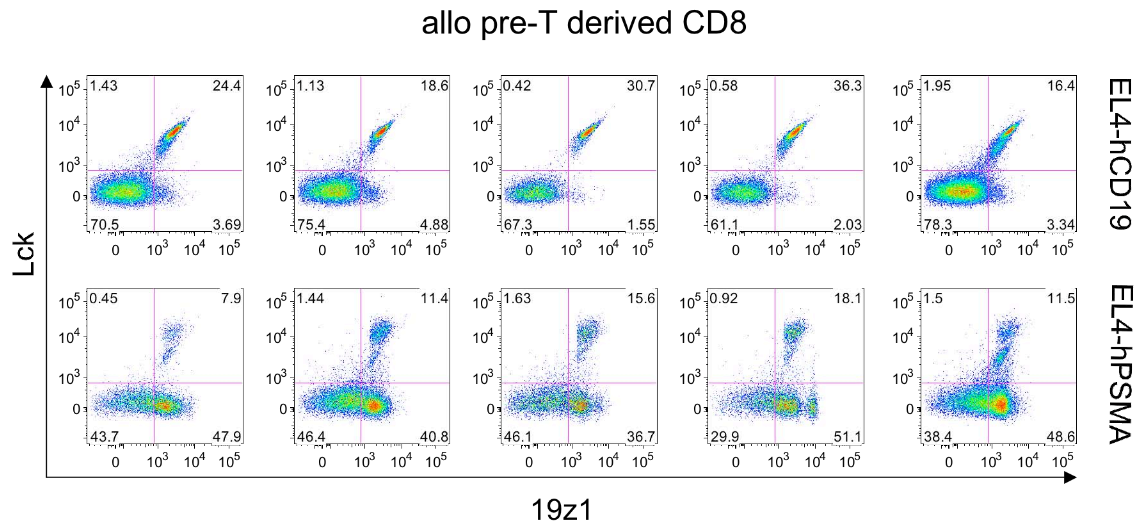
Supplementary Figure 8



Progeny of 19z1-expressing but not progeny of 19z1-negative T-cell precursors respond to stimulation with hCD19-expressing target cells

Lethally irradiated BALB/c recipients were transplanted with BALB/c HSCs and received additional C57BL/6(CD45.1)-derived *in vitro-generated* T-cell precursors transduced to express 19z1. Animals were immunized with irradiated A20-hCD19 cells on day 32 after HSCT. On day 40 after HSCT, animals were sacrificed and splenic T-cells were cultured overnight in the presence of soluble CD28-specific antibodies + irradiated EL4-hPSMA cells or EL4-hCD19 cells. Cells were stained for CD45.1, CD4, CD8, 19z1 and IFN-γ and analyzed for IFN-γ expression on C57BL/6-derived $CD4^{+}19z1^{-}$, $CD4^{+}19z1^{+}$, $CD8^{+}19z1^{-}$and $CD8^{+}19z1^{+}$ T-cells. Mean values + SEM are presented (*n* = 5).

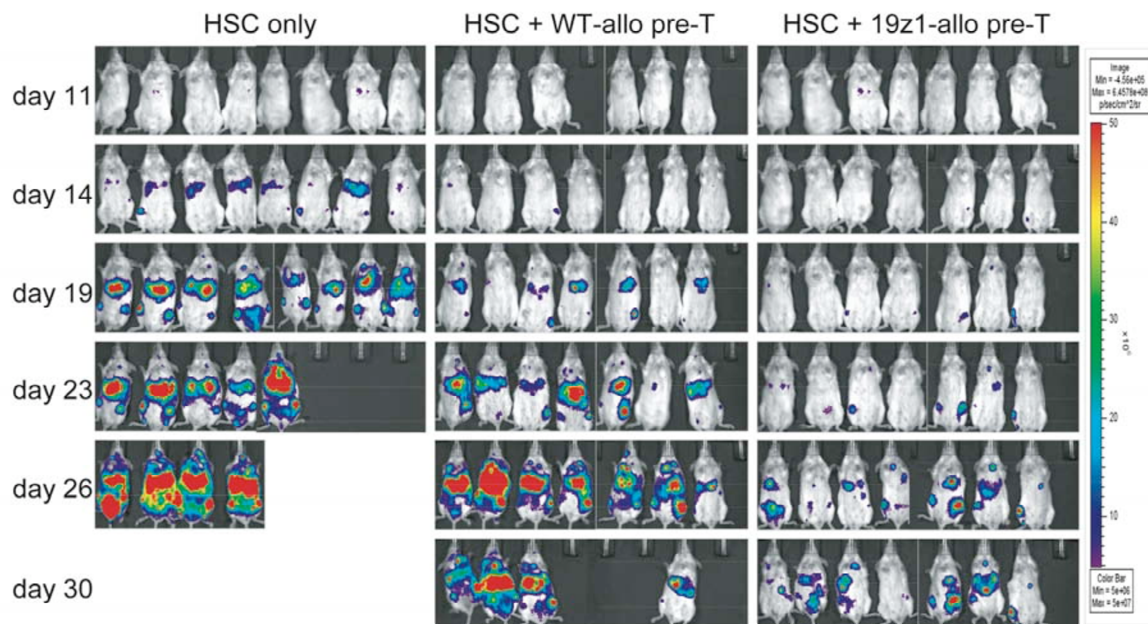
Supplementary Figure 9



Progeny of 19z1-expressing but not progeny of 19z1-negative T-cell precursors respond with increased Lck recruitment to stimulation with hCD19-expressing target cells

Lethally irradiated BALB/c recipients were transplanted with BALB/c HSCs and received additional C57BL/6(CD45.1)-derived *in vitro-generated* T-cell precursors transduced to express 19z1. Animals were immunized with irradiated A20-hCD19 cells on day 46 after HSCT. On day 56 after HSCT, animals were sacrificed and splenic T-cells were stimulated by irradiated EL4-hPSMA cells or EL4-hCD19 cells. Cells were stained with antibodies specific for CD45.1, CD8, 19z1 and Lck and analyzed for intracellular Lck expression on C57BL/6-derived $CD8^{+}19z1^{-}$and $CD8^{+}19z1^{+}$ T-cells (n = 5).

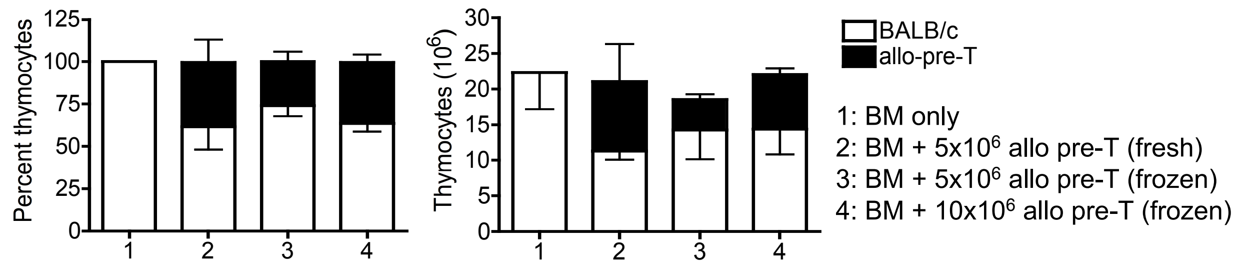
Supplementary Figure 10



Anti-tumor activity of adoptively transferred allogeneic T-cell precursors can be genetically enhanced

Lethally irradiated BALB/c recipients were transplanted with BALB/c HSCs only or received additional C57BL/6-derived T-cell precursors (unmanipulated or genetically engineered to express 19z1) on day 0. All mice received 3.3 x 10^5 A20-TGL-hCD19 tumor cells i.v. on day 0, and tumor growth was monitored by *in vivo* BLI. Pseudo-color images superimposed on conventional photographs on six time points after HSCT are presented (*n* = 7-8).

Supplementary Figure 11



Cryopreserved T-cell precursors engraft in the thymus of irradiated recipients

Lethally irradiated BALB/c recipients were transplanted with BALB/c HSCs; control mice received HSCs only, the pre-T groups received additional C57BL/6 derived T-cell precursors ($5x10^6$ fresh or $5x10^6$ cryopreserved or $10x10^6$ cryopreserved cells). Thymi were harvested on day 14 after transplantation and donor and host progenies were determined by total cellularity and multicolor flow cytometric analysis using Ly9.1 and CD45-specific antibodies. Mean cell numbers + SEM are presented (*n* = 4).

Supplementary Methods

Cells and cell lines. Single cell suspensions were prepared from spleen and thymus according to standard protocols. Harvest media consisted of RPMI-1640 supplemented with 10 % heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 g/ml streptomycin and 2 mM L-glutamine. OP9-DL1, a bone marrow stromal cell line of (C57BL/6 x C3H)F_2-*op/op* origin transduced with DL1, was described previously(22). A20, a B cell lymphoma cell line of BALB/c origin, and Renca, a renal cell carcinoma cell line of BALB/c origin, were kindly provided by A. Houghton (Memorial Sloan Kettering Cancer Center). A20 was either used untransduced or retrovirally transduced, either to express a triple fusion protein consisting of Herpes simplex virus thymidine kinase, enhanced green fluorescent protein (eGFP) and firefly luciferase (TGL)[52], or to express human CD19 (transduction with SFG-hCD19 oncoretroviral vectors derived from gpg29 cells)[38], or to express both TGL and hCD19. Renca was retrovirally transduced to express TGL, as described above. The construction of EL4-hCD19 and EL4-hPSMA has been described previously[38]. Cell culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 g/mL streptomycin and 2 mM L-glutamine (for A20 and Renca), Dulbecco Modified Eagle’s Medium (DMEM, Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 g/mL streptomycin and 2 mM L-glutamine (for EL4) and αMEM supplemented with 20% heat-inactivated FBS, 100 U/mL penicillin, 100 g/mL streptomycin (for OP9-DL1). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Mice and BM/HSC Transplantation. Female C57BL/6 (H-2^b), BALB/c (H-2^d), and C57BL/6 ($CD45.1^+$) mice were obtained from The Jackson Laboratory. Female Abb/B2m targeted mutation mice on a C57BL/6 background were purchased from Taconic. Mice used for experiments were 8–12 weeks old, unless otherwise specified. BM cells were removed aseptically from femurs and tibias. Donor BM was depleted for lineage marker-positive cells using the EasySep Hematopoietic Progenitor Cell Enrichment kit (Stem Cell Technologies) and used either for flow cytometric HSC isolation or for Lin^- HSCT. BM cells were resuspended in DMEM and transplanted by tail vein injection (1×10^5 cells in 0.2 mL total volume) into lethally irradiated recipients (BALB/c: 850 cGy, C57BL/6: 1100 cGy total body irradiation from a ^{137}Cs source as a split dose with a 3 h interval between doses to reduce gastrointestinal toxicity). When purified HSCs were used for transplantation, 1×10^3 HSC in 0.2 mL DMEM were injected. In GVT experiments, animals received tumor cells intravenously in a separate injection on day 0. Mice were housed in sterilized micro-isolator cages and received normal chow and autoclaved hyper-chlorinated drinking water (pH 3.0). The Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee approved all protocols involving experiments with animals.

Flow cytometry and cell sorting. Cells were washed in FACS buffer (PBS/0.5% BSA /0.1% sodium azide) and 10^6 cells/mL were incubated for 15 m at 4° C with CD16/CD32 FcR block. Subsequently, cells were incubated for 15 m at 4° C with antibodies and washed twice with FACS buffer. The stained cells were resuspended in FACS buffer and analyzed on a LSR-II flow cytometer (Becton

Dickinson) with DIVA software (Becton Dickinson). Data were analyzed with Flowjo (Treestar). For isolation of HSCs, BM cells from donor mice were obtained as described above. Cells were incubated with a mix of FITC conjugated lineage antibodies (antibodies to CD3, NK1.1, Gr-1, CD11b, CD19, CD4, CD8) and with Sca-1 and c-kit-specific antibodies. HSC ($Lin^{-}Sca\text{-}1^{hi}c\text{-}kit^{hi}$) were isolated using a MoFlo cell sorter (DakoCytomation). Sorted cells were ≥ 95% pure.

Assessment of GVHD, GVT, in vivo BLI. The severity of GVHD was assessed with a clinical GVHD scoring system as previously described[29]. Briefly, ear tagged animals in coded cages were individually scored every week for five clinical parameters on a scale from zero to two: weight loss, posture, activity, fur and skin. A clinical GVHD index was generated by summation of the five criteria scores (zero–ten). Survival was monitored daily. Animals were sacrificed after HSCT for histopathological analysis of GVHD target organs (small bowel, large bowel, and liver). Organs were harvested, formalin-preserved, paraffin-embedded, sectioned and hematoxylin/eosin-stained. A semiquantitative score consisting of 19 to 22 different parameters associated with GVHD was calculated, as described previously[30]. In GVT experiments, bioluminescent signal intensity of tumor-bearing mice was determined twice weekly. 15 m after intra-peritoneal injection of 3 mg/mouse D-Luciferin (Xenogen), mice were anaesthetized and placed into the light tight chamber of an IVIS 200 bioluminescence imaging system (Xenogen). Grayscale photographic images of the mice were acquired first and then a low-level bioluminescent signal was recorded. Pseudo-color images showing the whole body distribution of bioluminescent signal intensity were superimposed on

the grayscale photographs and total flux (photons/s) was determined for individual mice. The cause of death was confirmed by necropsy and histopathology.

Retroviral transduction and expansion of T cell precursors. T cell precursors were developed *in vitro* from C57BL/6 BM derived HSCs using the OP9-DL1 culture system, as described above. Lentiviral vectors were produced by tripartite transfection of 293T cells with pRRL-hPGKpr-19z1-WPRE, pCMV R8.92, and pUCMD.G[53]. Vector supernatants were concentrated by ultra-centrifugation and 0.75-1.5 x 10^8 total TU used to transduce 5 x 10^5 T cell precursors (coculture day 4–6) over 2 days in 24-well tissue culture plates coated with 15 ug/ml retronectin and 10ug/ml $DL1^{ext-IgG}$.Transduced cells were then expanded for an additional 14-21 days by OP9-DL1 coculture.

Reagents and Antibodies. Mouse-specific CD16/CD32 FcR block (clone 2.4G2) and all of the following fluorochrome or biotin-labeled monoclonal antibodies to mouse antigens were obtained from BD Biosciences: Ly9.1 (clone 30C7), CD45.1 (clone A20), CD45 (clone 30-F11), $H\text{-}2^d$ (clone 34-2-12), CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8a (clone 53-6.7), DX5 (clone DX5), CD11b (clone M1/70), CD11c (clone HL3), NK1.1 (clone PK136), Gr-1 (clone RB6-8C5), CD19 (clone 1D3), CD44 (clone IM7), CD25 (clone PC61), CD28 (clone 37.52), IFN-γ (clone XMG1.1), Lck (clone MOL171), mouse Vβ TCR screening panel, c-kit (clone 2B8), Sca-1 (clone D7), rat IgG1-κ (clone R3-34). The anti-idiotype monoclonal antibody 19e3-PE is specific for 19z1 and was a generous gift of the Gene Transfer Facility at Memorial Sloan Kettering Cancer Center. $DL1^{ext-IgG}$ was a generous gift of Irwin D. Bernstein, Fred Hutchinson Cancer Research Center. Retronectin was

purchased from Takara Biomedicals. A fixation/permeabilization solution kit was also obtained from BD Biosciences. Diamidino-phenylindole (DAPI) (Molecular Probes) was used for dead cell discrimination. Brefeldin A was obtained from Calbiochem and recombinant mouse Flt3-ligand was purchased from R&D Systems, Inc. Recombinant human KGF was kindly provided by Amgen and recombinant human IL-7 was kindly provided by Cytheris.

Hematopoietic stem cell/OP9-DL1 cocultures. T lineage cells were generated *in vitro* as described previously with modifications[22, 26]. Briefly, HSC were isolated as described above and seeded on a 60-80% confluent monolayer of OP9-DL1 cells at densities ranging from 3-8 x 10^4 cells/well into six-well tissue culture plates. The tissue culture media was supplemented with 5 ng/mL IL-7 and 5–10 ng/mL Flt3-ligand. Every 4–5 days, cells were collected by forceful pipetting, filtered through a 70 m nylon mesh and seeded into a new tissue culture vessel containing a monolayer of OP9-DL1 cells. Cells were maintained as predominantly DN2 and DN3 T-cell precursors from day 14 of coculture on until they were used for experiments (the latest at day 28).

MLR. T-cells (seeded at a density of 1 x 10^5 T-cells/well) were cultured for five days with irradiated (2,000 cGy) BALB/c, C57BL/6 or CBA derived splenocytes as stimulators (2 x 10^5 cells/well) in a 96-well plate. ^{3}H-Thymidine was added for the final 18 hours of culture. Cells were harvested with a Filtermate 196 harvester (Packard, Meridan, CT) and after addition of Microscint-20 scintillation fluid (Packard), counts per minute were measured with a Topcount NXT microplate scintillation counter (Packard). Counts are presented as stimulation index (counts

in MLR/counts in spontaneous proliferation).

Intracellular cytokine staining. For unspecific stimulation, cells were incubated for 16 h in 24-well tissue culture plates coated with anti-CD3 (10 g/mL) in the presence of soluble anti-CD28 (10 µg/mL). Brefeldin A (10 µg/mL) was added after one hour of incubation. For antigen-specific stimulation, cells were stimulated for 16 h with irradiated A20 or A20-TGL cells (20,000 cGy) in the presence of soluble anti-CD28 (10 µg/mL), at a ratio of 2:1, or with irradiated EL4-hCD19 or EL4-hPSMA (7,500 cGy), at a ratio of 5:1. Brefeldin A (10 µg/mL) was added after one hour. Following stimulation, cells were harvested, washed, and stained with fluorochrome-conjugated antibodies to surface antigens. Subsequently, cells were fixed and permeabilized with fixation/permeabilization solution kit reagents according to the manufacturer's instructions and stained with IFN-γ, Lck or rat IgG1-κ (isotypic control)-specific antibodies. Cells were analyzed by multicolor flow cytometry as described above.

ELISPOT. ELISPOT assays measuring mouse IFN-γ were used to assess T-cell responses to stimulation with A20-TGL or EL4-TGL cells. Multiscreen-IP plates (Millipore) were coated with 100 l anti-mouse IFN-γ monoclonal antibody (10 mg/mL; clone AN18, MabTech) in PBS, incubated overnight at 4 °C, washed with RPMI/FCS to remove unbound antibody, and blocked with RPMI/FCS for 2 h at 37 °C. T-cells were plated at a density of $5x10^4$ $CD8^+$ T-cells per well and stimulated with irradiated A20-TGL or EL4-TGL cells (10^4 per well) in a final volume of 200 l/well. After incubation at 37 °C for 20 h, plates were washed with PBS/0.05% Tween, and incubated with 100 l per well biotinylated antibody to mouse IFN-γ (1 mg/mL; clone

RA-6A-2, MabTech). Plates were incubated for an additional 2 h at 37 °C followed by spot development. Spots were counted with an Automated ELISpot Reader System with KS 4.3 software (Carl Zeiss MicroImaging Inc.).

Statistics. All results in this manuscript are based on two-sided test statistics. A *P*-value < 0.05 was considered statistically significant. The Mann-Whitney U-statistic was used to compare flow cytometric data. *In vivo* data regarding survival, weight changes, and photon intensity determined by *in vivo* BLI were collected in studies assessing GVHD and GVT. For those studies, mice were randomly assigned to the treatment groups and the area under the curve (AUC) was used to summarize the weight and photon trajectory of each mouse under study. Not all the mice were followed for the full length of the study. The primary reason for censoring was death or sacrifice, and ignoring this type of informative censoring may result in a biased treatment comparison. To eliminate this bias, a test statistic was formed using the information up to the minimum follow up time for each cross treatment mouse pair. By eliminating the uneven censorship between mouse pairs in different groups, a test statistic can be constructed that has mean zero when the growth rates in the two groups are equal. The statistic is based on the average difference in the censored AUC curves between treatment groups[54]. The log rank test statistic was used to compare survival curves between groups. For the analyses of the imaging studies, the P-values were generated from a permutation test, using the AUC and log rank test statistics. The application of the permutation procedure was due to the small number of animals in these studies.

References

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