

Equilibrium between Host and Cancer Caused by Effector T Cells Killing Tumor Stroma

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Abstract

The growth of solid tumors depends on tumor stroma. A single adoptive transfer of CD8⁺ CTLs that recognize tumor antigen-loaded stromal cells, but not the cancer cells because of MHC restriction, caused long-term inhibition of tumor growth. T cells persisted and continuously destroyed CD11b⁺ myeloid-derived, F4/80⁺ or Gr1⁺ stromal cells during homeostasis between host and cancer. Using high-affinity T-cell receptor tetramers, we found that both subpopulations of stromal cells captured tumor antigen from surrounding cancer cells. Epitopes on the captured antigen made these cells targets for antigen-specific T cells. These myeloid stromal cells are immunosuppressive, proangiogenic, and phagocytic. Elimination of these myeloid cells allowed T cells to remain active, prevented neovascularization, and prevented tumor resorption so that tumor size remained stationary. These findings show the effectiveness of adoptive CTL therapy directed against tumor stroma and open a new avenue for cancer treatments. [Cancer Res 2008;68(5):1563–71]

Introduction

Tumors⁵ have elaborate mechanisms that prevent recognition and elimination by the immune system (1, 2). Down-regulation of MHC class I occurs frequently in many human cancers and affects adversely the clinical course of cancers and the outcome of T-cell-based immunotherapy (3–5). Mutations in the MHC class I genes themselves, abnormalities in their regulation, and/or defects in MHC class I-dependent antigen processing in cancer cells can underlie MHC class I down-regulation. The prominence of variant cancer cells with such defects in a tumor may be a consequence of immune selection. These changes abrogate the susceptibility of cancer cells to lysis by CTL and may explain the rapid progression and poor prognosis of cancers that exhibit MHC class I down-regulation. Thus, direct killing of cancer cells by CTL is not always feasible.

The failure of immune therapy for nonhematologic solid tumors is probably due to the complicated interactions of T cells, cancer cells, and the stroma (6–10). It has been shown that destruction of stromal cells within tumors by CTL is essential for eradication of

large well-established solid tumors (6, 11–15). Stromal cells within tumors are genetically much more stable than cancer cells, and stromal cells cross-present antigen released from cancer cells to CTL. This not only results in the elimination of stroma but also causes bystander killing of cancer cells, including antigen loss variants. Indeed, tumor growth was inhibited by passive transfer of CTL in H-2^b Rag1^{-/-} mice bearing H-2^k PRO4L cancer cells expressing high levels of K^b-restricted cancer antigen (13). However, it remains unclear how the interaction between CTL and tumor stroma controls tumor growth.

Cancer progression is associated with the accumulation of CD11b⁺ myeloid-derived suppressor cells (MDSC), which include Gr1⁺ immature cells and F4/80⁺ macrophages (16–20). The elimination, functional inhibition, or differentiation of Gr1⁺ host cells and MDSCs can restore CD8⁺ T-cell responsiveness and revert inflammation-dependent cancer promotion in tumor-bearing hosts (17, 21–24). In addition, a recent study has shown the importance of myeloid CD11b⁺/Gr1⁺ stromal cells in resistance to antiangiogenic therapies (25). Here, we show long-term arrest and stabilization of progressive growth of established tumors by a single adoptive transfer of T cells that can only destroy stromal cells cross-presenting cancer antigen. During this equilibrium between host and cancer, the transferred T cells persisted and continuously killed CD11b⁺/Gr1⁺ myeloid cells in the tumor stroma. Because of the tumor-promoting activities of these stromal MDSCs, elimination of these cells benefits the host. Therefore, targeting stromal cells by T cells points to an important new approach to cancer therapy.

Materials and Methods

Mice, cell lines, and reagents. C57BL/6 Rag1^{-/-} mice were purchased from The Jackson Laboratory. OT-1 Rag1^{-/-} mice were provided by A. Ma (University of Chicago, Chicago, IL). 2C Rag1^{-/-} mice were provided by J. Chen (Massachusetts Institute of Technology, Boston, MA). For bone marrow chimeras, recipient mice were irradiated with 9 Gy and, 1 h later, received 10⁷ bone marrow cells from donor mice. The bone marrow chimeric mice were injected with indicated cancer cells at least 4 wk after bone marrow transfer. All animal experiments were approved by institutional animal use committees of the University of Chicago. PRO4L cells have been previously described (6). The MC57-SIY-Hi (SIY-2Hi) and MC57-SIY-Lo (SIY-2Lo) have been previously described (13, 14, 26). MFLM-91U endothelial cells (27) were provided by A. Akesson (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The 2C-recognized peptide SIYRYGL was synthesized by H. Auer and S. Meredith (University of Chicago). OVA-derived peptide SIINFEKL was provided by Y. Fu (University of Chicago). Anti-CD8, Vβ8.1/8.2, Gr1, CD11b, IFN-γ, CD90, bromodeoxyuridine (BrdUrd), and isotope control IgG2a and IgG2b antibodies were obtained from BD PharMingen and anti-F4/80 antibody was from eBioscience. Arginase inhibitor N^w-hydroxyl-nor-L-arginine, inducible nitric oxide synthase inhibitor N^G-monomethyl-L-arginine, matrix

⁵ We use "tumor" to refer to the mass of cancer cells and nonmalignant stromal cells; "cancer cells" refer only to malignant cells in the tumor.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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metalloproteinase (MMP) inhibitor GM6001, and vascular endothelial growth factor (VEGF) inhibitor V1 were purchased from Calbiochem.

Generation of SIY and gp33 vectors and cells expressing these genes.

The generation of MFG-SIY-EGFP and MFG-gp33-EGFP vectors was described previously (13, 14, 26). Phoenix cells were transfected with pLEGFP, MFG-SIY-EGFP, or MFG-gp33-EGFP using SuperFect (Qiagen), and supernatants were used to infect PRO4L cells to generate PRO4L-EGFP, PRO4L-SIY-EGFP, and PRO4L-gp33-EGFP cell lines.

Preparation of single-cell suspensions from murine tumors. Tumors were surgically excised under sterile conditions, placed in RPMI 1640 containing 1% antibiotic/antimycotic, and transported on ice. Fresh tumor tissues were washed with PBS, minced into 1 to 2 mm pieces, and centrifuged at 1,800 rpm for 10 min. The supernatants were removed and the tissues were washed again in PBS by centrifugation at 1,800 rpm for 10 min. The tissues were resuspended in a digestion solution (1 g tissue/5 mL) containing 1 mg/mL trypsin-1 mg/mL collagenase D-0.25 mg/mL DNase I in HBSS and incubated for 45 min to 1 h in an atmosphere of 5% CO₂ at 37°C. The suspension was mixed every 15 min by pipetting to enhance tissue disruption. DMEM with 10% FCS and 1% antibiotic/antimycotic was then added to the cell suspension to stop enzymatic activity. The tissue digests were incubated in cold NH₄Cl for 2 min to lyse RBCs and subsequently filtered through 70- μ m nylon filter mesh. Cells were counted; >95% of cells were single and viable.

Multimerization of single-chain T-cell receptor. Single-chain high-affinity T-cell receptor (TCR) 2C-m67 containing a COOH-terminal 15-amino acid biotin substrate was cloned into pET-28a and expressed in *Escherichia coli* BL21 (DE3) cells (Stratagene) together with a plasmid containing the gene for the expression of BirA ligase enzyme (provided by J. Cronan, University of Illinois, Urbana, IL). Coexpression resulted in the *in vivo* biotinylation of the single-chain TCR. Inclusion bodies that contained the single-chain TCR were solubilized in 3 mol/L urea and guanidine HCl and refolded. Ni-NTA agarose (Qiagen) was used to isolate the single-chain TCR followed by size exclusion chromatography (Sephacryl S-200; Amersham Biosciences). Tetramerization of single-chain TCR was performed by the gradual addition of streptavidin-phycoerythrin (BD PharMingen) in aliquots to saturate its binding sites to a total biotin-conjugated m67 single-chain TCR/streptavidin molar ratio of 20:1 (to ensure formation of tetrameric complexes).

Cell isolation. Gr1⁺, CD11b⁺, and F4/80⁺ cells were isolated from spleens and tumors using magnetic microbeads and MiniMACS columns (Miltenyi Biotec). Briefly, cells were suspended in MACS buffer, and 5 \times 10⁶ cells were incubated with 5 μ g of biotinylated anti-Gr1, anti-F4/80 antibodies, or direct anti-CD11b microbeads for 15 min at 4°C. For Gr1⁺ and F4/80⁺ cell isolations, cells were washed with cold buffer to remove unbound antibodies and then incubated with streptavidin microbeads for 15 min at 4°C. Gr1⁺, CD11b⁺, and F4/80⁺ cell populations were isolated on MiniMACS columns according to the manufacturer's instructions. The purity of isolation was >90% as evaluated by flow cytometry. Consistent with previous observations (20, 28), >98% of Gr1⁺ cells were also CD11b⁺.

Analysis of cells by fluorescence-activated cell sorting. All the samples were initially incubated with 2.4G2 to block antibody binding to the Fc receptors. Single-cell suspensions were stained with 1 μ g of relevant antibodies and then washed twice with cold PBS. The percentages of CD11b⁺, Gr1⁺, or F4/80⁺ cells in spleen and tumor tissues of tumor-bearing mice at indicated day after tumor inoculation were analyzed. For analysis of viable tumor cell populations following 2C T-cell transfer, dead cells were identified by propidium iodide (PI) staining based on CD11b or Gr1 and CD11b expression. For tetramer staining, cells were incubated with allophycocyanin-conjugated anti-CD11b and SIY-K^b-specific m67 TCR tetramer for 45 min at room temperature. Samples were analyzed on a FACSCalibur and data were analyzed with FlowJo software.

⁵¹Cr release assay. To generate cytolytic 2C T cells, 10⁶ Tris-NH₄Cl-treated splenocytes from a 2C Rag1^{-/-} mouse were stimulated with 6 \times 10⁶ NH₄Cl-treated, irradiated (20 Gy) splenocytes from a BALB/c mouse in a 15 mL round-bottomed tube (Falcon 353033) containing 3 mL RPMI 1640

with 25 mmol/L HEPES supplemented with 10% FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 50 μ mol/L β -mercaptoethanol, 50 units/mL penicillin, and 50 μ g/mL streptomycin. After 5 d, ⁵¹Cr release assays were performed as previously described (13, 26).

BrdUrd incorporation assay. For *in vivo* proliferation analysis, tumor-bearing mice were injected i.p. with 1 mg BrdUrd in 200 μ L PBS. One hour after injection, tumors and lymph nodes were removed. Cancer cells in tumors were gated on K^k EGFP⁺ cells; T cells in draining lymph nodes were gated on CD8⁺ V β 8.1/8.2⁺ cells. BrdUrd incorporation was revealed with a BrdUrd Flow kit (BD Biosciences).

Adoptive transfer of T cells. For transfer of activated cells, 5 \times 10⁶ NH₄Cl-treated splenocytes from 2C transgenic mice were stimulated with 10 μ mol/L of the SIYRYYL peptide for 3 to 4 d. Activation was confirmed by the up-regulation of CD44 on the specific T cells. To generate SIY-immune lymphocytes, the B6C3F1 mice were immunized with PRO4L-SIY-EGFP cells (1 \times 10⁷). Nine days later, splenocytes were cultured for 5 d with the SIYRYYL peptide and 10 units/mL interleukin-2. The single-cell suspensions of T cells were injected i.v. into the retro-orbital plexus in a 0.2 mL volume.

Tumor challenge. Cultured cancer cells were trypsinized and washed once with DMEM. Cancer cells (2 \times 10⁶) in suspension were injected s.c. into the indicated mice. For coinjection experiments, an indicated number of PRO4L cells in suspension combined with the purified G1⁺CD11b⁺ cells at 10:1 ratio were injected s.c. into one flank of C57BL/6 Rag1^{-/-} mice. The size of tumor was determined by direct measurements of tumor dimensions (three orthogonal axes *a*, *b*, and *c*) at 3-d intervals using calipers. Tumor volumes were calculated as *abc*/2.

Statistical analysis. Tumor incidences in different groups of mice (coinjection experiments) were compared using Fisher's exact test. The statistical significance of tumor volumes or number of migrated cells in different groups was determined by Student's *t* test. Probability values >0.05 were considered nonsignificant.

Results

Long-term inhibition of tumor growth by T cells targeting only cancer stromal cells. 2C T cells were injected into OT-1 Rag1^{-/-} C57BL/6 mice; the next day, the mice were inoculated with H-2^b-negative cancer cells. 2C T cells arrested the subsequent growth of the PRO4L-SIY-EGFP tumors. Inhibition was antigen specific because PRO4L-EGFP tumors (antigen-negative cancer cells) grew out progressively and eventually killed the hosts (Fig. 1A, left). In OT-1 Rag1^{-/-} mice bearing bilateral established PRO4L-EGFP and PRO4L-SIY-EGFP tumors, 2C T cells did not affect the growth of control PRO4L-EGFP tumors but arrested the growth of the PRO4L-SIY-EGFP tumors (Fig. 1A, right).

To examine whether nontransgenic T cells also affected the growth of PRO4L-SIY-EGFP tumors, B6C3F1 mice were immunized with PRO4L-SIY-EGFP cells. The recovered SIY-immunized nontransgenic T cells arrested the growth of established PRO4L-SIY-EGFP tumors but not the PRO4L tumors expressing the gp33 control antigen (Fig. 1B), similar to what was observed with 2C transgenic T cells. Therefore, our observation that long-term inhibition of tumor growth by T cells targeting only cancer stromal cells can be extrapolated to nontransgenic T-cell systems. Finally, we inoculated s.c. PRO4L-SIY-EGFP cancer cells into chimeric mice that expressed either H-2^b or H-2^k on bone marrow or non-bone marrow-derived cells. After 14 days of tumor growth, mice received 2C T cells to examine which stromal cells had to express SIY-presenting K^b molecules to achieve tumor arrest. Figure 1C shows that bone marrow-derived stromal cells allowed more effective inhibition than non-bone marrow-derived stromal cells; however, tumor equilibrium was only achieved when both stromal compartments were targeted.

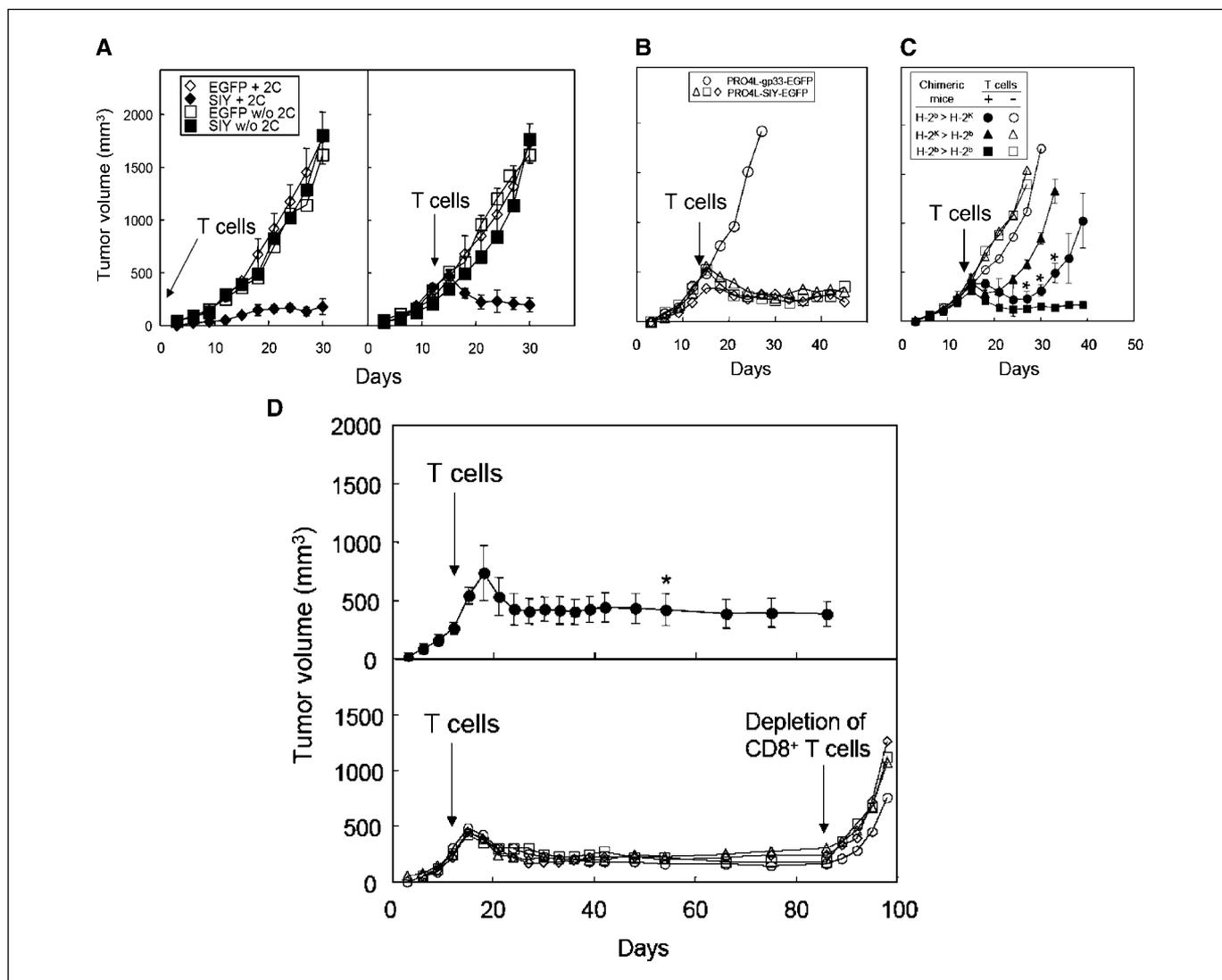


Figure 1. Long-term inhibition of tumor growth by T cells targeting cancer stroma alone. *A, left*, 2C T cells at 1×10^7 , or no T cells as a control, were injected i.v. into OT-1 Rag1^{-/-} mice; the next day, mice were injected s.c. with 2×10^6 PRO4L-EGFP and PRO4L-SIY-EGFP cells. Tumor volumes were monitored. Points, mean ($n = 4$); bars, SD. *Right*, OT-1 Rag1^{-/-} mice were injected s.c. with 2×10^6 PRO4L-EGFP and PRO4L-SIY-EGFP cells. After 14 d, mice were injected i.v. with 5×10^6 2C T cells or with no T cells. Tumor volume was monitored. Points, mean ($n = 4$); bars, SD. *B*, OT-1 Rag1^{-/-} mice were injected s.c. with 2×10^6 PRO4L-gp33-EGFP or PRO4L-SIY-EGFP cells. After 14 d, mice were injected i.v. with 1×10^7 SIY-immunized T cells. The generation of SIY-immune T cells is described in Material and Methods. *C*, C57BL/6 Rag1^{-/-} that received C3H Rag1^{-/-} bone marrow (H-2^b > H-2^k), C3H Rag1^{-/-} that received C57BL/6 Rag1^{-/-} bone marrow (H-2^k > H-2^b), and C57BL/6 Rag1^{-/-} that received C57BL/6 Rag1^{-/-} bone marrow (H-2^b > H-2^b) chimeric mice were s.c. injected with 2×10^6 PRO4L-SIY-EGFP cells. After 14 d, mice were injected i.v. with 1×10^7 SIY-immunized T cells. Tumor volume was monitored as indicated. Points, mean ($n = 3$); bars, SD. *, $P < 0.05$, compared with the H-2^k > H-2^b chimeric mice at different time points. *D, top*, OT-1 Rag1^{-/-} mice were injected s.c. with 2×10^6 PRO4L-SIY-EGFP cells in three independent experiments. After 14 d, mice were injected i.v. with 1×10^7 2C T cells. Tumor volumes were monitored. Points, mean ($n = 8$); bars, SD. *, from day 55 on, the data are pooled from only seven mice because the tumor escaped in one mouse (see text for analysis of this mouse). In the lower panel, four OT-1 Rag1^{-/-} mice were injected s.c. with 2×10^6 PRO4L-SIY-EGFP cells. After 14 d, mice were injected i.v. with 1×10^7 2C T cells. At 85 d, mice were injected i.p. with anti-CD8 mAb YTS 169.4.2. Each symbol represents an individual mouse.

We next tested whether targeting stromal cells effectively controlled tumor growth for extended times. As shown in Fig. 1D (top), the growth of PRO4L-SIY-EGFP tumors was inhibited or stabilized for more than 60 days following a single adoptive transfer of T cells. This was shown for a total of eight mice in three independent experiments. Only one of the eight tumors escaped 55 days after tumor challenge. Cancer cells cultured from a biopsy of this tumor still expressed high levels of SIY antigen measured by flow cytometry (Supplementary Fig. S1A). A second injection of T cells at day 70 reduced the size of this tumor dramatically without eliminating a residual tumor (data not shown). Cancer cells cultured from the biopsy before the second injection of T cells were injected

s.c. into three second hosts and produced progressively growing tumors; tumors regressed after T-cell transfer (Supplementary Fig. S1B), suggesting that these tumors remained susceptible to growth inhibition by T-cell-mediated stromal destruction. Thus, this escape was not due to a heritable cancer variant but probably due to lack of T cells.

Clearly, targeting tumor stroma alone caused long-term suppression of tumor growth, but viable cancer cells persisted in the small residual tumors. This was confirmed by injecting four additional mice with persistent tumors at day 85 with anti-CD8 antibody. Cancer cells grew out progressively following antibody treatment (Fig. 1D, bottom). Thus, targeting tumor stroma by T cells

caused remarkable regression of large tumors, which resulted in a small population of residual cancer cells and small tumors that did not grow. Because CTL failed to recognize and kill the cancer cells directly, CTL presumably achieved this effect indirectly through targeting tumor stromal cells.

Continuous turnover of T cells and cancer cells during equilibrium. Immunofluorescence staining of frozen sections from PRO4L-SIY-EGFP but not PRO4L-EGFP tumors showed extensive lymphocyte infiltration of predominant $V\beta 8.1/8.2^+$ $CD8^+$ T cells 6 days following naive 2C T-cell transfer (Supplementary Fig. S2A). Antigen-specific infiltration of $CD8^+$ T cells was confirmed by flow cytometry (Supplementary Fig. S2B, top). Furthermore, IFN- γ production by infiltrated T cells in PRO4L-SIY-EGFP tumors was markedly increased but was not increased in control PRO4L-EGFP tumors (Supplementary Fig. S2B, bottom). To examine whether adoptively transferred antigen-specific T cells retained effector

function during equilibrium, SIY and gp33 peptide-pulsed target cells were infused into these PRO4L-SIY-EGFP-bearing mice at days 40, 60, and 70. The ability of the mice to kill target cells *in vivo* at these times was unimpaired (Supplementary Fig. S2C, top) and killing was antigen specific because target cells were eliminated in PRO4L-SIY-EGFP-bearing mice but not in tumor-free or PRO4L-gp33-EGFP-bearing mice (Supplementary Fig. S2C, bottom). These data indicate that adoptively transferred T cells and/or their progeny persisted and functioned specifically during equilibrium.

Proliferation of cancer cells in tumors and antigen-specific T cells in draining lymph nodes was measured by BrdUrd incorporation. At day 12 before T-cell transfer, 11.2% of cancer cells incorporated BrdUrd. Following T-cell therapy, a smaller number of cancer cells (K^{k+} EGFP $^+$) consistently incorporated BrdUrd at days 35, 50, and 65 (Fig. 2A). In addition, at these times, similar percentages of BrdUrd $^+$ antigen-specific T cells were observed in

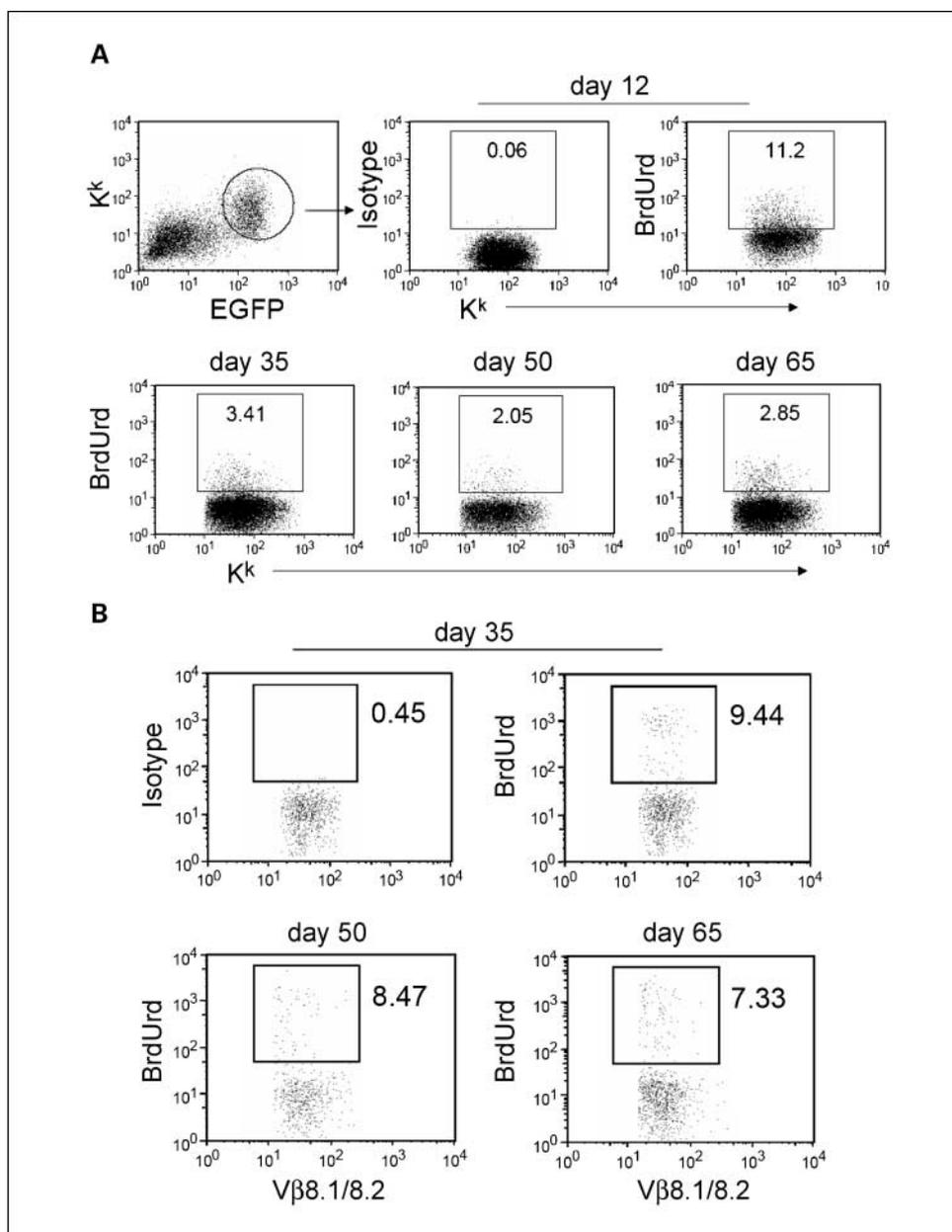
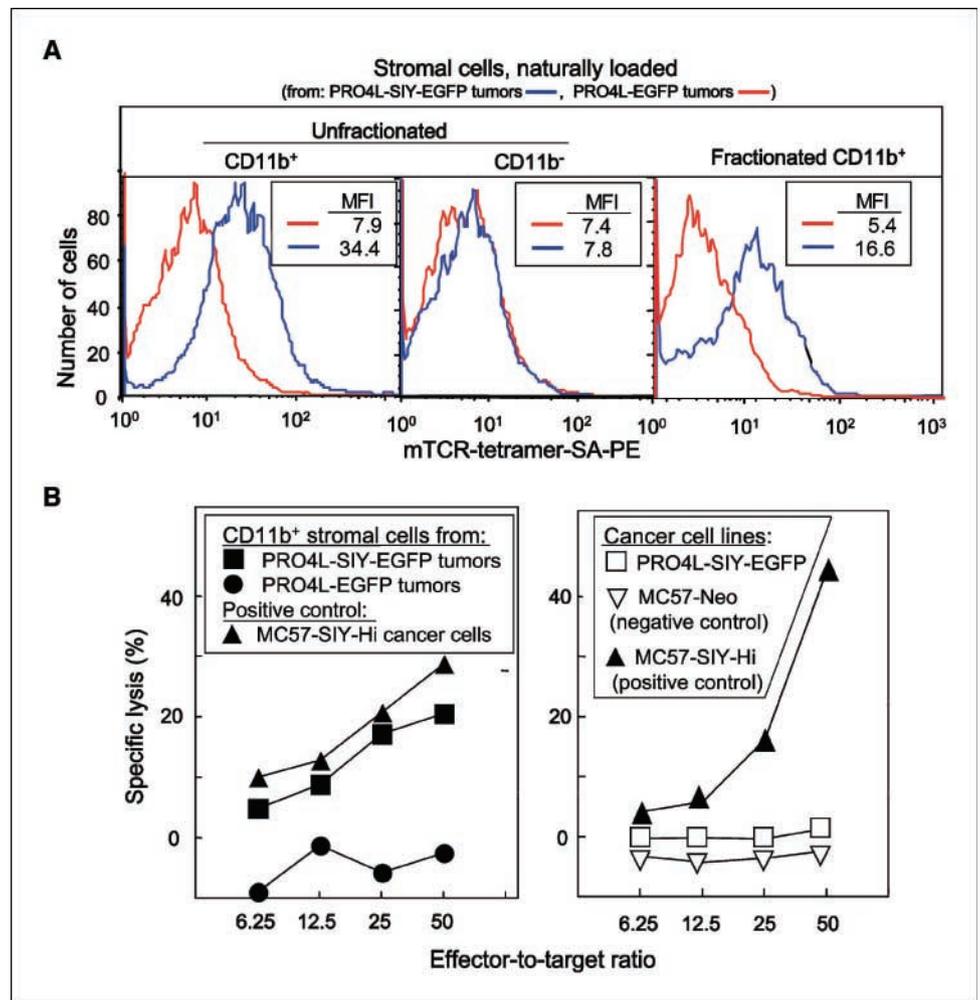


Figure 2. Continuous turnover of T cells and cancer cells during equilibrium. OT-1 Rag1 $^{-/-}$ mice were injected s.c. with 2×10^6 PRO4L-SIY-EGFP cells. After 14 d, 1×10^7 2C T cells were injected i.v. into these tumor-bearing mice. At days 12, 35, 50, and 65, the tumors (A) and draining lymph nodes (B) were excised and dispersed; single-cell suspensions were analyzed by flow cytometry for BrdUrd incorporation. Data are representative of two (A and B) independent experiments.

Figure 3. Recognition and destruction of tumor stroma cross-presenting antigen from surrounding cancer cells. **A**, detection of SIY-K^b pMHC on CD11b⁺ stromal cells from tumors induced by K^b-negative PRO4L-SIY-EGFP cancer cells. PRO4L-EGFP and PRO4L-SIY-EGFP cells were injected s.c. into opposite flanks of single C57BL/6 Rag1^{-/-} mouse, respectively. After 14 d, the tumors were excised and dispersed; single-cell suspensions were collected. Tumor-derived CD11b⁺ cells were positively selected using MACS columns. Cells before and after selection were analyzed by flow cytometry for staining with SIY-K^b-specific m67 TCR tetramers. The SIY-K^b-specific m67 TCR tetramer specifically bound to the purified CD11b⁺ cells but did not bind to CD11b⁻ cells from SIY-positive tumors. *MFI*, mean fluorescence intensity. **B**, in a 4.5-h ⁵¹Cr release assay, purified CD11b⁺ stromal cells from a 2-wk-old established PRO4L-SIY-EGFP tumor were lysed by 2C CTL cells, but CD11b⁺ stromal cells from a 2-wk-old SIY-negative PRO4L-EGFP tumor were not lysed. *Left*, MC57-SIY-Hi cells were used as positive controls; *right*, 2C T cells specifically lysed MC57-SIY-Hi cells but did not lyse PRO4L-SIY-EGFP cells. Data are representative of three (**A**) and two (**B**) independent experiments.



draining lymph nodes (Fig. 2B) and spleens (data not shown). These data indicate a state of dynamic equilibrium between CTL and cancer cells.

Persistent killing of tumor stroma by T cells is associated with the equilibrium. Using specific high-affinity TCR tetramers to detect peptide-MHC complexes on cell surfaces by flow cytometry (15), we found that the tetramers specifically bound to CD11b⁺ stromal cells but did not bind to CD11b⁻ stromal cells (Fig. 3A). Only CD11b⁺ cells from PRO4L-SIY-EGFP tumor showed staining (>3-fold) over background (Fig. 3A). Moreover, CD11b⁺ cells from both PRO4L-SIY-EGFP and PRO4L-EGFP tumors expressed similar levels of H-2K^b (data not shown). No positive staining using the tetramers was detected on CD11b⁺ cells in the PRO4L-EGFP or PRO4L-gp33-EGFP tumors (data not shown). Antigen-loaded CD11b⁺ stromal cells (tetramer staining positive) from PRO4L-SIY-EGFP tumors were lysed by T cells, and lysis was antigen specific because CD11b⁺ stromal cells from PRO4L-EGFP tumors were not lysed. PRO4L-SIY-EGFP cancer cells were not lysed by 2C T cells because they are H-2K^b negative (Fig. 3B). Therefore, the CD11b⁺ stromal cells that cross-presented antigen from the cancer cells were recognized and killed by specific CTL, but the cancer cells were not killed directly.

Whether stromal cells were destroyed in antigen-positive tumors following T-cell therapy was determined by PI staining. PRO4L-SIY-EGFP tumors contained more nonviable cells (PI positive)

6 days after 2C T-cell transfer than PRO4L-EGFP tumors (Fig. 4A). These nonviable cells in the PRO4L-SIY-EGFP tumor were largely CD11b⁺ (Fig. 4B). Similarly, adoptive transfer of 2C T cells caused >20-fold increase in nonviable Gr1⁺CD11b⁺ stromal cells from PRO4L-SIY-EGFP tumor compared with SIY-negative PRO4L-EGFP tumor (Fig. 4C and D). At days 40 and 60, comparable numbers of nonviable stromal CD11b⁺ (Fig. 5A) or Gr1⁺CD11b⁺ cells (Fig. 5B) were observed in SIY-positive tumors. This is consistent with the kinetics of distribution of Gr1⁺CD11b⁺ stromal cells in tumors following T-cell therapy (Fig. 5C). Therefore, during equilibrium, MDSCs in antigenic tumors are targeted continuously by specific CTL.

Tumor antigens are able to enter the circulation and may target stroma outside the tumor. However, as shown in Supplementary Fig. S3, antigen-specific tetramers failed to bind splenic Gr1⁺CD11b⁺ cells that still remained viable (PI⁻) during the equilibrium after T-cell transfer. Therefore, cross-targeting of T cells was mainly present in the tumor stroma but not peripherally presumably because peripheral stromal-type cells individually encountered so little antigen that peptide-antigen-MHC complexes were catabolized before the cells recruited recirculating CTL to their site.

Histology of PRO4L-SIY-EGFP tumors showed that the untreated tumor consisted of a homogenous well-vascularized mass of rapidly proliferating cancer cells with little evidence of apoptosis (Fig. 6A). In contrast, most of the mass of T-cell-treated tumor at

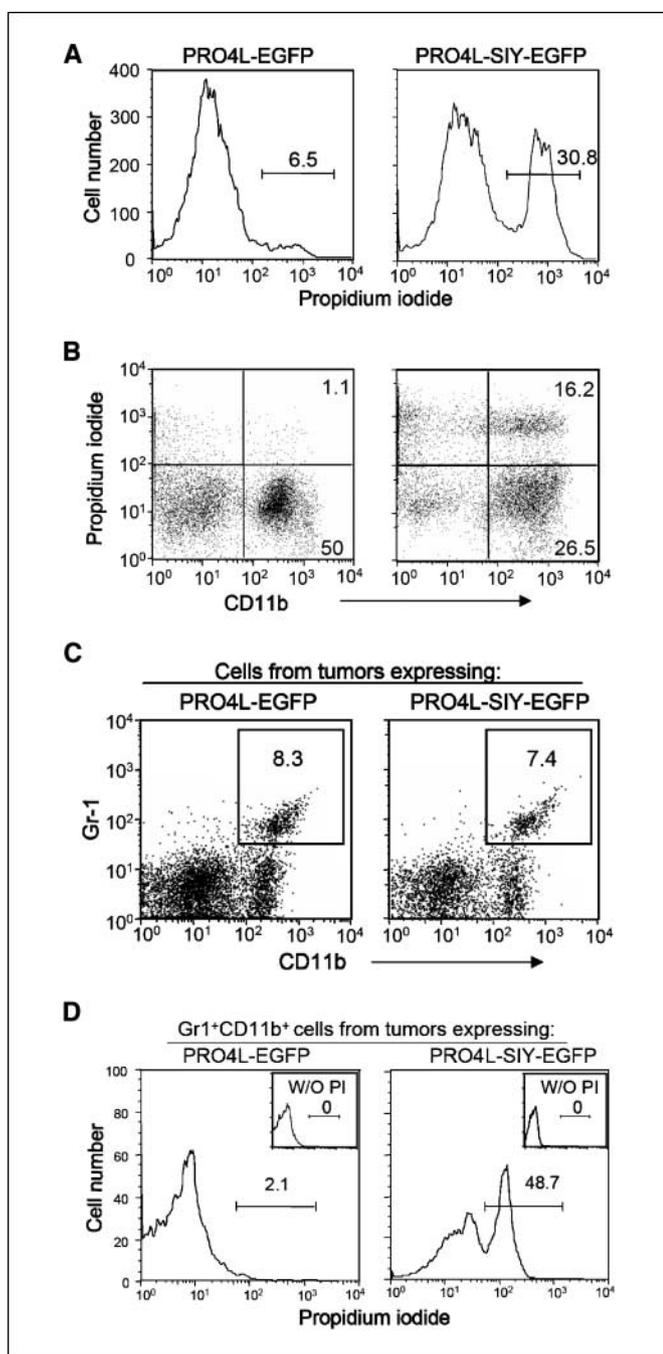


Figure 4. Destruction of tumor stromal cells by T cells. OT-1 $Rag1^{-/-}$ mice bearing PRO4L-EGFP or PRO4L-SIY-EGFP tumors were injected i.v. with 1×10^7 2C T cells at day 14. Tumor tissues were collected 6 d after 2C T-cell transfer. **A**, as determined by PI staining, PRO4L-SIY-EGFP tumors contained ~15-fold more nonviable cells than PRO4L-EGFP tumors. **B**, as determined by CD11b staining, PRO4L-SIY-EGFP tumors contained more nonviable stromal myeloid cells (PI⁺) than PRO4L-EGFP tumors. **C**, the percentage of Gr1⁺CD11b⁺ cells was similar in PRO4L-SIY-EGFP and PRO4L-EGFP tumors. **D**, as determined by Gr1 and CD11b staining, greater numbers of nonviable stromal myeloid cells (PI⁺) were found in PRO4L-SIY-EGFP tumors than in PRO4L-EGFP tumors.

day 70 was necrotic. A rim of viable and mitotic cancer cells surrounded this necrotic mass. Cancer cells were surrounded by lymphocytes directly adjacent to preexisting vasculatures in the outer area (Fig. 6B). A similar rim structure of surrounding necrotic tumor masses was observed at different times (i.e., at day 40, 50,

or 60) during equilibrium (data not shown), indicating a state of dynamic equilibrium between host and cancer.

Why killing of Gr1⁺/CD11b⁺ stromal cells promotes suppression of tumor growth. Gr1⁺/CD11b⁺ cells in tumor-bearing mice suppress T-cell responses, and here, we confirmed that either Gr1⁺ or CD11b⁺ cells from spleens or tumors suppressed antigen-specific CD8⁺ T-cell proliferation (Supplementary Fig. S4A and B). F4/80⁺ cells isolated from tumors almost completely blocked T-cell proliferation at 1:1 ratio. This inhibition was mediated by two major factors: increased production of nitric oxide and high arginase activity (Supplementary Fig. S4C).

Higher tumor incidence (Table 1) and tumor growth rate (Supplementary Fig. S5A) were observed when cancer cells were coinjected with splenic Gr1⁺CD11b⁺ cells from tumor-bearing mice than when cancer cells were injected alone or coinjected with Gr1⁺CD11b⁺ cells from tumor-free mice.

In tumor-bearing mice, splenic Gr1⁺ immature cells may differentiate into CD11b⁺ myeloid cells in tumors (20). Most CD11b⁺ cells that express F4/80 and infiltrated in tumors no longer express Gr1 (data not shown). We tested the effect of tumor-derived F4/80⁺ cells in an *in vitro* assay of morphogenesis in which MFLM-91U endothelial cells plated on Matrigel assemble into capillary-like structures. The formation of characteristic tubular structures was observed after 24-h incubation with conditioned medium of F4/80⁺ macrophages from tumors but not from spleens (Supplementary Fig. S5B). In the presence of MMP or VEGF inhibitor, the cells appeared isolated or assembled in clumps of rounded cells or short, thick cords attached to the Matrigel surface (Supplementary Fig. S5B). In addition, similar to endothelial cell culture medium, conditioned medium of F4/80⁺ cells from tumor tissues but not spleens stimulated cell migration. This induced cell migration was significantly reduced by addition of MMP or VEGF inhibitor (Supplementary Fig. S5C). Thus, F4/80⁺ cells in tumors may promote tumor angiogenesis and migration by MMP and VEGF. Together, these results strongly support the proposition that tumor-derived Gr1⁺/CD11b⁺ cells promote tumor growth and elimination of these stroma cells by T cells promotes suppression of tumor growth.

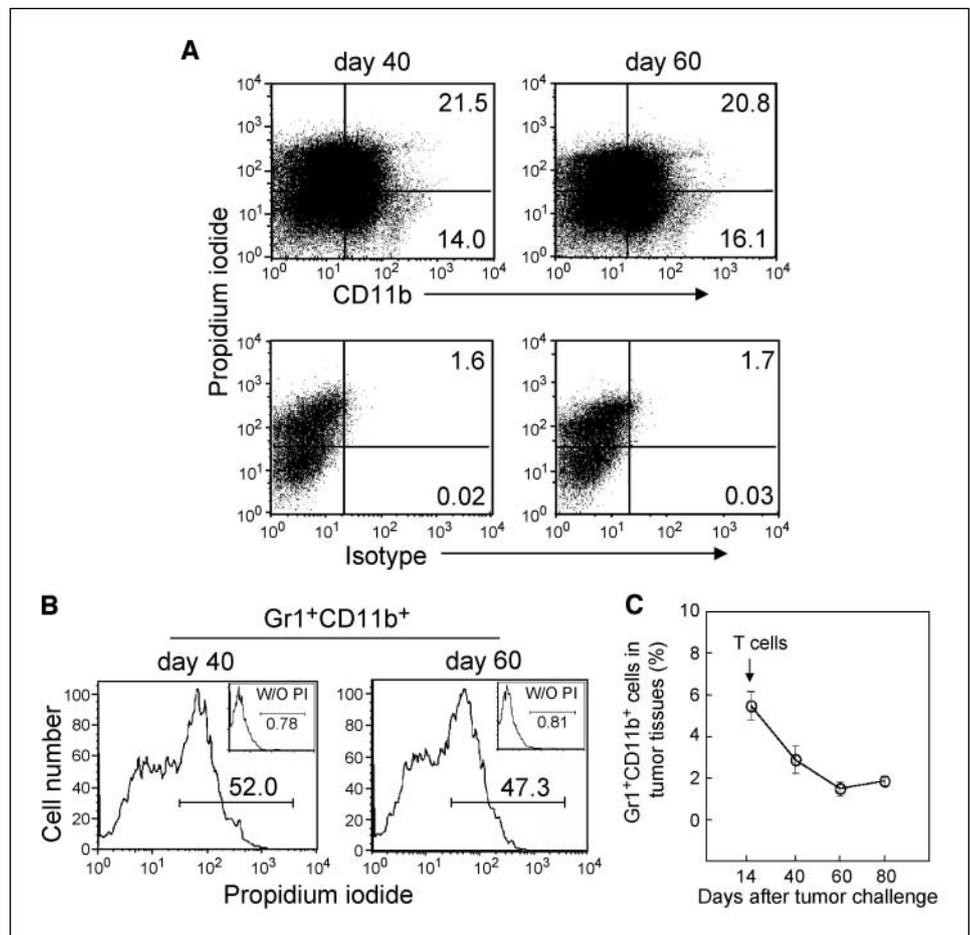
Discussion

The salient finding of our study is that a single T-cell transfer can cause long-term equilibrium between host and cancer by targeting only stromal cells that present a tumor antigen. The efficacy was not dependent on T cells from TCR transgenic mice because antigen-specific T cells from normal mice were also effective. Furthermore, the observed long-term arrest of tumor growth is also unlikely to be dependent on the particular tumor model we used because a human cancer that grows aggressively in $Rag1^{-/-}$ mice is also arrested for extended times by targeting the tumor-specific antigen released into the stroma.⁶

T-cell-mediated destruction of stromal MDSC may be the reason for allowing the transferred T cells to remain effective despite the large tumor burden. So, the histologic appearance of arrested tumors (lack of resorption, persistence of T cells, and lack of neovascularization) may be a consequence of T cells eliminating antigen cross-presenting CD11b⁺ myeloid stromal cells. This very heterogeneous cell population is known to contain macrophages,

⁶ B. Zhang and H. Schreiber, unpublished data.

Figure 5. Persistent elimination of stromal cells by T cells. OT-1 Rag1^{-/-} mice bearing PRO4L-SIY-EGFP tumors were injected i.v. with 1×10^7 2C T cells at day 14. Tumor tissues were collected at days 40 and 60. As determined by PI staining, tumors contained comparable numbers of nonviable (PI⁺) CD11b⁺ (A) and Gr1⁺CD11b⁺ (B) stromal cells. C, the kinetics of infiltration of Gr1⁺CD11b⁺ cells in tumor tissues following T-cell therapy was assessed by flow cytometry. Points, mean ($n = 3$); bars, SD.



MDSCs, and bone marrow-derived endothelial precursor and other proangiogenic cells such as pericytes.

We have shown previously that targeting both cancer cells directly as well as targeting stromal cells cross-presenting antigen released from the cancer cells are needed for eradication of large established solid tumors (13–15). For aggressively growing rapidly lethal cancers that cannot be excised, long-term arrest therefore would be an acceptable goal. Tumor eradication is obviously preferable to tumor arrest. However, cancer cells may not always be targeted, for example, when they have lost MHC class I molecules. Thus, this mechanism of growth arrest should extend to cancers that have lost or down-regulated the appropriate presenting MHC molecule, have defective antigen processing, or develop resistance to T-cell-mediated lysis or apoptosis (3, 4).

Despite continuous proliferation of the cancer cells, we have not observed that cancers can escape this powerful growth arrest by outgrowth of antigen loss variants. One reason may be that T cells are targeting only stromal cells that are generally genetically stable, although chromosome and epigenetic abnormalities have been described (29–33). Although cancer cells have remarkable genetic instability, they are not targeted directly and therefore not selected against. The importance of stroma being stable in expressing the appropriate MHC class I is emphasized by our finding that cancer escaped in chimeric mice in which either the bone marrow-derived or non-bone marrow-derived stroma expressed a MHC class I molecule inappropriate for cross-presenting the tumor-specific antigen. Cancers escaped stromal killing probably by using

stromal cells that could not cross-presented the antigens released from the cancer cells and therefore resistant to T-cell-mediated destruction. Indeed, we found that tumors that escaped in chimeric mice predominantly had stromal cells that were not able to present the antigens.⁷

Tumor stroma serves no beneficial purpose to the host but is necessary for tumor growth and therefore represents an ideal target. We previously reported that depletion of Gr1⁺CD11b⁺ cells *in vivo* using anti-Gr1 antibody enhanced CD8⁺ T-cell-mediated immune responses and resulted in the rejection of inocula of progressor tumor cells (16, 18, 21, 34). This is consistent with the results of subsequent studies showing that Gr1⁺CD11b⁺ bone marrow-derived cells give rise to MDSCs that inhibit CD8⁺ T cells (17, 18, 20, 35), are proangiogenic (36, 37), and stimulate cancer cell growth (16, 21). In addition, the presence of Gr1⁺CD11b⁺ myeloid cells has recently been associated with resistance to anti-VEGF antibody treatments; combined therapy with anti-Gr1 antibody helped to reverse this resistance (25). However, using anti-Gr1 antibody in a therapeutic setting seems untenable due to wide expression of Gr1 marker. The use of pharmacologic agents such as all-*trans*-retinoic acid (38), cyclooxygenase-2 inhibitors (39), or phosphodiesterase-5 inhibitors (40) improved the efficacy of adoptive T-cell therapy by reducing MDSC function, but the

⁷ B. Zhang, unpublished data.

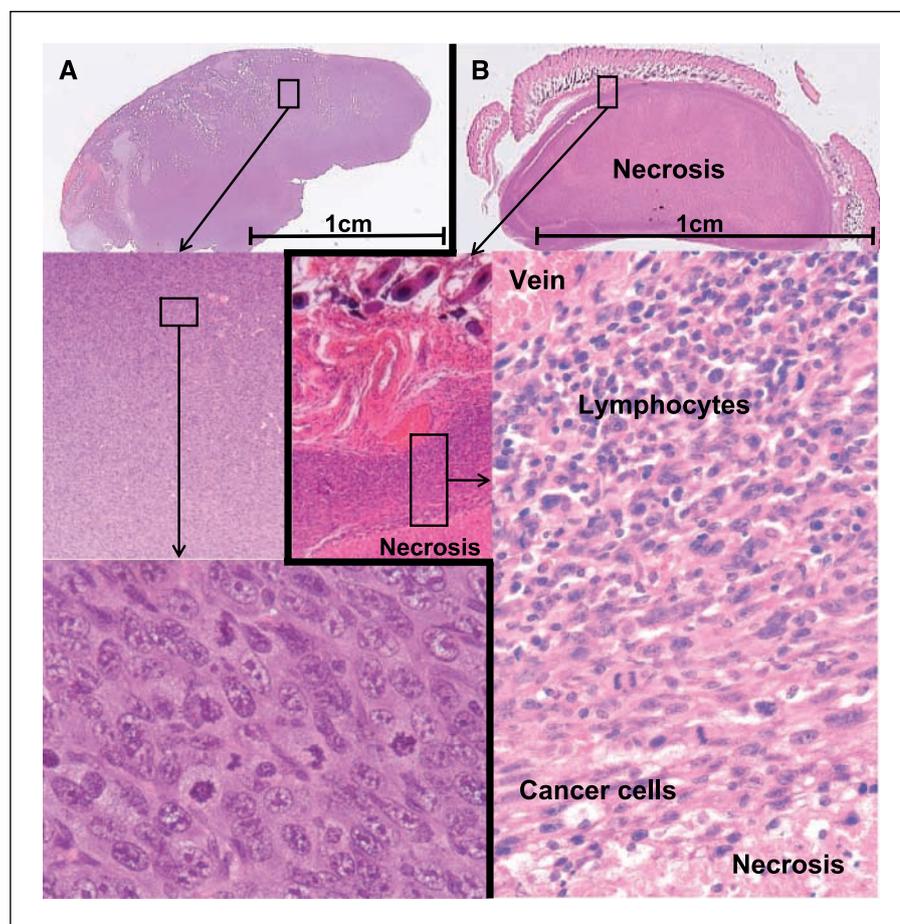


Figure 6. Histology of tumors during equilibrium. PRO4L-SIY-EGFP cells were injected s.c. into OT-1 Rag1^{-/-} mice. After 14 d, one mouse was left untreated (A), whereas the other (B) was treated with 2C T cells. Due to progressively growing tumor, the untreated mouse was sacrificed at day 20 and the tumor was excised for histology. The tumor in the treated mouse remained in equilibrium after initial shrinkage and was excised on day 70. Slides were stained with H&E.

discontinuation of these treatments could possibly restore immune suppression.

Researchers have used antigens directly expressed by stromal cells [VEGF receptor by endothelial cells (41), fibroblast activation protein by fibroblasts (42), or legumain by macrophages (43)] as targets for therapy. These self-antigens may be highly up-regulated but are not specific for tumor stroma. Vascular disrupting agents cause acute occlusion of tumor vessels leading to rapid and massive intratumoral necrosis (44); however, after this treatment, tumors rapidly regrow from the surviving rim at the periphery.

Antibodies neutralizing VEGF also arrest tumor growth, but only continuous treatments prevent escape (45). Our data do not address the question of how other components of tumor stroma or factors contribute to long-term inhibition of tumor growth, and important contributions by biological, metabolic, inflammatory, or angiostatic agents have been described (41, 46–48). However, our approach of stromal targeting described here is unique in that it is effective against large established solid tumors, requires a single transfer of T cells, and is tumor specific because the peptide-MHC class I molecule we target on the stromal cells depends on

Table 1. Coinjection of tumor-derived Gr1⁺CD11b⁺ cells increases incidence of tumor outgrowth in immunodeficient mice

Tumor challenge	Incidence of tumor outgrowth (%)*		
	Cancer cells only	Cancer cells and Gr1 ⁺ CD11b ⁺ cells from tumor-free mice	Cancer cells and Gr1 ⁺ CD11b ⁺ cells from tumor-bearing mice
Dose			
1 × 10 ⁴	0/6 (0)	0/8 (0)	4/8 (50)
5 × 10 ⁴	1/6 (17)	2/8 (25)	8/8 (100) [†]
2.5 × 10 ⁵	6/6 (100)	8/8 (100)	8/8 (100)

*C57BL/6 Rag1^{-/-} mice were injected s.c. with PRO4L cells and sorted Gr1⁺CD11b⁺ cells at 10:1 ratio at indicated doses from tumor-bearing mice in 200 μL PBS on the back. PRO4L cells alone and Gr1⁺CD11b⁺ cells from tumor-free Rag1^{-/-} mice were used as controls. Tumor outgrowth was assessed 30 d after tumor challenge. The data were pooled from two independent experiments.

[†]P < 0.05, compared with the groups "Cancer cells only" or "Cancer cells and Gr1⁺CD11b⁺ cells from tumor-free mice" at a dose of 5 × 10⁴.

the release of tumor-specific antigen from the cancer cells. These antigens are locally picked up by stromal cells in the tumor microenvironment, preventing any significant bystander killing of normal tissues outside of the cancer. Indeed, there was no evidence that CD11b⁺ cells peripheral to the tumor presented antigen or were killed by T cells.

Our findings still need to be confirmed in intact mice bearing transplanted or autochthonous tumors. However, there has been progress in the development of human T cells suitable for adoptive cellular immunotherapy, particularly when used in the lymphopenic patients (49, 50). The OT-1 Rag1^{-/-} mice we use may simulate such lymphodepleted patients. Although none of currently available stromal targeting strategies eliminates all cancer cells, our approach succeeds in obtaining long-term equilibrium of well-established cancers after a single T-cell transfer. Such growth arrest in patients

would be an admirable achievement for many cancers and could also be used as an adjuvant to other therapies. Thus, further research is needed to explore how the principles and concepts developed in this study will be applicable to human cancers.

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