

# Induced sensitization of tumor stroma leads to eradication of established cancer by T cells

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**Targeting cancer cells, as well as the nonmalignant stromal cells cross-presenting the tumor antigen (Ag), can lead to the complete destruction of well-established solid tumors by adoptively transferred Ag-specific cytotoxic T lymphocytes (CTLs). If, however, cancer cells express only low levels of the Ag, then stromal cells are not destroyed, and the tumor escapes as Ag loss variants. We show that treating well-established tumors expressing low levels of Ag with local irradiation or a chemotherapeutic drug causes sufficient release of Ag to sensitize stromal cells for destruction by CTLs. This was shown directly using high affinity T cell receptor tetramers for visualizing the transient appearance of tumor-specific peptide-MHC complexes on stromal cells. Maximum loading of tumor stroma with cancer Ag occurred 2 d after treatment and coincided with the optimal time for T cell transfer. Under these conditions, tumor rejection was complete. These findings may set the stage for developing rational clinical protocols for combining irradiation or chemotherapy with CTL therapy.**

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The promise of exploiting the specificity of the T cell response for treating cancer has been questioned. Immunotherapy may fail because (a) the cancer cells do not express MHC appropriately, (b) a physical stromal barrier (1–5) produced by the host–cancer interaction prevents access of immune effectors to the cancer, (c) immune regulation prevents activation/proliferation of effector T cells, or (d) variants emerge during cancer progression that are no longer susceptible to specific effector molecules or cells. Antigen (Ag)-specific CTLs may kill cancer cells very effectively even when they express low levels of Ag (6, 7). However, established tumors produced by cancer cells expressing high levels of Ag are cured by adoptively given CTLs, whereas tumors produced by cancer cells expressing low levels of Ag and treated similarly regress initially but then grow progressively because of the emergence of Ag loss variants (8, 9).

Our recent (8, 9) and present experiments show that targeting tumor stroma by adoptive

CTL therapy is highly effective for treating established cancers. The success of CTL therapy depended on the destruction of stroma of tumors produced by cancer cells expressing high levels of Ag. We suggested that the killing of stroma occurred because these stromal cells picked up sufficient Ag to cross-present it and be killed by CTLs. With the elimination of critical stroma, Ag-negative variant cancer cells in the tumor were also killed. If this is the case, then CTL therapy should also be effective against tumors produced by cancer cells expressing low levels of Ag if the tumor stroma was loaded with sufficient cancer cell Ag.

Irradiation or chemotherapy causes apoptosis and necrosis of cancer cells; therefore, we tested whether these procedures could cause sufficient release of Ag from cancer cells expressing low levels of Ag to sensitize tumor stroma for killing by CTLs. For this objective, we developed a high affinity TCR tetramer for detecting minute quantities of the relevant tumor-specific peptide–MHC complexes (pMHCs) cross-presented by stromal cells. Indeed, either radiation or chemotherapy, given 2 d

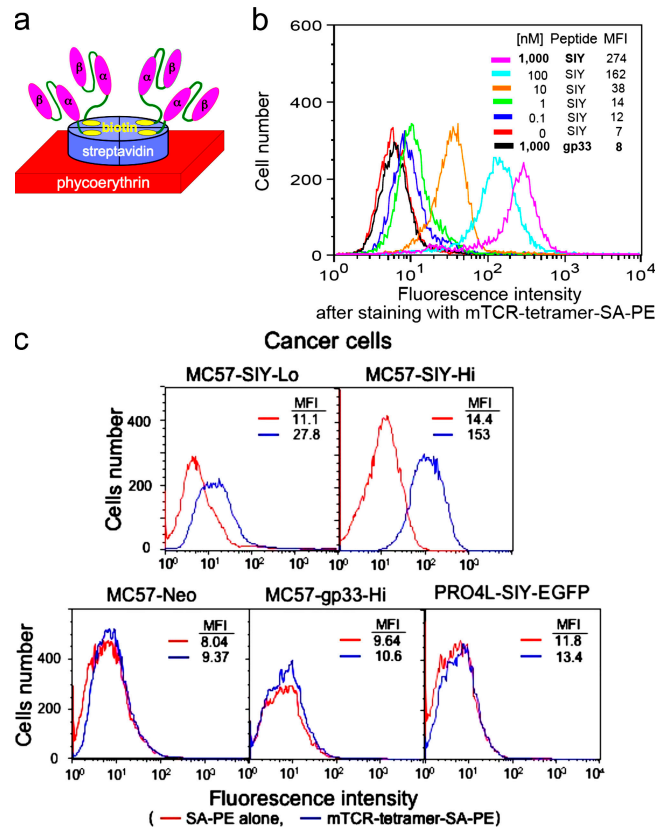
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before CTL therapy, sensitized cancer stroma for destruction by T cells so that the tumors were eradicated; i.e., variants did not grow out.

## RESULTS AND DISCUSSION

High affinity TCR tetramers were used for flow cytometry to visualize the presentation of the SIYRYYYGL (SIY) peptide used as tumor-specific Ag and presented on cell-surface K<sup>b</sup> as pMHC, because wild-type TCR tetramers cannot detect this complex (unpublished data). The single-chain m67 TCR consisted of the wild-type 2C V $\beta$  region, a 25-aa linker, and the mutant m67 V $\alpha$  region fused to a C-terminal peptide that contained the recognition site for biotinylation by the BirA enzyme. Each biotin attaches to the single biotin-binding site on a streptavidin (SA) molecule that occurs as a homotetramer and is chemically coupled to the fluorescent dye PE, thereby generating mTCR-tetramer-SA-PE (Fig. 1 a), thereby generating mTCR-tetramer-SA-PE (Fig. 1 a). The affinity of the engineered m67 TCR for the SIY-K<sup>b</sup> complex is  $\sim$ 16 nM, whereas the wild-type 2C TCR has  $>$ 1,000-fold lower affinity, similar to most other “natural” CD8-dependent TCRs reactive with peptide-self-MHC (10, 11). As shown in Fig. 1 b, differences in peptide concentrations in the range of 0.1 nM to 1  $\mu$ M could be clearly distinguished. The probe is specific with minimal background binding to cells loaded with the irrelevant gp33 peptides at a 1- $\mu$ M concentration (Fig. 1 b). Similar results were also observed in RMA-S cells (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20062056/DC1>). SIY-K<sup>b</sup> complexes were detected on MC57-SIY-Hi or on MC57-SIY-Lo cells. Complexes were not detected on parental MC57-Neo cells or on MC57-gp33-Hi cells that endogenously express high levels of irrelevant gp33 Ag (Fig. 1 c). K<sup>k</sup>-positive PRO4L cancer cells expressing high levels of SIY but lacking K<sup>b</sup> do not present the relevant SIY-pMHC complex on their cell surface (Fig. 1 c and Fig. S2). Cells stained with PE-conjugated SA (SA-PE) only or with an equimolar amount of an irrelevant m6 TCR tetramer were used as controls and exhibited background fluorescence intensity similar to that of the unstained cells (unpublished data). These results indicate that we have developed a reliable method to determine the levels of Ag-MHC complex on cell surfaces.

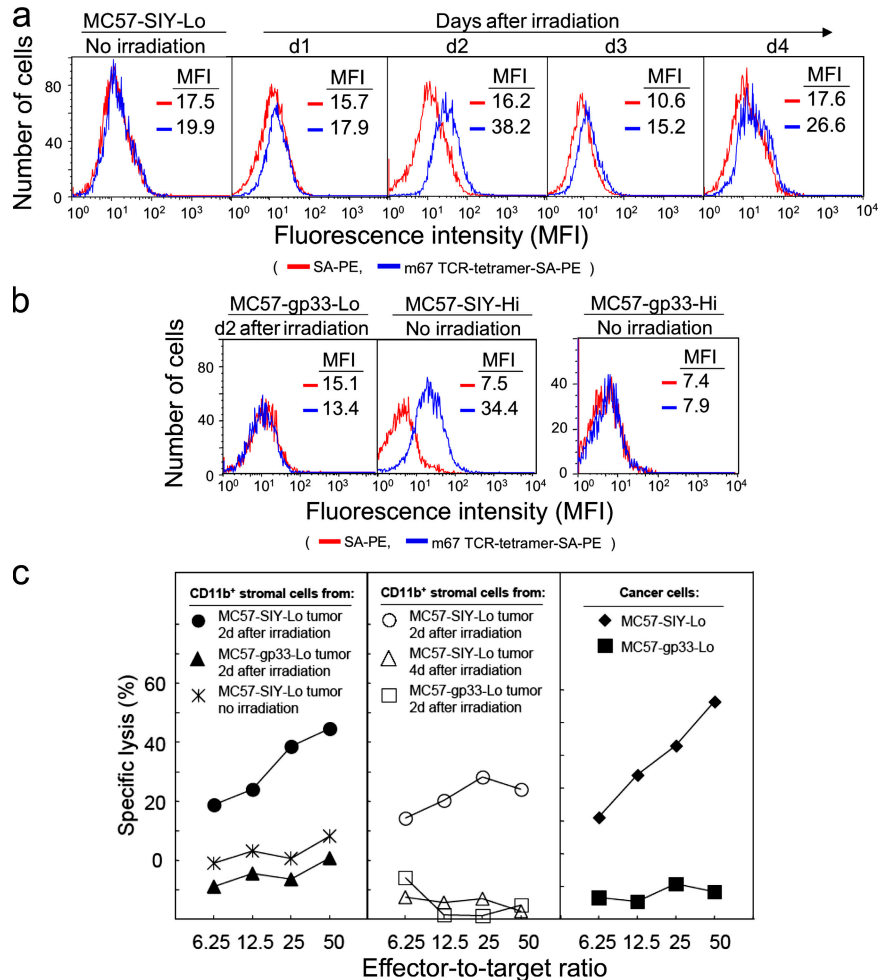
The CD11b<sup>+</sup> cells comprised  $>$ 20% of total cells in the 2-wk-old MC57 tumors. The CD11b<sup>+</sup> cells largely expressed CD45, F4/80, and MHC class II molecules but not CD11c, and therefore represented tumor-associated macrophages that may present tumor-derived Ag (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20062056/DC1>). To explore the kinetics of cross-presentation in vitro, we used J744 K<sup>b</sup> macrophages, peritoneal exude cells (PECs), Jaws II dendritic cells, and purified tumor-derived CD11b<sup>+</sup> stromal cells as APCs. As the source of Ag, cancer cells that were either necrotic (three serial freeze-thaw cycles) or apoptotic (induced by sorbitol) were incubated with APCs at a 1:1 ratio. Cross-presentation was determined by specific tetramer staining after incubation for 2, 4, 10, or 12 h. Maximal cross-presentation occurred at 4 h and decreased with very little



**Figure 1. High affinity TCR tetramer binding.** (a) Model of the SIY-K<sup>b</sup>-specific m67 TCR tetramer (see Results and discussion for details). (b) The TCR tetramer can detect SIY-K<sup>b</sup> complexes on T2K<sup>b</sup> cells artificially loaded with peptide at the indicated concentrations as low as 0.1 nM. (c) SIY-K<sup>b</sup> complexes were detected on MC57-SIY-Hi cells that express endogenously high levels of SIY Ag and MC57-SIY-Lo cells that express endogenously low levels of SIY Ag, but not on parental MC57-Neo or MC57-gp33-Hi cells that express endogenously high levels of irrelevant gp33 Ag. In addition, PRO4L-SIY-EGFP failed to be stained by the tetramers. Data are representative of two (b) or five (c) similar experiments. MFI, mean fluorescence intensity.

Ag remaining on the surface of APCs after 10 or 12 h of co-culture (Fig. S4), suggesting that tumor-derived APCs acquiring Ag from dead cancer cells in vivo might present the relevant pMHC complexes only transiently.

Tumor stroma from established MC57-SIY-Lo tumors was not stained by the TCR tetramer (Fig. 2 a). Also, the stromal cells from established MC57-SIY-Lo tumors were insensitive to lysis by specific T cells (Fig. 2 c, left). We then tested whether radiation or chemotherapy caused a “bolus” of tumor Ag release from MC57-SIY-Lo cells sufficient for uptake and presentation by CD11b<sup>+</sup> tumor stromal cells. High affinity TCR tetramers detected SIY-K<sup>b</sup> complexes on CD11b<sup>+</sup> stromal cells from the tumors of irradiated but not nonirradiated mice. To determine the time of maximal Ag cross-presentation by tumor-derived stroma in vivo, mice bearing 2-wk-old solid tumors were killed at 12 h and 1, 2, 3, and 4 d after local radiation. As shown in Fig. 2 a, maximal



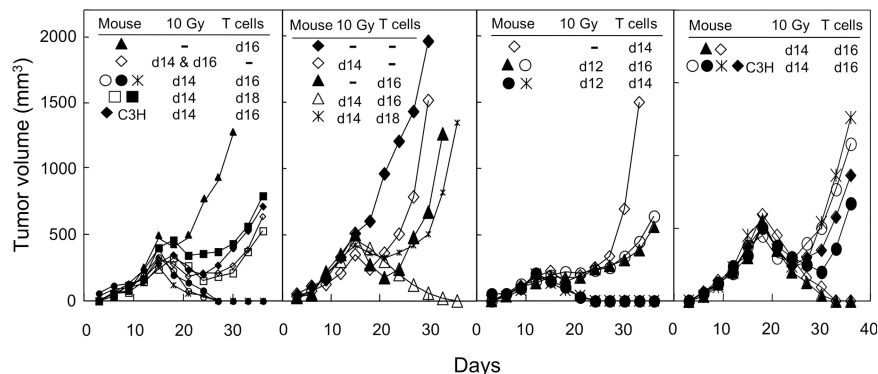
**Figure 2. Local radiation of tumors leads to the time-dependent stromal sensitization in established cancer.** (a)  $2 \times 10^6$  MC57-SIY-Lo or MC57-gp33-Lo cells were injected s.c. into the flanks of C57BL/6 Rag1<sup>-/-</sup> mice. Both cell lines express low levels of SIY or gp33 Ag. Where indicated, 15-d-old tumors were locally irradiated with 10 Gy. Tumors were excised at 12 h and 1, 2, 3, and 4 d. Single suspension cells were stained by APC-conjugated anti-CD11b antibody and m67 TCR tetramers. Cells were analyzed for tetramer staining by gating CD11b<sup>+</sup> cells. (b) As concurrent controls, CD11b<sup>+</sup> cells from locally irradiated MC57-gp33-Lo tumors were analyzed at 2 d after irradiation for tetramer staining.

SIY-K<sup>b</sup>-specific m67 TCR tetramer-bound CD11b<sup>+</sup> cells from a 2-wk-old MC57-SIY-Hi tumor but not those from a 2-wk-old MC57-gp33-Hi tumor are shown. (c) Effective sensitization of tumor-derived stroma by local irradiation of MC57-SIY-Lo tumors expressing low levels of SIY Ag, 2 but not 4 d after local irradiation, CD11b<sup>+</sup> stromal cells purified from 2-wk-old established MC57-SIY-Lo tumors were lysed by 2C TILs in a 4.5-h <sup>51</sup>Cr release assay. CD11b<sup>+</sup> stromal cells purified from a 2-wk-old antigenically irrelevant MC57-gp33-Lo tumor were not lysed. MC57-SIY-Lo and MC57-gp33-Lo cancer cells were used as concurrent controls. Data shown (a and b) represent one of two similar experiments. MFI, mean fluorescence intensity.

cross-presentation by MC57-SIY-Lo tumor stroma was found 2 d after irradiation and decreased thereafter with very little Ag remaining after 4 d. In contrast, SIY-K<sup>b</sup>-specific complexes were detected on CD11b<sup>+</sup> stromal cells from a 2-wk-old MC57-SIY-Hi tumor without irradiation (Fig. 2 b). CD11b<sup>+</sup> stromal cells from a 2-wk-old MC57-gp33-Hi tumor were negative, and there was no noticeable binding to CD11b<sup>+</sup> stromal cells from MC57-gp33-Lo tumors after irradiation (Fig. 2 b). Furthermore, MC57-SIY-Lo tumors were exposed to different doses of radiation, and the levels of SIY-K<sup>b</sup> complexes on the cell surface at a dose of 10 Gy, indicating that the increased cross-presentation

by CD11b<sup>+</sup> stromal cells was radiation dose dependent (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20062056/DC1>). The stromal cells derived from MC57-SIY-Lo cancer cells 2 d after irradiation were sensitized for destruction by CTLs, but stromal cells isolated 4 d after irradiation were not killed, consistent with the kinetics observed by staining (Fig. 2 c, left and middle). MC57-gp33-Lo stromal cells were not killed by CTLs when isolated 2 d after irradiation (Fig. 2 c, left and middle). MC57-SIY-Lo and MC57-gp33-Lo cancer cells were used as concurrent controls (Fig. 2 c, right).

We next determined whether radiation-induced sensitization of stroma from Lo-Ag tumors prevented escape of Ag



**Figure 3. Local radiation of tumors promotes cancer eradication by CTLs.**  $2 \times 10^6$  MC57-SIY-Lo cells per cell line were injected s.c. into OT-1 transgenic mice or a C3H Rag2<sup>-/-</sup> mouse, and the volume of the tumor (mm<sup>3</sup>) was measured every 3 d. After 14 d, the tumors were locally irradiated with 10 Gy, followed by adoptive transfer of  $5 \times 10^6$  preactivated 2C T cells at day 16 or 18. As controls, tumors were only treated at days 14 and/or 16

with local irradiation using 10 Gy, or tumor-bearing mice only received adoptive transfer of  $5 \times 10^6$  preactivated 2C T cells on day 16 but no irradiation. Each symbol represents an individual mouse. Four independent experiments are shown, with each in a separate panel. In addition, a fifth experiment (not depicted) confirmed that repeated irradiation with 10 Gy on days 14 and 16 (20 Gy total dose) did not cure any of the mice ( $n = 3$ ).

loss variants. As shown in Fig. 3, locally irradiating 14-d tumors once with 10 Gy followed by T cell transfer 2 d, but not 4 d, after irradiation led to complete eradication of well-established MC57-SIY-Lo tumors. These tumors, without irradiation, regularly escaped with an outgrowth of cancer variants after T cell therapy (unpublished data). Thus, the time interval between radiation and adoptive T cell therapy was crucial (Table I). Furthermore, MC57-SIY-Lo tumors growing in C3H Rag2<sup>-/-</sup> mice in which stroma is incapable of cross-presenting the tumor Ag were not rejected after irradiation and CTL therapy (Table I). Single (10 Gy) or repeated ( $2 \times 10$  Gy) local irradiation alone did not cure any of the mice. Thus, the radiation-induced effect depended on sensitization and destruction of tumor stroma. We next tested whether the effects observed after irradiation also applied to a chemotherapeutic agent, gemcitabine. Maximal loading of

the MC57-SIY-Lo tumor stroma occurred at 2 d after drug treatment (Fig. 4 a), and complete rejection was achieved when T cells were transferred at 2 but not at 4 d after gemcitabine treatment (Fig. 4 b and Table I).

Important work has been done on the synergy between immunotherapy and irradiation (references 12–15; for review see reference 16) or chemotherapy (references 14, 17, 18; for review see reference 19). However, unlike the present study, these studies have dealt with the induction of immune response or with direct effects of radiation or drugs on the killing of cancer cells. Recently, it was demonstrated that radiation, by activating the mammalian target of Rapamycin pathway, could rapidly increase the intracellular pool of peptides derived from rapidly degraded proteins and increase MHC class I expression and peptide presentation by the cancer cells, thereby increasing the sensitivity of cancer cells to

**Table I.** Stroma and timing are critical for complete elimination of established tumors

Treatment	Host	Tumor stroma	Rejection of tumors <sup>a</sup>	p-value
Irradiation + T cells 2 d later	OT-1	H-2 <sup>b</sup>	12/12	
Irradiation + T cells 4 d later	OT-1	H-2 <sup>b</sup>	0/9	0.001 <sup>b</sup>
Irradiation + T cells 2 d later	C3H Rag2 <sup>-/-</sup>	H-2 <sup>k</sup>	0/9	0.001 <sup>b</sup>
Gemcitabine + T cells 2 d later	OT-1	H-2 <sup>b</sup>	7/8	
Gemcitabine + T cells 4 d later	OT-1	H-2 <sup>b</sup>	0/6	0.001 <sup>c</sup>
Gemcitabine + T cells 2 d later	C3H Rag2 <sup>-/-</sup>	H-2 <sup>k</sup>	0/6	0.001 <sup>c</sup>

<sup>a</sup>Data are pooled from seven independent experiments. Mice shown in Fig. 3 and Fig. 4 b are included in this pool of data.

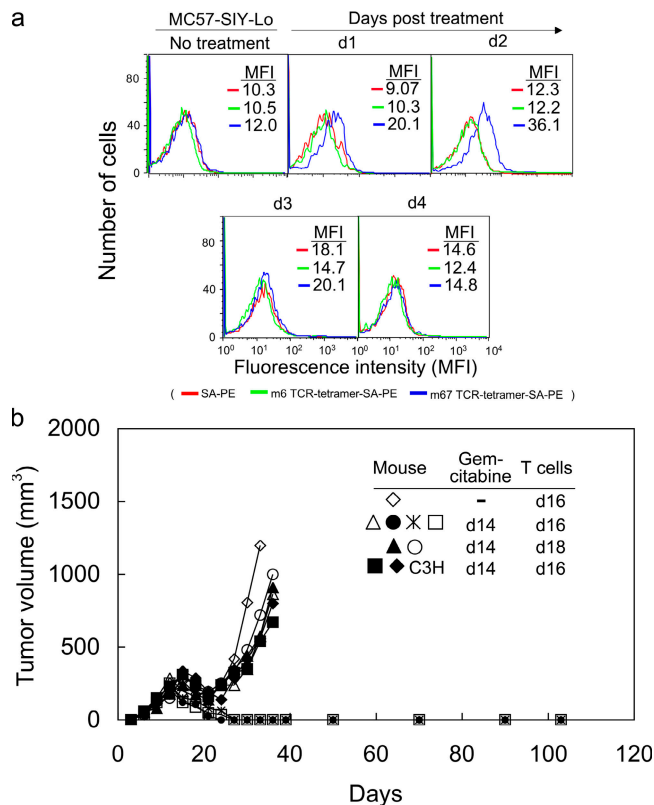
<sup>b</sup>Compared, respectively, with the group OT-1 irradiation + T cells 2 d later.

<sup>c</sup>Compared, respectively, with the group OT-1 Gemcitabine + T cells 2 d later.

direct killing by T cells (15). This enhancement of direct killing may be important and necessary, but not sufficient, for treating large, established solid tumors (8, 9). Indeed, our current results show that the direct effect of radiation on cancer cells is insufficient to eradicate cancer unless the tumor stroma is sensitized for destruction by T cells. Thus, after a combination of local radiation treatment (or systemic drug treatment) and adoptive T cell transfer, established cancers regularly escaped as Ag-loss variants when the stroma (H-2<sup>k</sup>) was unable to cross-present the Ag, or when T cells were transferred at a time when the stroma was no longer sensitized. In vitro, Ag loading of CD11b<sup>+</sup> cells peaked after a few hours (Fig. S4), whereas in vivo, the loading and cross-presentation of tumor Ag by CD11b<sup>+</sup> APCs peaked at 2 d after radiation or chemotherapy (Fig. 2 a and Fig. 4 a). The likely difference is that, in vitro, APCs were fed with already apoptotic or necrotic material, whereas in vivo, additional time is needed for apoptosis to occur after radiation or drug treatment before antigenic material is released. Our results do not address the question of whether the direct effects of radiation or chemotherapy on tumor stroma additionally contribute to tumor rejection, but such a contribution has been indicated by an earlier study using a chemotherapeutic agent (20).

These results support the concept that the curative combination of irradiation or chemotherapy and subsequent adoptive T cell immunotherapy of established solid tumors depends on the radiation or chemotherapeutic drugs releasing tumor Ag from cancer cells in the tumor, followed by sensitization and destruction of tumor stroma. One might also argue that T cell-mediated destruction of cells cross-presenting Ag violates an important basic safety mechanism. If, for example, Ag from virally infected cells sensitized normal (uninfected) surrounding tissue, CD8<sup>+</sup> T cell destruction of this tissue could be disastrous. However, the elimination of cells in proximity to infected cells could inhibit pathogen spread by sacrificing a rim of the healthy tissue while still limiting tissue damage locally to the infected area (5). This is consistent with our finding that destruction of cross-presenting tissue is completely Ag dose dependent. This point is furthermore made by recent findings showing that cross-presentation of intracellular peptides by transfer through gap junctions is limited to a few neighboring cells (21, 22). Although the spread of apoptotic and necrotic material from killed cancer cells most likely extends beyond neighboring cells, there was no destruction of normal stroma away from the tumor. Tumor stroma consists of nonmalignant cells, but this stroma is by no means a normal tissue. Tumor stroma contains activated fibroblasts (23, 24), recently formed immature and leaky capillaries, and many types of inflammatory cells comparable to a nonhealing wound (25). Ag pick-up and presentation is therefore likely to be very different in such a stroma. In fact, the advantage of our form of stromal targeting probably depends on not damaging or arresting stromal cells where there are no cancer cells.

The use of highly artificial experimental models allowed us to reveal principles, components, and interactions that



**Figure 4. Treating tumors with gemcitabine leads to the time-dependent stromal sensitization and helps achieve tumor eradication by CTLs.** (a) 14 d after tumor challenge, the tumor-bearing mice were treated i.p. with 200  $\mu\text{g/g}$  (body weight) gemcitabine. Tumor tissues were excised and dispersed, and single suspension cells were stained by APC-conjugated anti-CD11b and m67 TCR tetramer 1, 2, 3, and 4 d after drug treatment. As controls, cells were stained with an irrelevant m6 TCR-SA-PE tetramer (m6 specifically binds to the QL9 peptide on L<sup>d</sup>; reference 11) or SA-PE. Cells were subsequently analyzed by gating CD11b<sup>+</sup> cells. (b)  $2 \times 10^6$  MC57-SIY-Lo cells were injected s.c. into OT-1 transgenic mice or C3H Rag2<sup>-/-</sup> mice. After 14 d, the tumor-bearing mice were treated with 200  $\mu\text{g/g}$  gemcitabine i.p., followed by adoptive transfer of  $5 \times 10^6$  preactivated 2C T cells at day 16 or 18. The tumor-bearing control mice received  $5 \times 10^6$  preactivated 2C T cells at day 16 without drug treatment. Each symbol represents an individual mouse. Data shown (a and b) are representative of two similar experiments. MFI, mean fluorescence intensity.

would not have been discovered in usual models because of the lack of comparable high affinity TCR tetramers. The transfected Ag is not a self-Ag but a tumor-specific Ag; we believe that such Ags exist on the tumors and can be recognized with autologous T cells. In our study, we treated 2-wk-old established tumors in which the acute inflammation reaction of transplantation has vanished and which are virtually indistinguishable from nontransplanted autochthonous tumors by histopathologic examination (26). Therefore, although our results still need to be confirmed in hosts bearing autochthonous tumors, there is no reason to assume that the principles and concepts developed in this study will not be

applicable to established cancers in humans, as long as at least some sensitivity on chemotherapy or irradiation is present. Our experiments involved T cells transferred into the immunocompromised hosts, and there has been major progress in the development of human T cells suitable for adoptive cellular immunotherapy, particularly when used in a lymphopenic host environment (27); thus, monospecific TCR gene transduction of autologous T cells transferred into lymphodepleted patients (28) has become feasible. Our study shows that combination therapy is not simply based on adding two procedures, each of which kills cancer cells, but rather depends on taking advantage of a network of reactions requiring each component to be adjusted, for specific reasons, to optimal amounts, sequences, and timing.

## MATERIALS AND METHODS

**Mice, cell lines, and reagents.** C57BL/6 Rag1<sup>-/-</sup> mice were purchased from the Jackson Laboratory. M. Mescher (University of Minnesota, Twin Cities, MN) provided the OT-1 mice. The 2C Rag1<sup>-/-</sup> mice were provided by J. Chen (Massachusetts Institute of Technology, Boston, MA). Cultured cancer cells were trypsinized and washed once with plain DMEM, and  $2 \times 10^6$  cells were injected s.c. under the shaved back of mice. The tumor size was determined at 3-d intervals. Tumor volumes were measured along three orthogonal axes (*a*, *b*, and *c*) and calculated as tumor volume =  $abc/2$ . Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago. P. Ohashi (University of Toronto, Toronto, Canada), with the permission of H. Hengartner (University Hospital, Zurich, Switzerland), provided the MC57G methylcholanthrene-induced C57BL/6-derived fibrosarcoma. PRO4L cells have been previously described (2). H. Auer and S. Meredith (University of Chicago, Chicago, IL) synthesized the 2C-recognized peptide, SIY, and the P14-recognized peptide lymphocytic choriomeningitis virus-derived gp33 epitope, KAVYNFATM. All antibodies were purchased from BD Biosciences. Sorbitol was purchased from Sigma-Aldrich. J744 K<sup>b</sup> cells were provided by C.-R. Wang (University of Chicago, Chicago, IL).

**Generation of SIY and gp33 vectors and transfection/transduction of cells.** The generation of iSIY-LEGFP, igp33-LEGFP, MFG-SIY-EGFP, and MFG-gp33-EGFP vectors was previously described (7–9). MC57G was transfected with MerCreMer to generate MC57-Neo (Neo). Neo was transfected with iSIY-LEGFP or igp33-LEGFP, selected with 5 μg/ml puromycin, and cloned by limiting dilution to generate MC57-SIY-Lo or MC57-gp33-Lo, respectively. MC57-SIY-Lo or MC57-gp33-Lo cells were treated with 200 nM 4-hydroxytamoxifen for 4 d to generate MC57-SIY-Hi or MC57-gp33-Hi, respectively. Phoenix cells were transfected with pLEGFP, MFG-SIY-EGFP, or MFG-gp33-EGFP using Superfect (QIAGEN). Supernatants were used to infect PRO4L cells to generate PRO4L-EGFP, PRO4L-SIY-EGFP, or PRO4L-gp33-EGFP cell lines. PRO4L-EGFP and PRO4L-gp33-EGFP were used as controls for the effect of EGFP expression on tetramer staining.

**Preparation of single-cell suspensions from murine tumors.** Tumors were surgically excised under sterile conditions and placed in RPMI 1640 containing 1% antibiotic/antimycotic on ice, washed with PBS, minced into 1–2-mm pieces, and centrifuged at 1,800 rpm for 10 min, and supernatants were removed. After repeating this centrifugation, the tumor fragments were incubated for 45 min to 1 h in a digestion solution (5 ml/g tissue) containing 1 mg/ml trypsin, 1 mg/ml collagenase D, and 0.25 mg/ml DNase I in HBSS in an atmosphere of 5% CO<sub>2</sub> at 37°C. Pipetting of the samples every 15 min substantially enhanced tissue disruption. DMEM containing 10% FCS and 1% antibiotic/antimycotic was added to the cell suspension to stop the enzymatic activity. To lyse red blood cells, the tissue digests were briefly exposed to cold Tris NH<sub>4</sub>Cl, washed, and filtered through a 70-μm nylon filter mesh, resulting in a single-cell suspensions.

## Expression, purification, and multimerization of single-chain TCR.

High affinity TCR 2C-m67 was generated previously, using in vitro-directed evolution and yeast display (10). In brief, site-directed mutations in five successive codons of the CDR3α region (wild-type sequence GFASA) were introduced into the 2C single-chain TCR gene (Vβ-linker-Vα), and a library of mutants was expressed on the surface of the yeast. High affinity mutants were isolated by fluorescence-activated cell sorting using tetramers of the ligands SIYR-K<sup>b</sup> and dEV8-K<sup>b</sup>. Isolates with the CDR3α sequence LERPY were isolated from selections with both ligands. The 2C-m67 TCR that contained this CDR3α sequence was produced in soluble form and shown by various methods, including surface plasmon resonance, to have a dissociation constant value of 16 nM, a 2,000-fold increase compared with the wild-type 2C TCR (10).

Single-chain high-affinity TCR 2C-m67 containing a C-terminal 15-aa 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 M urea and guanidine HCl and refolded as previously described (29). Ni-NTA agarose (QIAGEN) was used to isolate the single-chain TCR, followed by size exclusion chromatography (Sephacryl S-200; GE Healthcare). Tetramerization of single-chain TCR was performed by the gradual addition of SA-PE (BD Biosciences) in aliquots to saturate its binding sites to a total biotin-conjugated m67 single-chain TCR/SA molar ratio of 20:1 (to ensure formation of tetrameric complexes).

**TCR tetramer staining and peptide-binding assay.** In tumor stromal experiments, samples were preincubated with 2.4G2 to block antibody binding to the Fc receptors. The CD11b<sup>+</sup> stromal cells were positively selected from collagenase-digested tumors using CD11b MicroBeads (Miltenyi Biotec). The CD11b marker was expressed by 75–80% of the purified cells. For tetramer staining, 10<sup>6</sup> fresh cultured cancer cells, purified CD11b<sup>+</sup> stromal cells, or tumor single-cell suspensions were incubated with 0.86 μg SIY-K<sup>b</sup>-specific m67 TCR tetramer in 100 μl of staining buffer in conjunction with mAb CD11b-APC. Cells were stained for 45 min to 1 h at room temperature. Cells stained with SA-PE or the molar equivalent amounts of PE-conjugated m6 TCR tetramer (specifically binding to QL9-L<sup>d</sup>) (11) were used as controls. Background fluorescence intensity was similar to that of the unstained cells (unpublished data). For the binding assay,  $5 \times 10^5$  T2 K<sup>b</sup> cells were incubated with the SIY or gp33 peptides at different concentrations (0.1–1,000 nM) for 2 h at 37°C and 5% CO<sub>2</sub> and stained with the SIY-K<sup>b</sup>-specific m67 TCR tetramer m67 for 1 h at room temperature. For Ag cross-presentation assays in vitro, purified tumor-derived CD11b<sup>+</sup> stromal cells, PECs induced by thioglycollate, J744 K<sup>b</sup> cells, or Jaws II cells ( $2 \times 10^5$  cells/well) were incubated in 48-well plates at 37°C overnight. An equal number of either apoptotic (sorbitol-treated) or necrotic (three consecutive freeze-thaw cycles) PRO4L-EGFP and PRO4L-SIY-EGFP cells were added as Ag donor cells and cocultured at 37°C. At the times indicated in the figures, cells were harvested using the plunger of a 1-ml syringe as a rubber policeman and analyzed by FACS for binding of the SIY-K<sup>b</sup>-specific m67 TCR tetramers. To exclude the possible interference of EGFP-positive cells, the cells were analyzed by gating the CD11b<sup>+</sup> population (tumor-derived stromal cells, J744 K<sup>b</sup> cells, or PECs) or EGFP-negative cells (Jaws II cells). Samples were analyzed on a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Inc.).

**<sup>51</sup>Cr release assay.** To generate cytolytic 2C T cells, 10<sup>6</sup> Tris NH<sub>4</sub>Cl<sup>-</sup>-treated splenocytes from a 2C Rag1<sup>-/-</sup> mouse were stimulated with  $6 \times 10^6$  NH<sub>4</sub>Cl-treated, irradiated (20 Gy) splenocytes from a BALB/c mouse in a 15-ml rounded bottom tube (BD Biosciences) containing 3 ml RPMI 1640 with 25 mM HEPES supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM β-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. After 5 d, <sup>51</sup>Cr release assays were performed as previously described (7, 8).

**Adoptive transfer of T cells.** For transfer of activated cells,  $5 \times 10^6$   $\text{NH}_4\text{Cl}$ -treated splenocytes from 2C transgenic mice were stimulated with  $10 \mu\text{M}$  of the SIY peptide for 3–4 d. Activation was confirmed by the up-regulation of CD44 on the specific T cells. The preactivated T cells were injected i.v. into the retroorbital plexus in a 0.1-ml volume.

**Local tumor irradiation and systemic chemotherapy.** Mice were irradiated using an x-ray generator (PCM 1000; Pantak) at a dose of 10 Gy. Each mouse was confined to a lead cover with its tumor-bearing flank exposed through an opening on the side, allowing the tumor to be irradiated locally. For systemic chemotherapy, tumor-bearing mice were injected i.p. with  $200 \mu\text{g/g}$  body weight gemcitabine (Eli Lilly and Company) 14 d after tumor challenge.

**Online supplemental material.** Fig. S1 illustrates the sensitivity of SIY-K<sup>b</sup>-specific TCR tetramer binding to RMA-S cells. Fig. S2 shows that PRO4L-SIY-EGFP cells express high levels of the SIY Ag comparable to those expressed by MC57-SIY-Hi cells. Fig. S3 shows characterization of tumor-derived CD11b<sup>+</sup> cells. Fig. S4 shows that APCs cross-present Ag from dead cancer cells rapidly and transiently. Fig. S5 shows that local radiation of tumors increases the Ag cross-presentation of stromal cells in a dose-dependent manner. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20062056/DC1>.

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