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IFN-γ– and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers

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Tumors elicit antitumor immune responses, but over time they evolve and can escape immune control through various mechanisms, including the loss of the antigen to which the response is directed. The escape of antigenloss variants (ALVs) is a major obstacle to T cell-based immunotherapy for cancer. However, cancers can be cured if both the number of CTLs and the expression of antigen are high enough to allow targeting of not only tumor cells, but also the tumor stroma. Here, we showed that IFN- γ and TNF produced by CTLs were crucial for the elimination of established mouse tumors, including ALVs. In addition, both BM- and non-BM-derived stromal cells were required to express TNF receptors and IFN- γ receptors for the elimination of ALVs. Although IFN- γ and TNF were not required by CTLs for perforin-mediated killing of antigen-expressing tumor cells, the strong inference is that tumor antigen-specific CTLs must secrete IFN- γ and TNF for destruction of tumor stroma.

Introduction

Cancers express antigens that are targets for specific CTLs (1, 2); however, tumor evasion by different mechanisms remains a significant obstacle to effective adoptive T cell therapy. Cancer cells may have lost or mutated the target antigen, lost or downregulated presenting MHC class I molecules, or altered their antigen-processing machinery (3–10). Many of these variants show heritable resistance because of the remarkable genetic instability of malignant cells (11). These subpopulations may escape eradication by CTLs and grow progressively.

Stroma (literally "bed" in Greek) is the connective tissue framework of an organ or tumor. In solid tumors, malignant cells are enmeshed in a stroma consisting of a complex network of microvasculature lined by endothelium, BM-derived, and other nonmalignant cells as well as extracellular matrix. Cancer cells must induce stroma to produce solid tumors to survive and replicate. For example, cancer cells embedded in stroma are 10- to 100-fold more tumorigenic than cancer cells alone (12-14). Often, the majority of the cells in a solid tumor are stromal cells; these nonmalignant cells are generally genetically stable, although epigenetic and chromosomal abnormalities have been previously described (15-19). Because stromal cells cannot escape as mutant variant cells, targeting the stroma of solid tumors is an important focus for various types of therapies using chemicals (such as small-molecule tyrosine kinase inhibitors), radiation, or biologicals (e.g., antiangiogenic agents, growth factor traps, immunizations, or gene delivery that blocks endothelial signaling) (20-24). In addition, T cell vaccines have been developed that target endothelial cells or activated fibroblasts in tumor stroma (25-27). However, targeting tumor stroma alone usually does not lead to eradication of large tumors; that is, antiangiogenic treatment is cytostatic, but alone it is usually not curative (20, 21, 28, 29).

We have shown that for the eradication of large established solid tumors, the stroma as well as the majority of the cancer cells in the tumor must be targeted and destroyed by CTL to prevent antigenloss variants (ALVs) from escaping. However, the mechanisms preventing the outgrowth of ALVs by T cells have not been identified. Our earlier experiments (8) showed that T cells lacking perforin failed to cause even temporary inhibition of tumor growth. In contrast, T cells that produced perforin but not IFN- γ reduced tumor size substantially, but growth resumed later, suggesting that IFN- γ might be important for preventing escape of ALVs possibly by acting on tumor stroma.

T cells secrete TNF and IFN- γ and upregulate FasL after encountering specific antigen. Our objective in the present study was to determine the role of these cytokines produced by adoptively transferred T cells in preventing tumor recurrence. We found that FasL-Fas interaction was not needed, but perforin-competent T cells must secrete TNF as well as IFN- γ , and both BM- and non-BM-derived stromal cells must express receptors for these cytokines for CTLs to eliminate ALVs by bystander killing.

Results

IFN- γ and TNF produced by transferred T cells are required for eradicating established tumors. To generate tumor-specific effector cells, spleen cells from SIY-immunized mice were activated in vitro with SIY peptide. These CTLs were infused i.v. into established MC57-SIY-Hi tumorbearing OT-1 mice (see Methods). MC57-SIY-Hi tumors grew rapidly in untreated control mice or in mice receiving CTLs from Pfr^{-/-} mice, whereas CTLs from WT mice caused complete tumor rejection (Figure 1, left, and Table 1), confirming our previous results (8). Adoptive transfer of CTLs from either IFN- $\gamma^{-/-}$ mice or TNF^{-/-} mice caused MC57-SIY-Hi tumors to initially regress but then grow progressively, whereas CTLs from FasL^{-/-} mice caused complete elimination of MC57-SIY-Hi tumors (Figure 1, middle, and Table 1). In addition, MC57-SIY-Hi tumors growing in IFN- $\gamma^{-/-}$ or TNF^{-/-} mice were eradicated by SIY-specific 2C CTLs (data not shown), suggesting that host-derived IFN- γ and TNF were not required.

Nonstandard abbreviations used: ALV, antigen-loss variant; IFN-γR, IFN-γ receptor; TNFR, TNF receptor.

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Figure 1

IFN- γ and TNF produced by T cells are needed for rejection of established tumors. OT-1 transgenic mice were injected s.c. with 2 × 10⁶ MC57-SIY-Hi cells; on day 14, the SIY-immune T cells from WT and Prf^{-/-} mice, as well as no T cells as controls (left), and the SIY-immune T cells from WT, TNF^{-/-}, and IFN- $\gamma^{-/-}$ mice (middle) were adoptively transferred into the tumor-bearing mice. Results were pooled from 3 experiments, each controlled by tumor-bearing mice treated with WT T cells. Right: OT-1 transgenic mice were injected s.c. with 2 × 10⁶ MC57-SIY-Hi cells plus 2 × 10³ MC57 cells. At day 14, the SIY-immune T cells from WT, TNF^{-/-}, and IFN- $\gamma^{-/-}$ mice were adoptively transferred into the tumor-bearing mice. The generation of SIY-immune T cells is described in Methods. Each curve represents an individual mouse.

To confirm that ALVs preexistent in established tumors were eliminated by T cells secreting IFN- γ and TNF, 2,000 antigennegative cancer cells (MC57 cells) as ALVs were mixed into 2×10^6 antigen-positive MC57-SIY-Hi cancer cells, inoculated s.c., and treated with adoptively transferred T cells on day 14, when the tumors were large. Transfer of CTLs generated either from IFN- $\gamma^{-/-}$ mice or from TNF^{-/-} mice resulted in the temporary inhibition of tumor growth followed by relapse, whereas transfer of CTLs from WT mice eradicated these tumors completely (Figure 1, right). These data demonstrate that IFN- γ and TNF produced by CTLs are required for preventing relapse after T cell-mediated destruction of established tumors. As we previously observed (8), perforin secretion by the transferred T cells is essential to destroy the bulk of antigen-positive tumor cells, but we could not detect a significant role of Fas/FasL signaling in our system.

SIY-expressing cancer cells induce SIY-specific T cells in IFN- $\gamma^{-/-}$ and TNF-/- mice. To test whether CD8⁺ T cells are primed in IFN- $\gamma^{-/-}$ and TNF-/- mice, we challenged WT, IFN- $\gamma^{-/-}$, and TNF-/- mice with MC57-SIY-Hi cancer cells. At 8 days after challenge, circulating anti-SIY CD8⁺ T cells were detected by peptide-MHC-dimer staining (Figure 2A); recovered T cells specifically responded to the SIY peptide but not to the irrelevant gp33 peptide (data not shown). IFN- $\gamma^{-/-}$ and TNF-/- mice produced the cytokine not knocked out at levels similar to those in WT mice; no IFN- γ was detected in IFN- $\gamma^{-/-}$ T cells and no TNF in TNF-/- T cells (Figure 2B). T cells from the WT host expressed both cytokines. IFN- γ - or TNF-expressing cells were not detected with an isotype control antibody (data not shown). These data suggest that antigen-specific T cells can be primed effectively in IFN- $\gamma^{-/-}$ or TNF-/- hosts.

T cells require neither TNF nor IFN- γ to kill antigen-positive targets. We next investigated the roles of Fas, IFN- γ , TNF, and perforin pathways for antigen-specific killing by CD8⁺ T cells in vivo. As targets, spleen cells from WT control or IFN- γ receptor–deficient (IFN- γ R^{-/-}), TNF receptor–deficient (TNFR^{-/-}), or lpr (i.e., Fas^{-/-}) mice were

pulsed with the gp33 or the SIY peptide and labeled with a low or a high concentration of CFSE. Cells of the 2 types of target populations were injected i.v. $(2 \times 10^7 \text{ cells})$ into Pfr^{-/-}, IFN- $\gamma^{-/-}$, TNF^{-/-}, or WT mice that had been immunized 8 days earlier with MC57-SIY-Hi cells to generate effector T cells. Nonimmunized mice were used as controls. After 24 hours, spleen cells were harvested from immunized and control mice and analyzed for SIY-specific loss of the injected labeled peptide-pulsed target cells. Immunized Pfr^{-/-} mice were severely compromised in their ability to kill SIY peptide-coated targets (52.9%; Figure 3). In contrast, immunized WT (94.7%), TNF-/- (94.9%), and IFN-γ-/-(95.3%) mice all displayed similarly effective antigen-specific killing in vivo. Furthermore, SIY peptide-pulsed target cells derived from WT, IFN-yR^{-/-}, TNFR^{-/-}, and lpr mice were similarly susceptible as targets in vivo. This indicated that SIY-specific CD8⁺ T cells do not require IFN-y/IFN-yR or TNF/TNFR signals or Fas/FasL engagement for antigen-specific killing in vivo. The remaining level of killing detected in the absence of perforin presumably represents the collective contribution of perforin-independent killing and may be IFN-yR, TNFR, or Fas dependent, as has been previously observed (30). This contribution of Fas-, IFN-y- and TNF-mediated killing is probably unmasked in the absence of the highly efficient perforin-mediated killing. However, our data indicate that neither of the TNF, IFN-γ, or FasL pathways are required for SIY-specific T cell killing in vivo and that killing by SIY-specific CD8⁺ T cells

in vivo was largely mediated by the perforin-dependent granule exocytosis pathway.

Expression of IFN-yR and TNFR on stromal cells is required for the successful elimination of cancer variants. In our model, targeting cancer cells as well as stromal cells was needed for perforin-mediated T cell rejection of tumors (ref. 8 and Figure 1, left). However, it remained unclear whether tumor rejection by T cells also required the action of IFN-y or TNF on stromal cells. To address this question, OT-1 WT, OT-1 IFN-yR^{-/-}, and OT-1 TNFR^{-/-} mice were injected s.c. with MC57-SIY-Hi or MC57-gp33-Hi cancer cells, and 14 days later SIY-specific 2C T cells were adoptively transferred. As shown in Figure 4A, SIY tumors but not gp33 tumors were rejected by WT mice. Interestingly, SIY tumors in TNFR^{-/-} or IFN- γ R^{-/-} mice regressed initially and then regrew (Figure 4A and Table 2). Cancer cells isolated from recurrent tumors of T cell-treated IFN-γR^{-/-} (Figure 4B, top) or TNFR^{-/-} mice (Figure 4B, bottom) were ALVs that had lost SIY-EGFP expression and were no longer recognized by 2C T cells (data not shown). Thus, for CD8⁺ T cell-mediated tumor rejection, IFN-γR or TNFR expression on cancer cells alone was not sufficient; stromal cells also had to express IFN-γR and TNFR to prevent relapse caused by ALVs.

Table 1

TNF and IFN- $\!\gamma$ produced by T cells are critical for complete elimination of established tumors

T cells	Host	Rejection of tumors	Р
WT	0T-1	10/10	-
FasL-/-	0T-1	5/5	-
TNF-/-	0T-1	0/7	<0.001^
IFN-γ ^{-/-}	0T-1	0/6	<0.001 ^A

Data were pooled from 7 independent experiments; the tumor growth curves of mice treated with TNF-/-, IFN- γ -/-, or WT T cells are shown in Figure 1 (*n* = 3 per group). ^AVersus WT.

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Figure 2

Injection of cancer cells expressing SIY antigen induces a SIY-specific T cell response in mice deficient in IFN-γ or TNF. (A) C57BL/6 WT, TNF-/-, or IFN-y-/- mice were injected s.c. with 2 × 10⁶ MC57-SIY-Hi cells. After 8 d, anti-SIY-specific CD8+ T cells were detected in peripheral blood lymphocytes of mice using SIY: Ig dimers. (B) Stimulated T cells from TNF-/- or IFN-γ-/- mice produced the cytokine not knocked out. At 9 days after tumor challenge, splenocytes were restimulated with 1 μg/ml of the SIYRYYGL peptide. After 6 hours, intracellular IFN-γ and TNF were examined in CD8+ T cells obtained from these mice. Numbers within plots denote the percent of cells in the indicated quadrant.

The stromal cell types necessary to prevent the outgrowth of ALVs in MC57-SIY-Hi tumors were subsequently determined by generating BM chimeras in which the respective BM-derived or non-BM-derived stromal cells expressed TNFR or IFN-yR $(TNFR^{-/-} \rightarrow WT, OT^{-1} TNFR^{-/-} BM \text{ to } OT^{-1} WT \text{ recipient; } WT \rightarrow$ TNFR^{-/-}, OT-1 WT BM to OT-1 TNFR^{-/-} recipient; IFN- $\gamma R^{-/-} \rightarrow$ WT, OT-1 IFN- $\gamma R^{-/-}$ BM to OT-1 WT recipient; WT \rightarrow IFN- $\gamma R^{-/-}$, OT-1 WT BM to OT-1 IFN-γR^{-/-} recipient). MC57-SIY-Hi tumors escaped rejection in IFN- $\gamma R^{-/-} \rightarrow WT$ and $WT \rightarrow IFN-\gamma R^{-/-}$ BM chimeric mice (Figure 5A). Similarly, MC57-SIY-Hi tumors also escaped rejection in TNFR^{-/-}→WT and WT→TNFR^{-/-} BM chimeric mice (Figure 5B), but were rejected in control $WT \rightarrow WT$ BM chimeric mice (Figure 5, A and B, and Table 3). Therefore, the elimination of ALVs by CTL most likely required both BM- and non-BM-derived stromal cells to express TNFR and IFN-yR during the effector phase of antitumor immune response.

Figure 3

TNF and IFN-y are not required for SIY-specific T cell killing in vivo. (A) Flow cytometric data showing representative examples of results. Left: SIY-pulsed (CFSE-high) or gp33-pulsed (CFSE-low) target cells from C57BL/6 WT, IFN- γ R^{-/-}, TNFR^{-/-}, or lpr mice were transferred into C57BL/6 WT mice. Right: SIY-pulsed or gp33-pulsed target cells from C57BL/6 WT mice were transferred into IFN-y-/-, TNF-/-, or Prf-/mice. Recipient mice were immunized with MC57-SIY-Hi cells 8 d prior to injection of target cells to generate host effector T cells. The unimmunized WT mice receiving target cells were used as controls (bottom right). Spleens were harvested 24 h later and analyzed for CFSE fluorescence. (B and C) Compiled data of percentage of killing. (B) n = 3 per group, pooled from 2 independent experiments. (C) n = 2 (WT and Ipr) or 4 (IFN- $\gamma R^{-/-}$ and TNFR^{-/-}), pooled from 2 independent experiments.

Discussion

We focused on the destruction of well-established 2-week-old solid tumors, about 1 cm in diameter (500 mm³), the minimum size that is usually detected clinically (31). Procedures that cause the rejection of small tumors emerging days after injection of cancer cells are usually ineffective for causing regression of large, established tumors when the artificially induced inflammation caused by tumor cell inoculation has disappeared (32). The outgrowth of variant cancer cells is the most frequent cause for failure of cancer therapy of large tumors, and outgrowth of cancer variants resistant to therapy is the Achilles heel







of cancer treatment, including T cell-based immunotherapy (31). Incomplete rejection and tumor recurrence due to variants is also a hallmark of other single-agent therapies (e.g., chemotherapy) (33).

A tumor about 1 cm in diameter contains about 109 cancer cells whether in humans or mice (31). Even if cancer cells had only the spontaneous mutation rate typical for nonmalignant cells of 10⁻⁵ to 10⁻⁶ mutational events per genetic locus per generation (34), this would mean that as a minimal estimate more than 1,000 variant cancer cells would not express or aberrantly express any given target gene. In our previous and present studies, cancer cells that escape and are found as recurrent tumor were always ALVs. In a reconstruction experiment, we inoculated 2,000 variant cancer cells (ALVs) along with 2×10^6 antigen-positive wild-type cancer cells. Assuming the ratio did not change during tumor growth before adoptive T cell therapy, about 106 ALVs (0.1%) were present in the 1-cm tumor (about 109 cancer cells total) at time of T cell transfer. Even though the number of ALVs is only a small fraction of the total number of cancer cells, still a very sizable number of ALVs must have been eliminated indirectly in an antigen-independent manner as bystanders. Whether killing of the overwhelming majority of sensitive antigen-positive cancer cells will also kill a few antigen-negative cancer cells was studied over 3 decades ago with discrepant conclusions (35-37). It is clear now that success depended on stromal cells being targeted.

Our study has defined IFN- γ and TNF released from CTL as key effector molecules for the bystander eradication of ALVs. TNFR and IFN- γ R on stromal cells were required, indicating that these cytokines acted on stromal cells. For tumor eradication, the majority of the cancer cells must be antigen positive and destroyed by perforin-secreting T cells (8). Thus, cancer cells and stroma are both essential, nonredundant targets for eradicating cancer. Cancer cell destruction can help the destruction of stroma by releasing antigen that sensitizes stromal cells to antigen-specific T cells (8, 38, 39) and by loss of essential growth and survival factors for stromal cells making stromal cells prone to destruction by TNF and IFN- γ .

Both BM- and non-BM-derived stromal cells must express the relevant haplotype K^b ; thus both must interact with T cells directly (8). This interaction is also likely to trigger TNF and IFN- γ release, and here we show that both stromal components had to express IFN- γ R and TNFR for eradication of ALVs. However, the stromal cells that trigger the cytokine release may not be the same cells that are targets of these cytokines, and the exact type of stromal cell targeted by TNF and/or IFN- γ remains to be identified.

Figure 4

Expression of IFN- γ R and TNFR on stromal cells is required for elimination of ALVs. (**A**) Antigenic cancer escape in mice lacking the receptor for either TNF or IFN- γ . OT-1 WT, TNFR--, or IFN- γ R-- mice were injected s.c. with 2 × 10⁶ MC57-SIY-Hi cells or MC57-gp33-Hi cells as controls. At day 14, the SIY-specific 2C T cells were adoptively transferred into these tumor-bearing mice. Each curve represents an individual mouse. (**B**) Tumors relapsing in receptor-deficient mice were ALVs. The regrowing MC57-SIY-Hi tumor cells from OT-1 IFN- γ R-- and OT-1 TNFR-- mice following T cell therapy were isolated at day 40. The parental MC57-Neo cells and MC57-SIY-Hi cells isolated from non-T cell-treated OT-1 IFN- γ R-- or OT-1 TNFR-- mice were used as controls. The levels of SIY antigen expression on those cancer cells were examined by flow cytometry using the EGFP fluorescence of the SIY-EGFP fusion protein.

Our experiments use hosts for tumor growth in which the BMor non-BM-derived host cells either carry or lack the receptor for TNF and IFN- γ . While host cells outside the tumor with these receptors may be necessary, we consider this extremely unlikely: T cells must see the specific antigen to secrete TNF and IFN- γ , and the cytokines are therefore likely to be released in the tumor, not elsewhere in the periphery, although stromal cells in draining LNs that have captured tumor antigen could be an additional site.

Although we have not addressed the interaction of TNF and IFN-γ in our model, it is tempting to suggest that TNF and IFN-γ produced by CTLs target endothelial cells in tumor angiogenesis to prevent survival of ALVs. TNF or IFN-γ alone inhibits migration and proliferation of endothelial cells (40-42). Moreover, TNF and IFN-γ synergize in endothelial activation by upregulating endothelial leukocyte adhesion molecule-1 (43) and in apoptosing angiogenic endothelial cells by reducing activation of $\alpha_{v}\beta_{3}$ integrin on endothelial cells (44). Vessel damage and destruction could kill cancer and their ALVs by anoxia. Alternatively, stromal recognition and/or destruction may also be associated with the local induction of various types of non-antigen-specific tumoricidal effector cells that kill ALVs in an antigen-independent manner. For example, hyperactivated nonspecific T cells or macrophages activated by IFN- γ (45) have powerful antitumor activities by releasing TNF (46, 47). While we found complete tumor rejection in TNF-/- mice (data not shown), it is possible that ALVs may be eliminated by activated macrophage effector molecules other than TNF (48). Although our previous studies (8) showed that NK and NKT cells are unlikely effectors in this process, more evidence is needed for this conclusion. We are developing imaging technology to investigate in vivo the localization, migration, and action of T cells within large tumors, their effects on stroma and vessels, and whether leukocyte/ALV interaction precedes the destruction of the variants.

Table 2

TNFR and IFN- γ R expression on stromal cells is critical for complete elimination of established tumors

Host (OT-1)	T cell	Rejection of tumors	Р
WT	2C	6/6	-
TNFR-/-	20	0/6	0.002 ^A
IFN-γR ^{-/−}	20	0/7	0.001 ^A

Data were pooled from 4 independent experiments; Figure 4A shows the tumor growth curves of 2 of these experiments. ^AVersus WT.





A number of recent and older publications (49-57) dissected the roles of perforin, IFN-y, and TNF in experimental settings different from ours. Our experiments focus on what is required for eradication of large, established, solid cancers; i.e., the prevention of outgrowth of ALVs. TNF is one of very few cytokines that cause dramatic destruction of large, established tumors (58). For induction of necrosis in large, established tumors by paratumoral injection of recombinant TNF, TNFRs needed to be expressed only on non-BM stromal cells (59). However, the resulting necrosis usually spares a rim of the tumor, from which the cancer regrows (60). Therefore, these studies do not contradict our findings that TNFR on both BM- and non-BM-derived stromal cells are required for eradication of established cancers. Interestingly, recurrence from the surviving margins of the tumor following injection of TNF can be prevented when IFN- γ is also injected around the lesion (61). This may help explain our present finding that TNF and IFN-γ were both needed to achieve tumor eradication. Local injection of TNF carries a high risk of lethal shock and is not applicable to cancers that have spread to multiple sites. By contrast, antigen-specific T cells localize to the tumors and release cytokines without evidence of systemic toxicity. Thus, we have demonstrated a crucial T cell-cytokine-stroma-variant cancer cell axis of interactions that is essential for the eradication of established tumors. This provides critical insight into the mechanisms of tumor escape and will help in the design of effective strategies against established cancers.

Methods

Mice, cell lines, and reagents. C57BL/6 WT, Prf^{-/-} (perforin^{-/-}), TNF^{-/-}, TNFR^{-/-}, IFN-γ^{-/-}, IFN-γR^{-/-}, gld (FasL^{-/-}), and lpr (Fas^{-/-}) mice were all purchased from The Jackson Laboratory. M. Mescher (University of Minnesota, Minneapolis, Minnesota, USA) provided the OT-1 mice. The 2C Rag1^{-/-} mice were provided by J. Chen (Massachusetts Institute of Technology, Boston, Massachusetts, USA). For BM chimeras, recipient mice were irradiated with 9 Gy and 1 h later received 10⁷ BM cells of donor mice. The BM chimeric mice were injected with the indicated cancer cells at least 4 weeks after BM transfers. Animal experiments were approved by the IACUC of the University of Chicago. P. Ohashi (University of Toronto, Toronto, Ontario, Canada), with permission of H. Hengartner (University Hospital Zurich, Zurich, Switzerland), provided the MC57G methylcholanthrene-induced, C57BL/6-derived fibrosarcoma. H. Auer and S. Meredith (University of Chicago) synthesized the 2C-recognized peptide SIYRYYGL and the P14-

Figure 5

Tumor rejection requires BM- and non–BM-derived stromal cells expressing TNFR and IFN- γ R. (**A**) Tumor growth curves showing the escape in IFN- γ R^{-/-} mice. IFN- γ R^{-/-} →WT, WT →WT, and WT→IFN- γ R^{-/-} mice were generated. (**B**) Tumor growth curves showing the escape in TNFR^{-/-} mice. TNFR^{-/-} →WT, WT→WT, and WT→TNFR^{-/-} mice were generated. Chimeric mice were injected s.c. with 2 × 10⁶ MC57-SIY-Hi cells. After 14 days, 5 × 10⁶ preactivated 2C T cells, or no T cells as a control, were transferred to these tumor-bearing mice, and tumor volume was monitored. Data are shown in Table 3, which shows the compiled results of further experiments.

recognized peptide LCMV-derived gp33 epitope KAVYNFATM. Brefeldin A (BFA), ionomycin, and PMA were purchased from Sigma-Aldrich. All antibodies and K^b-IgG Dimer X were purchased from BD Biosciences — Pharmingen. CFSE was purchased from Invitrogen.

Generation of SIY and gp33 vectors and transfection/transduction of cells. The generation of iSIY-LEGFP and igp33-LEGFP vectors has been described previously (8, 38, 62). MC57G was transfected with MerCreMer to generate MC57-Neo (Neo). Neo was transfected with iSIY-LEGFP or igp33-LEGFP, selected with 5 μ g/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 days to generate MC57-SIY-Hi or MC57-gp33-Hi, respectively.

Analysis of cells by FACS. For analysis of IFN- γ and TNF production, cells were incubated with no peptide, 50 ng/ml PMA plus 5 µg/ml ionomycin, 1 µg/ml SIY, or 1 µg/ml gp33 in the presence of 10 µg/ml BFA in cRPMI at 37°C. After 5–6 h, the cells were fixed with 4% paraformaldehyde and permeablilzed with 0.5% saponin in PBS with 1% BSA and 0.1% azide. The cells were stained with allophycocyanin-conjugated anti-CD8, PE-conjugated anti-SIY T cells, K^b-IgG Dimer X was loaded with a 40 M excess of the SIY peptide or the mutant p68 peptide, and peripheral blood lymphocytes were stained according to the manufacturer's instructions (BD Biosciences). Samples were analyzed on a FACSCalibur apparatus, and data were analyzed using FlowJo software.

Cytotoxicity assay in vivo. Analysis of tumor antigen–specific effector CTL activity in vivo was performed by an adaptation of the method of Oehen et al. (63). Briefly, C57BL/6 spleen cells were resuspended in PBS and divided into 2 equal populations, one of which was labeled with the SIY, and the other with the gp33 peptide at a concentration of 1 µg/ml for 60 min at 37°C. The cells were then labeled with CFSE at a final concentration of 5 µM for SIY peptide–pulsed cells (CFSE-high) and 0.5 µM for gp33 peptide–pulsed cells (CFSE-low). The cells were mixed at a ratio of 1:1, and a total of 2×10^7 cells were injected i.v. into recipient animals. Draining LNs, contralateral LNs (CLNs), and spleens were then harvested 24 h after

Table 3

TNFR and IFN- γ R expression on BM- and non-BM–derived stromal cells is required for complete elimination of established tumors

BM chimeric mouse	T cell	Rejection of tumors	Р
WT→WT	2C	6/6	_
WT→TNFR ^{_/_}	2C	0/6	0.002 ^A
TNFR─/─→WT	2C	0/6	0.002 ^A
WT→IFN-γR ^{_/_}	2C	0/6	0.002 ^A
IFN-γR ^{_/_} →WT	2C	0/6	0.002 ^A

Data were pooled from 2 independent experiments; Figure 5 shows the tumor growth curves of 1 of these experiments. ^AVersus WT \rightarrow WT.

adoptive transfer, and CFSE fluorescence intensity was analyzed by flow cytometry. Gating on CFSE⁺ cells, the percent killing was calculated as follows: 100 - ([(% SIY peptide pulsed in immunized/% gp33 pulsed in immunized)/(% SIY peptide pulsed in unimmunized/% gp33 peptide pulsed in unimmunized)] × 100).

Tumor challenge and adoptive transfer of T cells. Cultured cancer cells were trypsinized and washed once with plain DMEM, and 2×10^6 cells were injected s.c. under the shaved backs of mice. For mixing experiments, mice were injected s.c. with 2×10^6 MC57-SIY-Hi cells plus 2×10^3 MC57 cells. The size of tumor was determined at 3-day intervals. Tumor volumes were measured along 3 orthogonal axes (*a*, *b*, and *c*) and calculated as *abc*/2. For transfer of T cells, 1×10^7 NH₄Cl-treated splenocytes from 2C transgenic mice were harvested. To generate SIY-immune lymphocytes (8), the indicated mice were immunized against the SIY peptide. Nine days later, splenocytes were cultured for 5 d with the SIYRYYGL peptide and 10 U/ml IL-2. The single-cell suspensions of T cells were injected i.v. into the retro-orbital plexus in a 0.2 ml volume.

Statistics. Tumor rejection rates in different groups of mice were compared using Fisher's exact test. A *P* value less than 0.05 was considered significant.

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