

A Nonimmunogenic Sarcoma Transduced with the cDNA for Interferon γ Elicits CD8⁺ T Cells against the Wild-type Tumor: Correlation with Antigen Presentation Capability

By Nicholas P. Restifo, Paul J. Spiess, Stephen E. Karp, James J. Mulé, and Steven A. Rosenberg

From the Surgery Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Summary

To be recognized by CD8⁺ T lymphocytes, target cells must process and present peptide antigens in the context of major histocompatibility complex (MHC) class I molecules. The nonimmunogenic, low class I-expressing, methylcholanthrene (MCA)-induced murine sarcoma cell line, MCA 101, is a poor presenter of endogenously generated viral antigens to specific CD8⁺ T lymphocytes and cannot be used to generate tumor infiltrating lymphocytes (TIL). Since interferon γ (IFN- γ) has been shown to upregulate three sets of molecules important for antigen processing and presentation, we retrovirally transduced wild-type MCA 101 (101.WT) tumor with the mIFN- γ cDNA to create the 101.NAT cell line. Unlike 101.WT, some clones of retrovirally transduced 101.NAT tumor expressed high levels of class I, and could be used to generate CD8⁺ TIL. More importantly, these TIL were therapeutic in vivo against established pulmonary metastases from the wild-type tumor. Although not uniformly cytotoxic amongst several separate cultures, these TIL did specifically release cytokines (IFN- γ and tumor necrosis factor- α) in response to 101.WT targets. 101.WT's antigen presentation deficit was also reversed by gene modification with mIFN- γ cDNA. 101.NAT had a greatly improved capacity to present viral antigens to CD8⁺ cytotoxic T lymphocytes. These findings show that a nonimmunogenic tumor, incapable of generating a CD8⁺ T cell immune response, could be gene-modified to generate a therapeutically useful immune response against the wild-type tumor. This strategy may be useful in developing treatments for tumor histologies not thought to be susceptible to T cell-based immunotherapy.

Some tumor cells in mouse and humans present peptide/MHC class I complexes recognizable by T cells. CD8⁺ CTL can be generated that specifically lyse human and murine tumor cells in vitro and can eliminate established tumor in vivo (1–12). Many tumors, however, are thought to be nonimmunogenic (3, 13). Although there are many possible explanations for nonimmunogenicity, it has recently been demonstrated that a nonimmunogenic murine tumor poorly presented endogenously generated viral antigens in the context of MHC class I, despite the intracellular presence of these antigens in high quantities (14). Furthermore, in a mutant lymphoma cell line with a known antigen presentation defect, tumor antigens were not presented, while in its parent, nonmutated line they were (15). These findings suggested that a tumor might evade recognition by CD8⁺ T cells by failing to present tumor antigens that were not absent, as Hewitt et al. hypothesized (13), but that were in-

stead hidden intracellularly, and not presented on the cell surface.

Our understanding of the molecular mechanisms that might be involved in the evasion of recognition by CD8⁺ T cells has grown significantly over the past several years. The specificity of T cell recognition of a cognate partner cell occurs via the interaction of the TCR with a MHC molecule to which a peptide is bound. Some peptide/MHC complexes that are designated antigenic trigger a T cell response that can consist of proliferation, upregulation of surface molecules, activation of lytic machinery, and/or secretion of cytokines. The peptides presented by class I molecules are usually fragments of endogenous proteins that are cleaved, frequently to an ultimate size of eight or nine amino acids (16–20). This task is possibly achieved, in part, by a molecular complex known as the proteasome (21, 22). Peptide fragments may be transported across the membrane of the endoplasmic retic-

ulum (ER)¹ or some post-ER compartment by specialized proteins, related to the products of the multi-drug resistance genes, designated TAP 1 and 2 (23–26). Both the putative peptide transporter proteins and the proteasome component molecules appear to be very closely associated with the MHC region on chromosome 17 in the mouse, or on chromosome 6 in the human (21–26). Thus, there may be an antigen presentation kit consisting of the three groups of molecules apparently required for endogenous antigen presentation: proteasome, peptide transporter, and class I H chain with β_2 -microglobulin. It is significant that the known components of this equivalent of a eukaryotic operon for antigen presentation are all upregulatable by IFN- γ (27).

In the experiments reported here, we set out to study the effects of insertion of the cDNA for mIFN- γ on antigen processing and on presentation in a tumor. The nonimmunogenic murine methylcholanthrene (MCA)-induced sarcoma, MCA 101, has a very poor ability to present endogenously generated viral antigens (14). This tumor grows rapidly and lethally in nonimmunosuppressed hosts (3, 8). Furthermore, of seven MCA-induced tumors generated and characterized extensively in our laboratory (3, 6, 7, 8, 14, 28), only MCA 101 can neither act as an immunogen *in vivo* nor generate CTL *in vitro* (3, 8). In the current studies, we retrovirally transduced these cells with the cDNA coding for mIFN- γ . Our aim was not to use IFN- γ in these experiments for its effects on the immune system, but instead, for its effects on the antigen presentation capabilities of a tumor cell. We show that MCA 101 can be converted from a poor presenter of endogenously generated antigens to a tumor capable of efficiently presenting antigens. Furthermore, this conversion is correlated with a change in the tumor's ability to elicit tumor-infiltrating lymphocyte (TIL) cells. Whereas wild-type tumor does not elicit TIL cells, tumor retrovirally transduced with the cDNA for IFN- γ does. Most importantly, these TIL are active against the low class I-expressing wild-type MCA 101. These experiments represent the first reported to use functional assays to study the antigen presentation capabilities of a tumor before and after gene modification, and correlate this modification with *in vivo* behavior.

Materials and Methods

Tumor and Animals. MCA-induced sarcomas (101 and 102) (3) were generated in our laboratory in 8-wk-old female C57BL/6n (B6) mice (Animal Production Colonies, Frederick Cancer Research and Development Facility, National Institutes of Health, Frederick, MD) by intramuscular injection of 0.1 ml of 0.1% 3-MCA in sesame seed oil. Tumor lines were passaged in B6 mice, and all tumors were of early transplantation passage (passage 3–7). Tumors were harvested from mice, triple-enzyme digested with 0.1% collagenase,

0.002% DNase, and 0.01% hyaluronidase (all from Sigma Chemical Co., St. Louis, MO), and maintained in monolayer culture in complete medium (CM) containing RPMI 1640, 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (all from Biofluids Inc., Rockville, MD), 5×10^{-5} M 2-ME (Aldrich Chemical Co., Milwaukee, WI), and 0.03% (100 mM) glutamine (National Institutes of Health Media Unit, Bethesda, MD).

Retroviral Transduction of Tumor Lines. A long-term cultured line of MCA 101, was cloned by limiting dilution techniques. All 41 clones tested expressed low levels of class I. One of these clones was designated 101.WT and was plated at 10^6 cells in CM in a 75-cm² tissue culture flask (Falcon; Becton Dickinson Labware, Oxnard, CA) and allowed to adhere overnight. To create the bulk-transduced cell line 101.NAT, viral supernatants were added to washed adherent 101.WT tumor cells in the presence of polybrene (8 μ g/ml) for 12 h at 37°C, as described previously (29). CM was added the next morning. The retroviral construct used was obtained from E. Gilboa (Memorial Sloan-Kettering Cancer Center, New York, NY) and has been described previously under the designation DC/TKIFN- γ (30), but which was also referred to as NAT/IFN- γ by the Gilboa group, and is simply called NAT in this manuscript. Briefly, the construct was derived from the genome of the Moloney murine leukemia virus and contained the mIFN- γ gene, which was under the transcriptional control of the thymidine kinase promoter, and the neomycin resistance gene (Neo R) gene, which was under the transcriptional control of the LTR from the Moloney murine leukemia virus. The bulk-transduced cell line 101.NAT was cloned and two high class I-expressing clones (101.22H and 101.28H), and two low class I-expressing clones (101.18L and 101.25L) were selected for further work. A bulk control cell line transduced with the Neo R gene alone and called 101.LXSN was generated in the same way, except that the retrovirus used was designated LXSN. This virus is the Moloney murine leukemia virus backbone with the Neo R gene alone, and was obtained from Dr. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). Cells were selected in 1 mg/ml (0.5 mg/ml active) G-418 sulfate (Geneticin; Gibco Laboratories, Grand Island, NY) for 2 wk and then cloned at 0.2 cells/well. Successful introduction of cDNA was verified by Southern blotting after selection of the transduced cells in G-418. mIFN- γ activity in supernates of transduced tumor cells was measured by ELISA (Quantikine; R&D Systems, Inc., Minneapolis, MN).

FACS[®] Analysis. Cell surface class I expression was measured by FACS[®] analysis using a FACScan[®] 440 (Becton Dickinson & Co., Mountain View, CA). Fresh tumors were harvested and triple-enzyme digested *vide supra* before staining with appropriate antibodies for FACS[®]. Cultured tumor cell lines were harvested with 0.02% EDTA, washed, then stained for 30 min with culture supernatant from the hybridoma 28.8.6s (anti-Kb and Db) (31), obtained from Dr. D. Sachs (Massachusetts General Hospital, Boston, MA), or with the isotype-matched (IgG2a) control antibody (Becton Dickinson & Co.) followed by goat anti-mouse FITC-conjugated antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN). FACS[®] analyses were standardized with Calibrite flow cytometer beads (Becton Dickinson & Co.).

Generation of TIL and Anti-viral Effector Cells. TIL were generated from tumors that were injected subcutaneously and harvested ~2 wk later. They were enzymatically digested as described above, then washed twice with HBSS (Biofluids Inc.). Thy 1.2-bearing cells within the tumor digest were separated by immunobeads using magnetic beads coated with Thy 1.2 antibody and cultured in 20 U/ml of rhIL-2 (Cetus Corp., Emeryville, CA) as previously

¹ Abbreviations used in this paper: CM, complete medium; ER, endoplasmic reticulum; LAK, lymphokine-activated killer cells; MCA, methylcholanthrene; MCN, mean channel number; Neo R, neomycin resistance; NP, nucleoprotein; PR8, wild-type influenza virus A/PR/8/34; TATA, tumor-associated transplantation antigen; TIL, tumor-infiltrating lymphocytes; Vac, vaccinia virus; WT, wild-type.

described (7, 28). Antiviral polyclonal responder T cell populations were generated as described previously (14). Briefly, female 6-wk-old B6 mice were primed with 20 hemagglutinin U of PR8 viral allantoic fluid. After at least 2 wk, spleens were removed, dispersed, and cultured in IMDM with 7.5% heat-inactivated FCS (Biofluids Inc.). Splenocytes were then cultured for 7 d in the absence of IL-2, either with synthetic peptide at 1 $\mu\text{g}/\text{ml}$ ($\sim 0.6 \mu\text{M}$), or with PR8-infected spleen cells (14).

In Vivo Tumor Therapy Experiments. 8–12-wk-old C57BL/6n mice were irradiated with 500 rad and then injected intravenously with 3×10^6 wild-type MCA 101, 101.22H, or 101.28H (high class I-expressing clones of the mIFN- γ gene-modified cell line 101.NAT) tumor cells in 1 ml of HBSS to induce pulmonary metastases. On day 3, TIL were injected at doses specified in the text. Where specified, mice were treated intraperitoneally with 10,000 U rIL-2 in 0.5 ml HBSS twice daily for five consecutive days. On day 14, after tumor injection, all mice were ear tagged, randomized, and killed. Pulmonary metastases were enumerated in a coded, blinded fashion as described previously (3). If pulmonary metastases exceeded 250, they were deemed too numerous to count (TNTC).

Viruses and Infection of Tumor Lines. 3×10^6 tumor cells were washed three times in RPMI 1640, then placed in 1 ml of RPMI with 0.1% BSA (Sigma Chemical Co.), and 30 mM Hepes (Biofluids Inc.) at pH 6.8. Cells were then infected for 90 min at 37°C with 20 PFU/cell of recombinant vaccinia virus constructs containing either the influenza A/PR/8/34 nucleoprotein (NP) gene, or a non-cross-reactive control gene, neuraminidase (NA) (32). After infection, target cells were incubated in CM for 3 h at 37°C, then labeled with ^{51}Cr as described below. The production of the viral antigens, as a measure of infection by vaccinia, was quantified by FACS[®] with the antivaccinia mAb designated TWII, kindly provided by Drs. J. Yewdell and J. Bennink (National Institute of Allergy and Infectious Diseases, Bethesda, MD), followed by goat anti-mouse FITC-labeled antibody (GAMF) (Becton Dickinson & Co.). Cell membranes were rendered permeable by fixation with 0.5% paraformaldehyde for 20 min at room temperature, stained with TWII mAb in the presence of 0.1% saponin (Sigma Chemical Co.) in PBS, then stained with FITC-labeled goat anti-mouse antibody as a second reagent as described previously (14).

^{51}Cr Release Assays and Cytokine Release Assays. 4 h- ^{51}Cr release assays were performed as described previously (6). Briefly, 2×10^6 tumor targets in 0.5 ml of CM were labeled with 200 μCi of ^{51}Cr (New England Nuclear, Boston, MA) for 90 min. For studies of endogenous presentation, cells were infected with virus before ^{51}Cr labeling as detailed above. Labeled tumor cells were coincubated with antiviral effector CTL lines or TIL for 4 h. Supernatants were harvested and counted with a gamma counter (LKB Instruments, Inc., Gaithersburg, MD). Percent lysis was calculated as follows: $100 \times [(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximal cpm} - \text{spontaneous cpm})]$. Effector recognition of tumor targets by measuring cytokine release was done using tumors as described previously (7). Briefly, 5×10^5 effector cells were incubated with 10^6 stimulator cells for a period of 18–24 h in 24-well tissue culture plates. Supernatants were then harvested and assayed for cytokine production.

Results

Transfection of MCA 101 with mIFN- γ . We have recently demonstrated that a murine MCA-induced sarcoma, MCA 101, was a poor presenter of endogenously generated viral

antigens in the context of MHC class I molecules to CD8⁺ CTL. This defect was found to be reversed by incubation with exogenous mIFN- γ (14). The question remained as to whether MCA 101 was nonimmunogenic because it lacked tumor-associated transplantation antigens (TSTA), or because of its failure to present some postulated, but as yet unidentified, TSTA.

Since the amelioration of MCA 101's ability to present endogenous antigens after treatment in vitro with exogenous mIFN- γ was transient, and correlated with the duration of class I expression (data not shown), we hypothesized that retroviral transduction of MCA 101 with the cDNA for mIFN- γ would have a prolonged effect on the processing and presentation of endogenous antigen by virtue of the stable insertion of the gene. Thus, a Moloney leukemia retroviral construct, designated NAT, containing both the mIFN- γ gene and the Neo R gene was used. Bulk MCA 101 was cloned by limiting dilution to generate 41 different clones, all of which expressed low quantities (mean channel number [MCN] <10) of surface MHC class I molecules. We then retrovirally transduced one of these wild-type clones, designated 101.WT, with the cDNA for mIFN- γ , and selected it in G-418 for 2 wk to create the cell line designated 101.NAT. In parallel, 101.WT was transduced with a Moloney leukemia virus vector, designated LXS_N, containing the Neo R gene without the IFN- γ gene, to create the cell line 101.LXS_N, which was treated in exactly the same way in all respects as 101.NAT. Although the bulk-transduced tumor line 101.NAT secreted levels of mIFN- γ that were consistently <5 U/10⁶ cells per 24 h, as measured by ELISA, it did express much higher levels of surface class I molecules as measured by FACS[®] analysis than the Neo R bulk-transduced cells 101.LXS_N and 101.WT (Fig. 1). This pattern of very low or unmeasurable mIFN- γ production with greatly increased class I production is not dissimilar to that seen by Gansbacher et al. (30). As shown in Fig. 1, increased class I expression was seen in both fresh and cultured tumor lines. 101.WT and 101.LXS_N showed two populations of cells in fresh preparations. These data were interpreted to indicate that the lower class I expressing cells in these preparations were tumor cells and that the higher class-I expressing cells were likely to be infiltrating T cells and stromal cells. Extensive immunohistochemical studies of subcutaneous nodules, as well as pulmonary metastases of MCA 101, have been done using the same antibody as was used for FACS[®] analysis (28.8.6s). In these studies, MCA 101 tumor cells were found to retain their low expression of class I molecules in vivo (Dr. Jeffrey S. Weber, National Cancer Institute, Bethesda, MD, personal communication). Expression of the Neo R gene was evidenced by selection of the transduced cell line in 1 mg/ml of G418. Tumor cells that did not receive the Neo R gene could not be grown in G418 (data not shown). Integration of the Neo R gene was shown by Southern transfer at 3 mos after transduction (not shown).

Presentation of Viral Antigens by mIFN- γ Gene-modified MCA 101 to Antiviral CTL. In an effort to test the effect of IFN- γ gene transduction on the ability of MCA 101 to present endogenous antigens, we infected 101.WT, 101.LXS_N, and

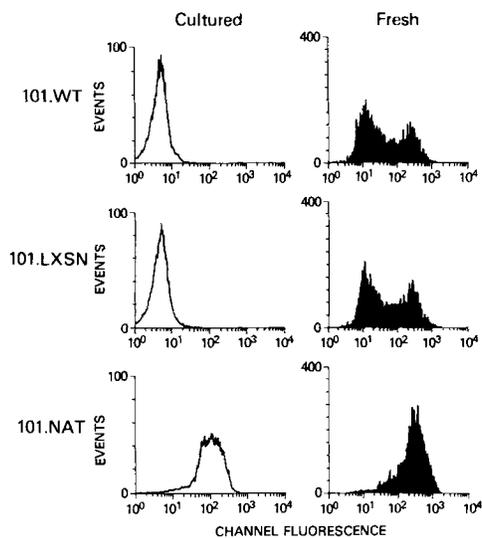


Figure 1. FACS[®] profiles of the following three bulk tumor cell lines: 101.WT (wild-type MCA 101), 101.LXSN (MCA 101 transduced with the Neo R gene alone), which was made from a clone of 101.WT, and 101.NAT (MCA 101 transduced with the Neo R gene and with IFN- γ), which was also made from the same clone of 101.WT as 101.LXSN. Profiles: (left) cultured tumor lines; (right) fresh tumor preparations, which have been harvested from subcutaneous sites in mice and triple enzyme-digested as described in Materials and Methods. Visible in the profiles of the fresh preparations is a bimodal curve (top). The high-expressing cells in these curves probably represent stromal cells along with infiltrating host immune cells.

101.NAT with wild-type influenza A/PR/8/34 (PR 8), then subjected them to killing by appropriately directed CTL. Gene-modified 101.NAT was killed more efficiently in this assay (25% at an E/T of 30:1) than were 101.WT and 101.LXSN (<5% at an E/T of 30:1). However, FACS[®] data showed poor infection of 101.NAT compared with the other two cell lines (data not shown), suggesting that the endogenous mIFN- γ gene made these tumor cells less susceptible to infection by the PR8 virus. This observation is consistent with the observed suppressive effect of exogenous IFN- γ on viral infection with influenza A (33).

To overcome this problem, we repeated our experiments using vaccinia viruses genetically engineered to contain influenza A genes. Effector cells were splenocytes from B6 mice stimulated in vivo with Vac-NP and in vitro with the NP peptide (amino acids 365–380) (34, 35). As shown in Fig. 2, wild-type MCA 101 (101.WT) was not killed when it was sham infected (no virus, NV), when it was infected with the control vaccinia virus containing the NA gene, or when it was infected with the vaccinia virus engineered to express the influenza A NP gene. IFN- γ gene-modified tumor cells (101.NAT) were also not killed when sham infected (NV) or when infected with a control virus (Vac-NA), but were specifically killed when they were infected with the Vac-NP virus. Like 101.WT, 101.LXSN (not shown) did not present viral antigens, indicating that the improvement in 101.NAT's ability to present antigen was not due simply to the effects

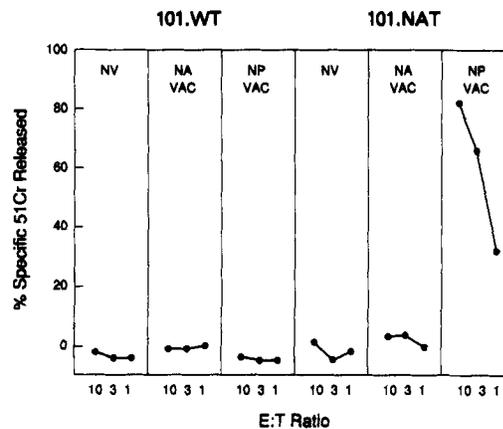


Figure 2. ⁵¹Cr release assay in which wild-type 101.WT, (left), or IFN- γ gene-modified 101.NAT, (right), are subjected to killing by CTL directed against the influenza A NP gene product. Cells were either sham infected (NV), infected with an engineered vaccinia virus containing the influenza A hemagglutinin gene as a control (Coni Vac), or infected with an engineered vaccinia virus containing the influenza A nucleoprotein gene (NP Vac). Only the mIFN- γ gene-modified MCA 101 tumor infected with the appropriate virus was killed in this assay. This finding demonstrates that the modification of MCA 101 with the gene for IFN- γ abrogates its inability to present endogenous antigen.

of retroviral transduction. It thus appeared that by inserting the mIFN- γ gene into a bulk population of MCA 101, we could convert it from a poor presenter of antigen to a cell line capable of presenting antigen to a similar extent as our most immunogenic tumors (see reference 14). This finding led us to hypothesize that MCA 101 might be capable of presenting its own tumor antigens in vivo.

Generation of TIL from mIFN- γ Gene-modified MCA 101. Generation of successful cultures of TIL from wild-type MCA 101 has never been achieved in our laboratory. To test whether the clone being used in the present studies was similar to the parental line, tumors were implanted subcutaneously in syngeneic B6 mice, and 10–20 d later TIL were harvested. Two of two blind attempts at the establishment of TIL cultures from wild-type MCA 101 (101.WT) or MCA 101 transduced with the Neo R gene alone (101.LXSN) yielded no successful TIL cultures. Although bulk-transduced 101.NAT tumor at first appeared to be successful, in two of two blind attempts the TIL cultures were ultimately overgrown with tumor. We hypothesized that some low class I-expressing tumor clonotypes, which may be the rare cells visible in Fig. 1, existed in the bulk population, and that they were able to overgrow the culture before TIL could be established.

To obtain pure cultures of uniformly high class I-expressing transductants, the bulk-transfected MCA 101 (101.NAT) was cloned. We found that the insertion of the cDNA for IFN- γ caused increased expression of steady-state surface class I as measured by FACS[®] analysis on some, but not all, of the tumor clones tested (Fig. 3). Two high (H) class I-expressing clones, 101.22H and 101.28H, and two low (L) class I-expressing clones 101.18L and 101.25L, were selected from bulk-

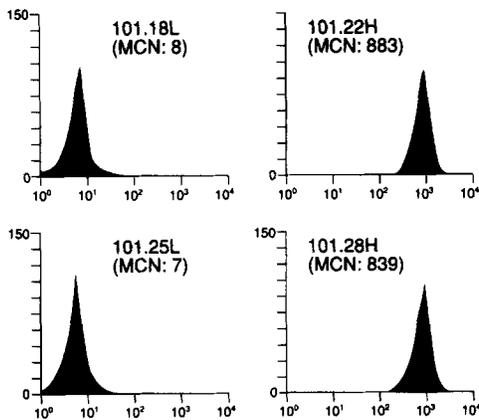


Figure 3. Neither the bulk-transduced tumor, nor any of the clones from the bulk-transduced 101.NAT produced measurable levels of IFN- γ by ELISA. However, bulk-transduced cells using the IFN- γ vector expressed much higher levels of surface class I than those cells transduced with a control vector expressing only the Neo R gene. FACS[®] profiles of two cell lines cloned from the bulk-transduced MCA 101 tumor after selection in G418. With a MCN of 8, clone 18L was among those clones expressing the lowest levels of class I after staining with the mAb 28.8.6s (which stains both K^b and D^b). FITC labeled goat anti-mouse Ab was used as a second reagent. Clone 22H, on the other hand, was among the highest class I expressers with a MCN of 882. The experiment shown was repeated twice with similar results over a period of several weeks, showing stability of the phenotype.

transduced 101.NAT. Despite their variable levels of class I expression, none of these clones secreted >5 U of mIFN- $\gamma/10^6$ cells/ml per 24-h ELISA. These clones were then implanted subcutaneously into syngeneic B6 mice. There were no significant differences between the growth rates of high- and low-class-I expressing tumors in vivo (data not shown).

When subcutaneous tumors were harvested after 10–20 d, Thy 1.2⁺ cells were obtained by immunobeading of single cell suspensions of tumor cells. In two of two blind attempts, we could not grow TIL from low class I-expressing tumor clones (101.18L or 101.25L) of the mIFN- γ -transduced

101.NAT. On the other hand, in six of six attempts, we could consistently grow TIL from high class I-expressing tumor clones of mIFN- γ -modified 101.NAT. Like other murine TIL generated in our laboratory, the TIL cultures used in vivo in Fig. 4 were found to be exclusively CD8⁺ by FACS[®] (data not shown). These antitumor CTL were then expanded in vitro in 20 U/ml of rhIL-2 and tested in vivo and in vitro.

In Vivo Effectiveness of TIL Generated from mIFN- γ Gene-modified MCA 101. In an effort to test whether the TIL generated from high class I-expressing mIFN- γ -transduced clones could be active in an adoptive immunotherapy model against established wild-type tumor in vivo, we used a 3-d lung metastases model (3). In these experiments (Fig. 4), mice were injected intravenously with fresh 101.WT or 101.22H tumor cells. On day 3, mice were treated with either saline alone, IL-2 (10,000 U) in saline twice daily for 5 d, or the same dosage of IL-2 plus varying dosages of TIL. Mice were killed on day 14 when their lungs were harvested and counted in a blind fashion for the number of pulmonary tumor nodules.

As shown in Fig. 4, TIL generated from the mIFN- γ gene-modified 101.22H tumor were effective against pulmonary metastases from the 101.22H tumor. Most significant, however, was the effectiveness of these CD8⁺ TIL against the wild-type MCA 101 tumor. In the experiment shown, the average number of pulmonary metastases from 101.WT were reduced from 200 in the IL-2 alone group to 48, 8, and 0 at 10^6 TIL, 3×10^6 , and 10^7 TIL, respectively. TIL generated from mIFN- γ gene-modified, high class I-expressing tumor clone 101.22H were therapeutically effective against the unmodified tumor in three other repeats of the same experiment.

Specificity of Anti-MCA 101 TIL In Vitro. Four ⁵¹Cr release assays were performed on the TIL generated from 101.22H tumor. Only two of four TIL cultures obtained from mIFN- γ gene-modified tumor were specifically cytotoxic. However, the two cultures that were cytotoxic demonstrated relative tumor specificity (Fig. 5). Two TIL cultures that were not lytic against fresh tumor preparations in vitro were still therapeutic in vivo. This finding was consistent with previous data (9) that showed that CD8⁺ TIL that were therapeutic in vivo were not in every case cytotoxic.

TIL generated from IFN- γ -modified tumor were treated for release of cytokines after coincubation with appropriate tumor cells. TIL derived from 101.22H tumor were tested by exposure to a variety of stimuli. The results shown in Fig. 6 made use of the same cells as were used one day earlier in the therapy experiment depicted in Fig. 4. Maximal release was gauged when TIL were stimulated with an anti-CD3 antibody, 2C11. Background levels of TNF- α and IFN- γ when TIL were incubated alone (without stimulation) were relatively low for both cytokines (Fig. 6). Fresh tumor preparations alone were found to secrete <5 U of IFN- $\gamma/10^6$ cells/ml per 24 h, with the exception of 101.22H which produced 6.3 U IFN- $\gamma/10^6$ cells/ml per 24 h. Production of TNF was variable by fresh tumor preparations, but was uniformly <20 U/ 10^6 cells/ml per 24 h. Values obtained from tumor alone were subtracted from values obtained from tumor

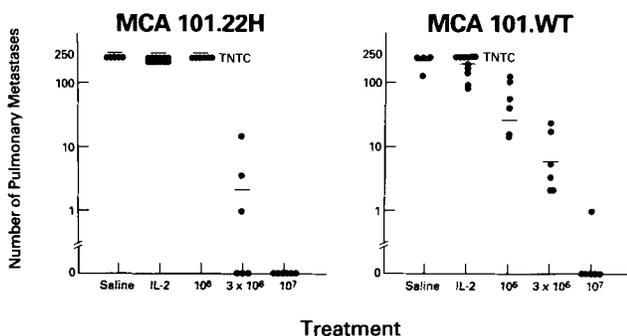


Figure 4. In this experiment, tumor was injected on day 0 to create lung metastases. On day 3, mice received one of several treatments listed. On day 14, the number of pulmonary metastases were enumerated and are expressed. CD8⁺ TIL generated from a high class I-expressing mIFN- γ -transduced tumor clone (101.22H) are effective not only against the gene-modified tumor, but against the wild-type tumor (101.WT) as well.

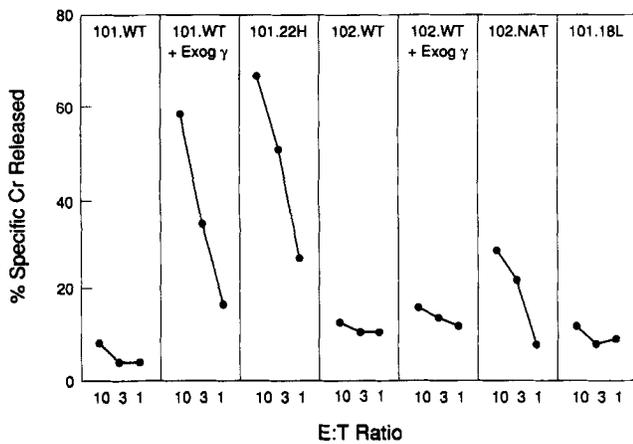


Figure 5. To determine whether the TIL generated from the mIFN- γ gene-modified MCA 101 tumor (101.22H) were tumor specific, a 4-h ^{51}Cr release assay was done using a variety of targets including wild-type MCA 101 (101.WT), 101.WT after a 48-h pretreatment with 200 U/ml of mIFN- γ (101.WT + Exogenous γ), IFN- γ gene-modified MCA 101 clone 22H, which is a high expresser of class I (101.22H), or a control tumor, MCA 102 (102.WT) treated exactly as was MCA 101. As an additional control, we included a clone of MCA 101 (101.18L), which is IFN- γ gene modified in the same way as was clone 101.22H, but which expresses low levels of class I. 101.WT + Exogenous γ and 101.22H were lysed relatively more efficiently than controls. This finding indicates that tumor-associated antigen is being recognized which is relatively specific for 101.WT, and which requires relatively high class I expression for killing in a 4-h ^{51}Cr release assay.

plus TIL. The most important finding was that 101.WT stimulated release of IFN- γ , as well as 101.22H, and release of TNF- α was almost as great when stimulated with 101.WT as with 101.22H. 102.WT, another MCA-induced sarcoma, was prepared in exactly the same way and used as a control. The primary purpose of this control was to see whether the cytokine release for the 101 tumor was specific. Control tumor 102.WT stimulated substantially less cytokine release from these TIL than did 101.WT or 101.22H. These results indi-

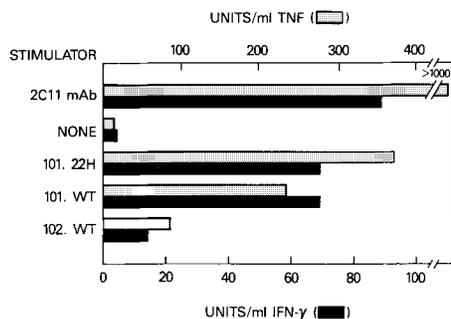


Figure 6. TIL generated from 101.22H were tested for the capacity to release TNF and IFN- γ to the tumor stimulators listed. 2C11 mAb (anti-CD3) was used to test maximal release, and CTL alone (None) was used to test spontaneous release. This graph shows that 101.WT stimulated TIL to release cytokines (mIFN- γ and mTNF- α) nearly as well as its mIFN- γ gene-modified counterpart 101.22H. As in the case of the cytotoxicity assay depicted in Fig. 5, these TIL were found to be relatively, but not absolutely specific (note some release of cytokines against the 102.WT tumor).

cated significant specificity in the response and furthermore suggested a possible mechanism for the action of the TIL cells in vivo. These experiments were repeated with high class I-expressing, transduced tumor clone 101.28H with similar results. Thus, TIL generated from mIFN- γ , gene-modified tumor clones could be triggered to secrete, in a relatively tumor-specific fashion, IFN- γ and TNF- α against not only high class I-expressing tumor clones, but also against the low class I-expressing-wild-type tumor.

Discussion

A major problem with cellular immunotherapies of cancer in humans has been the limited ability to generate therapeutic cells with antitumor properties in vivo. A recent approach to this problem has been to exploit the immunomodulatory effects of genetic modification of tumor cells with cytokine genes, including the IFN- γ gene (30, 36-38). The MCA 101 murine sarcoma may be a model for many tumor histologies because it has no demonstrable immunogenicity (3, 14). We show here that retroviral-mediated transduction of wild-type MCA 101 with mIFN- γ cDNA enables the generation of CD8 $^{+}$ TIL, which cannot be generated from the wild-type tumor. Most importantly, these TIL were therapeutically active in vivo against both the gene-modified and the wild-type tumor in a 3-d lung metastases model. Although the 101.WT tumor was not consistently lysed by therapeutic TIL, it did stimulate the release of cytokines with relative specificity. Finally, mIFN- γ gene-modified MCA 101 tumor presented viral antigens much more efficiently than controls, suggesting a mechanism for these observations.

Although mIFN- γ transduced-tumor lines and clones secrete little, if any, mIFN- γ into culture supernatant, the effect of the cytokine could act primarily via intracellular IFN- γ receptors. Based on our experiments that show steady state increases in the expression of cell surface-class I molecules, and on the enhanced killing by CD8 $^{+}$ T cells in experiments using a defined antigenic system, we may infer that there is increased density of tumor antigen peptide/class I molecule complexes on the cell surface. This inference is strengthened by recent evidence which shows that antigen presentation may be enhanced at several important points by the mIFN- γ molecule. In addition to increasing class I H chain at the level of transcription initiation (39), IFN- γ has also been shown to increase steady state levels of TAP 1 and 2, the putative peptide transport proteins, as well as the gene products similar to the low-molecular mass polypeptide (LMP) or proteasome, which may be involved in cleaving proteins into peptides before feeding them into the ER or post-ER compartment where they bind to class I molecules (27). Finally, IFN- γ has been shown to be a potent upregulator of certain accessory molecules, ICAM-1 among them (40), and while not specifically addressed in the studies presented here, the effect of IFN- γ on these molecules may play an important role in the phenomena described here. The relative contributions of these mechanisms are currently being addressed in our laboratory.

Increased antigen density on mIFN- γ gene-modified MCA 101 may explain why a therapeutically useful CD8 $^{+}$ T cell

response can be obtained from high class I-expressing clones, and not from low class I-expressing mIFN- γ transduced clones, control Neo R-transduced clones, or from wild-type tumor. There is evidence in tumor immunology that an immunologically strong afferent tumor stimulus can elicit a response against an immunologically much weaker efferent stimulus (41). Alternatively, IFN- γ could act primarily through upregulation of class II expression on macrophages and dendritic cells, thereby enhancing antigen presentation to Th cells (42).

During tumorigenesis, tumors that poorly present endogenous antigens might enjoy a selective advantage since mutations of normal cell proteins and expression of oncogene products may yield antigenic peptides that could result in the lysis of the incipient cancer. It has recently been demonstrated that viruses can persist in neurons because they lack class I expression (43). However, the related concept that tumor cells can escape immunosurveillance by T cells of tumor cells is controversial, and the role of class I molecules in this process is unclear (44). In fact, there is evidence that absence of class I makes some tumors more susceptible to lysis by NK cells (45). Nevertheless, MCA 101 is an extremely virulent tumor that may have benefited during its genesis from its poor antigen-presenting abilities (14). The explanation of MCA 101's poor capability to present endogenous antigen, which appears to be entirely reversible with IFN- γ , may stem from its possession of a gene product not unlike the E1A protein

of the adenovirus (46), as has been suggested for small cell lung cancer (47). It is in fact known from other studies, that MCA 101 suppresses class I production at the level of transcription initiation (48).

Our findings may have strategic implications for the development of immunotherapies for cancer in humans. A candidate for this approach may be a human tumor that fails to present endogenously synthesized antigen because it has decreased class I synthesis and normal class I transport, which can be upregulated by treatment with hIFN- γ . This tumor could then be used to establish a line in vitro that would then be transduced with the gene for IFN- γ , subsequently reimplanted at a subcutaneous site, and then harvested several weeks later for TIL. Thus far, our studies in this direction (N. P. Restifo, unpublished observations) suggest that some human tumor histologies, such as small cell lung cancers, may have antigen presentation defects very similar to those described previously for MCA 101: specifically, those with low steady state expression of class I, but which have normal transport (14). Thus, our work with the murine tumor MCA 101 suggests that human tumor cells previously considered to be nonimmunogenic could be rendered better presenters of endogenous antigen by IFN- γ gene modification. This approach may be useful in generating CD8⁺ antitumor effector T cells against tumor histologies not previously thought to be susceptible to T cell-based immunotherapies of cancer.

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Address correspondence to Nicholas P. Restifo, Surgery Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

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