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# Resection of Solid Tumors Reverses T Cell Defects and Restores Protective Immunity<sup>1</sup>

Silvia Salvadori,\* Giorgio Martinelli,<sup>†</sup> and Karen Zier<sup>2\*‡</sup>

We have previously reported that CTL were demonstrable early after inoculation of CMS5 fibrosarcoma cells, but that they disappeared within 3 wk. These mice were unable to reject a challenge with CMS5 tumor cells. Other studies demonstrated cell surface phenotype and signaling abnormalities of cells within the spleen. Since we assumed that such an environment would make it more difficult to elicit antitumor immune responses via immunotherapy, we asked whether resection of the tumor could reverse these abnormalities. Although early after tumor cell inoculation tumor resection leads to the development of immunity, the effect at late time points has not been studied critically. To test this, mice were inoculated s.c. with CMS5 cells and after 28 days the tumors were resected. We observed a gradual normalization of the cellular phenotype of the spleen. In particular, there was a decrease in the number of Mac1<sup>+</sup>/Gr1<sup>high</sup> cells and an increase in the number of CD3<sup>+</sup> cells in the spleen within 24–48 h of tumor resection. By day 10, these values were normal. Levels of p56<sup>lck</sup> increased as well. The functional implications of these changes were illustrated by the reduced growth rate or the complete rejection of a challenge of tumor cells in the resected mice. Both CD4<sup>+</sup> and CD8<sup>+</sup> cells were involved in the restoration of tumor immunity. Our results suggested that tumor resection not only led to the reversal of immune suppression, but also unmasked a population of primed T cells able to mediate protective immunity. *The Journal of Immunology*, 2000, 164: 2214–2220.

T cell-mediated immune responses are believed to protect the organism against the outgrowth of primary tumors and metastases. The development of metastatic disease suggests that this defense sometimes fails, a hypothesis supported by the results demonstrating reduced functional ability by T cells from tumor-bearing hosts. Some of the possible underlying mechanisms to explain this phenomenon include ineffective signal transduction following ligation of the TCR (1, 2), the preferential secretion of Th2 cytokines favoring the development of Ab-mediated responses at the expense of those that are cell mediated (3), and poor Ag-presenting ability of macrophages in the tumor-bearing host (4). Previously, we reported that although mice initially developed an immune response against CMS5 tumor cells, it waned with time (5). T cells from these mice contained reduced levels of p56<sup>lck</sup>, p59<sup>fyn</sup>, and the CD3/TCR  $\zeta$  (2) and were defective in transducing signals following activation through the TCR compared with T cells from control mice (6). These signaling abnormalities resembled those associated with anergic T cells, suggesting a mechanism for unresponsiveness (7, 8). Taken together, our findings suggested the hypothesis that decreased signal transduction by T cells in the tumor-bearing hosts might play a role in their failure to mount an effective antitumor response.

The early resection of progressively growing tumors has long been used as a mean of generating antitumor immunity (9), but the effect of resection at late time points after immune suppression has

developed has not been studied. Since the loss of antitumor immunity correlated with tumor progression, we asked whether non-responsiveness would be reversed by tumor resection. We observed that in the absence of the tumor all aspects of immune suppression reversed, resulting in a reduction in the elevated levels of Mac1<sup>+</sup>/Gr1<sup>+</sup> cells in the spleen and an increase in the levels of signal transduction proteins needed for T cell activation. Most importantly, a population of T cells emerged that mediated protective immunity. These results suggested that the loss of antitumor immunity was not an irreversible event and that the presence of the tumor masked the existence of antitumor T cells. The implications of these conclusions for the immunotherapy of cancer are discussed.

## Materials and Methods

### *Mice and tumor cells*

BALB/c mice were obtained from Charles River (Boston, MA). CMS5 is chemically induced fibrosarcoma of BALB/c origin (H-2d) (10). For in vivo inoculation,  $0.5 \times 10^6$  freshly prepared tumor cells were injected s.c. in the back. In these experiments, groups contained three to five mice. Tumor growth was measured in millimeters as a mean diameter (longest surface length (*a*) and width (*b*), (*a* + *b*)/2). CMS4 is a chemically induced fibrosarcoma that is antigenically distinct from CMS5 (11). EL-4 is a thymoma of the H-2b haplotype used as a specificity control in cytotoxicity experiments. YAC-1 is MHC negative and is the prototypic target for NK cells.

### *mAbs and flow cytometry*

For phenotypic analysis, spleen cells pooled from mice (five mice per group) were reacted with 100 ng of fluoresceinated anti-CD3 (145-2C11, hamster IgG; PharMingen, San Diego, CA), anti-Gr1 (rat, IgG2b; PharMingen), or anti-Mac1 (rat, IgG2b; PharMingen). Irrelevant isotype-matched Abs were used as controls. Cells were reacted with the desired Ab for 30 min at 4°C. Samples were washed twice in FACS buffer (PBS with 3% FBS and 0.02% sodium azide). Samples were analyzed for fluorescence using a Coulter Profile II flow cytometer (Palo Alto, CA), following gating on the lymphocyte population for analysis of CD3<sup>+</sup> cells and on the nonlymphocyte population for the analysis of Mac1<sup>+</sup> and Gr1<sup>+</sup> cells.

For in vivo depletion studies, five mice per group were inoculated i.p. twice per week with 100  $\mu$ l of anti-CD4 ascites (GK 1.5 rat IgG2b; Harlan Bioproducts for Science, Madison, WI), with 100  $\mu$ l of anti-CD8 ascites

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(2.43, rat IgG2b), with both, or with 100  $\mu$ l of an IgG2b isotype control. The completeness of the depletion was tested via FACS analysis and was always greater than 99% for the appropriate subset; the level of the other subset was unaffected.

For *in vitro* T cell enrichment, cells were prepared from spleens pooled from mice (five mice per group) by negative depletion of non-T cells with mAbs against I-A<sup>d</sup> (MKD6) and the anti-heat stable Ag (J11d) (12, 13) and complement. An aliquot of cells was reacted with anti-CD3 mAb and analyzed via flow cytometry to assess the enrichment obtained. In general, greater than 85% of the cells expressed the T cell marker.

### Western blotting

Cells were washed and lysed in buffer composed of 20 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40, 200  $\mu$ M EDTA, 10 mM sodium pyrophosphate, and 100 mM sodium fluoride containing the protease inhibitors PMSF, iodoacetamide, leupeptin, aprotinin, and sodium vanadate. Lysates corresponding to  $4 \times 10^6$  enriched T cells/sample were separated on a 10% SDS-PAGE gel at 200 V for 45 min. Material was electroblotted to polyvinylidene difluoride membrane, blocked with 5% BSA, and immunoblotted with a rabbit anti-*Ick* antiserum (Santa Cruz Biotechnology, Santa Cruz, CA). The filter then was reacted with a secondary Ab conjugated with HRP and developed via enhanced chemiluminescence (DuPont-NEN, Boston, MA).

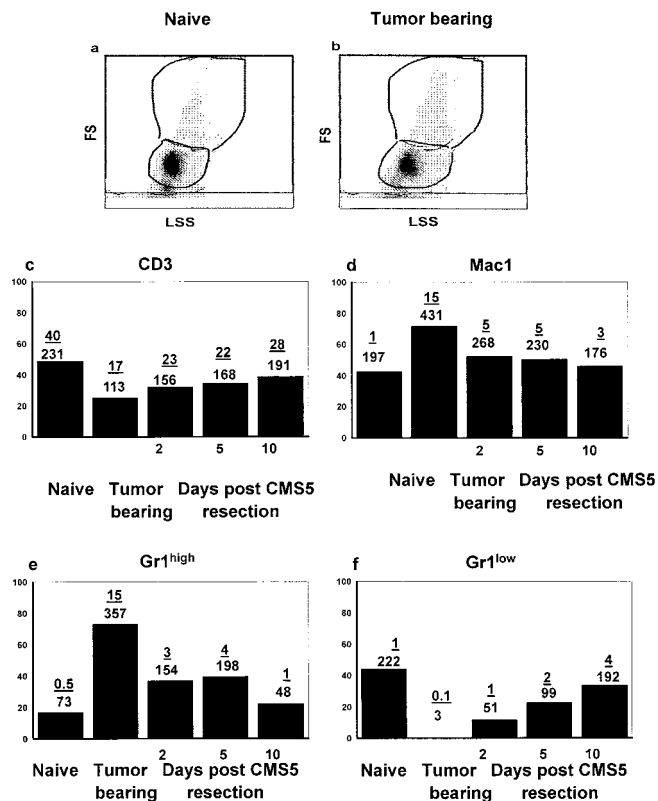
### Generation of CTL and the cell-mediated lympholysis assay

To prepare CTL, single cell suspensions of spleen cells devoid of erythrocytes were prepared and resuspended at  $2 \times 10^6$ /ml in RPMI medium (BioWhittaker, Walkersville, MD) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 2 mM L-glutamine, 25 mM HEPES buffer,  $5 \times 10^{-5}$  2-ME, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. These responder cells were stimulated for 6 days at a 10:1 ratio with  $\alpha$ -irradiated CMS5 cells (6000 rad). For these studies, CD8<sup>+</sup> T cell effectors were obtained by negative depletion with the anti-CD8 mAb 2.43 (rat IgG2b) and complement. Briefly, cells were resuspended at  $10^7$ /ml and incubated with the mAb or with PBS, as a control, for 30 min at 4°C. After washing the cells, they were incubated twice at 37°C with a 1/10 dilution of baby rabbit complement (Pel-Freez; Clinical System Division, Brown Deer, WI). At the end of the procedure, cells were resuspended in the original starting volume (13). For labeling, target cells were cultured for 1.5 h at 37°C in 6% CO<sub>2</sub> in the presence of 150  $\mu$ Ci of <sup>51</sup>Cr sodium chromate (DuPont-NEN). Cells were washed twice and counted, and 10,000 cells were distributed per well. Targets and effectors were mixed at several E:T ratios. After 18 h of incubation, supernatants were harvested from each well. Percent specific lysis was calculated as: [(cpm experimental cultures - cpm spontaneous release)/(cpm maximum release - cpm spontaneous release)]  $\times$  100.

## Results

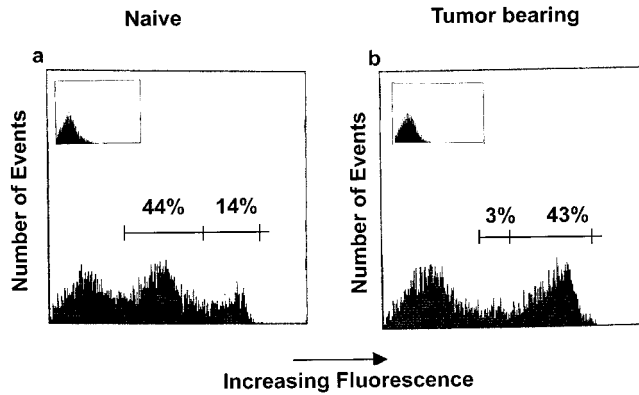
### The phenotype of spleen cells in tumor-bearing mice is altered following tumor resection

The absence of antitumor responsiveness by T cells *in vivo*, as well as their reduced levels of signaling proteins *in vitro*, have been associated with the presence of elevated numbers of Mac1<sup>+</sup> and/or Gr1<sup>+</sup> cells in the spleen (4, 14–18). To determine the phenotype of cells in the spleens following tumor resection, mice were inoculated s.c. with CMS5 tumor cells. After 28 days, tumors were resected. Spleens were harvested 2, 5, and 10 days later and analyzed for the presence of CD3<sup>+</sup>, Mac1<sup>+</sup>, and Gr1<sup>+</sup> cells using flow cytometry. Similar to other investigators (4, 14, 15, 19), we observed that on day 28, before tumor resection, spleens of the naive mice contained elevated numbers of nonlymphocytes compared with those of tumor-bearing mice (Fig. 1, *a* and *b*). Furthermore, decreased levels of CD3<sup>+</sup> cells and increased levels of Mac1<sup>+</sup> Gr1<sup>+</sup> cells were seen in tumor-bearing compared with naive controls (Fig. 1, *c–f*). In this figure, the absolute percentage of positive cells in the spleen (above the bars and underlined in the figure) was determined by multiplying the percentage of positive cells within the gate by the percentage of total splenocytes within the gate. The absolute numbers of cells ( $\times 10^3$ ) of the particular phenotype observed in the spleen are given by the numbers directly above the bars. These were determined by multiplying the absolute percentage of positive cells by the total cell number.



**FIGURE 1.** Change in phenotype of splenocytes in tumor-bearing mice following tumor resection: *a* and *b*, dot plots of lymphocyte and nonlymphocyte gates of splenocytes from naive (*a*) and tumor-bearing mice (*b*). *c–f*, Splenocytes of naive, tumor-bearing, and resected mice were stained with 100 ng of fluoresceinated anti-CD3 (*c*), anti-Mac1 (*d*), or anti-Gr1 (*e* and *f*) mAbs. The percentage of cells staining with the irrelevant isotype-matched controls has been subtracted from the values obtained with the specific Abs. Fluorescence was analyzed via flow cytometry. Cells were gated on lymphocytes for the detection of CD3<sup>+</sup> cells and on nonlymphocytes for the detection of Gr1<sup>+</sup> and Mac1<sup>+</sup> cells. The underlined numbers above the bars represent the absolute percentage of positive cells in the spleen. They were calculated by multiplying the percentage of positive cells within the gate by the percentage of total cells within the gate. The numbers directly above the bars ( $\times 10^3$ ) represent the absolute number of cells, calculated by multiplying the absolute percentage of positive cells by the total cell number. Similar results were obtained in three additional experiments.

Our results revealed two populations of Gr1 cells in splenocytes from naive mice, Gr1<sup>low</sup> and Gr1<sup>high</sup>, that were distinguished on the basis of staining intensity with anti-Gr1 mAb (Fig. 2*a*) (20). Interestingly, in the tumor-bearing host, the majority of Gr1<sup>+</sup> cells stained brightly for Gr1 (Gr1<sup>high</sup>), with very few Gr1<sup>low</sup> cells (Fig. 2*b*). A subsequent experiment using two-color flow cytometry demonstrated that all of the Gr1<sup>+</sup> cells were contained within the Mac1<sup>+</sup> population, but that some Mac1<sup>+</sup> cells were Gr1<sup>-</sup> (results not shown). Levels of B cells, determined by staining with the anti-CD45 mAb B220, were normal (results not shown). Surprisingly, within 48 h of tumor resection, we observed a dramatic change in the phenotype of cells within the spleen (Fig. 1, *c–f*). Specifically, levels of CD3<sup>+</sup> and Gr1<sup>low</sup> cells began to increase, while those of Mac1<sup>+</sup> and Gr1<sup>high</sup> began to decrease. Such changes could be seen as early as 24 h (results not shown). By day 10, all values were similar to those seen in mock-resected mice.



**FIGURE 2.** Populations of Gr1<sup>+</sup> cells differ in naive and tumor-bearing mice. Splenocytes of naive (a) and tumor-bearing mice (b) were gated for nonlymphoid cells and then stained with 100 ng of fluoresceinated anti-Gr1 mAb and analyzed for fluorescence via flow cytometry. Results obtained with the isotype control are shown in the boxes in the upper left corner.

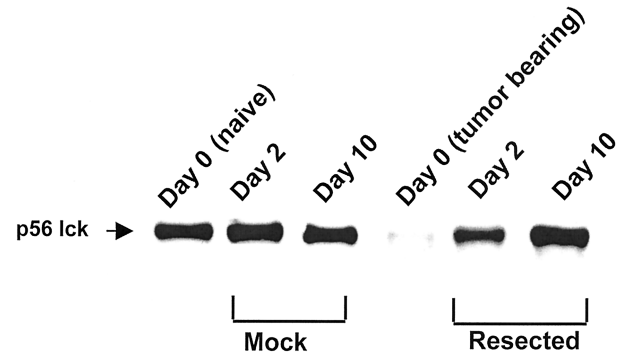
#### Normalization of the levels of p56<sup>lck</sup> in T cells from tumor-bearing mice

We have previously reported that one of the abnormalities in tumor-bearing hosts associated with their weak T cell responses is the diminished levels of signal transduction proteins, such as p56<sup>lck</sup>, p59<sup>fyn</sup>, and the CD3/TCR  $\zeta$ -chain (2). These abnormalities, however, were not observed in immune animals, whose levels of these proteins were similar to those of naive animals. Other investigators reported that following in vitro culture of T cells from tumor-bearing mice, levels of CD3/TCR  $\zeta$  reverted to normal (21). Whether protein levels can normalize in vivo, however, has not been tested. To examine whether levels of p56<sup>lck</sup> were influenced by tumor resection, mice were inoculated with CMS5 cells and after 28 days their tumors were resected. The mean size of the tumors, calculated as longest surface length (a) and width (b),  $(a + b)/2$ , was  $18 \pm 5$  mm. Control mice underwent mock resection. On days 2 and 10 after resection, spleens were pooled from each group and T cells isolated by negative selection of T cells with mAbs against I-A<sup>d</sup> (MKD6), the anti-heat stable Ag (J11d), and complement.

Using Western blotting, we observed that while T cells from unresected mice had the expected decreased levels of p56<sup>lck</sup> compared with control mice, following tumor resection levels of this protein begin to increase gradually. By day 10, levels of p56<sup>lck</sup> were indistinguishable from those in mock-resected mice (Fig. 3). Although it is formally possible that the increase in p56<sup>lck</sup> was due to the death of the abnormal T cells and an influx of normal T cells, the rapidity with which the response was observed suggests that levels of p56<sup>lck</sup> might have been restored in those cells in which they had been decreased.

#### Tumor resection restores cell-mediated immunity and unmask protective immunity

The normalization of the cellular composition of the spleen and the increased levels in signal transduction proteins would be biologically significant only if they influenced antitumor immunity. To test this, tumors were resected from mice 28 days after the inoculation of tumor cells. Ten days later, the mice were challenged with an s.c. inoculum of CMS5 or with CMS4, a tumor antigenically unrelated to CMS5. Fig. 4 shows that CMS5 cells grew rapidly in naive mice, while by 31 days no tumors had developed in resected mice. The antitumor response was specific, because resected mice failed to reject the challenge of CMS4 cells. Further-

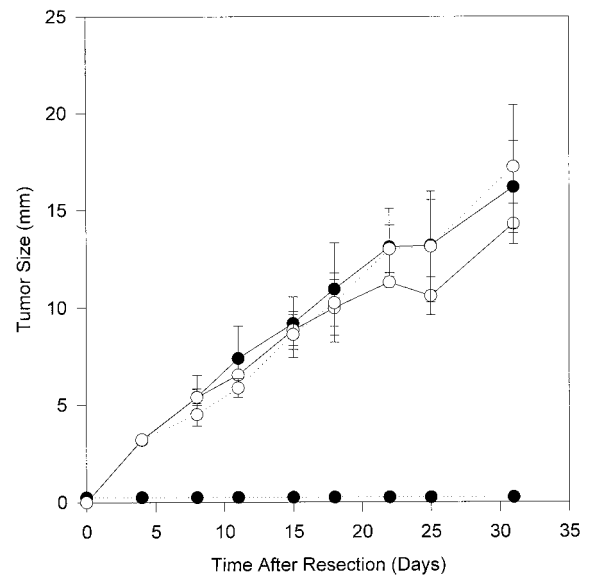


**FIGURE 3.** Normalization of the levels of p56<sup>lck</sup> after tumor resection. Cell lysates from enriched T cells ( $4 \times 10^6$  cell equivalent/lane) were separated on a 10% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and immunoblotted with an anti-p56<sup>lck</sup> polyclonal Ab, followed by a secondary Ab conjugated to HRP and developed via ECL. These results are representative of those obtained in three experiments.

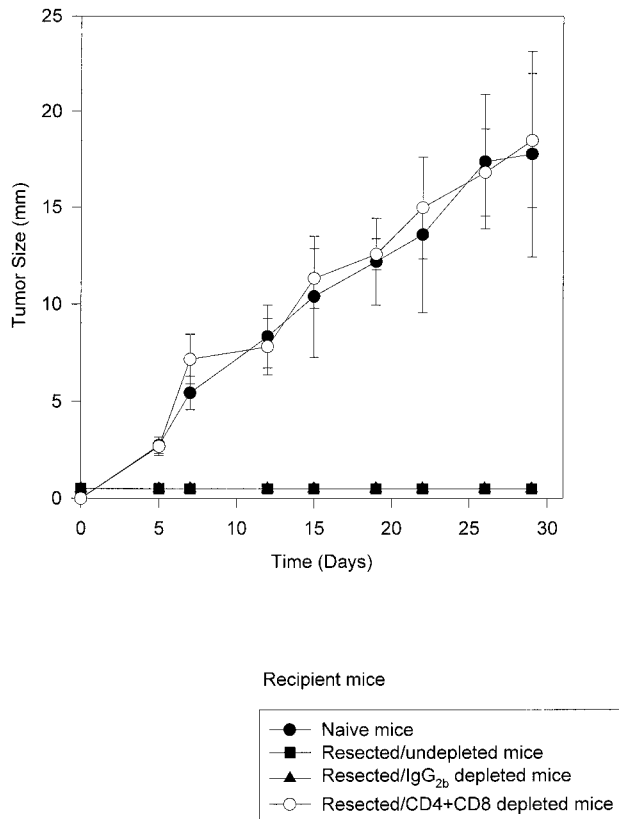
more, rejection depended upon the presence of T cells, because resected mice that were depleted of CD4<sup>+</sup> and CD8<sup>+</sup> T cells did not reject the challenge (Fig. 5).

#### Both CD4<sup>+</sup> and CD8<sup>+</sup> cells are required for the restoration of immunity

To determine the phenotype of the effector cell, tumor-bearing mice were resected, and on day 10, after immunity was restored, depleted for either CD4<sup>+</sup> or CD8<sup>+</sup> T cells. A group of mice was treated with an isotype control Ab. All mice were challenged with CMS5 cells and tumor growth followed with time. We observed



**FIGURE 4.** Tumor-specific protective immunity is unmasked by tumor resection. Tumor-bearing mice were resected 28 days after CMS5 inoculation. Ten days later, resected and naive mice were challenged s.c. with  $0.5 \times 10^6$  CMS5 or  $0.5 \times 10^6$  CMS4 cells. Tumor growth was followed with time.



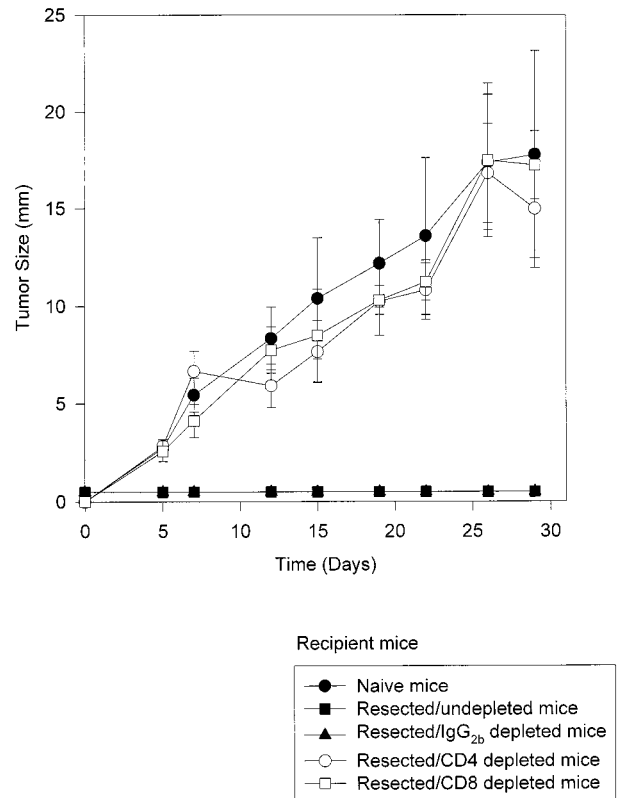
**FIGURE 5.** Resected mice require both CD4<sup>+</sup> and CD8<sup>+</sup> T cells to reject a tumor challenge. Tumor-bearing mice were injected i.p. with 100  $\mu$ l of anti-CD4 and anti-CD8 ascites or 100  $\mu$ l of an Ab isotype control 26 days after CMS5 inoculation, and then biweekly for the length of the experiment. On day 28, mice were resected and 10 days later challenged s.c. with  $0.5 \times 10^6$  CMS5 cells. Tumor growth was followed with time.

that none of the mice depleted for CD4<sup>+</sup> or CD8<sup>+</sup> cells was able to reject the tumor challenge (Fig. 6). In other experiments, from a total of 10 mice depleted for CD4<sup>+</sup> cells, none was able to reject the challenge, and only 2 of 10 mice depleted for CD8<sup>+</sup> cells were. These results, together with those of others, indicated that CD8<sup>+</sup> T cells are necessary, but not sufficient to reject the challenge, suggesting that CD4<sup>+</sup> cells play a very important role (22).

#### *Protection can be adoptively transferred with T cells from resected mice*

To test whether protection against tumor challenge could be adoptively transferred to naive mice, naive and resected mice were inoculated with  $0.5 \times 10^6$  CMS5 tumor cells. After 28 days, resected-immune or tumor-bearing mice were sacrificed, their spleens harvested, and the single cell suspensions enriched for T cells by negative depletion of non-T cells. Naive recipient mice were injected i.v. with  $30 \times 10^6$  T cells from tumor-bearing, from resected-immune mice, or with saline as a control. Twenty-four hours later, all mice received an s.c. challenge of CMS5 tumor cells. We observed that tumors developed in mice that received no cells or T cells from tumor-bearing mice, while mice that received the T cells from resected-immune mice were protected against tumor growth. Thus, protection against tumor challenge was transferable (Fig. 7).

Next, we asked the question whether T cells from tumor-bearing mice would return to a functionally responsive state in the host animal with time. To do that, naive mice were inoculated with  $0.5 \times 10^6$  CMS5 tumor cells. After 28 days, tumor-bearing mice

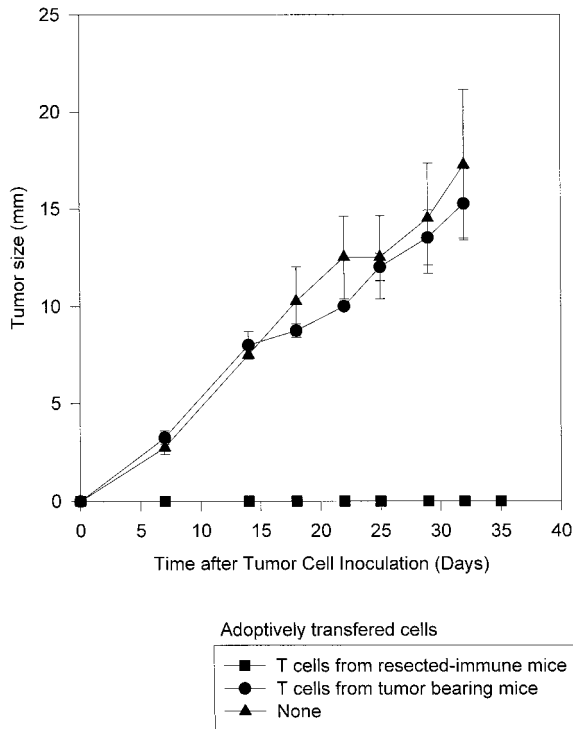


**FIGURE 6.** Resected mice require both CD4<sup>+</sup> or CD8<sup>+</sup> cells to reject a tumor challenge. Tumor-bearing mice were resected on day 28. Ten days later, groups of mice were inoculated with 100  $\mu$ l of anti-CD4 ascites, anti-CD8 ascites, or with an isotype control i.p. and then biweekly throughout the experiment. A group of mice was left undepleted. After 2 days, these mice, together with naive mice, were challenged s.c. with  $0.5 \times 10^6$  CMS5. Tumor growth was followed with time.

were sacrificed, their spleens harvested, and the single cell suspensions enriched for T cells. Naive recipient mice were divided in two groups and injected i.v. with the enriched T cells from tumor-bearing mice or with saline. Twenty-four hours later, and weekly for 3 wk, five mice from each group were challenged s.c. with tumor cells (Fig. 8). We observed that even when mice were challenged 3 wk after the adoptive transfer, tumors developed. This experiment suggested that protection to a tumor challenge is not conferred simply by transferring enriched T cells depleted for Gr1<sup>+</sup>/Mac1<sup>+</sup> cells from a tumor-bearing donor to a naive host. These results are consistent with the interpretation that APC loaded with tumor peptides may be necessary to restore antitumor responses by T cells.

#### *Resection restores CTL activity in vitro*

It is increasingly appreciated that in several systems, including ours, tumor-specific CTL are demonstrable early after tumor cell inoculation, but that they disappear with time (5, 13, 23). In previous studies, we observed that immunization with IL-2-secreting tumor cells induced a strong antitumor response in naive mice mediated largely by T cells (5, 13) and that the loss of *in vitro* CD8<sup>+</sup> CTL activity was associated with the failure of the mice to reject a challenge of viable tumor cells *in vivo* (5). To test whether tumor resection had restored CD8<sup>+</sup> CTL function, resected mice were challenged with CMS5 tumor cells. After 8 wk, spleens were harvested from both groups and restimulated *in vitro* with *x*-irradiated CMS5 tumor cells. Six days later, before testing for cytotoxicity, an aliquot of effector cells was treated with anti-CD8 Ab

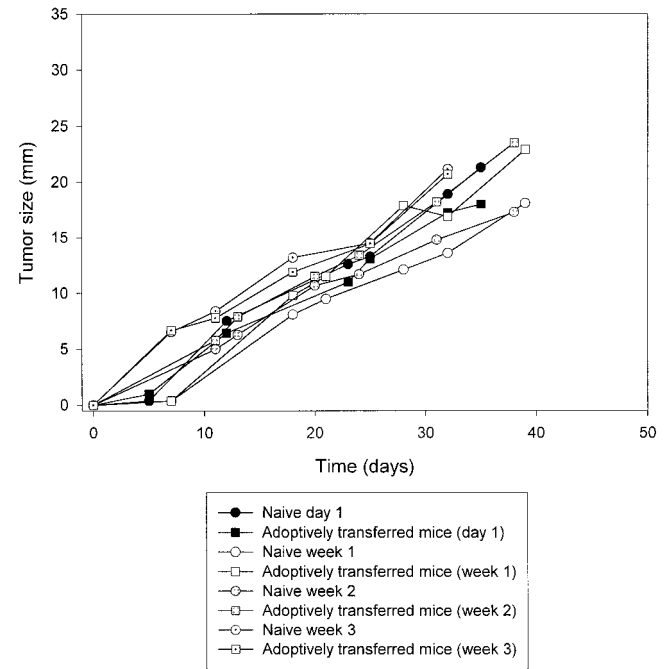


**FIGURE 7.** Protection against tumor challenge can be adoptively transferred to naive mice. Naive and resected mice were inoculated with  $0.5 \times 10^6$  CMS5 tumor cells. After 28 days, tumor-bearing and resected-immune mice were sacrificed, their spleens harvested, and the single cell suspensions were enriched for T cells by negative depletion of non-T cells (as described in *Materials and Methods*). A total of  $30 \times 10^6$  T cells from either tumor-bearing or resected-immune mice, or the same volume of saline alone, was injected into naive mice i.v. Twenty-four hours later, mice were challenged s.c. with CMS5 cells. Tumor growth was followed with time.

plus complement or with complement alone. Our results showed that CTL able to lyse specific tumor cells were detectable in cultures of splenocytes from mice challenged with CMS5 tumor cells (Fig. 9). No lysis of EL-4, an irrelevant target, or YAC-1, an NK target, was seen, consistent with the development of a response that was Ag specific, T cell mediated, and dependent upon the presence of CD8<sup>+</sup> T cells. In contrast, anti-CD8 treatment markedly reduced this cytotoxic activity. Taken together, these results suggested that tumor-bearing mice contained a population of long-lived antitumor T cells that was unmasked without any further immunization in the absence of the tumor.

## Discussion

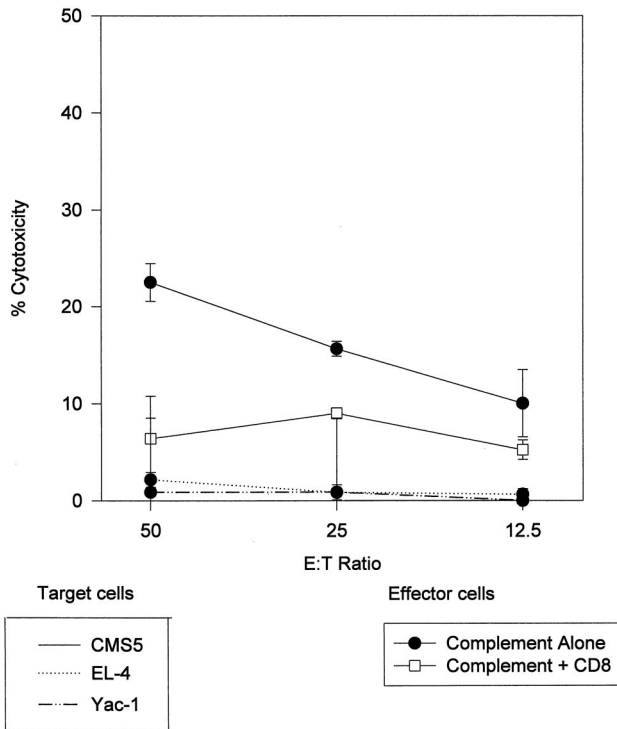
Reduced cell-mediated immunity is a serious problem in cancer patients. The basis for the development of this T cell unresponsiveness is unknown. In principle, it could be regulated by a mechanism that is reversible, such as anergy (24), or one that is irreversible, such as apoptosis (25). Although in some cases immune defects may be responsible for the failure to recognize a nascent tumor, experiments with mice have shown that tumor progression can occur in spite of the generation of initial antitumor immune responses (5, 23). Several mechanisms have been proposed to explain the failure of the immune response to contain tumor growth, including poor Ag-presenting ability of tumor cells (26, 27), the induction of peripheral tolerance or anergy to tumor cells that fail to deliver costimulatory signals (26–29), the emergence of Ag loss variants that do not display tumor-associated Ags (30), the secre-



**FIGURE 8.** Enriched T cells adoptively transferred from a tumor-bearing donor to a naive host do not confer protection to a subsequent tumor challenge. Naive mice were inoculated with  $0.5 \times 10^6$  CMS5 tumor cells. After 28 days, tumor-bearing mice were sacrificed, their spleens harvested, and the single cell suspensions were enriched for T cells by negative depletion of non-T cells (as described in *Materials and Methods*). A total of  $30 \times 10^6$  T cells or saline alone was injected into naive mice i.v. Twenty-four hours later and then weekly for up to 3 wk, mice (five per group) were challenged s.c. with CMS5 cells. Tumor growth was followed with time.

tion of soluble inhibitory factors such as TGF- $\beta$  and IL-10 by the tumor cells (31), loss of MHC expression preventing the presentation of peptides to potentially reactive T cells (32), or abnormal signal transduction by T cells following the binding of ligand (1, 2, 6). The development of an antitumor response is influenced heavily by several factors, including the mode of Ag presentation of tumor-associated Ags, which can be direct or indirect (33, 34). If this critical step is not sufficiently effective, the problem may not be the failure to make an antitumor response, but that the immune response made has been insufficient to contain tumor growth by the time it is down-regulated (5, 23). Thus, while it is likely that all of these mechanisms are operative under certain conditions, whether they are involved in the failure to prime antitumor effectors or in the down-regulation of an initial antitumor response is not known.

We and others have reported that there is a loss of signal transduction ability by T cells in tumor-bearing hosts (1, 2, 6). In a variety of systems, the infiltration of granulocytes in the spleen has been observed (4, 14–16) (Figs. 1 and 2). Saito's group reported that these cells could induce the loss of CD3 $\zeta$  as well as prevent Ag-specific T cell proliferation in vitro (15, 16). Of greatest interest to us are the mechanisms responsible for the failure to sustain an antitumor response after it has been initiated. T cells from late tumor-bearing mice lose the ability to differentiate into CTL following immunization with virus in vivo, to proliferate in response to stimulation with Ag in vitro (6, 13), or to secrete IL-2 in vitro after cross-linking the TCR (35). Although progress in understanding the signals required by T cells for activation has led to the rational design of immunotherapeutic approaches to stimulate the development of T cell-mediated antitumor immunity, the effectiveness of such vaccines might be compromised in tumor-bearing



**FIGURE 9.** Tumor resection restores CTL activity in vitro. Tumor-bearing mice were resected on day 28. Ten days later, mice were challenged s.c. with  $0.5 \times 10^6$  CMS5 cells. After 8 wk, the spleens were harvested and single cells restimulated with  $x$ -irradiated (6000 rad) CMS5 tumor cells. Following an additional 7 days of incubation in vitro, an aliquot of the culture was depleted for CD8<sup>+</sup> T cells (as described in *Materials and Methods*). At the end of the reaction, depleted and undepleted cells were tested at a variety of E:T cell ratios for their ability to lyse CMS5, YAC-1, and EL-4 <sup>51</sup>Cr-labeled target cells.

hosts that are unable to develop immunity even to viruses that elicit strong T cell responses (6). Nevertheless, patients with advanced disease often have exhausted other forms of therapy. Thus, the ability to reverse immune suppression is an important goal that requires an understanding of the regulation of T cell unresponsiveness.

In these studies, we identified several immunological abnormalities that were reversed by tumor resection. First, the levels of the Gr1<sup>+</sup> and Mac1<sup>+</sup> cells in the spleen were normalized (Fig. 2). The Gr1<sup>low</sup> population of cells that was largely absent in the day 28 tumor-bearing mouse increased and the cells in the Gr1<sup>high</sup> population decreased. High expression of Gr1 is found on mature granulocytes, while, at least in the bone marrow, cells expressing low levels of Gr1 are immature progenitors and myelocytes (36–38). While Mac1<sup>+</sup>/Gr1<sup>+</sup> cells have been shown to induce suppression (17, 18), we are not aware of other reports describing Gr1<sup>high</sup> and Gr1<sup>low</sup> cells in the spleen. Whether they represent different stages of differentiation of one cell or distinct subsets is currently under investigation. It is of interest that in some systems, T cell-mediated rejection of tumors genetically engineered to secrete G-CSF and/or IL-2 requires the infiltration of Gr1<sup>+</sup> cells into the tumor (37, 38). These investigators did not examine whether they were found in the spleens of the immune mice. Second, we demonstrated normalization of the levels of p56<sup>lck</sup> in T cells following tumor resection (Fig. 3). Third and most important from a biological perspective, following tumor resection, formerly immune suppressed mice specifically rejected a challenge of CMS5 tumor cells without any further immunization (Fig. 4). The mechanism involved both CD4<sup>+</sup> and CD8<sup>+</sup> cells, because mice depleted for CD4<sup>+</sup> cells

failed to reject their tumors, and mice depleted for CD8<sup>+</sup> cells also failed to reject their tumors (Fig. 6). Interestingly, adoptive transfer of enriched splenic T cells from tumor-bearing mice did not restore T cell responsiveness, even if the hosts were challenged with tumor cells as late as 21 days after adoptive transfer. These results suggest the hypothesis that professional APC loaded with tumor peptides may be required to obtain antitumor responses.

Our working hypothesis is that cytokines released by CD4<sup>+</sup> cells play an important role in tumor rejection, consistent with results of other groups. For example, the depletion of CD4<sup>+</sup> cells in already immune mice, before tumor challenge, prevents tumor rejection (39). In addition, following vaccination with an irradiated MHC class I<sup>-</sup> tumor vaccine, the rejection of class I<sup>-</sup> tumor cells requires CD4<sup>+</sup> cells, possibly to recruit APC or activate NK cells (40). Finally, it is increasingly appreciated that CD4<sup>+</sup> cells can produce cytokines such as the Th1 IFN- $\gamma$  and/or the Th2 IL-4 cytokines that then can recruit a variety of other effector cells, e.g., eosinophils, macrophages, to the tumor (22). The mechanism by which the tumor induces T cell suppression is unknown, but in our system it does not involve the secretion of a soluble cytokine that directly inhibits T cells (13). Also unclear is the relationship, if any, between the decrease in the Gr1<sup>high</sup> population, the return of p56<sup>lck</sup>, and the emergence of protective immunity. Answers to these questions await the results of further experimentation.

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