Interferon γ and Tumor Necrosis Factor Have a Role in Tumor Regressions Mediated by Murine CD8+ Tumor-infiltrating Lymphocytes

By Richard J. Barth, Jr., James J. Mulé, Paul J. Spiess, and Steven A. Rosenberg

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

Summary

We have investigated the mechanisms whereby adoptively transferred murine CD8+ lymphocytes mediate tumor regressions. Noncytolytic, CD8+ tumor-infiltrating lymphocytes (TIL) eradicated established lung tumors in irradiated mice. Many cytolytic and noncytolytic CD8+ TIL cultures specifically secreted interferon γ (IFN-γ) and tumor necrosis factor when stimulated with tumor cells in vitro. The effectiveness of TIL when adoptively transferred to mice bearing micrometastases correlated better with their ability to specifically secrete lymphokines than with their cytotoxicity in vitro. In 14 of 15 tests, therapeutically effective TIL specifically secreted IFN-γ in vitro, whereas only 1 of 11 ineffective TIL specifically secreted IFN-γ. In contrast, only 8 of 15 therapeutically effective TIL were cytolytic. Antibodies to TNF inhibited the effectiveness of two adoptively transferred TIL cultures. In five experiments, antibodies to IFN-γ abrogated the ability of four different CD8+ TIL cultures to mediate tumor regressions, indicating that secretion of IFN-γ is an essential part of the mechanism of action of TIL.

Early studies in mice investigating the mechanisms involved in syngeneic tumor regressions mediated by the transfer of T lymphocytes found a strong correlation between tests of in vitro cytotoxicity and in vivo antitumor effect (1–6). This correlation suggested that T cells with in vitro cytotoxicity were involved in tumor rejection in vivo and that the mechanism of rejection was direct cytolyis. However, the availability of mAbs to distinguish murine helper CD4+ and cytolytic CD8+ lymphocytes enabled investigators to deplete one or the other T cell subset and test each for their ability to cause tumor regressions in vivo. When cytolytic CD8+ lymphocytes were depleted, the resultant noncytolytic CD4+ lymphocytes were as capable as the nondepleted culture in eliciting rejection or preventing the outgrowth of leukemias (7), virally induced sarcomas (8), chemically induced sarcomas (9), and plasmacytomas (10) in murine models.

The possibility that these helper CD4+ cells were activating host cytolytic CD8+ lymphocytes to meditate tumor regression was investigated by using mice that were thymectomized, lethally irradiated, and then reconstituted with T cell–depleted, syngeneic bone marrow (B mice). The adoptive transfer of CD8-depleted splenocytes from tumor-immune mice to such B mice still eradicated the established tumor (11) and prevented the outgrowth of a subsequent tumor challenge (10). Although no cells that were cytotoxic to tumor could be cultured from the splenocytes of these cured mice, they were able to mediate a tumor-specific, delayed-type hypersensitivity (DTH)1 reaction when re-challenged with a tumor. These observations led investigators to propose that CD4+ lymphocytes may convey tumor resistance by initiating a DTH reaction at the tumor site, thus implicating macrophages, the cytotoxic cells in DTH reactions, as the final effectors of tumor killing. Further support for this hypothesis came from the observation that macrophage-activating factor (IFN-γ) was secreted in vitro in response to tumor stimulation by a CD4+ lymphocyte line that demonstrated antitumor efficacy in vivo (8). The secretion of IFN-γ by these CD4+ lymphocytes was implicated in the mechanism of tumor regression when it was found that concurrent treatment with a mAb against IFN-γ blocked the effectiveness of these cells in vivo (12).

Other investigators showed that cytolytic CD8+ clones (13–15) and CD8+ lymphocyte cultures (16–18), when administered with the T cell growth factor IL-2, also very effectively caused the regression of established tumors. However, there has been very little investigation on the mechanism by which CD8+ lymphocytes eradicate tumors. In this report, we demonstrate that the adoptive transfer of noncytolytic CD8+ lymphocytes can mediate the regression of established tumors in mice that were pre-irradiated to abolish host CTL

Abbreviations used in this paper: CM, complete media; DTH, delayed-type hypersensitivity; GM, granulocyte/macrophage; LAK, lymphokine activated killer; MCA, 3-methylcholanthrene; TIL, tumor-infiltrating lymphocytes.
Responses. Some CD8+ lymphocyte cultures specifically secrete IFN-γ and TNF when stimulated with a tumor; the therapeutic effectiveness of a large number of tumor-infiltrating lymphocyte (TIL) cultures correlates better with their ability to specifically secrete lymphokines than with their cytotoxic capacity in vitro. Finally, antibodies to IFN-γ and TNF-α inhibit the in vivo antitumor effectiveness of both cytolytic and noncytolytic CD8+ lymphocytes, thereby suggesting that a DTH reaction may be an important mechanism by which CD8+ lymphocytes mediate tumor regressions.

Materials and Methods

Mice. Female C57BL/6 mice (denoted B6) were obtained from the Animal Production Colonies of the National Cancer Institute, NIH (Frederick Facility), Frederick, MD, and from the Charles River Breeding Laboratories, Wilmington, MA. All mice were used at age 10 wk or older.

Tumors. The MCA 105, 203, 205, 207, and 233 tumors are weakly immunogenic, 3-methylcholanthrene (MCA)-induced fibrosarcomas of B6 origin (19). MCA 102 is a nonimmunogenic sarcoma. These tumors were generated in our laboratory and were passaged subcutaneously for 10 generations, at which time a cryopreserved vial from the first generation was thawed and transplanted. MC 38 is an immunogenic colon adenocarcinoma of B6 origin (17).

TIL Culture Conditions. TIL were cultured by immunoselection from freshly digested tumors using anti-Thy-1.2 mAb-coated magnetic beads as previously described (18, 20). TIL were then stimulated with irradiated tumor cells and B6 splenocytes on day 1 of culture and maintained in complete medium (CM) supplemented with 10–20 U/ml of rIL-2 (Cetus Corp., Emeryville, CA). Fresh CM containing rIL-2 was added to the culture every 3 d. Every 10 d, the cultures were stimulated with irradiated (3,000 rad) tumor cells at 5 × 10^6 tumor cells/well (Costar, Cambridge, MA). TIL clone 205-87 was generated as follows. TIL generated from a clone of MCA 205 were placed in 96-well, flat-bottomed microtiter plates (Costar) in CM + 20 U/ml rIL-2 at 0.3 cells/well. Irradiated (3,000 rad) tumor and TIL were added to each well at 2 × 10^5 cells/well and 10^5 cells/well, respectively. In multiple experiments, we have not observed proliferation of TIL after 3,000-rad irradiation. The media was changed every 4 d; at day 10, the culture was restimulated with irradiated tumor and TIL. On day 16, 3 of 192 wells were observed to have TIL colonies and these were expanded in CM + 20 U/ml rIL-2 with restimulation with irradiated tumor every 10 d. MC 38 TIL “cloids” were generated as follows. After immunobeading, TIL were plated at 100, 300, and 1,000 cells per well in 96-well, flat-bottomed microtiter plates in CM with 10–20 U/ml rIL-2 and were stimulated with 2 × 10^4 irradiated (3,000 rad) tumor cells/well and 10^4 irradiated (3,000 rad) B6 splenocytes/well. If wk later, cells were aspirated from plates in which half or less of the 96 wells contained proliferating cells and were expanded for in vivo use. Lymphokine-activated killer (LAK) cells were generated by incubating fresh B6 splenocytes in CM + 1,000 U/ml rIL-2 for 3–5 d, as previously described (18).

In Vitro Cytotoxicity Assays. 4-h 51Cr release assays were performed as previously described (18). Briefly, fresh tumor targets were labeled with 51Cr and incubated with TIL effectors at varying ratios for 4 h at 37°C. Supernatants were harvested and counted in a gamma counter. Percent lysis was calculated as follows: 100 × (experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm). One lytic unit is defined as the number of effector cells needed to cause 20% lysis of 10^4 fresh tumor target cells and is determined from the dose-response curves.

Fluorescence-activated Cell Sorting (FACS®) Analysis. Flow cytometry analysis of lymphocyte surface phenotypes was carried out by direct immunofluorescence using a FACS® 440 flow microfluorometer (Becton Dickinson & Co., Mountain View, CA). Pelleted single cell suspensions of 10^6 cells were incubated for 20 min at 4°C with 20 μl of appropriately titered, FITC-conjugated, anti-Lyt-2 (mAb 2.43; American Type Culture Collection [ATCC], Rockville, MD) or anti-L3T4 (mAb GK-1.5 ATCC) mAbs (Becton Dickinson & Co.) diluted in PBS containing 1% FCS and 0.1% sodium azide, washed, and analyzed.

Lymphokine Secretion and Detection. TIL were harvested and plated in 2 ml of CM plus 20 U/ml rIL-2 at 5 × 10^5 cells/well in a 24-well plate (Costar). TIL were stimulated with irradiated (3,000 rad) tumor cells at 5 × 10^6 tumor cells/plate (Costar, Cambridge, CA). Fresh CM containing rIL-2 was added to the culture every 3 d. Supernatants were harvested and counted in a 24-well plate (Costar). 10^6 freshly digested and washed tumor cells were added in 100 μl of CM to each well. The TIL plus tumor mixture was incubated at 37°C for 18 h, and then the supernatant was aspirated, centrifuged at 1,500 rpm to remove any cells, decanted, and frozen at −70°C. Aliquots were then thawed and tested in duplicate in an IFN-γ ELISA (Amgen, Thousand Oaks, CA) and a TNF ELISA (Genzyme, Boston, MA). The IFN-γ ELISA used a solid phase, specific anti-murine IFN-γ mAb, and then a biotinylated second, anti-murine IFN-γ mAb was added. The TNF ELISA used a solid phase hamster mAb specific for murine TNF. A goat polyclonal anti-murine TNF second antibody was then used, followed by a horseshad peroxidase–conjugated donkey anti-goat IgG. The antibodies detect both mouse TNF-α and TNF-β, but the assay is specifically calibrated for TNF-α. This ELISA has been determined not to detect rIL-1, rIL-2, rIL-3, recombinant granulocyte/macrophage (rGM)-CSF, and IFN-γ.

Adoptive Immunotherapy Models. For the experiments described in Fig. 1 and Tables 1, 3, 5, and 6, there were six mice in each therapy group. Some mice, where indicated, received 500-rad total body irradiation and then were injected intravenously with 5 × 10^6 tumor cells in 1 ml of HBSS (Biofluids, Rockville, MD) to induce pulmonary metastases. On day 3, 5 × 10^6 TIL were injected intravenously. Some mice were treated with 10,000 U rIL-2 in 0.5 ml of HBSS intraperitoneally twice a day on days 3–7. On day 15, all mice were ear tagged, randomized, killed, and pulmonary metastases were enumerated in a blinded fashion as previously described (21). A correlation exists between pulmonary metastasis reduction by TIL and prolongation of mouse survival (our unpublished results).

For the TIL “cloids” experiments described in Tables 8 and 9, three mice in each group received 500-rad total body irradiation, and 5 × 10^5 fresh tumor cells were injected to induce pulmonary metastases. On day 3, 10^6 TIL were injected and mice were treated with 10,000 U rIL-2 twice a day on days 3–7. On day 15, all mice were ear tagged, randomized, and pulmonary metastases were enumerated.

Antibody Blocking Experiments. For the anti–IFN-γ antibody blocking experiments described in Fig. 2, six mice in each therapy group were injected with 5 × 10^6 tumor cells on day zero to induce pulmonary metastases. On day 2, 200 μg of hamster anti–murine IFN-γ mAb (Genzyme) or 200 μg of hamster IgG (Organon Teknika, West Chester, PA) in 0.5 ml of PBS with 0.5% human serum albumin was injected intraperitoneally. This anti–IFN-γ mAb was shown to inhibit macrophage activation in mice infected with Toxoplasma gondii (22) and Listeria monocytogenes (23). 24 h later, various doses of TIL were injected and 10,000 U rIL-2 was given intraperitoneally twice a day on days 3–7. Mice were randomized and pulmonary metastases were counted on day 15.

For the anti–TNF antibody blocking experiments described in
Fig. 3, pulmonary metastases were induced and TIL plus rIL-2 therapy was identical to that in the anti-IFN-γ mAb blocking experiments. On day 2, 1 mg of rabbit anti-murine TNF antibody (raised in New Zealand white rabbits immunized to recombinant murine TNF-α [Genentech, South San Francisco, CA] as previously described [24]) or rabbit IgG (ICN Immunobiologicals, Costa Mesa, CA) in 0.5 ml of PBS with 0.5% human serum albumin was injected intraperitoneally. This anti-TNF antibody had been previously shown to be specific for TNF and to protect mice from a lethal challenge with endotoxin (24). We repeated this endotoxin protection experiment; 1 mg of anti-TNF antibody injected intraperitoneally 6 h before intraperitoneal endotoxin challenge (40 mg/kg) protected nine of nine mice, whereas two of nine mice treated with control rabbit IgG survived for 48 h. 1 mg of anti-TNF antisera or control rabbit IgG was injected intraperitoneally every morning on days 4–7. Mice were randomized and pulmonary metastases were counted on day 15.

Statistics. Statistical analyses of in vivo experiments were performed by the Wilcoxon rank sum test. The student's t test was used for group comparisons in Tables 7 and 9. Two-sided p values are presented in all experiments.

Results

Specifically Cytolytic TIL Are Specifically Effective In Vivo
We had previously shown that culture of TIL from antigenically distinct MCA-induced sarcomas in low-dose (10–20 U/ml) rIL-2 with tumor stimulation reproducibly led to TIL with cytolytic specificity in 4-h 51Cr release assays (18). Our current studies began by investigating whether such cytolytically specific TIL were also specifically effective in the treatment of pulmonary micrometastases. Five TIL cultures were derived from four different MCA-induced sarcomas in low-dose rIL-2 with tumor restimulation every 10 d. All TIL were CD8⁺, CD4⁻ by FACS® analysis. As shown in Fig. 1, two TIL cultures, grown from MCA 203 and 205, were simultaneously tested in a 4-h 51Cr release assay and specifically lysed their tumor of origin. Concurrently, 5 x 10⁶ of each of these TIL were adoptively transferred to groups of mice that had been intravenously injected with one of the two tumors 3 d earlier to induce pulmonary micrometastases. On days 3–7, 10,000 U rIL-2 was injected intraperitoneally twice a day and metastases were counted on day 15. As shown in

![IN VITRO](image-url)

![IN VIVO](image-url)

\[
\text{Table 1. Specifically Cytolytic TIL Are Specifically Effective In Vivo}
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| Effector cells | IL-2 | 105 | 205 | 207 |
|----------------|------|-----|-----|-----|
| 105 TIL        | +    | 1 (1) | >250 | 227 (22) |
| 205 TIL        | +    | 215 (35) | 62 (9) | >250 |
| 207 TIL        | +    | 245 (5) | >250 | 32 (5) |
| None           | +    | >250 | >250 | >250 |

* As determined in a 4-h 51Cr release assay.
† 5 x 10⁶ TIL were adoptively transferred to 500-rad pre-irradiated mice bearing 3-d pulmonary micrometastases. On days 3–7, 10,000 U rIL-2 was given intraperitoneally twice a day, and metastases were enumerated on day 15.
§ Significantly different than rIL-2 alone group (p < 0.002).

Fig. 1. Each TIL significantly reduced the number of pulmonary metastases in mice bearing the relevant tumor, but had no effect on the number of pulmonary metastases in mice bearing the other tumor. This experiment was repeated with three different TIL cultures, from MCA 105, 203, and 205 (Table 1). Again, each TIL was significantly effective against only that tumor that it specifically lysed in vitro. These studies...
Three different TIL cultures were used as positive controls; their ages at the time of the assay were 60, 62, 65, and 120 d.

tPercent cytolyis of MCA 205 target in 4-h 51Cr release assays at an E/T ratio of 50:1.

Suggested that there was a correlation between in vitro cytolysis and in vivo efficacy of cytolytic CD8+ TIL.

Nonlytic, CD8+, CD4- TIL Effectively Mediate Tumor Regressions in Pre-irradiated Mice.

To test whether the in vitro property of cytolysis was a necessary characteristic of therapeutically effective TIL, we examined TIL that did not demonstrate significant tumor lysis in 4-h 51Cr release assays. When murine TIL are grown in low-dose (10 U/ml) rIL-2 with repeated tumor stimulation, approximately one third of the resultant, rapidly proliferative, CD8+, CD4- cultures will be nonlytic (<10% lysis of relevant fresh tumor targets at an E/T ratio of 50:1 in a 4-h 51Cr release assay) (18). The cytolytic capacity of culture 205-73, a nonlytic, CD8+, CD4- TIL culture from the MCA 205 tumor, is shown in Table 2. In five separate 51Cr release assays over a period of 3 mo, there was no significant lysis of MCA 205 tumor by TIL culture 205-73, whereas other 205 TIL cultures or LAK cells lysed the target cell in each assay. In long-term assays, when 205-73 TIL were co-incubated with MCA 205 tumor at a 2:1 TIL/tumor ratio for 5 d, tumor cells overgrew the TIL culture, indicating that this culture had no detectable short- or long-term lytic capability.

In two separate experiments, these nonlytic 205-73 TIL were adoptively transferred to mice bearing 3-d pulmonary micrometastases. On days 3–7, 10,000 U rIL-2 was given intraperitoneally twice a day and metastases were enumerated on day 15. As shown in Table 3, in both experiments these nonlytic TIL significantly reduced the number of pulmonary micrometastases. Therefore, it is not necessary for a CD8+ lymphocyte to be lytic in order to mediate effective in vivo regression of an established tumor. Furthermore, these nonlytic TIL were effective even when the mice were pre-irradiated with 500-rad total body irradiation, which abrogates host lymphocyte responses (25). Therefore, these CD8+ nonlytic TIL are not activating host CTL, but instead may be mediating tumor regression through their effects on radiation-resistant host effector cells.

Some CD8+ TIL Specifically Secrete IFN-γ and TNF When Stimulated with Tumors. These results led us to investigate whether TIL, when stimulated with tumor, secreted lymphokinases that might be able to modify the tumor or host effector cells to lead to tumor regression. As shown in Table 4, three different TIL cultures were simultaneously stimulated with weakly immunogenic relevant or irrelevant tumors, and the amount of IFN-γ secreted into the media was assayed. The relevant tumor induced secretion of at least 10 times more IFN-γ than that secreted by unstimulated cells and at least three times more IFN-γ than that secreted by TIL stimulated with irrelevant tumors. Very little, if any (0–2 U), IFN-γ was detected in the supernatants of tumors incubated without the addition of TIL. We also observed specific secretion of TNF by some TIL in response to tumor stimulation (Table 5). When freshly digested tumors were incubated

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Table 2. Lack of Lysis of MCA 205 by TIL Culture 205-73

| TIL 205-73 culture age | Effector TIL 205-73 | Another 205 TIL Cx* | LAK |
|------------------------|---------------------|---------------------|-----|
| 32                     | 1†                  | ND                  | 30  |
| 55                     | 7                   | 37                  | 77  |
| 88                     | –1                  | 34                  | ND  |
| 91                     | 5                   | 20                  | ND  |
| 118                    | –3                  | 30                  | 49  |

* Three different TIL cultures were used as positive controls; their ages at the time of the assay were 60, 62, 65, and 120 d.
† Percent cytolyis of MCA 205 target in 4-h 51Cr release assays at an E/T ratio of 50:1.

Table 3. Treatment of 3-d Pulmonary Metastases with Nonlytic TIL Culture 205-73

| Treatment | Pre-irradiation | Exp. 1* | Exp. 2† |
|-----------|-----------------|---------|---------|
| HBSS      | –               | >250    | >250    |
| HBSS + IL-2§ | 180 (23) | >250    |
| HBSS + IL-2§ | +               | >250    |
| 205-73 TIL + IL-2§ | – | 8 (2)§ | 8 (2)§ |
| 205-73 TIL + IL-2§ | + | 12 (3)§ | 25 (3)§ |

* TIL culture 205-73 was 60 d old at the time of adoptive transfer of 5 x 10⁵ TIL/mouse.
† TIL culture 205-73 was 90 d old at the time of adoptive transfer of 5 x 10⁵ TIL/mouse.
§ On days 3–7 after tumor injection, 10,000 U rIL-2 was injected intraperitoneally twice a day.

Table 4. Specific Secretion of IFN-γ by TIL in Response to Tumor Stimulation

| TIL culture | Tumor of origin of TIL | MCA tumor stimulator* |
|-------------|------------------------|-----------------------|
| 105–53      | None                   | 105                   |
| 233–92B     | 105                    |
| 233–90D     | 233                    |

* All TIL cultures at 2.5 x 10⁵ cells/ml were stimulated simultaneously with 5 x 10⁵ tumor cells/ml. Supernatants were collected at 18 h and assayed by ELISA.
Table 5. *In Vitro* Lymphokine Secretion and Cytolysis by TIL that Were Effective In Vivo

| TIL    | Tumor of origin | Percent reduction of pulmonary metastases | IFN-γ secretion | TNF secretion | Cytolysis |
|--------|-----------------|------------------------------------------|-----------------|---------------|-----------|
|        |                 |                                          | U/2.5 x 10⁴ cells/ml | pg/2.5 x 10⁴ cells/ml |             |
|        |                 |                                          | None | Rel. | Irr. | Irr. | None | Rel. | Irr. | Irr. | Rel. | Irr. | Irr. |
| 90A    | 233             | 79                                       | 0    | 48   | 16   | 15  | 130  | 205  | 140  | 195  | 4    | 13   | 8    |
| 90B    | 233             | 99                                       | 1    | 55   | 9    | 9   | 150  | 205  | 155  | 180  | 21   | 10   | 1    |
| 90D    | 233             | 77                                       | 1    | 25   | 8    | 4   | 55   | 190  | 290  | 165  | 6    | 0    | -4   |
| 91D    | 233             | 99                                       | 3    | 45   | 9    | 6   | 180  | 250  | 235  | 195  | 6    | 2    | -2   |
| 91D    | 233             | 50                                       | 1    | 15   | 5    | 5   | 70   | 50   | 70   | 100  | 8    | 11   | 3    |
| 92B    | 233             | 99                                       | 0    | 18   | 2    | 1   | 150  | 155  | 85   | 35   | 12   | 4    | 1    |
| 92B    | 233             | 99                                       | 0    | 50   | 7    | 2   | 195  | 195  | 170  | 130  | 20   | 10   | 3    |
| 92B    | 233             | 75                                       | 0    | 41   | 9    | 2   | 25   | 75   | 75   | 10   | 16   | -5   | -1   |
| 93A    | 233             | 75                                       | 2    | 70   | 19   | 8   | 10   | 480  | 0    | 0    | 52   | 9    | 2    |
| 93A    | 233             | 75                                       | 1    | 53   | 6    | 17  | 20   | 905  | 140  | 200  | 45   | 0    | 4    |
| 93B    | 233             | 75                                       | 4    | 65   | 21   | ND  | 200  | 1,330 | 300  | 83   | 3    | 2    | 1    |
| 93B    | 233             | 75                                       | 4    | 80   | 17   | 11  | 50   | 1,020 | 45   | 40   | 24   | -6   | ND   |
| 93B    | 233             | 75                                       | 6    | 53   | 12   | 7   | 25   | 825  | 185  | 50   | 42   | 8    | 3    |
| 93B    | 233             | 75                                       | 1    | 21   | 3    | 2   | 150  | 0    | 0    | 12   | 8    | ND   | |

* TIL were transferred to mice bearing pulmonary micrometastases as described in Materials and Methods. Percent reduction of metastases was calculated by comparing the number of metastases in the group treated with TIL + rIL-2 to the group treated with rIL-2 alone.

1 Secretion of lymphokines was measured in response to tumor stimulators as described in Materials and Methods. Specific lymphokine secretors are boxed. The relevant (Rel.) tumor is the tumor of origin of the TIL; irrelevant (Irr.) tumors are other syngeneic, weakly immunogenic MCA-induced sarcomas.

2 Percent lysis in a 4-h ⁵¹Cr release assay at an E/T ratio of 50:1. Lysis of target by LAK cells was >20% in all experiments. Specifically cytolytic cultures are boxed.

In IFN-γ-containing media, there was no increased secretion of TNF, indicating that the TNF detected in these assays was being secreted by TIL rather than by macrophages that may have been present in the fresh tumor stimulator cells. Secretion of IFN-γ and TNF by TIL is rapid (90% of maximum levels are secreted in 8 h) and is a reproducible characteristic of the culture over time (26). Optimization of the conditions for lymphokine secretion by murine TIL is presented elsewhere (26).

TIL Lymphokine Secretion Correlates with In Vivo Efficacy. To investigate what role, if any, lymphokine secretion by TIL has in their in vivo efficacy, we simultaneously tested TIL for lymphokine secretion and cytolysis in vitro and adoptively transferred these TIL into mice bearing pulmonary micrometastases. 26 paired in vitro and in vivo tests were done on 13 different TIL cultures and one TIL clone (205-87) from three different MCA-induced sarcomas. All TIL were CD8⁺, CD4⁻ by FACS® analysis and were proliferating well at the time of adoptive transfer.

In 15 of these paired tests, therapy with TIL plus rIL-2 significantly (p < 0.05) reduced the number of pulmonary metastases compared with a group treated with rIL-2 alone; the in vitro properties of these TIL are presented in Table 5. In 11 tests, therapy with TIL plus rIL-2 did not significantly reduce the number of pulmonary metastases compared with a group treated with rIL-2 alone; the in vitro properties of these therapeutically ineffective TIL are presented in Table 6. In each experiment where a TIL culture was found to be ineffective, there was another group of mice bearing metastases from the same tumor in which the metastases were significantly reduced by other effective TIL (i.e., a positive control group).

As shown in Table 5, when effective TIL were not stimulated with a tumor, they constitutively released little IFN-γ. Stimulation with a relevant tumor induced secretion of 10-50-fold more IFN-γ than that secreted by unstimulated cells, and two- to ninefold more IFN-γ than that secreted in response to stimulation by irrelevant tumor. Secretion of IFN-γ was considered specific if the amount secreted in response to relevant tumor stimulation was greater or equal to three times the amount secreted by two irrelevant tumors and >15 U/2.5 x 10⁵ cells/ml; such values are highlighted by enclosure in boxes in Tables 5 and 6. Secretion of TNF was considered specific if the amount secreted in response to relevant tumor stimulation was greater or equal to three times the amount secreted by two irrelevant tumors and >100 pg/2.5 x 10⁵ cells/ml; these values are highlighted by boxes in Tables 5 and 6. TIL were considered specifically cytolytic...
Table 6. *In Vitro Lymphokine Secretion and Cytolysis by TIL that Were Ineffective In Vivo*

| TIL | Tumor of origin | Percent reduction of pulmonary metastases | IFN-γ secretion | TNF secretion | Cytolysis |
|-----|----------------|------------------------------------------|-----------------|---------------|-----------|
|     |                |                                          | U/2.5 × 10^5 cells/ml | pg/2.5 × 10^5 cells/ml |           |
|     |                |                                          | Rel. Irr. Rel. Irr. | Rel. Irr. Irr. |           |
| 89  | 233            | -25                                      | 1 6 3 2           | 35 20 0 45     | -1 6 -1   |
| 89  | 233            | -5                                       | 1 1 2 1           | 45 10 235 40   | 1 9 -3    |
| 90A | 233            | -5                                       | 6 [40] 13 12      | 65 110 285 130 | -4 20 13  |
| 90B | 233            | -2                                       | 2 15 5 18         | 50 65 330 130  | 2 11 23   |
| 91A | 233            | -27                                      | 11 15 14 15       | 70 40 10 80    | -1 -1 -3  |
| 91A | 233            | 16                                       | 8 9 11 9          | 50 0 65 0      | -9 5 0    |
| 91B | 233            | -39                                      | 2 9 9 6           | 40 35 0 70     | 7 23 18   |
| 91C | 233            | -39                                      | 1 3 2 1           | 60 65 30 80    | 4 1 2     |
| 91D | 233            | 6                                        | 3 5 3 11          | 70 70 50 100   | 4 11 ND   |
| 84  | 205            | 0                                        | 2 7 4 2           | 180 150 180 180| 14 3 ND   |
| 86B | 205            | 5                                        | 2 12 3 ND         | 140 125 180 ND | 3 2 1     |

* TIL were transferred to mice bearing pulmonary micrometastases as described in Materials and Methods. Percent reduction of metastases was calculated by comparing the number of metastases in the group treated with TIL + rIL-2 to the group treated with rIL-2 alone. In each experiment, a positive control TIL culture reduced the number of metastases 99% compared with rIL-2 alone.

† Secretion of lymphokines was measured in response to tumor stimulators as described in Materials and Methods. Specific lymphokine secretors are boxed. The relevant (Rel.) tumor is the tumor of origin of the TIL; irrelevant (Irr.) tumors are other syngeneic, weakly immunogenic MCA-induced sarcomas.

§ Percent lysis in a 4-h 51Cr release assay at an E/T ratio of 50:1. Lysis of target by LAK cells was >20% in all experiments. Specifically cytolytic cultures are boxed.

Table 7. *Summary of In Vitro Lymphokine Secretion and Cytolysis by TIL Simultaneously Tested for In Vivo Efficacy*

| No. of tests | IFN-γ secretion | TNF secretion | Cytolysis |
|--------------|-----------------|---------------|-----------|
|              | U/2.5 × 10^5 cells/ml | pg/2.5 × 10^5 cells/ml |           |
| Effective TIL | 15              | 45 (5)        | 416 (105) | 26 (6)   |
| Ineffective TIL | 11             | 11 (3)        | 63 (15)  | 2 (2)    |
| p valueⅠ     | <0.0001         | 0.009         | 0.002     |

* Secretion of lymphokines in response to stimulation by relevant tumor as described in Materials and Methods. Data are the mean (SEM).

† Percent lysis of relevant tumor at an E/T ratio of 50:1 in a 4-h 51Cr release assay. Data are the mean (SEM).

§ Effective TIL + rIL-2 significantly (p < 0.05) decreased the mean number of pulmonary metastases compared with a group treated with rIL-2 alone.

Ⅰ Student's t test for the difference between means of effective and ineffective TIL.

if the relevant tumor lysis was >20% and irrelevant tumor lysis was <10% at an E/T ratio of 50:1 in a 4-h 51Cr release assay; these values are also highlighted by boxes in Tables 5 and 6.

The data in Tables 5 and 6 are summarized in Table 7. Effective TIL secreted significantly more IFN-γ (45 ± 5 vs. 11 ± 3 U/2.5 × 10^5 cells/ml, p < 0.0001) and TNF (416 ± 105 vs. 63 ± 15 pg/2.5 × 10^5 cells/ml, p = 0.009) and were significantly more lytic (26 ± 6 vs. 2 ± 2% lysis at an E/T ratio of 50:1, p = 0.002) than ineffective TIL.

The relationship between specific lymphokine secretion and cytolysis by TIL and in vivo effectiveness is summarized in Table 8. Specific secretion of IFN-γ was associated with in vivo effectiveness in 14 of 15 tests. There was only one case of an effective TIL that did not specifically secrete IFN-γ; in this case, secretion of IFN-γ in response to a relevant tumor was twice that seen in response to an irrelevant tumor (Table 5). In 10 of 11 tests on ineffective TIL, there was low or nonspecific IFN-γ secretion. Since 9 of 15 effective TIL did not specifically secrete TNF and 7 of 15 effective TIL were marginally lytic or only lytic, neither specific secretion of TNF nor specific cytolysis appeared to be necessary for in vivo effectiveness. However, if an IFN-γ-secreting culture also specifically secreted TNF or was specifically cytolytic, this ap-
Table 8. Relationship between Lymphokine Secretion and Cytolysis in Vitro and Efficacy of TIL In Vivo

| In vitro parameter | In vivo effectiveness |
|--------------------|----------------------|
|                    | Effective* | Ineffective |
| No. of tests       |            |             |
| IFN-γ secretion    | Specific†  | 14           |
|                    | Nonspecific| 1           |
| TNF secretion      | Specific†  | 6           |
|                    | Nonspecific| 9           |
| Cytolysis          | Specific†  | 8           |
|                    | Nonspecific| 7           |

* The mean number of pulmonary metastases in mice treated with effective TIL + rIL-2 was significantly less than those treated with rIL-2 alone by Wilcoxon rank sum test.
† Secretion of IFN-γ (and TNF) was considered specific if the amount secreted in response to relevant tumor stimulation was greater or equal to three times the amount secreted by two irrelevant tumors and >15 U/2.5 x 10⁵ cells/ml (and >100 pg/2.5 x 10⁵ cells/ml).
§ Cytolysis was considered specific in a 4-h ⁵¹Cr release assay if relevant tumor lysis was >20% and irrelevant tumor lysis was <10% at an E/T ratio of 50:1.

Table 10. Relationship between Lymphokine Secretion and Cytolysis in Vitro and Efficacy of MC 38 TIL Cloids In Vivo

| In vitro parameter | In vivo effectiveness |
|--------------------|----------------------|
|                    | Effective* | Ineffective |
| No. of different cloids tested |            |             |
| IFN-γ secretion    | Specific†  | 23           |
|                    | Nonspecific| 0           |
| TNF secretion      | Specific†  | 21           |
|                    | Nonspecific| 2           |
| Cytolysis          | Specific†  | 14           |
|                    | Nonspecific| 9           |

* Effective TIL cloids decreased the mean number of pulmonary metastases at least 25% compared with the group treated with rIL-2 alone.
† Secretion of IFN-γ (and TNF) was considered specific if the amount secreted in response to relevant tumor stimulation was greater or equal to three times the amount secreted by two irrelevant tumors and >15 U/2.5 x 10⁵ cells/ml (and >100 pg/2.5 x 10⁵ cells/ml).
§ Cytolysis was considered specific in a 4-h ⁵¹Cr release assay if relevant tumor lysis was >20% and irrelevant tumor lysis was <10% at an E/T ratio of 10:1.

23 TIL "cloids" were effective in reducing lung metastases and 10 were ineffective (Table 9). Effective TIL "cloids" secreted significantly more IFN-γ (60 ± 4 vs. 19 ± 5 U/2.5 x 10⁵

Table 9. Summary of In Vitro Lymphokine Secretion and Cytolysis by TIL Cloids from MC 38 Simultaneously Tested for In Vivo Efficacy

| No. of cloids tested | IFN-γ secretion*          | TNF secretion*          | Cytolysis†         |
|----------------------|---------------------------|-------------------------|--------------------|
|                      | U/2.5 x 10⁵ cells/ml      | pg/2.5 x 10⁵ cells/ml   |                    |
| Effective TIL "cloids" | 23                        | 60 (4)                 | 465 (64)          |
| Ineffective TIL "cloids" | 10                        | 19 (5)                 | 77 (51)           |
| p value‡             | <0.0001                   | 0.0008                  | 0.0004            |

* Secretion of lymphokines in response to stimulation by relevant tumor as described in Materials and Methods. Data are mean (SEM).
† Percent lysis of relevant tumor at an E/T ratio of 10:1 in a 4-h ⁵¹Cr release assay.
‡ Effective TIL cloids + rIL-2 decreased the mean number of pulmonary metastases at least 25% compared with the group treated with rIL-2 alone.
§ Student’s t test for the difference between means of effective and ineffective TIL.
Cells/ml, p < 0.0001) and TNF (465 ± 64 vs. 77 ± 51 pg/2.5 × 10^5 cells/ml, p = 0.0008) were significantly more lytic (28 ± 2 vs. 12 ± 3% lysis at an E/T ratio of 10:1) than ineffective TIL "cloids". The relationship between specific cytokine secretion or cytolysis by these TIL "cloids" and in vivo effectiveness is summarized in Table 10. All 23 effective TIL effectiveness when compared with TIL plus hamster IgG control (TIL + and-IFN-γ vs. TIL + hamster IgG: Exp. 1, p = 0.02; Exp. 4, p = 0.025). Therefore, in five of five experiments with four different TIL cultures from three different sarcomas, anti-IFN-γ mAb significantly inhibited the effectiveness of TIL. Of note, in three of these experiments, highly cytolytic TIL were inhibited by anti-IFN-γ mAb. The data from this series of experiments and the experiments summarized in Tables 7 and 9 strongly suggest that secretion of IFN-γ is a necessary part of the mechanism whereby both lytic and nonlytic TIL eradicate tumor cells in vivo.

Anti-TNF Antibody Inhibits Some Effective TIL In Vivo. Concurrently, during some of these experiments, the same TIL were adoptively transferred to mice that had been treated with anti-TNF antibody. As shown in Fig. 3, three TIL cultures, two of which specifically secreted TNF in response to tumor stimulation and one that did not, were transferred to mice bearing pulmonary micrometastases. Anti-TNF antibody or control rabbit IgG (1 mg) was given 6 h before TIL transfer and daily on days 4-7. On days 3-7 10,000 U rIL-2 was injected intraperitoneally twice a day. In two separate experiments, although TIL culture 105-53 specifically secreted TNF in vitro, anti-TNF antibody had no significant effect on the therapeutic efficacy of this TIL (Fig. 3, Exps. 1 and 2). In contrast, in two experiments using TIL clone 205-87, which also specifically secreted TNF in vitro, anti-

Anti-IFN-γ mAb Inhibits Effective TIL In Vivo. IFN-γ was specifically secreted in 14 of 15 tests on TIL cultures that were therapeutically effective and in 23 of 23 tests on effective TIL "cloids". To further delineate the role of IFN-γ secretion in the therapeutic effectiveness of TIL, TIL that specifically secreted IFN-γ were adoptively transferred to mice, bearing pulmonary micrometastases, that had been treated with anti-IFN-γ mAb. In these experiments (Fig. 2, Exps. 1-5), TIL were tested for lymphokine secretion and cytolysis on the day before the induction of pulmonary micrometastases. 2 d after metastasis induction, 200 µg of hamster anti-murine IFN-γ mAb or 200 µg of hamster IgG was injected intraperitoneally. 24 h later, 5 × 10^6 TIL were injected intravenously (except in Exp. 5, where 2 × 10^6 TIL were injected). On days 3-7, 10,000 U rIL-2 were given intraperitoneally twice a day, and on day 15, the mice were randomized and lung metastases were counted.

The TIL in all five experiments specifically secreted IFN-γ (Fig. 2). The TIL in Exps. 1, 2, and 5 also specifically secreted TNF and were specifically cytolytic. The TIL in Exps. 3 and 4 nonspecifically secreted low amounts of TNF and were marginally cytolytic. Treatment of mice that received control hamster IgG with TIL plus rIL-2 in all experiments significantly decreased the mean number of pulmonary metastases compared with the group receiving rIL-2 alone (Wilcoxon rank sum test for TIL + Hamster IgG vs. IL-2: Exp. 1, p = 0.002; Exp. 2, p = 0.03; Exp. 3, p = 0.003; Exp. 4, p = 0.002; Exp. 5, p = 0.002). In Exps. 2, 3, and 5, anti-IFN-γ mAb completely inhibited the effectiveness of the TIL (TIL + anti-IFN-γ mAb vs. IL-2, p = NS). In Exps. 1 and 4, TIL still had some effect when administered with anti-IFN-γ mAb, but anti-IFN-γ mAb significantly inhibited their effectiveness when compared with TIL plus hamster IgG control antibody (TIL + hamster IgG vs. TIL + anti-IFN-γ: Exp. 1, p = 0.02; Exp. 4, p = 0.025). In these experiments (Fig. 2, Exps. 1-5), TIL that specifically secreted IFN-γ were adoptively transferred to mice, bearing pulmonary micrometastases, that had been treated with anti-IFN-γ mAb. In these experiments (Fig. 2, Exps. 1-5), TIL were tested for lymphokine secretion and cytolysis on the day before the induction of pulmonary micrometastases. 2 d after metastasis induction, 200 µg of hamster anti-murine IFN-γ mAb or 200 µg of hamster IgG was injected intraperitoneally. 24 h later, 5 × 10^6 TIL were injected intravenously (except in Exp. 5, where 2 × 10^6 TIL were injected). On days 3-7, 10,000 U rIL-2 were given intraperitoneally twice a day, and on day 15, the mice were randomized and lung metastases were counted.

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**LYMPHOKINE SECRETION, CYTOLYSIS**

**IN VITRO**

| EXP. # | TIL 105-53 | TIL 205-87 | TIL 205-87 |
|--------|-------------|-------------|-------------|
| TNF    | 10-100      | 50-100      | 25-100      |
| IFN-γ  | 0-100       | 0-100       | 0-100       |
| % Lysis| 0-25        | 0-25        | 0-25        |

**IN VIVO EFFICACY**

![Graphs of tumor metastasis and TNF secretion](image)

**Figure 3.** Effect of anti-TNF antibody on TIL effectiveness. On day -1, TIL were tested for lymphokine secretion and cytolysis as described in Materials and Methods. TNF is expressed as picograms per 2.5 × 10⁵ cells/ml; IFN-γ is expressed as units per 2.5 × 10⁵ cells/ml; percent lysis is measured in a 4-h ⁵¹Cr release assay at an E/T ratio of 50:1. Pulmonary metastases were induced by intravenous injection on day 0. On day 3, 1 mg of anti-TNF antibody or rabbit IgG control was injected intraperitoneally. 6 h later, 5 × 10⁶ TIL were transferred, and 10,000 U rIL-2 was given intraperitoneally twice a day on days 3–7. 1 mg of anti-TNF antibody or rabbit IgG control was given intraperitoneally on days 4–7. The mean ± SEM number of metastases on day 15 is plotted. By Wilcoxon rank sum analysis, Exps. 1 and 2: TIL + rabbit IgG vs. TIL + anti-TNF, p = 0.037; Exps. 4 and 5: TIL + rabbit IgG vs. IL-2, p = 0.002; TIL + anti-TNF vs. IL-2, p = NS.

TNF antibody completely abrogated the effectiveness of the TIL (Fig. 3, Exps. 4 and 5: TIL + rabbit IgG vs. IL-2, p = 0.002; TIL + anti-TNF vs. IL-2, p = NS). When a third TIL culture, 233-92B, which did not specifically secrete TNF, was tested, TIL plus anti-TNF antibody were effective, but significantly less so than TIL plus rabbit IgG control (Fig. 3, Exp. 3: TIL + anti-TNF vs. TIL + rabbit IgG, p = 0.037). Thus, it appears that for some but not all TIL, secretion of TNF may play a role in tumor eradication.

**Discussion**

The in vitro cytolytic specificity of murine TIL (Fig. 1 and Table 1) indicates that antigens are detected by TIL on some MCA-induced tumors and not on others. The observation that the therapeutic effectiveness of these TIL in vivo is similarly specific (Fig. 1 and Table 1) suggests that the antigenic determinant identified in vitro is also detected in vivo and is a determinant of importance in the eradication of tumor by TIL. However, the correlation between in vitro cytotoxicity and in vivo effectiveness does not prove that the mechanism of tumor regression in vivo is direct cytolysis by adoptively transferred cells. In fact, the demonstration that a noncytolytic CD8⁺ TIL culture was effective in vivo (Tables 2 and 3) indicates that cytotoxicity is not a necessary requirement for CD8⁺ lymphocytes to mediate tumor regressions. The observation that these nonlytic TIL were effective even when a host CTL response was ablated with 500-rad irradiation indicates that these TIL are not activating host CTL, but instead may be mediating tumor regression through their effects on radiation-resistant host effector cells such as macrophages.

These results led us to investigate whether TIL secreted lymphokines, which might activate host effector cells. There is evidence that certain lymphokines are secreted by cytolytic T cells. Murine CD8⁺ lymphocyte clones have been shown to secrete IFN-γ in response to stimulation with allogeneic cells (27), Con A (28), and anti-CD3 mAb (29). Anti-CD3 mAb-stimulated clones were also found to secrete TNF, GM-CSF, and IL-3 (29). Furthermore, one group has reported that a cytolytic CD8⁺ lymphocyte clone specifically reactive with a murine malignant glioma will secrete IFN-γ in vitro when stimulated with tumor cells (13).

The data in Tables 8 and 10 show that IFN-γ was specifically secreted in 14 of 15 tests on TIL cultures that were simultaneously shown to be therapeutically effective, and in 23 of 23 tests on effective TIL “clones”. When bulk TIL were analyzed, in 10 of 11 tests, ineffective TIL did not specifically secrete IFN-γ. In contrast, only 8 of 15 effective TIL cultures were specifically cytolytic (Table 7). These data indicate that secretion of IFN-γ may be a better predictor of the in vivo antitumor efficacy of a TIL culture than cytotoxicity. We are currently evaluating whether a similar relationship exists between the pattern of lymphokine secretion and therapeutic efficacy of human TIL cultures.

The high correlation observed between IFN-γ secretion in vitro and therapeutic efficacy suggests that the secretion of IFN-γ is a necessary part of the mechanism of tumor eradication by CD8⁺ lymphocytes. The five experiments (Fig. 2) demonstrating that anti-IFN-γ mAb inhibits the effectiveness of four different TIL cultures from three different tumors strongly support the hypothesis that secretion of IFN-γ is an essential part of the mechanism whereby cytolytic CD8⁺ lymphocytes mediate tumor regression.

The role of TNF in the mechanism of tumor eradication by CD8⁺ lymphocytes has also been addressed by these experiments. The observation that many TIL that did not specifically secrete TNF were effective in vivo (Tables 4 and 7) indicates that specific secretion of TNF is not necessary for the in vivo antitumor effectiveness of CD8⁺ lymphocytes. However, the constitutive release of low levels of TNF...
by these TIL may, in concert with other lymphokines, have
a role in tumor regression. Since TIL that specifically secreted
TNF were therapeutically effective in six of six tests on TIL
cultures and in 21 of 22 tests on TIL clots, the specific secre-
tion of TNF may be a sufficient condition for in vivo effec-
tiveness. Of note, all of these effective TIL also specifically
secreted IFN-γ.

The effect of anti-TNF antibody on TIL effectiveness in
vivo (Fig. 3) demonstrates that for one TIL clone (205-87),
TNF secreted either by TIL, or by host effector cells acti-
vated by TIL, plays an integral role in tumor eradication.
It is possible that the anti-TNF antibody abrogated TIL effec-
tiveness by binding to and eliminating TNF-secreting TIL.
However, we feel that this is highly unlikely since we have
not been able to demonstrate binding of the anti-TNF antici-
body used in the experiments reported here to activated mac-
rophages or stimulated TIL (our unpublished observations).
The lack of effect of anti-TNF antibody on TIL culture 105-
53, although this culture specifically secretes TNF, indicates
that for this culture TNF secretion does not appear to be
a part of the mechanism of tumor regression.

The specific local secretion of IFN-γ or TNF by TIL at
the site of a tumor may lead to tumor regression in several
ways. There is in vitro evidence that IFN-γ and TNF are
directly cytotoxic to some tumors (30, 31). However, nei-
ther lymphokine is cytotoxic at high concentrations in vitro
to the MCA sarcomas we have used (our unpublished obser-
vations). Furthermore, when supernatants from TIL that were
high lymphokine secretors were incubated with multiple MCA
targets in 18-h 51Cr release assays, no cytotoxicity was ob-
erved (our unpublished observations). Therefore, it is un-
likely that lymphokines secreted by TIL are directly cyto-
toxic in vivo. IFN-γ has also been implicated in the generation
of CTL from resting precursors (32, 33). Although the mice
used in the antibody blocking experiments described here were
not pre-irradiated, the ability of TIL to effectively eradicate
tumors in pre-irradiated animals (Tables 1 and 3) makes it
unlikely that IFN-γ activation of host CTL plays an impor-
tant role in tumor eradication.

Specific local secretion of IFN-γ or TNF by TIL may modulate
the tumor to increase its lysability by CTL. IFN-γ has been
shown in vitro and in vivo to upregulate MHC class I an-
tigens on the sarcomas used in our experiments (34, 35). This
increase in MHC class I expression may explain the observa-
tion that MC 38 tumor cells treated with IFN-γ and TNF
in vitro were lysed by a TIL that failed to lyse untreated tumor
cells, whereas nonspecific LAK lysis of the target was un-
affected (35). Furthermore, administration of IFN-γ and TNF
with these TIL plus rIL-2 enhanced the in vivo effectiveness
of the TIL (35). Therefore, through modification of the tumor,
secreted IFN-γ and TNF may enhance a cytolytic mecha-
nism of tumor eradication by TIL.

The local secretion of IFN-γ by a CD8+ lymphocyte at
a tumor site may also lead to tumor regression by initiating
a DTH reaction. Cloned murine CD8+ lymphocytes have been
shown to be capable of mediating DTH reactions (36).
The importance of IFN-γ in the initiation of DTH has been
established through the use of anti-IFN-γ antibodies to block
DTH in mice (37) and to prevent lymphocyte recruitment
at the site of LPS-induced DTH in the rat (38). Adherence of
lymphocytes to endothelial cells in the post-capillary venule,
the first step in the migration of lymphocytes out of the blood
to sites of inflammation, is markedly stimulated by IFN-γ
in vitro (39). Thus, secretion by TIL of IFN-γ at the site
of a tumor may serve as a homing signal for lymphocyte
recruitment. Furthermore, local secretion of IFN-γ (and TNF)
at a tumor site may induce the priming (and triggering) steps
necessary for the activation of cytotoxic macrophages (40-42).
The levels of IFN-γ present in supernatants of TIL that have
been stimulated by tumor in vitro (i.e., 20 U/ml) are sufficient
to activate tumoricidal macrophages (26, 41). Also consist-
ent with a role for macrophages as the effectors of CD8+
lymphocyte-mediated tumor regressions are the results of ex-
periments in which tumor-bearing mice are pre-treated with
carageenan or Trypan blue, which are toxic to macrophages
but not lymphocytes. Adoptive immunotherapy with immune
splenocytes was ineffective when mice were pre-treated with
these agents (43).

A role for macrophages in a DTH reaction as at least a
part of the mechanism by which CD8+ lymphocytes me-
diate tumor regressions has important biological implications.
It has been proposed that immunotherapy failures, resulting
from the treatment of tumors composed of cells that may
have considerable antigenic heterogeneity with antigen-specific
agents (e.g., TIL), may be due to the selection of resistant
cells with quantitative or qualitative differences in antigen
expression (44). The nonselective destructive capabilities
of macrophages have made it extremely difficult to select, in
vitro, tumor cells resistant to macrophage-mediated lysis (45).
Therefore, if macrophages are the effector arm of a process
initiated by a specific TIL, it would be unlikely that differ-
ences in antigen expression would account for immunotherapy
failures.

Knowledge of the characteristics of adoptively transferred
cells that are important determinants of their ability to eradi-
cate tumor cells in vivo may help in the development of more
effective adoptive immunotherapy strategies. We are currently
investigating different methods of TIL culture to optimize
the growth conditions for TIL that are high lymphokine
secretors. Furthermore, the transduction of genes coding for
lymphokines involved in the process of tumor eradication into
TIL may allow higher concentrations of lymphokines to be
released at the tumor site, which may enhance the immune-
mediated process of tumor destruction. We have adoptively
transferred TIL to melanoma patients that were transduced
with a gene conferring Neo/mycin resistance and demonstrated
that transduced TIL localize to tumor deposits and persist
in the host (46). Recently, Miyatake and co-workers (47, 48)
transduced IFN-γ cDNA into a cloned CTL specific for a
murine glioma, and observed that the transduced clone was
more effective than the parent in Winn assays and in the im-
munotherapy of subcutaneous tumors. We are actively inves-
tigating the properties of human TIL transduced with the
cDNA for TNF and other lymphokines.
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Address correspondence to James J. Mulé, Surgery Branch, National Cancer Institute, Building 10, Room 2B46, National Institutes of Health, Bethesda, MD 20892.

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References

1. Chauvenet, P.H., C.P. McArthur, and R.T. Smith. 1979. Demonstration in vitro of CTL with apparent specificity toward tumor specific transplantation antigens on chemically induced tumors. J. Immunol. 123:2575.

2. Burton, R.C., and N.L. Warner. 1977. In vitro induction of tumor specific immunity. IV. Specific adoptive immunotherapy with CTL induced in vitro to plasmacytoma antigens. Cancer Immunol. Immunother. 2:91.

3. Fernandez-Cruz, E., B. Halliburton, and J.D. Feldman. 1979. In vivo elimination by specific effector cells of an established syngeneic rat Moloney virus induced sarcoma. J. Immunol. 123:1772.

4. Kedar, E., M. Schwartzbach, Z. Raanan, and S. Hefetz. 1977. Demonstration in vitro of CTL with apparent specificity toward tumorspecific transplantation antigens on chemically induced tumors. J. Immunol. 123:2575.

5. Rosenau, W., and D.L. Morton. 1966. Tumor specific inhibition of growth of MCA induced sarcomas in vivo and in vitro by sensitized isologous lymphoid cells. J. Natl. Cancer Inst. 36:825.

6. Greenberg, P.D., M.A. Cheever, and A. Fefer. 1981. Eradication of disseminated murine leukemia by chemoinmunotherapy with cytoxan and adoptively transferred immune syngeneic Lyt-1-2- lymphocytes. J. Exp. Med. 145:946.

7. Prat, M., M.F. DiRenzo, and P. Comoglio. 1983. Characterization of T lymphocytes mediating in vivo protection against RSV-induced murine sarcomas. Int. J. Cancer. 31:757.

8. Prat, M., S. Berti, M. Amedeo, S. Landolf, and P.M. Comoglio. 1987. Monoclonal antibody against IFN gamma abrogates in vivo tumor immunity against RSV-induced murine sarcomas. J. Immunol. 138:4530.

9. Bhan, A.K., L.L. Perry, H. Cantor, R.T. McCluskey, B. Benacerraf, and M.I. Greene. 1981. The role of T-cell subsets in the rejection of MCA induced sarcoma (S1509a) in syngeneic mice. Am. J. Pathol. 102:20.

10. Fujiwara, H., M. Fukuyawa, T. Yoshioka, H. Nakajima, and T. Hamaoka. 1984. The role of tumor specific Lyt-1-2- T cells in eradicating tumor cells in vivo. J. Immunol. 133:1671.

11. Greenberg, P.D., D.E. Kern, and M.A. Cheever. 1985. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1-2- T cells. J. Exp. Med. 161:1122.

12. Prat, M., S. Berti, M. Amedeo, S. Landolf, and P.M. Comoglio. 1987. Monoclonal antibody against IFN gamma abrogates in vivo tumor immunity against RSV-induced murine sarcomas. J. Immunol. 138:4530.

13. Yamasaki, T., H. Handa, J. Yamashita, Y. Watanabe, Y. Namba, and M. Hamaoka. 1984. Specific adoptive immunotherapy with tumor specific CTL clone for murine malignant gliomas. Cancer Res. 44:1776.

14. Dailey, M.O., E. Pillemer, and I.L. Weissman. 1982. Protection against syngeneic lymphoma by a long lived cytotoxic T cell clone. Proc. Natl. Acad. Sci. USA. 79:5384.

15. Rosenberg, M., and S.A. Rosenberg. 1984. Generation of lytic and proliferative lymphoid clones to syngeneic tumor: in vitro and in vivo studies. J. Natl. Cancer Inst. 72:1161.

16. Greenberg, P.D. 1986. Therapy of murine leukemia with cyclophosphamide and immune Lyt-2- cells: cytolytic T cells can mediate eradication of disseminated leukemia. J. Immunol. 136:1917.

17. Spiess, P.J., J.C. Yang, and S.A. Rosenberg. 1987. In vivo antitumor activity of tumor infiltrating lymphocytes expanded in R1-2. J. Natl. Cancer Inst. 79:1067.

18. Barth, R.J., S.N. Bock, J.J. Mulé, and S.A. Rosenberg. 1990. Unique murine tumor associated antigens identified by tumor infiltrating lymphocytes. J. Immunol. 144:1531.

19. Shu, S., and S.A. Rosenberg. 1985. Adoptive immunotherapy of newly induced murine sarcomas. Cancer Res. 45:1657.

20. Yang, J.C., D. Perry-Lalley, and S.A. Rosenberg. 1990. An improved method for growing murine TIL with in vivo antitumor activity. J. Biol. Response Modif. 9:149.

21. Wexler, H. 1966. Accurate identification of experimental pulmonary metastases. J. Natl. Cancer Inst. 36:641.

22. Suzuki, Y., M.A. Orellana, R.D. Schreiber, and J.S. Remington. 1988. IFN gamma: the major mediator of resistance against Toxoplasma gondii. Science (Wash. DC). 240:516.

23. Buchmeier, N.A., and R.D. Schreiber. 1985. Requirement of endogenous IFNgamma production for resolution of Listeria monocytogenes infection. Proc. Natl. Acad. Sci. USA. 82:7404.

24. Fraker, D.L., H.N. Langstein, and J.A. Norton. 1989. Passive immunization against tumor necrosis factor partially abrogates interleukin 2 toxicity. J. Exp. Med. 170:1015.

25. Anderson, R.E., and Warner, N.L. 1976. Ionizing radiation and the immune response. Adv Immunol. 24:215.

26. Barth, R.J., J.J. Mulé, A.L. Asher, M.G. Sands, and S.A. Rosenberg. 1991. Unique murine tumor-associated antigens identified by TIL: tumor specific secretion of IFN gamma and TNF. J. Immunol. In press.

27. Kelso, A., and A.L. Glassbrook. 1984. Secretion of IL-2, MAF, IFN and CSF by alloreactive T lymphocyte clones. J. Immunol. 132:2924.

28. Guerne, P., P. Piguet, and P. Vassali. 1984. Production of IL-2, IL-3 and IFN by mouse T lymphocytes of Lyt-2+ and -2- phenotype. J. Immunol. 132:1869.

29. Gajewski, T.F., and F.W. Fitch. 1990. Anti-proliferative effect of interferon gamma: the major mediator of resistance against Toxoplasma gondii, Science (Wash. DC). 240:516.

30. Tyring, S., G.R. Klimpel, W.R. Fleischmann, and S. Baron. 1991. Direct cytolysis by partially purified preparations of immune IFN. Int. J. Cancer. 30:59.
31. Sugarman, B.J., B.B. Aggarwal, P.E. Hass, I.S. Figani, M.A. Palladino, and H.M. Shepard. 1985. Recombinant \( \text{IFN-\( \alpha \) :} \) effects on proliferation of normal and transformed cells in vitro. *Science (Wash. DC)*. 230:943.

32. Maraskovsky, E., W. Chen, and K. Shortman. 1989. IL-2 and IFN gamma are 2 necessary lymphokines in the development of CTL. *J. Immunol.* 140:1210.

33. Giovarelli, M., A. Santoni, C. Jemma, T. Musso, A. Giuffrida, G. Cavallo, S. Landolfo, and G. Forni. 1989. Obligatory role of IFN gamma in induction of lymphokine activated and T lymphocyte killer activity, but not in boosting of natural cytotoxicity. *J. Immunol.* 141:2831.

34. Weber, J.S., and S.A. Rosenberg. 1988. Modulation of murine tumor MHC antigens by cytokines in vivo and in vitro. *Cancer Res.* 48:5818.

35. Fox, B.A., P.J. Spiess, A. Kasid, R. Puri, J.J. Mulé, J.S. Weber, and S.A. Rosenberg. 1990. In vitro and in vivo antitumor properties of a T cell clone generated from murine TIL. *J. Biol. Resa Mod.* 9:499.

36. Lin, Y, and B.A. Askonas. 1981. Biological properties of an influenza A virus specific killer T cell clone. *J. Exp. Med.* 154:225.

37. Fong, T.A.T., and T.R. Mosmann. 1989. The role of IFN gamma in DTH mediated by TH1 clones. *J. Immunol.* 143:2887.

38. Issekutz, T.B., J.M. Stoltz, and Pvd. Meide. 1988. Lymphocyte recruitment in DTH. The role of IFN gamma. *J. Immunol.* 140:2989.

39. Issekutz, T.B. 1990. Effects of 6 different cytokines on lymphocyte adherence to microvascular endothelium and in vivo lymphocyte migration in the rat. *J. Immunol.* 144:2140.

40. Meltzer, M. 1981. Macrophage activation for tumor cytotoxicity: characterization of priming and trigger signals during lymphokine activation. *J. Immunol.* 127:179.

41. Pace, J.L., SW. Russell, B.A. Torres, H.M. Johnson, and P.W. Gray. 1983. Recombinant mouse IFN gamma induces the priming step in macrophage activation for tumor cell killing. *J. Immunol.* 130:2011.

42. Schultz, R.M., and W.J. Kleinschmidt. 1983. Functional identity between murine IFN gamma and macrophage activating factor. *Nature (Lond.)*. 305:239.

43. Mulé, J.J., M. Rosenstein, S. Shu, and S.A. Rosenberg. 1985. Eradication of a disseminated syngeneic mouse lymphoma by systemic adoptive transfer of immune lymphocytes and its dependence upon a host component. *Cancer Res.* 45:526.

44. Topalian, ST., A. Kasid, and S.A. Rosenberg. 1990. Immunoselection of a human melanoma resistant to specific lysis by autologous TIL. *J. Immunol.* 144:4487.

45. Fogler, W.E., and I.J. Fidler. 1985. Nonselective destruction of murine neoplastic cells by syngeneic tumorricidal macrophages. *Cancer Res.* 45:14.

46. Rosenberg, S.A., P. Aebersold, K. Cornetta, A. Kasid, R. Morgan, R. Moen, E. Karson, M. Lotze, J. Yang, S. Topalian, M. Merino, K. Culver, A. Miller, R. Blaese, and W. Anderson. 1990. Gene transfer into humans: immunotherapy of patients with advanced melanoma, using TIL modified by retroviral gene transduction. *N. Engl. J. Med.* 323:570.

47. Nishihara, K., S. Miyatake, T. Sakata, J. Yanashita, H. Kikuchi, Y. Kawade, Y. Wu, Y. Namba, M. Hanaoka, and Y. Watanabe. 1988. Augmentation of tumor targeting in a line of glioma specific mouse CTL by retroviral expression of mouse IFN gamma cDNA. *Cancer Res.* 48:4730.

48. Miyatake, S., K. Nishihara, H. Kikuchi, J. Yamashita, Y. Namba, M. Namaoka, and Y. Watanabe. 1990. Efficient tumor suppression by glioma specific murine CTL transfected with the IFN gamma gene. *J. Natl. Cancer. Inst.* 82:217.