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

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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 cytolysis. 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 mediate 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

[1] *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/plate (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 × 10^4 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 rIL-2/ml 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 10^5 irradiated (3,000 rad) B6 splenocytes/well. 1 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 ^{51}Cr release assays were performed as previously described (18). Briefly, fresh tumor targets were labeled with ^{51}Cr 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). 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 horseradish peroxidase–conjugated donkey anti-goat Ig. 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 rIFN-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 × 10^5 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 "cloid" 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 × 10^5 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 ^{51}Cr 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 ^{51}Cr release assay and specifically lysed their tumor of origin. Concurrently, 5×10^6 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 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

Table 1. *Specifically Cytolytic TIL Are Specifically Effective In Vivo*

Effector cells		In vitro MCA tumor target: 105	205	207
		*$LU_{20}/10^6$ cells**		
105 TIL		100	<0.1	<0.1
205 TIL		<0.1	10	<0.1
207 TIL		<1	<1	>250
LAK		105	125	100
Effector cells	**IL-2**	**In vivo MCA tumor metastases: 105**	**205**	**207**
		mean no. of metastases (SEM)‡		
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 ^{51}Cr release assay.
‡ 5×10^6 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 enumerted on day 15.
§ Significantly different than rIL-2 alone group ($p < 0.002$).

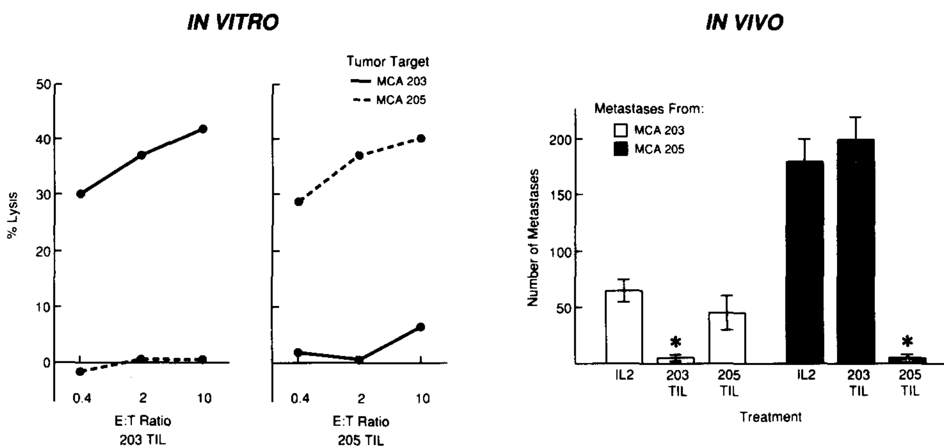

Figure 1. Specifically cytolytic TIL are specifically effective in vivo. TIL lysed autologous tumor but not another syngeneic sarcoma in a 4-h ^{51}Cr release assay. Concurrently, 5×10^6 TIL were adoptively transferred to mice bearing 3-d pulmonary metastases. 10,000 U rIL-2 was given to all groups twice a day on days 3–7, and metastases were counted on day 15. Treatment with each TIL significantly reduced the number of metastases in mice bearing autologous tumor ($p \leqslant 0.004$), but had no effect on the irrelevant tumor.

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

205–73 TIL culture age	Effector		
	TIL 205-73	Another 205 TIL Cx*	LAK
d			
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 cytolysis of MCA 205 target in 4-h ^{51}Cr 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 ^{51}Cr 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 ^{51}Cr 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 ^{51}Cr 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 Tumor. These results led us to investigate whether TIL, when stimulated with tumor, secreted lymphokines 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

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

Treatment	Pre-irradiation	Exp. 1*	Exp. 2‡
		No. of metastases (SEM)	
HBSS	−	>250	>250
HBSS + IL-2§	−	180 (23)	>250
HBSS + IL-2§	+	>250	>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 × 10^6 TIL/mouse.
‡ TIL culture 205–73 was 90 d old at the time of adoptive transfer of 5 × 10^6 TIL/mouse.
§ On days 3–7 after tumor injection, 10,000 U rIL-2 was injected intraperitoneally twice a day.
‖ Significantly different from the group treated with rIL-2 alone ($p < 0.002$).

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

TIL culture	Tumor of origin of TIL	MCA tumor stimulator*			
		None	105	233	205
		U/2.5 × 10^5 TIL/ml			
105–53	105	2	44	6	3
233–92B	233	0	7	50	2
233–90D	233	3	9	45	6

* All TIL cultures at 2.5 × 10^5 cells/ml were stimulated simultaneously with 5 × 10^5 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[§]		
			None	Rel.	Irr.	Irr.	None	Rel.	Irr.	Irr.	Rel.	Irr.	Irr.
			U/2.5 × 10^5 cells/ml				*pg/2.5 × 10^5 cells/ml*						
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
90D	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
53	105	75	2	70	19	8	10	480	0	0	52	9	2
53	105	99	1	42	20	5	5	350	110	65	33	−2	10
53	105	74	1	53	6	17	20	905	140	200	45	0	4
87	205	99	4	65	21	ND	200	1,330	300	ND	83	1	ND
87	205	96	4	80	17	11	50	1,020	45	40	24	−6	ND
87	205	94	6	53	12	7	25	825	185	50	42	8	3
93B	205	82	1	21	3	2	150	0	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.

‡ 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 ^{51}Cr 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 × 10^5 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 × 10^5 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§		
			None	Rel.	Irr.	Irr.	None	Rel.	Irr.	Irr.	Rel.	Irr.	Irr.
			U/2.5 × 10⁵ cells/ml				*pg/2.5 × 10⁵ cells/ml*						
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 ^{51}Cr 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.

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 ^{51}Cr 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 nonlytic, 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 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 × 10⁵ cells/ml	*pg/2.5 × 10⁵ cells/ml*	
Effective TIL§	15	45 (5)	416 (105)	26 (6)
Ineffective TIL	11	11 (3)	63 (15)	2 (2)
p value‖		<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 ^{51}Cr 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.
‖ Student's t test for the difference between means of effective and ineffective TIL.

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	1
	Nonspecific	1	10
TNF secretion	Specific‡	6	0
	Nonspecific	9	11
Cytolysis	Specific§	8	0
	Nonlytic	7	11

* 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 × 10^5 cells/ml (and >100 pg/2.5 × 10^5 cells/ml).
§ Cytolysis was considered specific in a 4-h ^{51}Cr 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	5
	Nonspecific	0	5
TNF secretion	Specific‡	21	1
	Nonspecific	2	9
Cytolysis	Specific§	14	1
	Nonlytic	9	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 × 10^5 cells/ml (and >100 pg/2.5 × 10^5 cells/ml).
§ Cytolysis was considered specific in a 4-h ^{51}Cr release assay if relevant tumor lysis was ≥20% and irrelevant tumor lysis was <10% at an E/T ratio of 10:1.

peared to be sufficient for in vivo effectiveness (in six of six tests that showed specific TNF secretion, the TIL were effective; TIL were effective in eight of eight tests that showed cytolytic specificity).

To further investigate and substantiate these relationships, TIL "cloids" were derived from the MC 38 adenocarcinoma by plating dilute concentrations of TIL immediately after immunobeading in 96-well plates as described in Materials and Methods. In three separate experiments, a total of 33 "cloids" were simultaneously tested against MC 38 and irrelevant MCA 102 for in vitro lymphokine secretion and cytolysis and were adoptively transferred to mice bearing MC 38 pulmonary micrometastases. Stimulations for lymphokine secretion were performed as previously described for the bulk TIL cultures and the same criteria for specific lymphokine secretion were used. Likewise, the same criteria for specific cytolysis were used, although TIL "cloids" were tested at an E/T ratio of 10:1. A TIL "cloid" was considered effective if the mean number of pulmonary metastases in the treated group was at least 25% less than the number in the group treated with rIL-2 alone. The mean and median percent reductions in pulmonary metastases for the 23 effective TIL "cloids" tested were 87 and 99%, respectively.

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 × 10^5

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 × 10^5 cells/ml	*pg/2.5 × 10^5 cells/ml*	
Effective TIL "cloids"§	23	60 (4)	465 (64)	28 (2)
Ineffective TIL "cloids"	10	19 (5)	77 (51)	12 (3)
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 ^{51}Cr 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.

LYMPHOKINE SECRETION, CYTOLYSIS *IN VITRO*

EXP. # **MCA Tumor Stimulator or Target**

1. TIL 105-53

	None	105	233	205
IFN-γ	2	70	19	8
TNF	10	480	0	0
% Lysis	-	52	9	2

2. TIL 105-53

	None	105	205	207
IFN-γ	1	53	6	17
TNF	20	905	140	200
% Lysis	-	45	0	4

3. TIL 205-93B

	None	205	105	233
IFN-γ	1	21	3	2
TNF	150	0	0	0
% Lysis	-	12	-8	ND

4. TIL 233-92B

	None	233	105	205
IFN-γ	0	41	9	2
TNF	250	75	75	10
% Lysis	-	16	-5	-1

5. TIL 205-87

	None	205	105	203
IFN-γ	4	80	17	11
TNF	50	1020	45	40
% Lysis	-	24	ND	-6

***IN VIVO* EFFICACY**

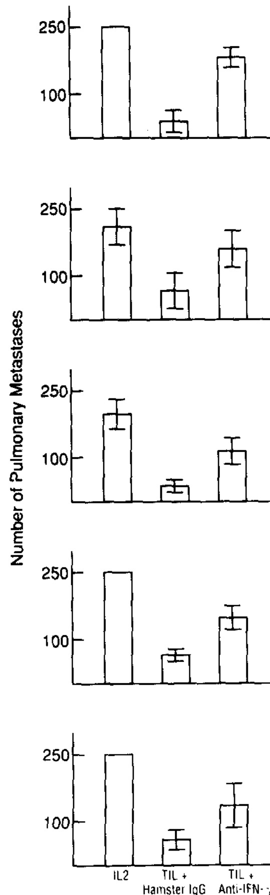

Figure 2. Inhibition of TIL effectiveness by anti-IFN-γ mAb. On day −1, TIL were tested for lymphokine secretion and cytolysis as described in Materials and Methods. IFN-γ is expressed as units per 2.5 × 10^5 cells/ml; TNF is expressed as picograms per 2.5 × 10^5 cells/ml; percent lysis is measured in a 4-h ^{51}Cr release assay at an E/T ratio of 50:1. Pulmonary metastases were induced by intravenous injection on day 0. On day 2, 200 μg of anti-IFN-γ mAb or hamster IgG control was injected intraperitoneally. 5 × 10^6 TIL were adoptively transferred on day 3, and 10,000 U rIL-2 was given intraperitoneally twice a day on days 3–7. The mean ± SEM number of metastases on day 15 is plotted. Treatment of mice that received control hamster IgG with TIL + rIL-2 in all experiments significantly decreased the number of pulmonary metastases compared with the group receiving rIL-2 alone (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 TIL (TIL + anti-IFN-γ vs. IL-2, p = NS). In Exps. 1 and 4, anti-IFN-γ inhibited TIL effectiveness when compared with TIL + hamster IgG control (TIL + anti-IFN-γ vs. TIL + hamster IgG: Exp. 1, p = 0.02; Exp. 4, p = 0.025).

cells/ml, $p < 0.0001$) and TNF (465 ± 64 vs. 77 ± 51 pg/2.5 × 10^5 cells/ml, p = 0.0008) and 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 "cloids" specifically secreted IFN-γ. All but one of the TIL "cloids" that specifically secreted TNF (21 of 22) were effective. Similarly, all but one of the TIL "cloids" that were specifically cytolytic (14 of 15) were effective.

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). 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-

LYMPHOKINE SECRETION, CYTOLYSIS *IN VITRO*

EXP. # MCA Tumor Stimulator or Target

1. TIL 105-53

	None	105	233	205
TNF	10	480	0	0
IFN-γ	2	70	19	8
% Lysis	-	52	9	2

2. TIL 105-53

	None	105	205	207
TNF	20	905	140	200
IFN-γ	1	53	6	17
% Lysis	-	45	0	4

3. TIL 233-92B

	None	233	105	205
TNF	25	75	75	10
IFN-γ	0	41	9	2
% Lysis	-	16	-5	-1

4. TIL 205-87

	None	205	105	203
TNF	50	1020	45	40
IFN-γ	4	80	17	11
% Lysis	-	24	ND	-6

5. TIL 205-87

	None	205	105	203
TNF	25	825	185	50
IFN-γ	6	53	12	7
% Lysis	-	42	8	3

***IN VIVO* EFFICACY**

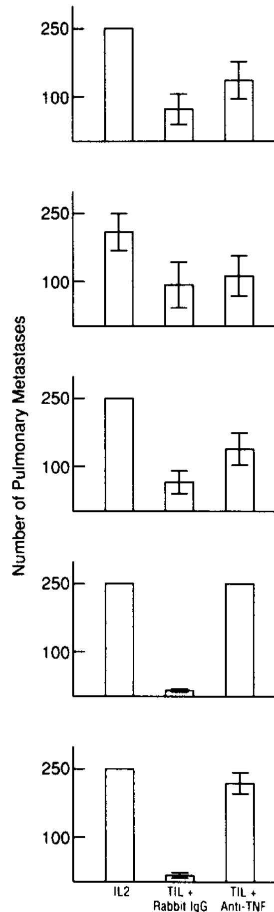

Figure 3. Effect of anti-TNF antibody on TIL effectiveness. On day −1, TIL were tested for lymphokine secretion and cytolysis as described in Materials and Methods. TNF is expressed as picograms per 2.5×10^5 cells/ml; IFN-γ is expressed as units per 2.5×10^5 cells/ml; percent lysis is measured in a 4-h ^{51}Cr 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, 5×10^6 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 = NS; Exp. 3: 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 "cloids". 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 cloids, the specific secretion of TNF may be a sufficient condition for in vivo effectiveness. 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 activated by TIL, plays an integral role in tumor eradication. It is possible that the anti-TNF antibody abrogated TIL effectiveness 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 antibody used in the experiments reported here to activated macrophages 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, neither lymphokine is cytotoxic at high concentrations in vitro to the MCA sarcomas we have used (our unpublished observations). Furthermore, when supernatants from TIL that were high lymphokine secretors were incubated with multiple MCA targets in 18-h ^{51}Cr release assays, no cytotoxicity was observed (our unpublished observations). Therefore, it is unlikely that lymphokines secreted by TIL are directly cytotoxic 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 important role in tumor eradication.

Specific local secretion of IFN-γ or TNF 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 antigens on the sarcomas used in our experiments (34, 35). This increase in MHC class I expression may explain the observation 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 unaffected (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 mechanism 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 consistent with a role for macrophages as the effectors of CD8$^+$ lymphocyte-mediated tumor regressions are the results of experiments 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 mediate 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 differences in antigen expression would account for immunotherapy failures.

Knowledge of the characteristics of adoptively transferred cells that are important determinants of their ability to eradicate 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 Neomycin 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 immunotherapy of subcutaneous tumors. We are actively investigating the properties of human TIL transduced with the cDNA for TNF and other lymphokines.

We thank Drs. H. N. Langstein and J. A. Norton for their generous gift of the anti-TNF antibody, and Dr. A. L. Asher for the use of clone 205-87.

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Received for publication 24 October 1990 and in revised form 11 December 1990.

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