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Therapeutic Effects of Tumor-Reactive Type 1 and Type 2 CD8$^+$ T Cell Subpopulations in Established Pulmonary Metastases[1]

Mark J. Dobrzanski, Joyce B. Reome, and Richard W. Dutton[2]

Cytolytic CD8$^+$ T cells fall into two subpopulations based on cytokine-secretion. Type 1 CD8$^+$ cells (Tc1) characteristically secrete IFN-γ, whereas type 2 CD8$^+$ cells (Tc2) secrete IL-4 and IL-5. We assessed the relative therapeutic effects of adoptively transferred OVA-specific Tc1 and Tc2 CD8$^+$ cells in mice bearing established OVA-transfected B16 melanoma lung metastases. Both Tc1 and Tc2 subpopulations mediated a reduction in lung tumor growth that subsequently prolonged survival times in mice with both early (day 7) and more advanced (day 14) levels of tumor development. CD8$^+$ T cell populations recovered from spleens of tumor-bearing mice receiving Tc1 or Tc2 cells showed markedly enhanced tumor Ag-specific cytolytic and cytokine-releasing activities that correlated with delays in tumor cell growth and progression. Initially, both tumor-reactive Tc1 and Tc2 effector cells accumulated at the tumor site with nearly equal frequency. Tc1 cells persisted, whereas Tc2 cell numbers progressively diminished over time. Titration of Tc1 and Tc2 effector cells showed that protection was dose dependent with the former being 5-fold more effective. Tc2 cells achieved a comparable reduction in lung tumor cell growth at higher concentrations of cell transfer. Tc1 effectors from IFN-γ-deficient mice were less therapeutically effective than wild-type mice, but there was no significant reduction in activity between corresponding Tc2 populations. We speculate that the effectiveness of Tc1 and Tc2 cells may depend on different mechanisms. These studies suggest a potential role for Tc1 and Tc2 CD8$^+$ subpopulations in tumor regression and immunotherapy. ***The Journal of Immunology,*** **1999, 162: 6671–6680.**

CD8 T cells are a major immunological effector cell population mediating resistance to cancer. In addition to their cytolytic activity, CD8$^+$ T lymphocytes can be further classified into two distinct effector cell types based on their cytokine-secreting profiles following Ag encounter (1–5). Type 1 CD8 T cells (Tc1)[3] produce IL-2, IFN-γ, and TNF-α, whereas type 2 CD8 T cells (Tc2) predominantly secrete IL-4, IL-5, and IL-10. Such cytokines not only have diverse inhibitory effects on tumor cells themselves but also affect the nature and development of the immune response by other cells toward progressively growing tumors. Both populations of effector CD8 cells are cytolytic and kill predominantly by the perforin pathway (6–8). However, Tc1 but not Tc2 CD8 effector cells do exhibit some Fas-mediated killing (6, 8). Aside from their cytolytic properties, these distinct cytokine-secreting CD8 subpopulations can also have direct effects on tumor cell populations by noncytolytic mechanisms. Several studies have shown that cytokines, such as IL-4, IFN-γ, and TNF-α, can modulate surface Ag expression and growth kinetics among many tumor cell populations, including several types of disseminated malignancies (7, 9–11). Such effects may include tumor growth inhibition and enhanced tumor immunogenicity by induction of elevated levels of surface MHC class I expression. Alternatively, it has been suggested that cytokines secreted by such polarized CD8$^+$ T cell subpopulations have additional secondary effects that influence the expression of adhesion molecules, chemokine receptors, and other immune cell surface molecules that may aid in proximal lymphocyte activation, cell trafficking, and differentiation (2, 9, 11–14). Such "cellular interplay" can potentially affect both the nature and outcome of antitumor immune responses and tumor progression. Therefore, it seems probable that such CD8$^+$ subpopulations, with their distinct cytokine-secreting profiles, may have differing modes of inducing effective tumor immunity.

Studies in animal models have defined many of the underlying principles, and provided many insights for the development, of adoptive T cell therapy as a viable modality for the treatment of certain human cancers. However, studies examining the requirements for effective T cell function following adoptive transfer suggest that the inability of such T cells to respond at sites of tumor growth may be a major determinant of therapeutic efficacy. Several factors may modify effective antitumor responses by T cells following adoptive transfer, including proper expression of MHC Ags on tumor cell surfaces, the recruitment and induction of other antitumor effector cell populations, and the production or availability of adequate amounts of immunoenhancing cytokines, including IL-2, IFN-γ, and IL-4 (9, 15, 16). It is likely that the multifunctional properties afforded by Tc1 or Tc2 CD8 effector cells can effectively overcome such barriers and promote effective antitumor immunity.

Using a poorly immunogenic OVA Ag-expressing B16 melanoma lung metastases model, we assessed the therapeutic effects of adoptively transferred OVA Ag-specific Tc1 and Tc2 CD8 effector cells in mice bearing established pulmonary malignancy. Systemic transfer of tumor-reactive Tc1 and Tc2 effector cells resulted in the local accumulation of transferred cells at the tumor site that induced tumor regression and subsequently enhanced survival times

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[3] Abbreviations used in this paper: Tc1, CD8$^+$ CTL-producing type 1 cytokines; Tc2, CD8$^+$ CTL-producing type 2 cytokines; B16-OVA, OVA-expressing B16 melanoma; OT-I.IFN-γ mice, IFN-γ knockout mice.

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in mice with established pulmonary tumors. Although therapeutic efficacy appeared greater in mice receiving Tc1 effector cell transfer, systemic antitumor responses induced by both Tc1 and Tc2 effector cell subpopulations were highly tumor Ag specific and appeared to be mediated by different mechanisms of action. Tc1 effector cells generated from IFN-γ knockout mice (OT-1.IFN-γ mice) were less therapeutically effective than those from wild-type OT-I mice, whereas there was no significant reduction in activity between corresponding Tc2 populations. Furthermore, the level of effectiveness by adoptively transferred Tc1 and Tc2 effector cells appeared to be influenced by the stage of tumor development in the lung. We discuss the potential therapeutic roles of tumor-reactive Tc1 and Tc2 effector cell subpopulations in effective antitumor immunity and tumor regression.

Materials and Methods

Animals

Female C57BL/6 mice, 6 to 10 wk of age, were obtained from the Animal Breeding Facility at the Trudeau Institute, Saranac Lake, NY. The OT-I mouse strain, on a C57BL/6 background (H-2^b), was originally obtained from Dr. Michael Bevan (University of Washington, Seattle, WA). These mice express a transgenic TCR Vα2 specific for the SIINFEKL peptide of OVA in the context of MHC class I, H2-K^b (17). Homozygous IFN-$\gamma^{-/-}$ knockout mice, expressing the TCR Vα2 transgene (OT-I.IFN-γ), were generated by backcrossing OT-I mice onto IFN-γ knockout mice (H-2^b). Animals were maintained and treated according to animal care committee guidelines of the National Institutes of Health, Bethesda, MD, and Trudeau Institute.

Tumor cells

The weakly immunogenic OVA-transfected B16 melanoma tumor cell line (B16-OVA) that is syngeneic to the C57BL/6 background was kindly provided by Drs. Edith Lord and John Frelinger (Rochester, NY). EL4 and the derivative OVA-expressing EG.7-OVA cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Spleen and lung cell preparation

Spleens were collected from mice, and single-cell suspensions were prepared, washed twice in HBSS, and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 2 mM pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated FCS (Life Technologies). CD8-enriched T cells were obtained by treating with anti-CD4 (RL172.4), anti-heat-stable Ag (J11D), and anti-MHC class II (D3.137, M5114, CA4) mAbs and complement. For preparation of single-cell suspensions from lung parenchyma, lungs were flushed in situ with HBSS via cannulation of the heart to remove residual intravascular blood pools. Minced lung tissues were incubated for 1 h at 37°C on a rocker platform, in 1.5 ml/lung RPMI 1640 supplemented with DNase I (50 U/ml; Sigma, St. Louis, MO), collagenase I, type 4197 (250 U/ml; Sigma), and 5% FCS. Following incubation, digested lung tissues were mechanically dispersed through stainless steel mesh screens in RPMI 1640–5% FCS. After three washes in RPMI 1640–5% FCS, lymphoid cells were resuspended in RPMI-10% FCS to attain a cell concentration of 1×10^7 viable cells/ml.

Generation of OVA-specific CD8 effector T cells

To obtain effector cells to OVA peptide, single-cell suspensions from spleen and lymph nodes of OT-I mice were washed twice in HBSS and resuspended in RPMI 1640–10% FCS. CD8-enriched T cells were obtained by passing lymphoid cell suspensions through nylon wool columns (4) and treating with anti-CD4 (RL172.4), anti-heat-stable Ag (J11D), anti-MHC class II (D3.137, M5114, CA4) mAbs, and complement. Small resting CD8 T cells were harvested from Percoll gradients (Sigma) and resuspended to appropriate cell concentrations in culture media. Naive CD8 cells were typically 90% pure as demonstrated by immunofluorescent Ab staining. APCs were enriched from spleens of normal C57BL/B6 (B6) mice by anti-Thy-1.2 (HO13.14 and F7D5), anti-CD4 (RL172.4), and anti-CD8 (3.155) mAbs and complement. T cell-depleted APCs were pulsed with OVA peptide (10 M) for 30 min at 37°C and treated with mitomycin C (50 g/ml, Sigma) for an additional 30 min at 37°C. For Tc1 effector cell generation, naive CD8 T cells from OT-I trangenic mice (2×10^5 cells/ml) were stimulated with mitomycin C-treated OVA peptide-pulsed APCs (6×10^5 cells/ml) in the presence of IL-2 (20 U/ml, X63.IL-2 supernatants), IL-12 (2 ng/ml, kindly provided by Dr. Stanley Wolf, Genetics Institute, Cambridge, MA), and anti-IL-4 mAb (200 U/ml, X63.Ag.IL4 supernatants). Alternatively, for Tc2 effector cell generation, naive CD8 T cells from OT-I trangenic mice (2×10^5 cells/ml) were stimulated with mitomycin C-treated OVA peptide-pulsed APCs (6×10^5 cells/ml) in the presence of IL-2 (20 U/ml), IL-4 (200 U/ml, X63.IL-4 supernatants), and anti-IFN-γ mAb (20 g/ml, XMG1.2). Effector cell cultures were incubated for 4 days with additional IL-2 (20 U/ml) added to the cultures on day 2 to promote CD8 cell expansion of Tc1 or Tc2 populations.

Adoptive immunotherapy model

Syngeneic B6 mice were injected i.v. with 5×10^5 B16-OVA melanoma cells to establish pulmonary metastases, and 7 or 14 days following tumor challenge, mice were treated i.v. with various doses of either Tc1 or Tc2 OVA-specific effector T cells. At weekly intervals after therapy, mice were sacrificed for enumeration of pulmonary metastatic nodules. Control groups received no treatment. Metastases on freshly isolated lungs appeared as discrete black pigmented foci that were easily distinguishable from normal lung tissue. The number of pulmonary metastases in treatment and control groups was counted in a blind fashion and reported as the mean ± SEM. Metastatic foci too numerous to count were assigned an arbitrary value of >250.

Assay for cytolytic activity

Cytolytic T cell activity was determined by a standard ^{51}Cr release assay as described previously (4). Briefly, syngeneic EG.7-OVA or EL4 target cells were radiolabeled with 200 μCi of $Na_2{}^{51}CrO_4$ (ICN Radiochemicals, Irvine, CA) for 1 h at 37°C, washed, and resuspended in RPMI 1640–10% FCS. CD8$^+$ T cells were combined with tumor target cells (1×10^4 cells/well) at various E:T ratios in 96-well U-bottom plates (Costar, Cambridge, MA) and incubated for 4 h at 37°C with 5% CO_2. Culture supernatants were harvested and counted in a Wizard automatic gamma counter (WALLAC, Gaithersburg, MD). Spontaneous release of ^{51}Cr was determined by incubation of targets in the absence of effectors, whereas maximum release of ^{51}Cr was determined by incubation of targets in 1% Triton X-100. Results are expressed as the percent specific release and was calculated as [(experimental − spontaneous)/(maximum − spontaneous)] × 100.

Results are also expressed as lytic units/10^6 effector cells. One lytic unit was defined as the number of effector cells required to cause 30% lysis of 10^4 target cells.

Assay for T cell cytokine-releasing activity

Detection of secreted cytokines from supernatants of T cell cultures following restimulation has been described previously (1, 4). Briefly, CD8-enriched T cells (1×10^6/ml) from tumor-bearing mice were restimulated with either mitomycin C-treated EG.7-OVA or EL4 tumor cells (1×10^6/ml) for 24 and 48 h in 1-ml volumes. Culture supernatants were harvested and assessed for cytokine content by cytokine-specific ELISA. Murine IL-5 and IL-4 were measured with anti-IL-5 (TRFK5) and anti-IL-4 (TRFK4) mAbs, respectively. IFN-γ was detected by anti-IFN-γ mAbs R46A2 and XMG1.2. Standard curves were constructed with purified IL-4 (X63.IL-4 supernatants), IL-5 (X63.IL-5 supernatants), and IFN-γ (X63.IFN-g supernatants). Values for T cells or stimulator cells cultured in media alone were negligible.

Flow cytometric analysis

Single-cell suspensions of either spleen or processed murine lung were washed three times in a fluorescent Ab buffer consisting of 1% BSA and 0.02% sodium azide in 0.01 M PBS, pH 7.2. CD8 lymphocytes expressing the TCR Vα2 transgene were phenotyped by their expression of surface markers using direct immunofluorescence staining techniques. Lymphocytes (10^6) were mixed with 100 μl of fluorescent Ab buffer containing 1 μg of both cytochrome-conjugated anti-CD8 (PharMingen, San Diego, CA) and fluorescein-conjugated anti-Vα2 (PharMingen, clone B20.1) mAbs and incubated for 20 min on ice. Stained cell preparations were than washed three times in fluorescent Ab buffer and analyzed by multiparameter flow cytometry using a Becton Dickinson FACScan (San Jose, CA). Ten thousand cells were analyzed per sample with dead cells excluded on the basis of forward light scatter. Surface marker analysis was performed using Cell Quest software, and the percentages and absolute numbers of positive cells were determined.

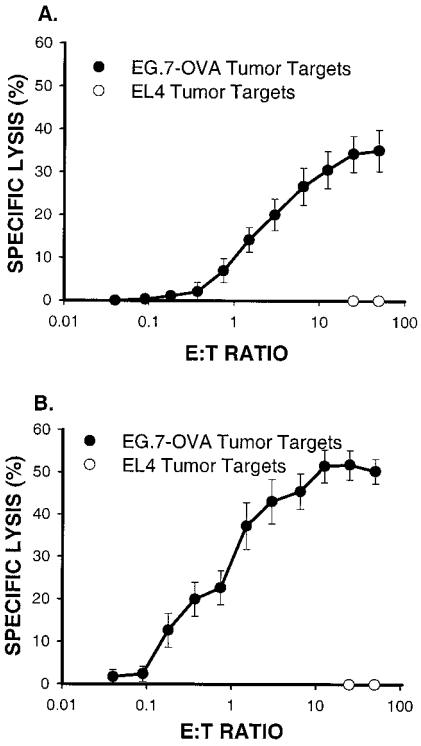

FIGURE 1. OVA Ag-specific cytolytic activity by Tc1 and Tc2 CD8 effector cells in vitro. Tc1 and Tc2 effector cell populations were generated as described in *Materials and Methods.* Cytolytic activity was assessed in a standard 4-h ^{51}Cr release assay against OVA-expressing EG.7 or parent EL4 tumor cell lines at various E:T ratios. Spontaneous release in all assays were <15%. Data are expressed as the mean ± SEM of five independent experiments.

Statistical analysis

For statistical analysis, the two-tailed Student *t* test was used.

Results

Phenotypic characterization of in vitro-generated OVA-specific Tc1 and Tc2 effector T cells

CD8 Tc1 and Tc2 effector T cells were generated in vitro from OVA-specific TCR transgenic OT-I mice as described in *Materials and Methods.* Effector cells were harvested, and their Ag-specific cytolytic potentials were evaluated in a standard 4-h chromium release assay. As shown in Fig. 1, both Tc1 and Tc2 effector cells demonstrated tumor Ag-specific cytolytic activity to OVA-expressing tumor cell targets. At E:T ratios of 50:1, Tc2 effector cells showed a mean specific lysis of 50.0 ± 3.5%, whereas Tc1 cell populations were comparatively lower at 36.0 ± 3.0%. Moreover, when cytotoxicity was assessed in lytic units/10^6 effector cells, Tc2 effector cell populations were nearly 10-fold greater than that of Tc1 effector cell populations (100 LU/10^6 effector cells *vs* 10 LU/10^6 effector cells, $p < 0.001$). In contrast, both Tc1 and Tc2 effector cell populations showed negligible lytic activity to non-OVA-expressing EL4 parental tumor cell targets, confirming that killing was highly Ag specific.

To ascertain whether these Tc1 and Tc2 effector cells are polarized and have stable cytokine-secreting phenotypes, freshly generated effector cell populations were washed and restimulated with mitomycin C-treated OVA-expressing EG.7 tumor cells for 24 and 48 h. As shown in Table I, Tc1 effector cell populations produced substantial amounts of IFN-γ with no detectable levels of IL-4 or IL-5 when cocultured with EG.7-OVA tumor cells. In contrast, Tc2 populations released considerable amounts of IL-5 and IL-4 in similarly restimulated cultures. Although Tc2 cell populations did acquire the ability to secrete some IFN-γ upon restimulation with OVA-expressing tumor cells, these amounts were nearly 50-fold less than that of Tc1 populations (12,224 ± 649 u/ml *vs* 260 ± 64 U/ml, respectively). The secretion of these cytokines by both Tc1 and Tc2 effector cell populations was OVA Ag specific, given that control mitomycin C-treated EL4 parent cell lines did not stimulate detectable levels of cytokine release from either effector cell population (Table I).

Flow cytometric analysis showed that both Tc1 and Tc2 effector cell populations expressed similar patterns of cell surface Ag markers that are characteristic of the effector cell phenotype. As shown in Fig. 2, both effector cell populations were $CD8^+CD4^-$ and expressed up-regulated levels of CD44 and CD25. Although both Tc1 and Tc2 effector cells showed a noticeable down-regulation in the levels of CD62L surface Ag expression, the former showed slightly higher levels than that of the latter. Aside from the surface Ag marker profiles characteristic of effector cell phenotypes, both Tc1 and Tc2 cell populations expressed down-regulated levels of the transgenic Vα2 TCR, typical of increased cell activation. In contrast, naive CD8 T cells were characteristically $CD44^{low}$, $CD62L^{high}$, and $CD25^{low}$ with higher levels of transgenic Vα2 TCR expression (Fig. 2).

Table I. *OVA Ag-specific cytokine-releasing activity by Tc1 and Tc2 effector cell populations*[a]

Effector Cell Population	Restimulation Time (h)	Restimulation with	Cytokine Production[b] IFN-γ (U/ml/10^6 cells)	IL-4 (pg/ml/10^6 cells)	IL-5 (U/ml/10^6 cells)
Tc1	24	EG.7-OVA	12,224 ± 649	0	0
		EL4	0	0	0
	48	EG.7-OVA	13,942 ± 1,370	0	0
		EL4	0	0	0
Tc2	24	EG.7-OVA	260 ± 64	2,270 ± 52	31,165 ± 1,100
		EL4	0	0	0
	48	EG.7-OVA	1,150 ± 280	8,010 ± 400	38,940 ± 1,000
		EL4	0	0	0

[a] OVA Ag-specific Tc1 and Tc2 effector cells were generated in vitro from naive CD8 T cells of OVA-TcR trangenic OT-I mice as described in *Materials and Methods*. Freshly generated effector cells were harvested and restimulated with mitomycin C-treated EG.7-OVA or parental EL4 tumor cells for 24 or 48 h. Supernatants were harvested and analyzed for cytokines by ELISA.

[b] Data are expressed as the mean ± SEM of five independent experiments.

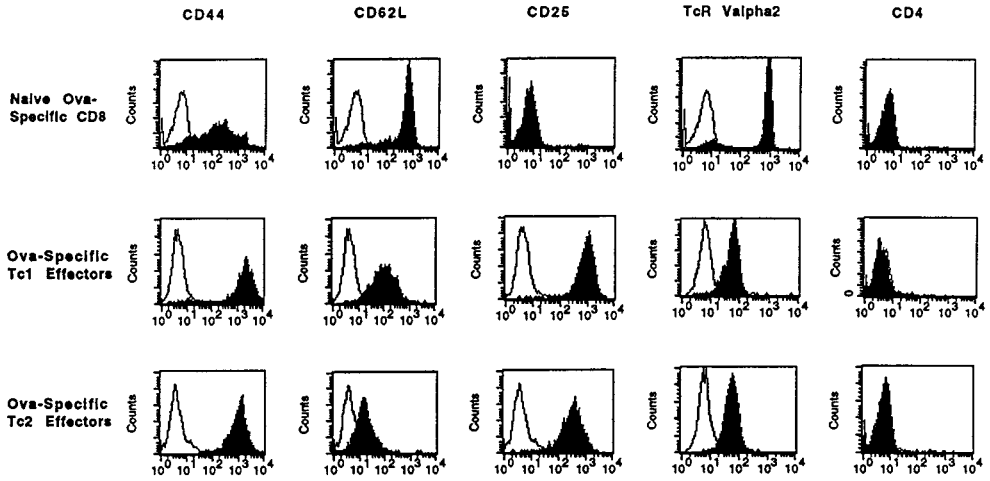

FIGURE 2. Flow cytometric analysis of cell surface Ag expression among OVA Ag-specific Tc1 and Tc2 effector cell populations. OVA Ag-specific CD8 effector cells were generated in vitro from naive CD8 T cells of OT-I TcR trangenic mice as described in *Materials and Methods.* Cells were duel labeled with FITC-anti-CD8 and PE-labeled mAbs specific for CD44, CD62L, CD25, TcR Vα2, or CD4. Lymphocytes were distinguished by their forward light scatter/side scatter profiles, and FITC-CD8$^+$ populations were gated and analyzed for expression of PE label. Isotype controls are shown as unshaded curves.

Enhanced tumor regression by adoptively transferred Tc1 and Tc2 effector cells in mice with advanced levels of established pulmonary tumors

Since Tc1 and Tc2 effector cell populations showed highly tumor Ag-specific cytolytic and cytokine releasing activities in vitro, the therapeutic efficacy of Tc1 and Tc2 effector cell populations at different levels of tumor development were assessed in an experimental OVA Ag-expressing B16 melanoma lung metastases model. Pulmonary metastasis were induced in normal syngeneic C57BL/6 mice by i.v. injection of 5×10^5 B16-OVA melanoma cells. Seven days later, when disseminated micrometastases were established, 2×10^6 OVA Ag-specific Tc1 or Tc2 effector cell populations were systemically transferred into tumor-bearing mice, and the numbers of lung metastases were evaluated at weekly intervals. Treatment of tumor-bearing mice with either Tc1 or Tc2 effector cell populations caused an effective reduction in the number of pulmonary metastases when compared with animals receiving no treatment. In animals bearing 7-day established tumors, Tc1 or Tc2 effector cell therapy markedly reduced established lung tumor colonies by nearly 95% and 80%, respectively (Table II). The mean number of lung metastases in mice receiving Tc1 effector cell therapy was significantly lower than that of untreated tumor-bearing control mice (3.3 ± 0.3 vs 69.0 ± 17.8; $p < 0.01$). Moreover, corresponding groups of tumor-bearing mice receiving Tc2 effector cells showed a similarly significant decrease in the number of established pulmonary tumor foci. This reduction in pulmonary tumor growth among animals receiving either effector cell therapy was observed for up to 30 days post-tumor challenge. Lung tumors among untreated animals grew progressively, with all mice exhibiting >250 tumor foci by day 30 post-tumor challenge (Table II). To determine whether successful Tc1- or Tc2-mediated antitumor effects can be extended to more advanced levels of established pulmonary metastases, similar numbers of effector cells were transferred into animals bearing 14 day-established B16-OVA pulmonary tumors. Both Tc1 and Tc2 effector cells were relatively ineffective in reducing metastatic disease during the first week following therapy with the numbers of lung tumor foci being nearly equal to that of corresponding tumor-bearing control mice receiving no therapy (Table II). However, a progressive reduction in the numbers of lung metastases was noted at wks 2 and 3 following single-dose effector cell transfer with either Tc1 or Tc2 cells. Twenty-one days following Tc1 or Tc2 effector cell therapy, mice with advanced 14-day-established tumors showed a nearly 7- and 13-fold decrease in the numbers of pulmonary metastases, respectively, when compared with control groups. The mean number of lung metastases after 35 days post-tumor challenge in mice receiving Tc1 or Tc2 effector cell transfer on day 14 was significantly lower when compared with corresponding tumor-bearing control mice (38.5 ± 3.5 and 20.0 ± 3.0, respectively, vs >250; $p < 0.001$).

Although metastatic lesions were noticeably reduced with the adoptive transfer of effector cells, minimal residual disease among treated animals was still apparent. Survival studies were determined to further evaluate the therapeutic efficacy of Tc1 and Tc2 effector cells and establish the extent of residual disease following treatment. As shown in Table III, the mean survival time of mice receiving Tc1 or Tc2 effector cell therapy 7 days after B16-OVA tumor challenge was significantly ($p < 0.001$) prolonged when

Table II. *Therapeutic efficacy of adoptively transferred Tc1 or Tc2 effector cells at different levels of tumor development*[a]

Therapy (i.v.)	Days Posttherapy	Mean No. of Lung Metastases ± SEM[b] 7-Day-established tumor	14-Day-established tumor
Tc1	7	3.3 ± 0.3**	110.3 ± 4.3
	14	32.3 ± 14.1**	45.3 ± 0.3*
	21	39.0 ± 4.0*	38.5 ± 3.5*
Tc2	7	14.0 ± 1.1**	82.0 ± 18.4
	14	21.7 ± 6.1**	68.0 ± 2.9*
	21	27.3 ± 4.3*	20.0 ± 3.0*
None	7	69.0 ± 17.8	105.3 ± 7.5
	14	113.7 ± 13.5	>250
	21	>250	>250

[a] Mice ($n = 10$/group) were injected i.v. with 5×10^5 B16-OVA tumor cells. Tc1 or Tc2 effector cells were adoptively transferred into mice bearing established 7-day (early tumor development) or 14-day (late tumor development) metastases.

[b] Lungs ($n = 3$/group) were harvested at 7-day intervals after therapy and tumor foci were counted as described in *Materials and Methods*. p value for treated animals vs untreated animals: *, $p < 0.001$; **, $p < 0.01$.

Table III. *Prolonged survival times among mice with established pulmonary tumors after Tc1 or Tc2 effector cell therapy*[a]

Therapy (i.v.)	Mean Survival Time ± SEM (Days)	% Increase in Mean Survival Time[b]
Tc1-day 7	45.7 ± 1.6*,**	66.8
Tc2-day 7	45.2 ± 2.0*,**	64.9
Tc1-day 14	37.5 ± 1.2*	36.9
Tc2-day 14	34.6 ± 0.7*	26.3
None	27.4 ± 0.8	

[a] Mice ($n = 10$/group) were injected i.v. with 5×10^5 B16-OVA tumor cells. Tc1 or Tc2 effector cells were adoptively transferred into mice bearing established 7-day (early tumor development) or 14-day (late tumor development) metastases.

[b] Percent increase in the mean survival times (MST) for each group was calculated by (Treated MST − nontreated MST/nontreated MST) × 100. p value for treated animals vs untreated animals: *, $p < 0.001$; p value for day 7-treated animals vs day 14-treated animals: **, $p < 0.002$.

compared with that of untreated mice. The mean survival times of mice receiving Tc1 and Tc2 treatment were 45.7 ± 1.6 and 45.2 ± 2.0 days, respectively. In contrast, the mean survival time of untreated tumor-bearing control mice was 27.4 ± 0.8 days post-tumor challenge. Although, Tc1 and Tc2 effector cell-mediated tumor growth inhibition appeared transient in animals with 7-day-established tumors, survival times were substantially increased by >65% when compared with that of untreated control mice. When survival rates among animals with more advanced 14-day-established lung tumors were accessed, Tc1 and Tc2 effector cell-treated mice showed a nearly 25–35% increase in survival times when compared with that of untreated tumor-bearing mice (37.5 ± 1.2 and 34.6 ± 0.7 days, respectively, *vs* 27.4 ± 0.8 days; $p < 0.002$) (Table III). Naive CD8 T cells from OT-I mice had no effect on survival or reduction in tumor cell growth among mice bearing established pulmonary metastases (data not shown).

Therapeutic efficacy and specificity of adoptively transferred Tc1 and Tc2 effector cells in mice with established pulmonary tumors

Since the therapeutic efficacy of adoptive immunotherapy is proportional to the numbers of transferred immune cells, we quantitatively analyzed the antitumor effects of Tc1 and Tc2 effector cell populations by transfer of different effector cell concentrations and monitoring survival times in mice with established pulmonary tumors. As shown in Fig. 3, mice receiving doses of 10–50 × 10^5 of either Tc1 or Tc2 effector cells showed an increase in survival time when compared with that of untreated control tumor-bearing animals. Moreover, transfer of 25-fold less Tc1 effector cells, at numbers as low as 0.4 × 10^5, resulted in a similarly effective prolongation in survival times among mice bearing 7-day established tumors. In contrast, groups of mice receiving a similar dose of Tc2 effector cells showed no therapeutic effect and only when given a 5-fold higher Tc2 effector cell number (2 × 10^5) did animals start to show modest increases in survival times. These results suggest that, on a per cell basis, Tc1 effector cells were nearly 5-fold more effective than Tc2 effector cells since the former demonstrated greater therapeutic effects at cell numbers as low as 0.4 × 10^5 when transferred into tumor-bearing mice. All mice receiving no treatment succumbed to progressively growing tumor within 32 days post-tumor challenge (Fig. 3). Concomitantly, we assessed the immunological specificity of OVA Ag-specific Tc1 and Tc2 effector cell populations. As shown in Fig. 3*C*, transfer of cell numbers as high as 50 × 10^5 of either Tc1 or Tc2 effector cells into mice challenged with the non-OVA-expressing B16 parent line showed no detectable therapeutic effect in survival times when compared with control untreated B16 tumor-bearing mice.

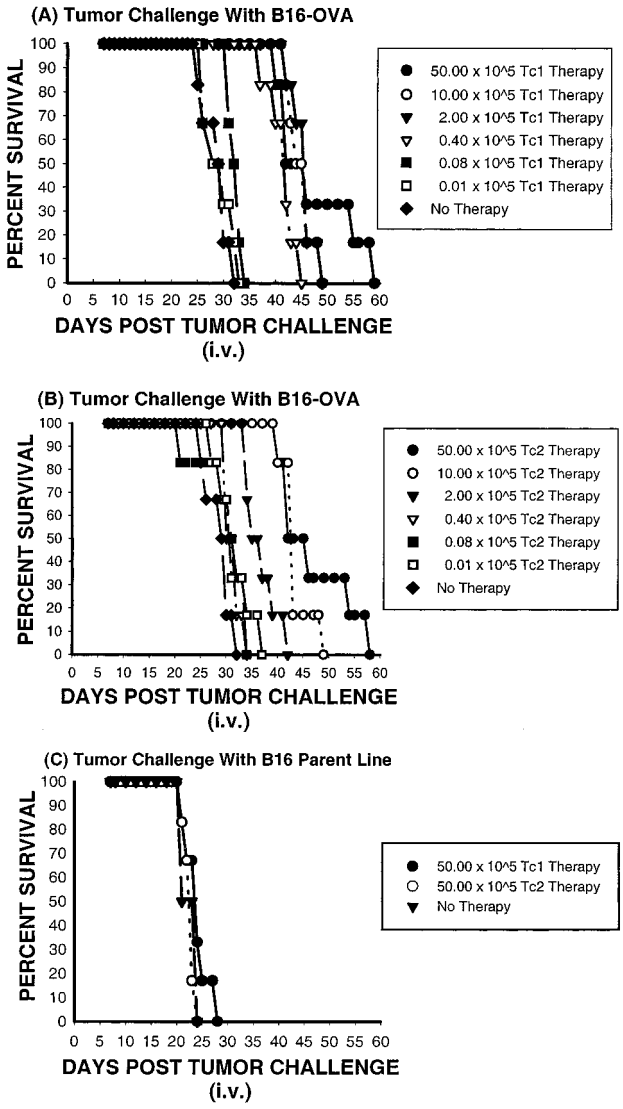

FIGURE 3. Therapeutic efficacy and specificity of OVA Ag-specific Tc1 and Tc2 effector cells in mice bearing established lung tumors. Syngeneic mice ($n = 6$/gp) were injected i.v. with 5 × 10^5 B16-OVA tumor cells. Seven days later, various doses of OVA Ag-specific Tc1 (*A*) or Tc2 (*B*) effector cells were adoptively transferred into tumor-bearing mice, and survival was monitored. In *C*, mice ($n = 6$/group) were injected with 5 × 10^5 non-OVA Ag-expressing B16 parental tumor cells. Seven days later, 5 × 10^6 OVA Ag-specific Tc1 or Tc2 effector cells were adoptively transferred into tumor-bearing mice, and survival was monitored. Results are representative of two similar experiments.

Decreased therapeutic efficiency by IFN-γ-deficient Tc1 but not Tc2 effector cell populations in mice with established pulmonary tumor

Since Tc2 effector cell populations were found to secrete low, yet detectable, levels of IFN-γ upon restimulation with tumor Ag, we assessed the role of effector cell-derived IFN-γ in both Tc1 and Tc2 effector cell-mediated antitumor responses. OVA Ag-specific Tc1 and Tc2 effector cell subpopulations, generated from OT-I.IFN-γ mice, were transferred into syngeneic C57BL/6 tumor-bearing mice, and survival times were monitored as previously described. As shown in Table IV, mean survival times among

Table IV. *Role of effector cell-derived IFN-γ on day 7 effector cell therapy following B16-OVA tumor challenge*[a]

Effector Cell Therapy	Mean Survival Time ± SEM (Days)
Wild-type Tc1	54.0 ± 3.5*
OT-I.IFN-γ Tc1	42.8 ± 1.5*,**
Wild-type Tc2	58.6 ± 8.3*
OT-I.IFN-γ Tc2	45.2 ± 2.2*
Untreated	30.8 ± 0.5

[a] Mice (n = 8/group) were injected i.v. with 5×10^5 B16-OVA tumor cells. OVA-specific Tc1 or Tc2 effector cells, generated from either OT-I.IFN-γ or wild-type OT-I mice, were adoptively transferred into mice bearing 7-day established lung metastases. p value for treated animals vs untreated animals: *, $p < 0.008$; p value for IFN-γ-deficient effector cell treatment vs corresponding wild-type effector cell treatment: **, $p < 0.05$.

tumor-bearing mice receiving IFN-γ-deficient Tc1 or Tc2 effector cell therapy were substantially prolonged when compared with that of untreated mice. However, the former was significantly ($p < 0.05$) less effective than that of corresponding groups of tumor-bearing mice receiving wild-type Tc1 effector cell therapy (42.8 ± 1.5 days vs 54.0 ± 3.5 days). This suggests that IFN-γ may play a substantial role in Tc1 effector cell-mediated therapeutic responses. In contrast, heightened tumor recipient survival times among groups of mice treated with IFN-γ-deficient Tc2 effector cell populations were not significantly ($p < 0.20$) different from that of corresponding groups of tumor-bearing mice receiving wild-type Tc2 effector cell therapy (45.2 ± 2.2 days vs 58.6 ± 8.3 days), suggesting that Tc2 effector cell-derived IFN-γ does not play a significant role in Tc2 effector cell-mediated therapy (Table IV).

Distribution of OVA Ag-specific Tc1 and Tc2 CD8 effector T cells following adoptive transfer into mice with established pulmonary tumor

OVA Ag-specific effector CD8 T cells from OT-I mice, that express the TCR transgene Vα2, were generated in vitro and transferred into tumor-bearing mice as previously described. To assess the local and systemic distribution of such effector cells following adoptive transfer in mice bearing pulmonary tumors, multicolor flow cytometry was performed on lung and spleen tissues, respectively. As shown in Fig. 4*A*, the number and frequency of systemic CD8 T cells expressing the TCR Vα2 transgene were highest in spleens 7 days after either Tc1 or Tc2 effector cell treatment in mice with 7-day-established pulmonary tumors. However, the frequency and absolute cell numbers of detectable transgene-positive CD8 T cells progressively declined by wk 2 after therapy and thereafter decreased by nearly 3.5- and 5-fold by wk 3 for both Tc1 and Tc2 populations, respectively. Similarly, the frequencies and cell numbers of transgene-positive CD8 T cells in lungs of mice receiving Tc1 and Tc2 effector cells were greatest at 7 days after therapy. However, transferred Tc1 effector cells remained similarly elevated at the site of tumor growth for up to 3 wk post-therapy, whereas Tc2 effector cell populations progressively diminished to nearly equal levels of endogenous recipient T cell population numbers by wk 2 and 3 after therapy. Although equal numbers of Tc1 and Tc2 effector cells were transferred into mice 7 days after tumor challenge, the cell number and frequency of the former were consistently greater than that of the latter in both lungs and spleen at all time points tested in these animals. Endogenous CD8/TCR Vα2 T cell population levels never appeared greater than 1.0% in both spleen and lung tissues of untreated tumor-bearing control mice at all time points tested (Fig. 4). Similar results were obtained in corresponding groups of mice with more advanced 14-day-established lung tumors (data not shown).

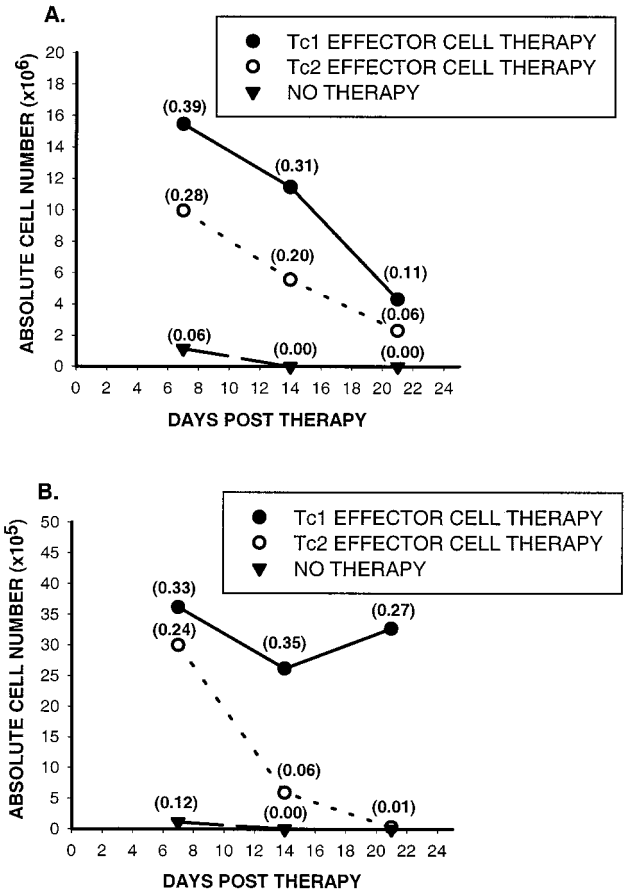

FIGURE 4. Kinetics of the frequency of Vα2 transgene-positive CD8 T cells in spleen and lung following adoptive transfer into mice with established pulmonary tumor. OVA Ag-specific Tc1 and Tc2 effector cells, which express the TcR Vα2 transgene, were generated in vitro and transferred into 7-day-established tumor-bearing mice as described in *Materials and Methods*. Spleen (*A*) and lung (*B*) cells were harvested from mice at 7, 14, and 21 days posttherapy and duel labeled with FITC-anti-CD8 and PE-anti-Vα2 mAbs. Lymphocytes, distinguished by their forward light scatter/side scatter profiles, were analyzed by flow cytometry. The absolute cell number for the population shown was calculated as the percentage of cells × the total numbers of cells per tissue. Numbers in parentheses indicate the frequency of transgene-positive CD8 cells and were defined as the absolute cell number of transgene-positive CD8 cells per absolute cell number of total CD8 T cells for each time point. Results are representative of three independent experiments.

Characterization of systemic tumor Ag-specific T cell responses induced by adoptively transferred Tc1 and Tc2 effector cells in mice bearing established pulmonary tumors

Systemic cytolytic activity of T cells from spleens of tumor-bearing mice following adoptive transfer of OVA Ag-specific Tc1 or Tc2 effector T cells were assessed at weekly intervals following therapy in a standard 4-h chromium release assay. As shown in Table V, CD8-enriched T cells from spleens containing both donor and recipient cells of 14-day tumor-bearing mice treated 7 days earlier with either OVA Ag-specific Tc1 or Tc2 effector cells showed markedly enhanced Ag-specific CTL responses to OVA-expressing EG.7 tumor cell targets. Systemic OVA Ag-specific CTL responses among groups of mice receiving Tc1 therapy were significantly ($p < 0.005$) greater than that of corresponding Tc2

Table V. *OVA Ag-specific functional responses by splenic CD8 T cells from mice receiving Tc1 or Tc2 effector cell therapy*[a]

Therapy	Days Posttherapy	Stimulator or Target Cell	Cytokine Production[b] IFN-γ (U/ml/10^6 cells)	IL-4 (pg/ml/10^6 cells)	IL-5 (U/ml/10^6 cells)	% Specific Lysis ± SEM[c] (E:T Ratio 100)
Tc1	7	EG.7-OVA	851.0	260.0	0.0	22.4 ± 0.8
		EL4	0.0	0.0	0.0	0.0
	14	EG.7-OVA	938.0	8.0	0.0	15.0 ± 1.0
		EL4	0.0	0.0	0.0	0.0
	21	EG.7-OVA	0.0	0.0	0.0	0.0
		EL4	0.0	0.0	0.0	0.0
Tc2	7	EG.7-OVA	575.0	350.0	11,625.0	15.3 ± 0.6
		EL4	0.0	0.0	0.0	0.0
	14	EG.7-OVA	0.0	40.0	1,095.0	0.0
		EL4	0.0	0.0	0.0	0.0
	21	EG.7-OVA	0.0	10.0	0.0	0.0
		EL4	0.0	0.0	0.0	0.0
None	7	EG.7-OVA	0.0	0.0	0.0	0.0
		EL4	0.0	0.0	0.0	0.0
	14	EG.7-OVA	0.0	0.0	0.0	0.0
		EL4	0.0	0.0	0.0	0.0
	21	EG.7-OVA	0.0	0.0	0.0	0.0
		EL4	0.0	0.0	0.0	0.0

[a] Mice were injected i.v. with 5×10^5 B16-OVA tumor cells. After 7 days, 2×10^6 Tc1 or Tc2 effector cells were adoptively transferred into mice bearing established 7-day pulmonary tumors.

[b] Spleens ($n = 2$/group) were harvested at weekly intervals and CD8-enriched T cell populations were cultured for 48 h with mitomycin C-treated EG.7-OVA or parental EL4 tumor cells. Supernatants were harvested and analyzed for cytokines by ELISA.

[c] Spleens ($n = 2$/group) were harvested at weekly intervals and tumor-specific cytolytic activity of CD8-enriched T cell populations were assessed in a standard 5-h chromium release assay.

effector cell-treated mice (22.4 ± 0.8 vs 15.3 ± 0.6). Cytolytic responses to control non-OVA-expressing EL4 tumor cell targets were negligible, confirming that killing was Ag specific and NK cell independent. Fourteen days after therapy, CD8-enriched T cells from spleens of mice receiving Tc1 therapy showed progressively lower OVA Ag-specific CTL responses that were not detectable by day 21 posttherapy. In contrast, OVA Ag-specific CTL responses among corresponding T cell populations from mice receiving Tc2 effector cell therapy appeared to diminish at earlier time points with negligible CTL activity at days 14 and 21 following therapy. Cytolytic responses by CD8-enriched T cell populations from spleens of tumor-bearing control mice receiving no treatment were undetectable at all time points tested (Table V). Similar results were obtained in separate experiments in mice with more advanced 14-day established pulmonary disease (data not shown). Since potential antitumor immune responses may be mediated by cytokines secreted by effector T cells (9), we analyzed the tumor Ag-specific cytokine-releasing activities of systemic CD8 T cells from spleens of tumor-bearing mice receiving Tc1 or Tc2 effector cell therapy. As shown in Table V, CD8-enriched T cells from spleens of 14-day tumor-bearing mice treated 7 days earlier with OVA Ag-specific Tc1 effector cells showed enhanced levels of IFN-γ and IL-4 cytokine production to EG.7-OVA tumor cells when compared with that of corresponding untreated tumor-bearing control mice. Although IFN-γ- and IL-4 cytokine-secreting T cell responses among mice receiving Tc1 effector cell therapy persisted for up to 14 days posttherapy, levels of both cytokines eventually subsided to undetectable levels by day 21 posttherapy. IL-5 cytokine production among these same animals was undetectable at all time points tested. Cytokine-releasing activities to control non-OVA-expressing EL4 tumor cells were negligible, confirming that T cell-mediated cytokine-secreting potentials were Ag specific. Although corresponding cell populations from spleens of tumor-bearing mice receiving Tc2 effector cell therapy showed similarly elevated levels of IFN-γ production 1 wk after treatment, IFN-γ cytokine levels diminished over time and were eventually undetectable by days 14 and 21 posttherapy. In contrast, these same animals produced markedly higher levels of IL-4 and IL-5 at both 7 and 14 days after Tc2 effector cell transfer than did untreated or Tc1-treated mice. However, both IL-4 and IL-5 cytokine producing levels by these CD8 T cells were substantially lower and negligible by day 21 posttherapy, respectively. Cytokine-releasing activity by CD8-enriched T cell populations from spleens of untreated tumor-bearing control mice were negligible at all time points tested (Table V). Data shown are one representative experiment of three that were performed with similar results. Similar results were obtained in separate experiments in mice with more advanced 14-day-established pulmonary disease (data not shown).

Discussion

Our goal was to analyze the therapeutic effects of adoptively transferred tumor Ag-specific Tc1 and Tc2 CD8 effector cell populations in mice bearing established pulmonary malignancy. The number and growth of B16-OVA lung metastases were significantly reduced by both Tc1 and Tc2 effectors, and survival times were prolonged in mice with both early (day 7) and more advanced (day 14) levels of tumor development. CD8 T cell populations recovered from the spleens of tumor-bearing mice receiving Tc1 or Tc2 effector cells showed markedly enhanced tumor Ag-specific cytolytic and cytokine-releasing activities that correlated with delays in tumor cell growth and progression.

Effective tumor rejection by adoptively transferred cell populations may be dependent on several potential direct and indirect mechanisms. First, both effector CD8 T cell populations can directly eradicate tumor cells through cognate interactions that may involve either perforin-mediated or Fas-mediated lytic mechanisms (6–8, 18, 19). As a result, tumor-associated Ags may be

released and reexpressed by host APC that may enhance host immune responses at sites proximal and distal to tumor growth. Second, release of Tc1 and Tc2 cytokines, such as IFN-γ and IL-4, have been shown to directly inhibit tumor cell growth (20–22), enhance Ag presentation through up-regulation of MHC Class I among both tumors and host APC (22–24), and influence expression of specific immunoenhancing effector cell surface molecules that effectively facilitate antitumor responses and tumor rejection (22–27). Alternatively, other T cell-derived cytokines, such as IL-10, have been shown to potentiate tumor cell growth (28, 29). Third, Tc1 and Tc2 effector cells can induce Ag nonspecific inflammatory responses that may indirectly aid in cytolytic and/or cytostatic antitumor effects. Local release of cytokines, such as Tc2-derived IL-4/IL-5 and Tc1-derived IFN-γ, have been shown to mediate the selective recruitment and localization of macrophages, NK cells, and granulocytes that may facilitate tumor growth inhibition and/or enhance tumor Ag presentation (22, 30–34). In either instance, differential utilization of discrete cytokine profiles by Tc1 and Tc2 effector cell populations can induce tumor rejection with different mechanisms and potentially affect both the nature and outcome of effective antitumor responses and tumor progression. Investigations to elucidate effector cell-mediated mechanisms and their contribution to host immunity in tumor eradication are currently under way.

The therapeutic efficacy of Tc1 and Tc2 cell populations administered to tumor-bearing mice with established pulmonary malignancy was evaluated. Effector cell titration studies revealed that in vivo antitumor effects by both Tc1 and Tc2 effector cells are dose dependent, with the former exhibiting greater therapeutic activity at lower effector cell concentrations. Thus, on a per cell basis, Tc1 effector cells were more efficacious. Differences in therapeutic effects among the two CD8 effector cell populations may be attributed to many possible quantitative and/or qualitative differences among each cell populations in vivo. Since the accumulation of effector cells at the tumor site may be important, we determined the distribution of Tc1 and Tc2 effector cells in lungs of tumor-bearing mice at various intervals following therapy and found a local accumulation of both donor Tc1 and Tc2 CD8 cells. Both Tc1 and Tc2 effector cell populations were detectable at the tumor site with nearly equal frequency and cell number within the first week following therapy. However, while Tc1 effector cells maintained similar frequency levels for up to 3 wk following therapy, Tc2 effector cell numbers and frequencies progressively diminished over time, suggesting that Tc1 effector cell populations appear to be more effective in eliciting antitumor immunity with extended effector cell-to-tumor cell interaction times. Such quantitative differences among effector cell populations at the tumor site may be attributed to local tumor-induced inflammatory responses that may modulate various effector cell surface Ag receptors responsible for localization and trafficking properties of such cells in vivo (35, 36). Conversely, inherent quantitative and qualitative differences in the expression of select cell surface receptors may exist between Tc1 and Tc2 cell populations. As in the case with Th1 and Th2 effector cells, differences in various factors, such as chemokine receptor expression, affinity, and surface density, have been suggested to influence effector T cell recirculation and functional capacity (37–39). Such differences may exist in corresponding CD8 Tc1 and Tc2 cell populations in response to progressively growing tumor in the lung. We have observed differences, however, in the expression levels of the CD62L adhesion/homing Ag among Tc1 and Tc2 cell populations in vitro, in that the former expressed greater levels than those of the latter. Although its significance remains unclear, it has been suggested that the levels of cell surface CD62L expression may have profound effects on lymphocyte-mediated antitumor function in vivo (40, 41).

Another explanation for differences in effector cell frequencies at the tumor site may be that one or the other of the effector cell populations is intrinsically more sensitive in its ability to be anergized (42, 43) or differentially more susceptible to activation-induced cell death (44). As with Th1/Th2 effector cells (44, 45), different CD8 effector cell populations may preferentially express heightened levels of activation-induced cell death-related cell surface receptors, such as Fas, which when engaged by tumor-derived FasL, could induce T cell death and depletion (46–48). In either instance, such effector cell reactions may result in rapid exhaustion of select Ag-specific effector cell responses at the site of disease and/or progressively growing tumor in vivo (49–51). The basis for this difference will be further investigated.

Tumors, such as the B16 melanoma, have been shown to express low levels of class I surface Ag (52, 53) which could potentially result in the failure of appropriate T cell Ag recognition and activation. Tc1 effector cells may enhance tumor Ag presentation and T cell expansion/survival time by the secretion of endogenous cytokines, such as IFN-γ and IL-2, that may subsequently maintain T cell survival and tumor-reactive responses for longer periods of time. The adoptive transfer of CD8 effectors from OT-I mice crossed to IFN-γ-deficient mice showed that these Tc1 effectors were less effective than the wild type, as anticipated. Tc2 CD8 effectors from IFN-γ-deficient mice, however, were not significantly less effective than cells from OT-I wild-type mice, suggesting that Tc2 effector cells must act by an effector cell-derived IFN-γ-independent mechanism. Tc2 effector cells may facilitate tumor rejection through indirect mechanisms that initiate and recruit nonspecific antitumor immune responses, such as that of macrophages, NK cells, and eosinophils which may, in part, delay progressive tumor growth. Investigations on the role of individual Tc1/Tc2-derived cytokines in recruiting host antitumor responses are currently under way.

Although metastatic lesions in effector cell-treated mice were greatly reduced and, in fact, appeared eliminated in lungs of "cured" mice receiving lower doses of tumor (data not shown), minimal residual disease among these animals was still apparent. These observations were in agreement with those obtained using a different tumor Ag model reported by Prevost-Blondel et al. (54), in which adoptively transferred tumor-reactive TIL cells were able to initiate, but not sustain tumor-specific CTL responses sufficient to clear s.c. tumor challenge. Survival studies in our pulmonary model showed a marked increase in the mean survival rates among mice with early vs late stages of tumor development following effector cell treatment, suggesting that effective single dose Tc1 and Tc2 effector cell therapy may, in part, be dependent on the nature and level of tumor cell development in vivo. Differences in effector cell efficacy among animals with early (day 7) or more advanced (day 14) stages of tumor maturation may be attributed to tumor growth kinetics and tumoricidal activity by effector T cell populations in vivo (55), e.g., the inability of transferred effector cells to keep up with more established tumors that rapidly proliferate and lead to enhanced tumor outgrowth in vivo. The persistence or absence of repeated tumor Ag restimulation may lead to tumor-induced tolerance or anergy of adoptively transferred tumor-reactive effector T cells in tumor-bearing recipients (51, 55). Alternatively, tumor-reactive effector T cells may efficiently eradicate tumor cells, but may select for tumor Ag negative variants in vivo which would diminish effector cell recognition and enhance variant tumor cell outgrowth and progression (56, 57). Additionally, other tumor-related mechanisms contributing to the active

immunosuppression of tumor-reactive effector cells have been described and involve release of tumor-derived soluble suppressor factors, such as TGF-β and prostaglandins (9); generation of host T cells with suppressor function (58, 59); discordant structural alterations in TCR-mediated signal transduction pathways, particularly in late tumor-bearing hosts (55, 60, 61); induction of CD95-mediated T cell apoptosis by Fas ligand-expressing tumor cells (46–48); and down-regulation of tumor cell surface costimulatory factors that result in induction of T cell anergy (62).

In summary, we relate 1) that substantial numbers of systemically transferred Tc1 and Tc2 effector cell populations preferentially accumulated at the site of tumor challenge, however, with differing frequencies of retention and/or prolonged localization, 2) single-dose transfer of tumor-reactive Tc1 and Tc2 effector cells induced tumor regression and subsequently enhanced survival times in mice with established pulmonary tumors, 3) systemic antitumor responses induced by both Tc1 and Tc2 effector cell subpopulations were highly tumor Ag specific and appeared to be mediated by different mechanisms of action, 4) the level of effectiveness by adoptively transferred Tc1 and Tc2 effector cells appeared to be influenced by the stage of tumor development in the lung. Future studies will be directed at elucidating the underlying mechanisms and cellular interactions involved in generating and maintaining long term Tc1 and Tc2 effector cell-mediated tumor immunity.

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