Tc1 and Tc2 Effector Cell Therapy Elicit Long-Term Tumor Immunity by ContrastingMechanisms That Result in Complementary Endogenous Type 1 Antitumor Responses

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J Immunol 2004; 172:1380-1390; doi: 10.4049/jimmunol.172.3.1380
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Adoptive T cell immunotherapy has been shown to be effective for the treatment of patients with certain metastatic cancers. Recent clinical studies have shown that the selection, expansion, and infusion of high avidity, melanoma-reactive CD8 T cell subpopulations derived from either peripheral blood or tumors of patients with late stage disease can induce either partial or complete tumor regression (1–4). Therapeutic efficiency by such treatment is dependent in part on the ability of tumor Ag-specific T cells to persist long-term and retain antitumor function in vivo. Ideally with respect to effective tumor therapy and vaccine development, such treatment would not only induce tumor regression and prevent tumor relapse in cancer patients, but also initiate and amplify effective endogenous recipient T cell-tumor cell interactions.

Clinical trials employing cellular adoptive immunotherapy in patients with advanced stages of melanoma have shown that infused tumor peptide-specific CD8 T cell clones can be identified among tumor infiltrates by tetramer staining (1, 3–10). Phenotypic and functional analysis of these subpopulations and their immune repertoires indicate that although tumor Ag-specific T cell populations can persist in cancer patients, they may be ineffective and/or unresponsive to specific tumor Ag in vivo. This may be due in part to alterations in T cell signal transduction (11, 12), inhibition through NK-like cell receptors on CD8 T cells (13, 14), or the presence of immunosuppressive cytokines and regulatory T cells (9, 15). Alternatively, others have shown that endogenous CD4 helper cells and/or exogenous cytokines such as IL-2 can provide helper function to augment tumor eradication by adoptively transferred tumor Ag-specific CD8 T cells (3, 9, 16–22). This suggests that in vivo persistence and maintenance of such CD8 effector cells are dependent on endogenous CD4 Th cell responses. Furthermore, others have shown that Th1 CD4 T cell subpopulations producing IFN-γ appear crucial to the optimal generation and durability of specific CTL in vivo and may aid in part with recruitment of adoptively transferred effector cells into the tumor environment (23–26). These observations provide a further impetus to characterize endogenous T effector cell subpopulations that arise and/or persist among populations of tumor-reactive T cells that are associated with in vivo tumor regression.

As in the case of CD4 T cells, CD8 T lymphocytes can be further classified into distinct effector cell types based on their cytokine-secreting profiles after tumor Ag encounter (27–29). Type 2 CD8 T cells (Tc2) preferentially secrete IL-4, IL-5, and IL-10 and kill predominantly by the perforin pathway, whereas type 1 CD8 T cells predominantly secrete IFN-γ and kill by either perforin- or Fas-mediated mechanisms. Although both effector cell subpopulations have been identified in human peripheral blood and in patients with various clinical disorders (30–33), their roles and effects on endogenous antitumor immune responses and tumor eradication of established malignancy remain relatively undefined. Using an OVA-transfected B16 lung metastases model, we have previously shown that either adoptively transferred Tc1 or Tc2 effector cell immunotherapy mediates long-term tumor immunity by different mechanisms that subsequently potentiate endogenous recipient-derived type 1 antitumor responses. The Journal of Immunology, 2004, 172: 1380–1390.
effector cells promote long-term survival among high proportions (80–90%) of mice with established malignancy (34). In the current study we extended our observations to assess the potential direct and indirect mechanisms involved with both Tc1 and Tc2 effector cell-mediated, long-term tumor immunity and their subsequent effects on endogenous T cell responses in long-term surviving mice treated for established pulmonary malignancy. We show that induction of effector cell-mediated, long-term tumor immunity was dependent in part on potentially different mechanisms involving effector cell-derived IL-4/IL-5 and IFN-γ for Tc2 or Tc1 effector cells, respectively, whereas effector cell-derived perforin was not necessary for either effector cell therapy. Moreover, both Tc1 and Tc2 effector cell therapies potentiated endogenous recipient-derived, long-term antitumor immune responses by inducing 1) local T cell-derived chemokines associated with type 1-like immune responses; 2) memory recipient-derived OVA tetramer-positive CD8 T cells that were CD44+/Ly6C−, and CD95 and perforin− which predominately produced IFN-γ and TNF-α; and 3) heightened numbers of highly activated recipient-derived Th1 and Tc1 T cell subpopulations expressing CD25, CD69, and CD95 cell surface activation markers. Moreover, both Tc2 and Tc1 effector cell therapies are dependent in part on recipient-derived IFN-γ and TNF-α for long-term survival and protection. These observations in conjunction with our previous studies show that Tc1 and Tc2 effector cells elicit long-term tumor immunity by contrasting mechanisms that are accomplished by complementary recipient-derived type 1 antitumor responses.

Materials and Methods

Animals

Female C57BL/6 mice, 6–10 wk of age, were obtained from the Animal Breeding Facility at Trudeau Institute. The OT-I mouse strain (Thy 1.2) on the C57Bl/6 background (H-2b) was originally obtained from Dr. M. Bevan (University of Washington, Seattle, WA). These mice express a transgenic TCR Vβ2 and Vγ5 specific for the SIINFEKL peptide of OVA in the context of MHC class I, I, H-2Kk (35). Perforin−/− (IL-4−/−, IL-5−/−, IFN-γ−/−, TNF-α−/−, and B6.PL-Thy 1.1 mice on a B6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous perforin−/− (OT-I.PKO), IL-4−/− (OT-I.LL-4), IL-5−/− (OT-I.LL-5), and IFN-γ−/− (OT-IFN-γ−/−) knockout mice expressing the TCR Vβ2 and Vγ5 transgenes were generated by backcrossing OT-I mice onto specified syngeneic knockout mice (H-2b) for more than three generations. Animals were maintained and treated according to animal care committee guidelines of the National Institutes of Health and Trudeau Institute.

Tumor cells

The weakly immunogenic OVA-transfected B16 tumor cell line (B16-OVA) that is syngeneic to the C57Bl/6 background was provided by Drs. E. Lord and J. Freihering (University of Rochester, Rochester, NY).

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 L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated FCS (Life Technologies). CD8-enriched T cells were obtained by treating mice with anti-CD4 (R1.1.2), anti-heat-stable Ag (J11D), and anti-MHC class II (D3.137, M5114, CA4) mAbs for 30 min at 4°C. The cell-depleted APCs were pulsed with OVA peptide (10 μM) for 30 min at 37°C and treated with mitomycin C (50 μg/ml; Sigma-Aldrich) for an additional 30 min at 37°C. For Tc1 effector cell generation, naive CD8 T cells from OT-I transgenic mice (2×10⁶ cells/ml) were stimulated with mitomycin C-treated, OVA peptide-pulsed APCs (6×10⁵ cells/ml) in the presence of IL-2 (20 U/ml), IL-4 (2 U/ml), IL-12 (2 ng/ml; Sigma-Aldrich), and anti-IFN-γ mAb (20 μg/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 or B6.PL/Thy 1.1 mice were injected i.v. with 1×10⁶ B16-OVA melanoma cells to establish pulmonary metastases. Seven days after tumor challenge, mice were treated i.v. with 2×10⁶ Tc1 or Tc2 OVA-specific effector T cells, and survival times were monitored daily (34). Control groups of mice received no treatment. Mice surviving primary tumor challenge were rechallenged with similar cell numbers of either B16-OVA or parental B16 tumor cells, and survival times were monitored as previously described. 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 treated and untreated control groups was counted in a blind fashion. Metastatic foci too numerous to count were assigned an arbitrary value of ≥250. Alternatively, cytospin preparations of cells from lung homogenates were fixed with methanol and stained with eosin and methylene blue (Fisher Scientific). Although tumor cells appeared heterogeneous in size, they were easily differentiated as predominately larger cells with an elevated nuclear to cytoplasm ratio, and in some cases containing black pigment. Counts were performed on a total of 200–300 cells on coded slides.

Flow cytometric analysis

Single cell suspensions of processed murine lung were washed three times in a fluorescent Ab buffer (FAB) consisting of 1% BSA and 0.02% sodium azide in 0.01 M PBS, pH 7.2. Recipient immune cell populations were phenotyped by their expression of surface markers using direct immunofluorescence staining techniques. Lymphocytes (10⁶), pretreated with FcR block, were incubated for 20 min on ice with 100 μl of FAB containing 1 μg of various mAbs conjugated to biotin, PE, FITC, CyChrome, or Tri-color. For biotinylated mAbs, streptavidin-allophycocyanin or streptavidin-CyChrome was used as a second-step reagent. The mAbs used include anti-CD90.1 (Thy 1.1; BD Pharmingen, San Diego, CA; clone HIS51), anti-CD90.2 (Thy 1.2; BD Pharmingen; clone 53-2.1), anti-CD8 (Caltag Laboratories, Burlingame, CA), anti-CD4 (BD Pharmingen), anti-CD44 (BD Pharmingen; clone IM7), anti-Ly6c (BD Pharmingen), anti-CD25 (BD Pharmingen), anti-CD69 (BD Pharmingen), and anti-CD95 (BD Pharmingen). Stained cell preparations were then washed three times in FAB and analyzed by multiparameter flow cytometry using a FACS-Calibur.
Whitney rank-sum test was used.

MHC tetrameric reagents and analysis

The construction of folded MHC class I-peptide complexes and their tetramerization have been described previously (36). Tetramers were generated by the Molecular Biology Core Facility at Trudeau Institute. The OVA tetramer is Kb folded with the SIINFEKL peptide derived from OVA. Tetramer titration was determined using spleen and lymph node cell preparations from OT-I mice expressing a transgenic TCR specific for the Kb/ SIINFEKL epitope. No cross-reactivity was detected in studies using cell preparations from OT-I mice mixed with wild-type C57BL/B6 or non-strain-related BALB/c mice. T cell-enriched populations from either spleen or lungs of either effector cell-treated or untreated tumor-bearing mice were incubated with FcR block for 10 min and stained with PE-conjugated tetrameric reagent for 1 h at 4°C, followed by staining with Tricolor anti-CD8 and FITC-Thy 1.1 or FITC-Thy 1.2 mAbs on ice for 20 min. Stained samples were washed and analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Immunocytometry Systems).

Intracellular cytokine staining

Single cell suspensions were obtained from lungs of normal age-matched, effector cell-treated or untreated tumor-bearing mice as described above. Cells were stimulated with either PMA (10⁻⁷ M) and ionomycin (1 µg/ml) or plate-bound anti-CD3 for 5 h. Two hours before harvesting, brefeldin A (10 µg/ml) was added to cultures to retain cytoplasmic cytokines. Cells were pretreated with FcR block, followed by FITC-conjugated anti-CD90.1 (Thy 1.1; BD Pharmingen; clone HS51), FITC-conjugated anti-CD90.2 (Thy 1.2; BD Pharmingen; clone 53-2.1), or CyChrome-conjugated anti-CD8 or anti-CD4 (BD Pharmingen). Subsequently, cells were fixed with 2% paraformaldehyde, followed by intracellular staining in permeabilization buffer containing 0.5% saponin and 1% BSA in PBS and PE-conjugated IFN-γ, IL-4, IL-10, or TNF-α (BD Pharmingen) mAbs. Cells were washed and resuspended in 1% BSA/PBS solution and analyzed by flow cytometry. Data were analyzed using CellQuest software (BD Biosciences).

Cytokine/chemokine mRNA expression in lung tissue

Lungs were flushed in situ with HBSS via cannulation of the heart to remove residual intravascular blood pools. Total RNA from either unstimulated or anti-CD3-restimulated cell cultures or whole lungs of effector cell-treated or untreated tumor-bearing mice were prepared by tissue homogenization in TRIzol reagent (Life Technologies). mRNA levels were quantitated using the RibOQuant Multiprobe RNase protection assay system (BD Pharmingen) with the mCK-1, mCK-3, and mCK-5 cytokine/chemokine mRNA detection probe sets. Bands were detected using the Molecular Imager FX with the Quantity One Software analysis program (Bio-Rad, Hercules, CA) and normalized against the L32 housekeeping gene as relative units.

Tumor cell phenotype and cell death

Tumor cell cultures were harvested and washed once in PBS at various time intervals, and total cellular RNA was prepared with TRIzol reagent (Life Technologies). RNase protection assays were performed using the mCK-2 mRNA detection probe set and analyzed as previously described. Apoptotic cell death of the tumor cells was measured by flow cytometry using FITC-conjugated annexin V and propidium iodide (apoptosis detection kit; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Statistical analysis

For statistical analysis, two-tailed Student’s t test or nonparametric Mann-Whitney rank-sum test was used.

Results

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

CD8+ Tc1 or Tc2 effector T cells were generated in vitro from OVA-specific TCR transgenic OT-1 mice as described in Materials and Methods. As shown in earlier studies, both effector cell populations demonstrated potent tumor Ag-specific cytolytic activity to OVA Ag-expressing tumor cells (EG.7-OVA) in vitro that was predominantly mediated by the perforin pathway (28, 37). Moreover, Tc2 populations released considerable amounts of IL-5 (>20,000 U/ml/10⁶ cells in 24 h) and IL-4 (>20 ng/ml/10⁶ cells in 24 h), with low, yet detectable, levels of IFN-γ upon restimulation with OVA-expressing tumor cells. In contrast, Tc1 effector cell populations produced substantial amounts of IFN-γ (>12,000 U/ml/10⁶ cells in 24 h), with no detectable levels of IL-4 or IL-5. Flow cytometric analysis showed that both effector cell populations expressed similar patterns of cell surface Ag markers that are characteristic of the effector cell phenotype (37). Effector cell populations were CD8⁺ CD4⁻, Ly6C negative, OVA tetramer positive and expressed up-regulated levels of both CD44 and CD25 and down-regulated levels of CD62L.

Long-term tumor immunity by adoptively transferred Tc1 and Tc2 effector cells in mice with established pulmonary malignancy is partially attributed to select effector cell-derived cytokines and not to perforin

Previously, we have shown that both Tc1 and Tc2 effector cell populations promote long-term tumor immunity and survival in mice with established B16-OVA pulmonary metastases (34). Long-term tumor immunity was highly Ag specific and evident by resistance to lethal tumor rechallenge and detection of OVA Ag-specific CTL responses. In the current study we extend our observations to determine whether effector cell-derived perforin is required for the induction of either Tc1 or Tc2 effector cell-mediated, long-term protection. Normal syngeneic C57BL/6 mice (n = 6–8/group) were injected i.v. with 1 x 10⁵ B16-OVA tumor cells. Seven days later, when metastases were established, 2 x 10⁶ OVA Ag-specific Tc1 or Tc2 effector cells, generated from either OT-I or OT-I.PKO perforin knockout mice, were systemically transferred into tumor-bearing mice, and survival times were monitored daily. As shown in Fig. 1A, long-term survival times among tumor-bearing mice receiving either wild-type or perforin-deficient Tc1 effector cells were significantly prolonged (p < 0.001) compared with those of untreated tumor-bearing control mice. Single-dose treatment with either Tc1 or Tc1.PKO effector cell therapy prolonged survival times among 83% (five of six) and 87% (seven of eight) of tumor-bearing mice, respectively. In contrast, the mean survival time of untreated tumor-bearing control mice was 39 ± 5.2 days after tumor challenge. Eighty days after initial tumor cell challenge, surviving mice were rechallenged with 1 x 10⁶ B16-OVA tumor cells. All mice initially receiving effector cell therapy exhibited protection and long-term survival for >150 days after tumor rechallenge (Fig. 1). Corresponding groups of untreated control mice receiving B16-OVA tumor cells died within 45 days after tumor challenge, demonstrating that tumor cell preparations used for rechallenge experiments were lethal in naive mice (data not shown). Similarly, OVA Ag-specific Tc2 effector cells, generated from either OT-I or OT-I.PKO knockout mice, showed similar therapeutic effects (Fig. 1B). However, in corresponding studies using effector cells derived from specified cytokine knockout OT-I mice, long-term survival was dependent in part on Tc2 and Tc1 effector cell-derived IL-4, IL-5, and IFN-γ, respectively (Fig. 1, C and D). Collectively, this suggests that induction of effector cell-mediated, long-term tumor immunity was dependent in part on potentially different mechanisms involving effector cell-derived IL-4/IL-5 and IFN-γ for Tc2 or Tc1 effector cells, respectively, whereas effector cell-derived perforin was not necessary for either
in Fig. 3, the frequency of lung-derived CD4/CD44 high T cells among long-term surviving mice receiving effector cell therapy. As shown in Fig. 2, the frequency and cell numbers of CD8/CD44 high T cells among TC2 effector cell-treated mice coexpressing CD25, CD69, and CD95 were substantially elevated at local (lung) sites compared with those in normal age-matched control mice (34). In the current study local and systemic recipient T cell subpopulations from lungs and spleens of mice receiving effector cell therapy >200 days previously were further characterized for coexpression of surface markers relevant to stages of T cell activation and differentiation. Using Thy1 congenic mice, donor (Thy1.2) and tumor-bearing recipient (Thy1.1) T cell subpopulations were enumerated from lungs and spleens of effector cell-treated mice using multicolor flow cytometric analysis. As shown in Fig. 2, the frequency and cell numbers of CD8/CD44 high T cells among TC2 effector cell-treated mice coexpressing CD25, CD69, and CD95 were substantially elevated at local (lung) sites of tumor growth compared with those of corresponding tissues in normal age-matched control mice. However, the cell frequency levels of these cells in spleens of effector cell-treated mice were consistently lower compared with those of corresponding cells in lungs of these same animals. Moreover, the frequency and numbers of CD8/CD44 high cells coexpressing high levels of the memory cell marker Ly6c were nearly 2- to 3-fold higher in both spleen and lungs compared with those in age-matched controls (Fig. 2). Similar results were obtained in long-term surviving animals receiving TC1 effector cell therapy (data not shown).

In parallel studies we evaluated the accumulation and phenotypic characterization of activated recipient-derived CD4/CD44 high T cells at sites local or distal to tumor growth among long-term surviving mice receiving effector cell therapy. As shown in Fig. 3, the frequency of lung-derived CD4/CD44 high T cells coexpressing elevated levels of CD25, CD69, and CD95 after TC2 effector cell therapy >200 days previously were significantly higher than those of normal age-related control mice. Similarly, the frequency of corresponding cell subpopulations in spleens of these same mice were nearly 2- to 3-fold greater than those of normal control mice. Similar results were obtained in long-term surviving animals receiving TC1 effector cell therapy (data not shown).

Although metastatic lesions in effector cell-treated mice were greatly reduced and, in fact, appeared to be eliminated in lungs of long-term survivors, minimal residual disease among these animals was still apparent. Cytospins from single cell suspensions of lungs from effector cell-treated mice showed low, yet detectable, numbers of tumor present at times >150 days after tumor rechallenge (Fig. 2).

Assessment of recipient T cell cytokine-releasing profiles in lungs of long-term surviving mice treated with effector cell therapy

As up-regulated expression of select T cell-derived cytokines has been shown to have influential effects on antitumor immune responses and tumor progression in vivo (9, 20), we next assessed the cytokine-releasing profiles of recipient-derived CD4 (Thy1.1/CD4) and CD8 (Thy1.1/CD4) T cell subpopulations derived from lungs of long-term surviving mice receiving effector cell therapy. Type 1 (IFN-γ and TNF-α) and type 2 (IL-4 and IL-10) T cell cytokine profiles were assessed at the single cell level by intracellular staining and flow cytometry. As shown in Fig. 4, the frequency and cell number of both host-derived CD8/Thy1.1 and CD4/Thy1.1 T cells producing IFN-γ and TNF-α were predominately greater than those in corresponding host-derived T cells producing IL-4 and IL-10 in lungs of long-term surviving mice receiving TC2 effector cell therapy. Moreover, both type 1-like cytokine-producing recipient CD4 and CD8 T cell populations were noticeably elevated by nearly 2- to 3-fold compared with corresponding T cell populations in normal age-matched control mice. Concomitantly, the frequency and cell numbers of either intracellular IL-4 or IL-10 among corresponding T cells from these same treated animals were at low, yet significantly (p < 0.05) higher, levels compared with those in normal control mice. Similar results were obtained in long-term surviving animals receiving TC1 effector cell therapy (data not shown). Collectively, this suggests that treatment with either TC1 or TC2 effector cells elicits both systemic and local Th1 and TC1 IFN-γ- and TNF-α-producing T cell responses and tumor progression in vivo (9, 20), we next assessed the cytokine-releasing profiles of recipient-derived CD4 (Thy1.1/CD4) and CD8 (Thy1.1/CD4) T cell subpopulations derived from lungs of long-term surviving mice receiving effector cell therapy. 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cell responses in mice with established pulmonary disease, and that such responses may aid in part in promoting Tc1 or Tc2 effector cell-mediated, long-term tumor immunity and protection.

Identification of both donor and recipient-derived OVA tetramer-positive CD8 T cells among effector cell-treated, long-term tumor surviving mice

As long-term immunity and protection of class I-expressing tumors may be dependent in part on the localization and persistence of tumor Ag-specific CD8 T cells at the site of tumor growth, we next assessed the presence of OVA Ag-specific CD8 T cells among long-term surviving mice receiving effector cell therapy. Using Thy 1 congenic mice, donor (Thy 1.2) and recipient (Thy 1.1) OVA Ag-specific cells from lungs of long-term surviving mice were enumerated by OVA-Ag-specific tetramer staining and multicolor flow cytometric analysis. As shown in Fig. 5, OVA Ag-specific donor Thy 1.2/CD8 effector cells were present at similarly low, yet detectable, levels in the lungs of either Tc1 or Tc2 effector cell-treated, long-term surviving mice. In contrast, recipient tetramer-positive staining Thy 1.1/CD8 T cell frequencies from these same effector cell-treated animals showed markedly elevated levels, suggesting that either Tc1 or Tc2 effector cell therapy can induce recipient tumor Ag-specific CD8 T cell populations at sites of established tumor growth. Moreover, aside from quantitative differences, tetramer staining intensities among recipient CD8 T cell populations were significantly lower (p < 0.04) than those of donor tetramer-positive staining effector cells (mean intensity of tetramer staining: 333.0 ± 54.6) ex vivo, suggesting potential qualitative differences in tumor Ag binding affinities among the tumor Ag-specific CD8 T cell populations (5). Similar results were obtained in spleens of these same animals (data not shown). Detectable cell frequencies from

FIGURE 2. Tc1 or Tc2 effector cell therapies induce long-term tumor immunity, characterized by elevated levels of recipient CD8/CD44^high^ T cells coexpressing CD25, CD69, and Ly6C.Recipient mice (Thy 1.1) were injected i.v. with 1 × 10^7^ B16-OVA tumor cells. Seven days later, 2 × 10^6^ OVA Ag-specific Tc2 effector cells (Thy 1.2) were adoptively transferred into mice bearing established metastases. Eighty days later, surviving mice were rechallenged with similarly lethal numbers of B16-OVA tumor cells. At times >150 days after tumor rechallenge, spleen and lungs from individual animals were harvested, and single cell suspensions were made as described in Materials and Methods. Cells were stained with anti-Thy 1, anti-CD8, anti-CD44, and anti-CD25, -CD69, -CD95, or -Ly6C mAbs. Gates were set on Thy 1/CD8^+^ and CD44^high^ T cell populations, and activation marker staining profiles within these populations were assessed by multicolor flow cytometry. Data shown are from a representative experiment showing the percentages of lung- and spleen-derived Thy 1/CD8/CD44^high^ T cells coexpressing elevated levels of CD25, CD69, CD95, and Ly6C surface Ags in either effector cell-treated (A) or normal age-matched (B) mice. Histogram gates for all figures were adjusted to appropriate isotype controls and distinguish regions of nonspecific and specific Ab staining. Data are representative of five independent experiments. C. Absolute cell numbers were calculated as the percentage of positive-staining cells × the total number of monocytes per tissue. Results are expressed as the mean ± SEM of four to six mice per group in five independent experiments. *, p < 0.001; **, p < 0.01 (treated animals vs normal age-matched controls).
corresponding tissues among normal age-matched control mice were negligible (Fig. 5).

Phenotypic characterization of recipient-derived, OVA tetramer-positive CD8 T cells in effector cell-treated, long-term tumor surviving mice

As up-regulated expression of various cell surface markers may be indicative of T cell memory and/or differentiation (38) that may in part influence T cell-mediated antitumor immune responses, we next assessed the cell surface phenotypic characteristics of recipient OVA tetramer-positive Thy1.1/CD8 T cells at local (lung) sites of tumor growth among long-term surviving mice treated with effector cell therapy. As shown in Fig. 6, nearly all OVA tetramer-positive Thy1.1/CD8 T cells at local (lung) sites of tumor growth among long-term surviving mice treated with effector cell therapy were CD44high Ly6C high and expressed CD122 memory surface Ags. Histogram gates for all figures were adjusted to appropriate isotype controls and distinguish regions of nonspecific and specific Ab staining. Data are representative of two independent experiments. C. Absolute cell numbers were calculated as the percentage of positive-staining cells × the total number of monocytes per tissue. Results are expressed as the mean ± SEM of four to six mice per group in four independent experiments. *, p < 0.001; **, p < 0.01 (treated animals vs normal age-matched controls).

**FIGURE 3.** Tc1 or Tc2 effector cell therapies induce long-term tumor immunity characterized by elevated levels of recipient CD4/CD44 high T cells coexpressing CD25, CD69, and CD95. Long-term B16-OVA tumor-surviving mice were obtained as described in Fig. 2. At times >150 days after tumor rechallenge, spleen and lungs from individual animals in either Tc2 effector cell-treated (A) or normal age-matched (B) mice were harvested, and single cell suspensions were made as described in Materials and Methods. Cells were stained with anti-Thy 1.1, anti-CD4, anti-CD44, and anti-CD25, -CD69, or -CD95 mAbs. Gates were set on Thy 1.1/CD4 + and CD44high T cell populations, and activation marker staining profiles within these populations were assessed by multicolor flow cytometry. Numbers indicate the percentages of lung- and spleen-derived Thy 1.1/CD4/CD44 high recipient T cells coexpressing elevated levels of CD25, CD69, or CD95 surface Ags. Histogram gates for all figures were adjusted to appropriate isotype controls and distinguish regions of nonspecific and specific Ab staining. Data are representative of four independent experiments. C. Absolute cell numbers were calculated as the percentage of positive-staining cells × the total number of monocytes per tissue. Results are expressed as the mean ± SEM of four to six mice per group in four independent experiments. *, p < 0.001; **, p < 0.01 (treated animals vs normal age-matched controls).

Selected up-regulation of T cell-derived chemokine gene expression in lungs of mice exhibiting long-term tumor immunity after effector cell treatment

Chemokines have been shown to facilitate and aid in the generation of select antitumor immune responses (39). As treatment with either Tc1 or Tc2 effector cells resulted in accumulation and elevation of activated recipient-derived T cells that predominantly secreted cytokines associated with a type 1-like immune response (IFN-γ and TNF-α), we next investigated the expression of local T cell-derived chemokine genes in lungs of long-term surviving mice receiving effector cell therapy. Enriched T cells from lungs of either normal age-matched or effector cell-treated mice surviving beyond 150 days after tumor rechallenge were restimulated for 5 h with plate-bound anti-CD3 Ab, and chemokine gene expression...
was assessed by RNase protection assays as described in Materials and Methods. As shown in Fig. 7, restimulated T cells derived from lungs of either Tc1- or Tc2-treated animals showed a consistent and preferential elevation of the type 1-related chemoattractants IFN-inducible protein-10, macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, and lymphotactin compared with that of corresponding unstimulated cultures or normal age-matched controls (p < 0.05). In contrast, other type 1-associated chemokines, such as RANTES and monocyte chemoattractant protein-1, showed no significant (p > 0.05) differences in T cell gene expression between anti-CD3-restimulated and corresponding unstimulated cultures. This suggested that long-term tumor immunity elicited by either adoptively transferred Tc1 or Tc2 effector cells may in part differentially up-regulate and/or facilitate gene expression of select chemokines associated with type 1-like immune responses among resident lung T cells of effector cell-treated, long-term surviving mice.

**B16-OVA tumor cells are sensitive to TNF-α and IFN-γ in vitro**

As many tumors are potentially responsive to various cytokines, and our data show a marked accumulation of activated recipient-derived T cells predominantly producing IFN-γ and TNF-α at sites of local tumor growth, we next assessed whether B16-OVA was responsive to either TNF-α or IFN-γ. Previously, we have shown that the addition of exogenous IFN-γ, but not IL-4 or IL-5, can...
substantially inhibit B16-OVA tumor cell growth and increase tumor cell immunogenicity by up-regulating select tumor cell surface Ags and chemokine gene expression in vitro (40, 41). In the current study we extended our observations to investigate the direct effects of TNF-α in the absence or the presence of IFN-γ on B16-OVA tumor cell growth, phenotype, and survival. B16-OVA tumor cells were cultured in the presence or the absence of TNF-α and/or IFN-γ, harvested, and enumerated at various time intervals by standard cell count methods. In parallel cultures, total RNA was prepared, and RNase protection assays were performed as described in Materials and Methods. Chemokine mRNA was detected by RNase protection assays and normalized against the L32 housekeeping gene as relative units for comparative analysis. Normal age-matched mice served as controls.

FIGURE 5. Identification of recipient-derived OVA Ag-specific CD8 T cells elicited by either Tc1 or Tc2 effector cell therapies among surviving tumor-bearing mice. Surviving Tc1- or Tc2-treated tumor-bearing mice were obtained as described in Fig. 2. At times >150 days after tumor rechallenge, lungs from individual animals were harvested, and single cell suspensions were made as described in Materials and Methods. Lung cell suspensions were labeled with either anti-Thy 1.1 or anti-Thy-1.2, anti-CD8 and OVA tetramer. Gates were set on either donor Thy 1.1/CD8+ (A) or recipient Thy 1.1/CD8+ (B) T cells, and tetramer staining profiles within these populations were assessed by multicolor flow cytometry. Numbers indicate the percentages of total recipient (Thy 1.1) or donor (Thy 1.2) OVA tetramer-positive staining T cells per lung. Data are representative of three or four mice per group in five independent experiments. Normal age-matched Thy 1.1 mice served as controls.

FIGURE 6. Characterization of recipient-derived, tetramer-positive memory CD8 T cells in lungs of long-term tumor surviving mice receiving either Tc1 or Tc2 effector cell therapy. Lungs from long-term B16-OVA tumor-surviving mice (Thy 1.1) treated with either Tc1 or Tc2 effector cell therapy (Thy 1.2) were obtained as described in Fig. 5. Single cell suspensions were labeled with anti-Thy 1.1, anti-CD8, OVA tetramer, and anti-CD44, anti-Ly6C, or anti-CD122 CD8 T cell memory markers. Gates were set on Thy 1.1/CD8+ /OVA tetramer-positive cell populations, and memory marker-staining profiles within these populations were assessed by multicolor flow cytometry. Numbers indicate the percentages of lung-derived Thy 1.1/CD8+/tetramer-positive recipient T cells coexpressing elevated levels of CD44® , Ly6C® , and CD122 surface Ags. Data are representative of five independent experiments.
in vitro. These results merely suggest that these tumor cells are responsive to IFN-γ and TNF-α, and that apoptosis may in part be a potential TNF family-related death mechanism that is dependent on IFN-γ.

**Role of recipient-derived TNF-α and IFN-γ in effector cell-mediated, long-term tumor immunity and protection**

Because either Tc1 or Tc2 effector cell treatment resulted in an accumulation and elevation of activated recipient-derived T cells that predominantly secreted cytokines associated with a type 1-like immune response, we next investigated the role of host-derived IFN-γ and TNF-α in Tc1 or Tc2 effector cell-mediated, long-term tumor immunity. OVA Ag-specific Tc1 or Tc2 effector cell populations were generated as previously described and transferred into either wild-type or syngeneic cytokine gene knockout mice. Corresponding groups of untreated tumor-bearing mice served as controls. Results are representative of two independent experiments.

![Graph](image)

**FIGURE 8.** Role of recipient-derived TNF-α and IFN-γ in either Tc1 or Tc2 effector cell induced, long-term tumor immunity. Wild-type, TNF-α-deficient, or IFN-γ-deficient mice (n = 6–10/group) were injected i.v. with 1 × 10⁶ B16-OVA tumor cells. Seven days later, 2 × 10⁶ OVA Ag-specific Tc1 (A and B) or Tc2 (C and D) effector cells were adoptively transferred into specified groups of mice bearing established lung metastases, and survival times were monitored. Corresponding groups of untreated tumor-bearing mice served as controls. Results are representative of two independent experiments.

Effective tumor rejection by adoptively transferred T cells may depend on several potential direct and indirect mechanisms. Both Tc1 and Tc2 effector cell subpopulations have been shown in vitro to directly eradicate tumor cells through cognate interactions that involve predominately perforin-mediated lytic mechanisms. Interestingly, perforin derived from either polarized Tc1 or Tc2 effector cell subpopulations had little or no effect on enhancing survival among tumor-bearing mice in vivo. This is in agreement with other studies showing that perforin from nonpolarized CD8 T cell populations not only played little if any role in tumour-bearing wild-type mice receiving similar doses of Tc1 effector cell therapy. In parallel studies, transfer of these same effector cell populations into tumour-bearing IFN-γγ-/- knockout recipients showed a similarly marginal therapeutic effect compared with corresponding groups of untreated, tumour-bearing control knockout mice (Fig. 8B). Moreover, similar therapeutic effects were seen with corresponding groups of tumor-bearing mice treated with OVA Ag-specific Tc2 effector cells (Fig. 8C and D). Control groups of untreated tumor-bearing wild-type or cytokine knockout mice similarly died within 42 days after tumor challenge (Fig. 8). Collectively, this suggested that both Tc1 and Tc2 effector cell-mediated, long-term tumor immunity and protection were markedly dependent in part on host-derived IFN-γ and TNF-α.

**Discussion**

In the current study we assessed the therapeutic effects and potential influences of adoptively transferred, tumor-reactive Tc1 and Tc2 effector cells on endogenous T cell responses among mice bearing established pulmonary malignancy. We show that single-dose adoptive T cell immunotherapy with either Tc1 or Tc2 effector cell subpopulations induces considerable suppression, but not cure, of pulmonary metastases. However, long-term tumor immunity was evident by resistance to lethal tumor rechallenge that consequently prolonged survival times indefinitely among mice with established tumor. At early stages after therapy, long-term protection by Tc2 and Tc1 effector cells was dependent in part on effector cell-derived IL-4, IL-5, and IFN-γ, respectively, whereas effector cell-derived perforin was not necessary for either subpopulation. Moreover, single-dose T cell therapy with either Tc1 or Tc2 effector cells can potentiate effective endogenous recipient-derived, long-term antitumor responses by inducing 1) local T cell-derived chemokines associated with type 1 immune responses; 2) recipient-derived tetramer-positive CD8 memory T cells producing IFN-γ and TNF-α; and 3) accumulation of highly activated Th1 and Tc1 effector cell populations at the sites of tumor growth.

Effective tumor rejection by adoptively transferred T cells may be dependent on several potential direct and indirect mechanisms. Both Tc1 and Tc2 effector cell subpopulations have been shown in vitro to directly eradicate tumor cells through cognate interactions that involve predominately perforin-mediated lytic mechanisms. Interestingly, perforin derived from either polarized Tc1 or Tc2 effector cell subpopulations had little or no effect on enhancing survival among tumor-bearing mice in vivo. This is in agreement with other studies showing that perforin from nonpolarized CD8 T cell populations not only played little if any role in T cell-mediated antitumor responses (42–44), but also had the potential to regulate various CD4 and CD8 T cell-mediated immune responses in vivo (45). However, our results do show that in the absence of effector cell-derived IL-4, IL-5, and IFN-γγ-/- knockout recipients showed a similarly marginal therapeutic effect compared with corresponding groups of untreated, tumour-bearing control knockout mice (Fig. 8B). Moreover, similar therapeutic effects were seen with corresponding groups of tumor-bearing mice treated with OVA Ag-specific Tc2 effector cells (Fig. 8C and D). Control groups of untreated tumor-bearing wild-type or cytokine knockout mice similarly died within 42 days after tumor challenge (Fig. 8). Collectively, this suggested that both Tc1 and Tc2 effector cell-mediated, long-term tumor immunity and protection were markedly dependent in part on host-derived IFN-γ and TNF-α.
Although some experimental tumor models have demonstrated either favorable or deleterious contributions by type 2 cytokines in tumor rejection (25, 46–50), multiple models have suggested a dominant role for type 1 T cell responses in promoting optimal tumor rejection (51–55). Even though both donor-derived Tc1 and Tc2 effector cell numbers diminished to nearly nondetectable levels over time, both effector cell subpopulations promoted long-term tumor immunity that was characterized in part by the presence of chemokines associated with type 1-like immune responses and elevated levels of activated endogenous CD4 and CD8 T cells secreting IFN-γ and TNF-α at sites of tumor growth. The local emergence of such recipient-derived Tc1 and Th1 effector cell subpopulations after either Tc1 or Tc2 effector cell therapy may aid in part in controlling local tumor growth and further participate in regulating tumor relapse and recurrence. Our results show that B16-OVA tumor cells are both sensitive and responsive to IFN-γ and TNF-α in vitro. Addition of the latter in the presence of IFN-γ produced not only a marked decrease in tumor cell growth, but also an increase in the proportion of tumor cells undergoing apoptosis. This suggests that such direct synergistic cytokine-mediated effects can promote potential TNF family-related death mechanisms that are highly dependent on IFN-γ. Moreover, these findings were substantiated in vivo when long-term tumor immunity and protection were markedly dependent on recipient-derived IFN-γ and TNF-α in studies using effector cell-treated, tumor-bearing, cytokine knockout recipients. Collectively, this suggests that endogenous IFN-γ and TNF-α directly contribute in part to tumor cell death and/or acute tumor cell senescence that is initiated by effector cell-mediated immunotherapy in vivo.

Another interesting finding with therapeutic relevance was that both Tc1 and Tc2 effector cells promoted the emergence and persistence of substantial levels of endogenous OVA tetramer-positive Tc1 memory cells among long-term surviving mice with established tumor. It has been suggested that effective long-term tumor immunity is dependent on not only the quantity, but also the quality, of the memory T cells that are generated (9, 20). In our current study recipient-derived tetramer-positive CD8 T cells were CD44high, CD122+* and Lymph6high and predominately produced substantial amounts of IFN-γ and TNF-α. However, tetramer binding intensities among these cells were markedly lower than those of donor effector cell subpopulations. As earlier studies by Yee et al. (5) have shown that differences in T cell binding affinities to select tumor Ags correlated with differences in tetramer staining intensities among various tumor Ag-specific T cell clones, we suggest that the observed lower binding affinity by recipient-derived, tetramer-positive CD8 cells, as detected by flow cytometry, may potentially reflect a qualitatively less effective endogenous antitumor T cell response in vivo. Investigations to further elucidate and characterize the phenotype, functional efficiency, and contributions of such memory T cells in tumor senescence and eradication are in progress.

Although metastatic lesions in either Tc1 or Tc2 effector cell-treated mice were greatly reduced and, in fact, appeared to be eliminated in lungs of cured mice, minimal residual disease among these long-term surviving animals was still apparent. One possible explanation may be the emergence of select endogenous peptide-specific T cell populations after effector cell therapy. Consequently, such T cell repertoires may be ineffective, in that the absence of tumor antigen selects only the best tumor-reactive T cell clones for the treatment of patients with metastatic melanoma: In vivo persistence, migration, and antitumor effect of transferred T cells. Proc. Natl. Acad. Sci. USA 99:16168.


References