Article

Type 1 CD8$^+$ T Cells are Superior to Type 2 CD8$^+$ T Cells in Tumor Immunotherapy due to Their Efficient Cytotoxicity, Prolonged Survival and Type 1 Immune Modulation

Zhenmin Ye[1, 4], Chaoke Tang[1, 4], Shulin Xu[1], Bei Zhang[1], Xueshu Zhang[1], Terence Moyana[2], Jicheng Yang[3] and Jim Xiang[1, 5]

CD8$^+$ cytotoxic T (Tc) cells play a crucial role in host immune responses to cancer, and in this context, adoptive CD8$^+$ Tc cell therapy has been studied in numerous animal tumor models. Its antitumor efficacy is, to a large extent, determined by the ability of Tc cells to survive and infiltrate tumors. In clinical trials, such *in vitro*-activated T cells often die within hours to days, and this greatly limits their therapeutic efficacy. CD8$^+$ Tc cells fall into two subpopulations based upon their differential cytokine secretion. In this study, we *in vitro* generated that ovalbumin (OVA)-pulsed dendritic cell (DC_{OVA})-activated CD8$^+$ type 1 Tc (Tc1) cells secreting IFN-γ, and CD8$^+$ type 2 Tc (Tc2) cells secreting IL-4, IL-5 and IL-10, which were derived from OVA-specific T cell receptor (TCR) transgenic OT I mice. We then systemically investigated the *in vitro* and *in vivo* effector function and survival of Tc1 and Tc2 cells, and then assessed their survival kinetics after adoptively transferred into C57BL/6 mice, respectively. We demonstrated that, when compared to CD8$^+$ Tc2, Tc1 cells were significantly more effective in perforin-mediated cytotoxicity to tumor cells, had a significantly higher capacity for *in vivo* survival after the adoptive T cell transfer, and had a significantly stronger therapeutic effect on eradication of well-established tumors expressing OVA in animal models. In addition, CD8$^+$ Tc1 and Tc2 cells skewed the phenotype of CD4$^+$ T cells toward Th1 and Th2 type, respectively. Therefore, the information regarding the differential effector function, survival and immune modulation of CD8$^+$ Tc1 and Tc2 cells may provide useful information when preparing *in vitro* DC-activated CD8$^+$ T cells for adoptive T cell therapy of cancer. *Cellular & Molecular Immunology*. 2007;4(4):277-285.

Key Words: Tc1 cell, Tc2 cell, cytokine profile, cytotoxicity, survival, tumor therapy

Introduction

CD8$^+$ cytotoxic T lymphocytes (CTLs) play a crucial role in host immune responses to cancer. The ability of adoptive T cell immunotherapy to restore immunity and eradicate tumors has been shown in numerous animal models (1-5). However, the adoptive therapy is mostly limited to the treatment of early stage tumors or early lung metastases, but not well-established tumors. In clinical trials, CTLs derived from tumor-infiltrating lymphocytes, patient's peripheral blood T cells or CTL clones, have been used to treat such conditions as melanoma, and Hodgkin's disease (6-9). However, only limited numbers of patients have responded to the CTL therapy (objective response rates, ~30%) (10, 11). In general, the antitumor efficacy of transferred T cells is, to a large extent, determined by the ability of the T cells to survive and infiltrate into tumors (12). However, the fraction of transferred T cells that accumulate in tumors is disappointingly small (13).

In vivo CD8$^+$ T cell responses consist of three main phases (14) namely (i) a proliferation phase consisting of growth and differentiation of naïve CD8$^+$ T cells into effector T cells; (ii) a contraction phase characterized by a transition from the large population of effector T cells to a smaller population (≈ 5-10%) of memory T (Tm) cells (15) with

[1]Research Unit, Saskatchewan Cancer Agency, Departments of Oncology and Immunology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 4H4, Canada;

[2]Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Ontario K1H 8L6, Canada;

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[3]Cell and Molecular Biology Institute, College of Medicine, Soochow University, Suzhou 215132, China;

[4]Ye and Tang made the same contribution to the study.

[5]Corresponding to: Dr. Jim Xiang, Saskatoon Cancer Center, 20 Campus Drive, Saskatoon, Saskatchewan S7N 4H4, Canada; Tel: 306-655-2917, Fax: 306-655-2635, Email: jxiang@scf.sk.ca

$CD44^+CD62L^{high}CD127^+$ phenotype (16); and (iii) a memory phase with long-term maintenance of Tm cells in the host. When re-challenged with pathogen or tumor antigen, Ag-specific Tm cells, unlike naïve T cells, can respond swiftly by robust proliferation and upregulation of effector functions i.e. the so-called recall responses. *In vitro* activated $CD8^+$ T cells undergo a similar contraction phase of high-level activation-induced cell death (AICD) (17), such that only about 5-10% of infused T cells survive (6, 11, 17). In clinical trials, such *in vitro*-activated T cells, particularly with cloned T cells, often die within a few hours of adoptive transfer, and generally survive only a matter of days, which greatly limits their therapeutic efficacy (6, 9, 11, 18).

$CD8^+$ T cells can be polarized into two subpopulations based upon their differential cytokine secretion. Type 1 $CD8^+$ cytotoxic T (Tc1) cells secrete IFN-γ whereas type 2 cytotoxic T (Tc2) cells secrete IL-4, IL-5 and IL-10 (19). Tc1 cells, because of their capacity of efficiently migrating to inflamed tissues (20) where they secrete IFN-γ and kill their targets, are a major defense against virus-infected cells (20, 21). Tc2 cells were found in chronic human pathologies such as viral infections (22), cancer (23, 24), neurologic (25) and autoimmune diseases (26). The presence of Tc2 cells was correlated with disease severety and progration (22, 23, 25). In some circumstances, IL-10-secreting $CD8^+$ Tc cells may act as regulator T (Tr) cells (27, 28). In tumor immunology, Dobrzanski et al. have previously reported that both effector subpopulations display predominantly perforin-dependent cytolysis *in vitro* and furthermore that tumor-specific Tc1 cells were relatively more effective in reducing lung metastasis of B16 melanoma and subcutaneous Lewis lung carcinoma than Tc2 cells (29, 30). Recently, they have further shown that the Tc1 and Tc2 cells can also cure intradermally tansplanted B16 melanomas, but only in their very early stages (4). However, the survival and immune modulation of adoptively transferred Tc1 and Tc2 cells *in vivo* are unclear.

In this study, we developed a model system with a defined tumor antigen ovalbumin (OVA) using a OVA-transfected B16 melanoma cell line BL6-10_{OVA} and the OVA-specific T cell receptor (TCR) transgenic OT I mice, which provides a monoclonal source of T cells expressing OVA-specific TCR (31). We *in vitro* generated $CD8^+$ Tc1 and Tc2 cells secreting IFN-γ and IL-4/5/10, respectively, which were derived from OT I transgenic mice. We then systemically investigated the *in vitro* and *in vivo* effector function of Tc1 and Tc2 cells as well as the *in vivo* survival of Tc1 and Tc2 cells after T cells adoptively transferred into C57BL/6 mice, respectively. We also investigated the *in vivo* immune modulation of Tc1 and Tc2 cells.

Materials and Methods

Antibodies, cytokines, cell lines and animals

Biotin-conjugated anti-mouse CD4, CD8, CD25, CD44, CD45.1, CD62L, CD127, Fas, FasL, Vα2Vβ5 TCR, perforin and Bcl-2 antibodies (Abs) were all purchased from BD PharMingen (San Diego, CA). The fluorescein isothiocyanate (FITC)-conjugated avidin were purchased from Bio/Can Scientific (Mississauga, Ontario, Canada). R-phycoerythrin (PE)-labeled H-$2K^b$/$OVA_{257-264}$ tetramer, PE-labeled anti-IL-4 and anti-IFN-γ Abs, FITC-labeled anti-CD4 and anti-CD8 Abs as well as energy-coupled dye (ECD)-conjugated anti-CD45.1 Abs were all obtained from Beckman Coulter (San Diego, CA). The anti-IL-4 and anti-IFN-γ Abs, and the recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF), IL-2, IL-4 and IL-12 were purchased from R&D Systems (Minneapolis, MN). The chicken egg ovalbumin (OVA) protein, concanamycin A (CMA), emetin, PMA, ionomycin and lipopolysaccharide (LPS) were obtained from SIGMA (St. Louis, MO). OVA I ($OVA_{257–264}$, SIINFEKL) peptide (31) was synthesized by Multiple Peptide Systems (San Diego, CA). The mouse thymoma cell line EL4 and its derivative OVA-transfected cell line EG7 were obtained from American Type Culture Collection (ATCC, Rockville, MD). EG7 tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Gaithersburg, MD) containing 10% fetal calf serum (FCS) and G418 (0.5 mg/ml). C57BL/6 (B6, $CD45.2^+$) mice, B6.SJL-Ptpera (B6.1, $CD45.1^+$) mice as well as the OVA-specific T cell receptor (TCR) transgenic OT I and OT II mice having a transgenic Vα2Vβ5 TCR specific for OVA I epitope (SIINFEKL) in context of H-$2K^b$ and OVA II epitope (ISQAVHAAHAEINEAGR) in context of Ia^b (32) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Homozygous OT II/B6.1 mice were generated by backcrossing B6.1 mice onto OT II mice. Animal care was in accordance with the guidelines of the University of Saskatchewan.

Preparation of bone marrow-derived DC

The preparation of bone marrow (BM)-derived dendritic cells (DCs) was previously described. Briefly, BM cells prepared from femora and tibiae of normal C57BL/6 mice were depleted of red blood cells with 0.84% ammonium chloride and plated in DC culture medium I [DMEM plus 10% FCS, GM-CSF (20 ng/ml)]. Two days later, the nonadherent granulocytes, T and B cells were gently removed, and fresh media [DMEM plus 10% fetal calf serum (FCS), GM-CSF (20 ng/ml) and IL-4 (20 ng/ml)] were added. On day 6, the adherent cells were harvested and pulsed overnight at 37°C with OVA (0.3 mg/ml) and termed DC_{OVA}.

Preparation of OVA-specific $CD8^+$ T cell subsets

Naïve OVA-specific $CD8^+$ T cells were isolated from the spleens and lymph nodes of OT I mice, enriched by passage through nylon wool columns (C&A Scientific, Manassa, VA), and then fractionated by negative selection using anti-mouse CD4 (L3T4) paramagnetic beads (DYNAL Inc., Lake Success, NY) according to the manufacturer's protocols. The OVA-specific T cell subsets (Tc1 and Tc2 cells) were further generated by culturing naïve $CD8^+$ T cells (3×10^5 cells/ml) with irradiated (4,000 rad) DC_{OVA} (2×10^5 cells/ml) in 96-well plate in presence of IL-2 (20 U/ml), IL-12 (5 ng/ml) and anti-IL-4 Ab (5 μg/ml) or IL-2 (20 U/ml), IL-4 (20 ng/ml) and anti-IFN-γ Ab (10 μg/ml) (30), respectively. *In vitro*-

activated Tc1 and Tc2 cell subsets were harvested after 3 day's culture, purified using Ficoll-Paque (Sigma, St. Louis, MO) density gradient centrifugation, and then CD8 microbeads (Miltenyi Biotec, Auburn, CA).

Phenotypic characterization of $CD8^+$ Tc1 and Tc2 cells
The active T cell subsets were stained with a panel of Abs for CD8, CD25, FasL and TCR, and then analyzed by flow cytometry. For the intracellular cytokines, T cells were restimulated with 4,000 rad-irradiated EG7 tumor cells for 4 hours, and then processed using a "Cytofix/CytoPerm Plus with GolgiPlug" kit (BD PharMingen), with biotin-conjugated anti-perforin and anti-Bcl-2 Abs followed with FITC-conjugated avidin. Culture supernatants of the re-stimulated $CD8^+$ T cell subsets were analyzed for cytokine expression using ELISA kits (Endogen, Cambridge, MA), as previously reported (31). The results were normalized to the recombinant cytokine standard curves.

$CD8^+$ Tc1 and Tc2 cell survival in vitro and in vivo
Naïve C57BL/6 mice (6 per group) were *i.v.* injected with DC_{OVA}-activated $CD8^+$ Tc1 or Tc2 cells (1×10^7 cells per mouse). H-$2K^b$/$OVA_{257-264}$ tetramer staining assay was performed to examine the amount of OVA-specific $CD8^+$ Tc1 and Tc2 cells in mouse peripheral blood at different time points after the adoptive T cell transfer. The tail blood samples were incubated with PE-labeled H-$2K^b$/$OVA_{257-264}$ tetramer, and FITC-labeled rat anti-mouse CD8 Ab, and analyzed by flow cytometry (31).

$CD8^+$ Tc1 and Tc2 cell cytotoxicity in vitro
$CD8^+$ Tc1 or Tc2 cells were used as effector (E) cells, whereas the OVA-expressing EG7 tumor cells and the original EL4 tumor cells without OVA expression were used as target (T) cells. The target cells were radio-labeled by culturing these cells for 1 h in the culture medium in presence of 50 μl of sodium [^{51}Cr]-chromate (36 mCi/ml; Amersham, Arlington Heights, IL), and then washed twice with DMEM. Approximately 1×10^4 labeled target cells per triplicate well were mixed with effector cells at various E:T cell ratios, and then incubated for 6 h. For testing the killing mechanism, the effector cells were pre-incubated with concanamycin A (CMA) (1 μM) and emetin (5 μM) for 2 h to prevent perforin (CMA)- or Fas/FasL interaction (emetin)-mediated cytotoxicity (33). The percentage of specific lysis was calculated as: 100 × [(experimental cpm - spontaneous cpm) / (maximal cpm - spontaneous cpm)]. The maximal cpm release was determined by lysis of T cells with 0.25% Triton X-100.

In vivo immune modulation of Tc1 and Tc2 cells
$CD8^+$ Tc1 or Tc2 cells (1×10^7 cells per mouse) were *i.v.* transferred into C57BL/6 mice (4 mice per group). Twelve hours later, equal numbers of naïve OT II $CD4^+$ T cells were *i.v.* injected. After one day, mice were *i.v.* given a mixture of OVA protein (0.5 mg), OVA I peptide (50 μM) and LPS (10 ng). Six days later, mice were sacrificed and their splenocytes were assayed for intracellular cytokine production (ICP) after 6 h of PMA (50 ng/ml) and ionomycin (500 ng/ml) stimulation. Splenocytes were stained with FITC-anti-CD4 and ECD-anti-CD45.1. Cells were then fixed and cell membranes were permeable in Cytosix/Cytoperm solution (BD Biosciences) and stained with PE-anti-IFN-γ or PE-anti-IL-4 Abs for flow cytometric analysis (34). In another set of experiments, $CD8^+$ Tc1 and Tc2 cells (5×10^6 cells per mouse) were *i.v.* transferred into C57BL/6 mice (6 mice per group). Six days later, mice were sacrificed and their splenocytes were stained with PE-labeled H-$2K^b$/$OVA_{257-264}$ tetramer and FITC-labeled anti-CD8 Ab (31), and analyzed by flow cytometry.

Adoptive Tc1 and Tc2 cell immunotherapy model
C57BL/6 mice (eight per group) were *s.c.* injected with 2×10^6 EG7 tumor cells in their right thighs. At 7-8 and 10-11 days post-inoculation, tumors became ~4 and ~6 mm in diameter, respectively. To compare the immunotherapeutic effect between Tc1 and Tc2 cells, the above tumor-bearing mice were *i.v.* injected with $CD8^+$ Tc1 and Tc2 (5×10^6) cells, respectively. Animal mortality and tumor growth or regression was monitored daily for up to 10 weeks; for humanitarian reasons, all mice with tumors that achieved a size of 1.5 cm in diameter were sacrificed. Log rank test and Graphpad Prism software were used to compare the mouse survival data (35).

Results

Characterization of $CD8^+$ Tc1 and Tc2 cells
$CD8^+$ cytotoxic T cells derived from TCR transgenic OT I mice were generated *in vitro* by OVA-pulsed DC (DC_{OVA}) stimulation in the presence of IL-12/anti-IL-4 Ab and IL-4/anti-IFN-γ Ab, respectively. These $CD8^+$ T cells displayed expression of cell-surface CD8, CD25 and Vα2Vβ5 TCR (Figure 1A), indicating that they are OVA-specific active $CD8^+$ T cells. They all expressed a similar amount of cell-surface FasL and intracellular perforin. We then examined cytokine secretion of $CD8^+$ T cells in their culture supernatants by ELISA. As shown in Figure 1B, $CD8^+$ T cells derived from DC_{OVA} stimulation in presence of IL-12/anti-IL-4 Ab secreted IFN-γ (~3.4 ng/ml/10^6 cells/24 h), but not IL-4, IL-5 and IL-10, whereas $CD8^+$ T cells derived from DC_{OVA} stimulation in presence of IL-4/anti-IFN-γ Ab secreted IL-4 (~1.6 ng/ml/10^6 cells/24 h), IL-5 (~1.8 ng/ml/10^6 cells/24 h) and IL-10 (~2.6 ng/ml/10^6 cells/24 h), but not IFN-γ, indicating that $CD8^+$ T cells derived from DC_{OVA} stimulation in presence of IL-12/anti-IL-4 Ab and IL-4/anti-IFN-γ Ab are type 1 and type 2 $CD8^+$ cytotoxic T (Tc1 and Tc2) cells, respectively.

$CD8^+$ Tc1 cells are more effective in in vitro cytotoxicity for OVA-expressing tumor cells than Tc2 cells
To examine *in vitro* $CD8^+$ T cell cytotoxicity, we tested its impact on the cell's abilities to lyse ^{51}Cr-labeled OVA-expressing EG7 cells. As shown in Figure 2A, $CD8^+$ Tc1 cells displayed significant cytotoxicity for OVA-expressing

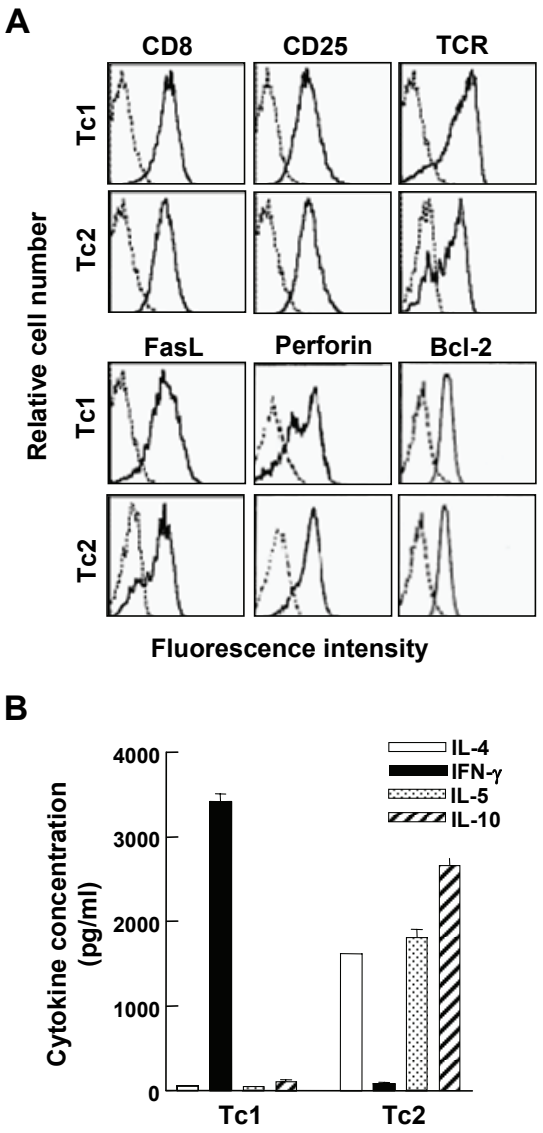

Figure 1. Phenotypic characterization of CD8$^+$ Tc1 and Tc2 cells. (A) Phenotypic analysis by flow cytometry. CD8$^+$ Tc1 and Tc2 cells were stained with biotin-conjugated anti-CD8, CD25, FasL and TCR Abs followed with FITC-avidin (solid lines), and then analyzed by flow cytometry. CD8$^+$ Tc1 and Tc2 cells were also permeabilized and stained for intracellular perforin and Bcl-2 using biotin-labeled anti-perforin and anti-Bcl-2 Abs followed with FITC-avidin (solid lines), and then analyzed by flow cytometry. Irrelevant isotype-matched biotin-conjugated Abs were used as controls (dotted lines). (B) Cytokine secretion of CD8$^+$ Tc1 and Tc2 cells. Culture supernatants of the re-stimulated CD8$^+$ T cell subsets were analyzed for cytokine expression using ELISA kits. Values represent the means of triplicates from two experiments.

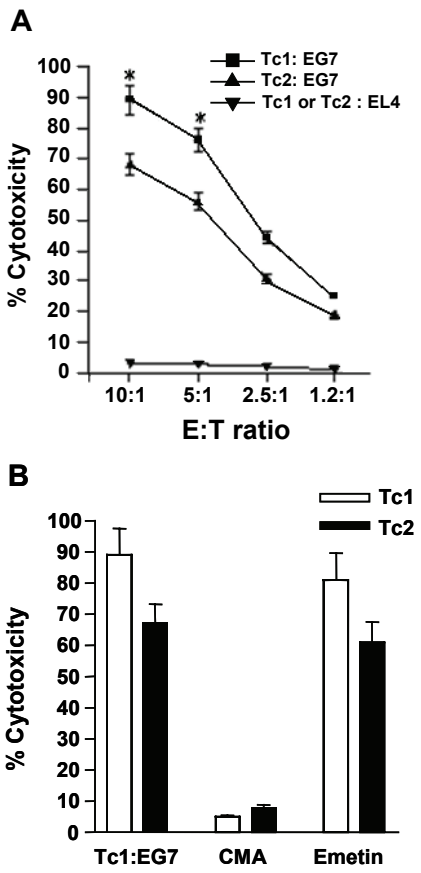

Figure 2. Cytotoxicity assay. (A) DC_{OVA}-activated CD8$^+$ Tc1 and Tc2 cells were used as effector (E) cells, whereas the OVA-expressing EG7 and the parental EL4 tumor cells were used as target (T) cells. The data are presented as the percent specific lysis of the target cells in a 6 h ^{51}Cr-release assay. Each point represents the mean of triplicate cultures. *, $p < 0.05$ versus cohorts of Tc2 cells (Student's t test). (B) In inhibition assays, the effector cells were preincubated with CMA and emetin for 2 h and then applied to the cytotoxicity assay. One representative experiment of three is depicted.

EG7 tumor cells (89% specific killing at an E:T cell ratio of 10), but not for the parental tumor cell line EL-4 without OVA expression, indicating that the cytotoxicity is OVA-tumor specific. However, CD8$^+$ Tc2 cells displayed a significantly decreased level of specific killing (i.e., 66% specific killing at an E:T cell ratio of 10) for EG7 tumor cells ($p < 0.05$), indicating that CD8$^+$ Tc1 cells are more effective in *in vitro* cytotoxicity for OVA-expressing tumor cells than CD8$^+$ Tc2 cells. In general, CD8$^+$ Tc cells kill the target cells through two distinct cytolytic pathways, the perforin-dependent granule exocytosis and the FasL/Fas interaction pathway (36). Perforin, in the presence of calcium, has the ability to insert functional pores into lipid bilayer membranes, polymerize and form structural alterations that can lead to cell lysis, whereas binding of FasL to Fas on CTLs initiates the death pathway of apoptosis in the Fas-bearing target cells. To study the killing mechanism, CMA and emetin were used to inhibit perforin- and FasL/Fas interaction-mediated cytotoxicity, respectively. Our data showed that both CMA and emetin demonstrated a dose-dependent inhibition of Tc cell cytotoxicity (data not shown). The treatment of EG7 tumor cells with CMA at 1 μM resulted in ~90% inhibition of both Tc1 and Tc2 cell-mediated cytotoxicity, whereas

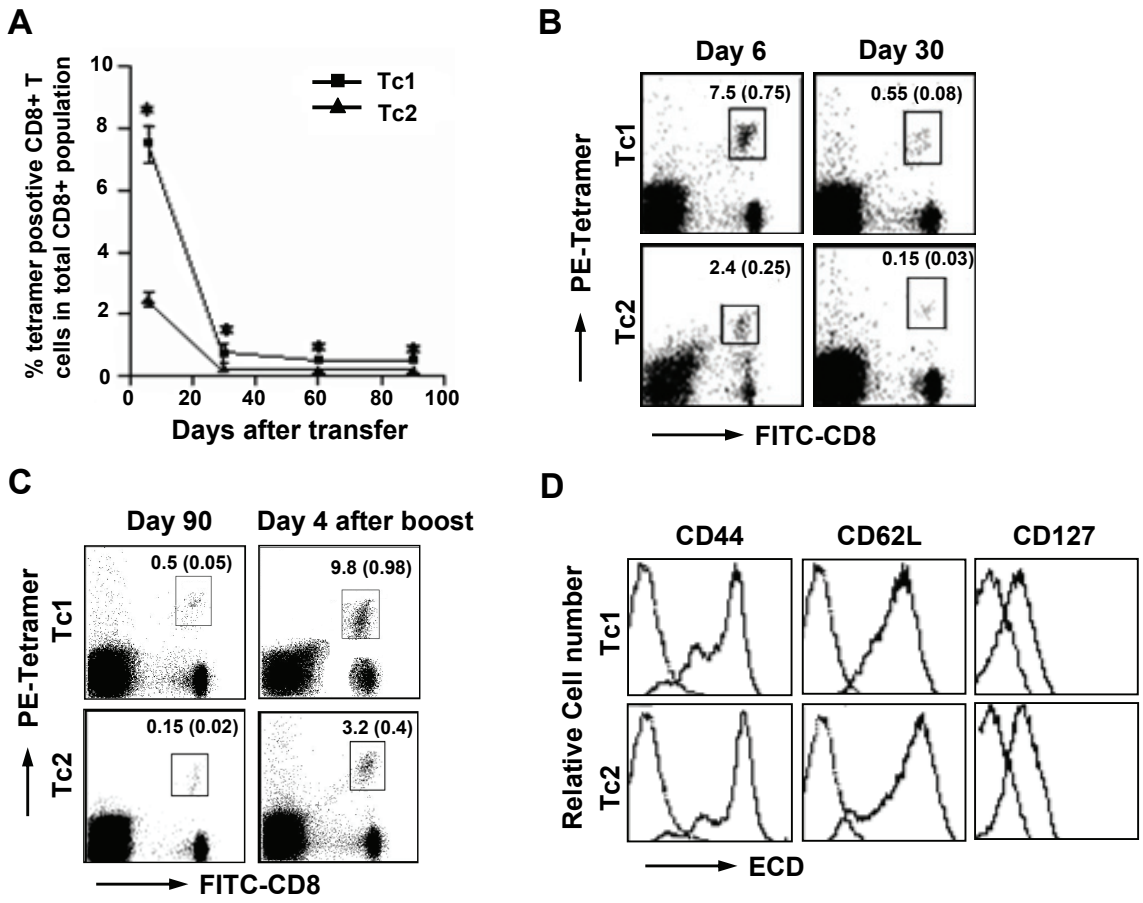

Figure 3. CD8$^+$ T cell survival *in vivo* after adoptive transfer. (A & B) CD8$^+$ Tc1 and Tc2 cells (10×10^6 cells per mouse) were *i.v.* injected into C57BL/6 mice, respectively. Mouse tail blood cells were stained with PE-H-2K^b/OVA I tetramer (PE-tetramer), FITC-anti-CD8 (FITC-CD8) Ab, and analyzed by flow cytometry at indicated time points after adoptive T cell transfer. The value in each panel represents the percentage of PE-tetramer-positive CD8$^+$ T cells versus the total peripheral CD8$^+$ T cell population. The value in parenthesis represents the standard deviation. *, $p < 0.05$ versus cohorts of Tc2 cells (Student *t* test). (C) Three months after adoptive transfer, the mice were *i.v.* boosted with DC_{OVA} (0.5×10^6 cells). Four days after the boost, mouse tail blood cells were stained with PE-tetramer and FITC-CD8, and then analyzed by flow cytometry. (D)The PE-tetramer-positive CD8$^+$ T cells detected on day 30 were also gated for further analysis of ECD-44, ECD-CD62L and ECD-CD127 expression by flow cytometry.The value in each panel represents the percentage of PE-tetramer-positive CD8$^+$ T cells versus the total peripheral CD8$^+$ T cell population. The value in parenthesis represents the standard deviation. The results presented are representative of three in the above different experiments.

treatment with emetin at 5 μM resulted in only ~8% inhibition (Figure 2B). This indicates that the perforin-mediated pathway plays a major role in both Tc1 and Tc2 cytotoxicity *in vitro*.

CD8$^+$ Tc1 cells have prolonged in vivo survival after adoptive T cell transfer

To examine *in vivo* T cell survival, CD8$^+$ Tc1 or Tc2 cells (10×10^6 cells per mouse) were *i.v.* transferred into C57BL/6 mice. The number of detected OVA-specific CD8$^+$ T cells in the mouse blood were evaluated at different time points after T cell transfer by flow cytometry. As shown in Figures 3A and 3B, the number of detected OVA-specific CD8$^+$ T cells in the mouse blood accounted for 7.5% and 2.4% of the total CD8$^+$ T cell population at day 6 after the transfer of Tc1 and Tc2 cells, respectively ($p < 0.05$). The numbers then gradually dropped to 0.55% and 0.15% ($p < 0.05$) after the first month, but remained stable at those levels at least 3 months, indicating that CD8$^+$ Tc1 cells have prolonged *in vivo* survival after adoptive T cell transfer. All the OVA-specific CD8$^+$ T cells in the blood expressed CD44, CD62L and CD127, indicating that they had become CD8$^+$ memory T (Tm) cells (16) 30 days after adoptive T cell transfer. To examine their functional activity, we performed a recall response by re-stimulation of the mice with DC_{OVA}. As shown in Figure 3C, CD8$^+$ Tm cells derived from CD8$^+$ effector Tc1 and Tc2 cells could all be greatly expanded ~20 fold, accounting for 9.8% and 3.2% and of the total CD8$^+$ T cell population 4 days after DC_{OVA} boost.

CD8$^+$ Tc1 and Tc2 cells modulate type 1 and type 2 immune response, respectively

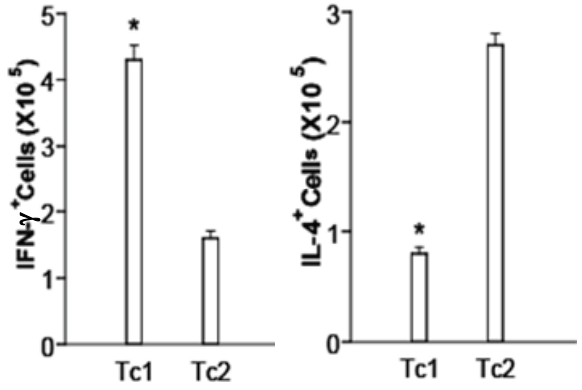

Figure 4. Tc1 and Tc2 effectors promote Th1 and Th2 cell development. $CD8^+$ Tc1 or Tc2 cells were adoptively transferred into C57BL/6 mice together with (12 h apart) naïve $CD4^+$ T cells derived from OT II/B6.1 mice. Mice were then immunized with a mixture of OVA protein, SIINFEKL peptide and LPS. Six days later, mouse splenocytes were tested for intracellular cytokine production (ICP) after stimulation with PMA/ionomycin. Splenocytes were stained with FITC-anti-CD4 Ab, ECD-anti-CD45.1 Ab and PE-anti-IFN-γ or IL-4 Ab, and analyzed by flow cytometry. Numbers of IFN-γ and IL-4 positive cells within the fraction of $CD4^+CD45.1^+$ T cells (grouped by flow cytometry) in each spleen were counted. *, $p < 0.05$ versus cohorts of Tc2 cells (Student's *t* test). One representative experiment of two is shown.

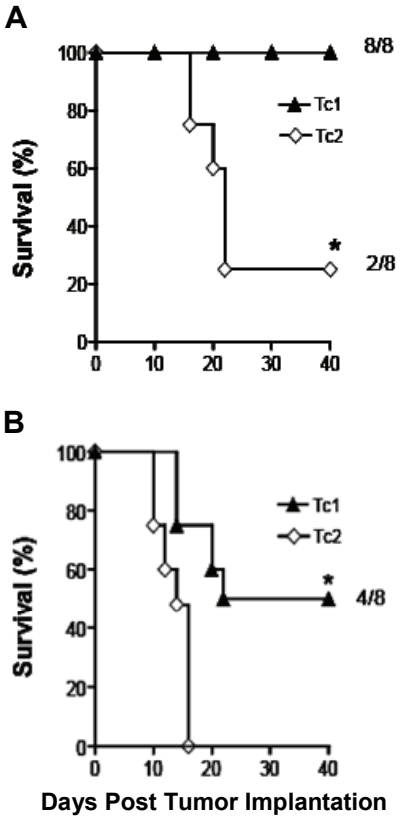

Figure 5. Inhibition of tumor growth in animal models. Mice (8 per group) bearing (A) early stage (~4 mm in diameter) or (B) well-established (~6 mm in diameter) tumors were given *i.v.* injection of $CD8^+$ Tc1 and Tc2 cells, respectively. Tumor growth was monitored and the tumor size (diameter) measured using a caliper. The evolution of the tumors in individual mice is depicted for their survival period. *, $p < 0.05$ versus cohorts of Tc2 cell immunization (Log rank test). One representative experiment of two is shown.

To test the capacity of immune modulation, $CD8^+$ Tc1 or Tc2 cells were transferred into C57BL/6 mice together with naïve $CD4^+$ T cells derived from OT II/B6.1 mice, and challenged with a mixture of OVA protein, SIINFEKL peptide and LPS. Animals were killed 6 days after antigen challenge, and the cytokine profile of expanded OT II/B6.1 $CD4^+CD45.1^+$ T cells was evaluated. As shown in Figure 4, $CD8^+$ Tc1 and Tc2 cells had 2.6-fold and 3-fold enrichment of OVA-specific $CD4^+$ T cells producing IFN-γ and IL-4, respectively, indicating that $CD8^+$ Tc1 and Tc2 cells favor polarization of *in vivo* ongoing responses toward a Th1 and Th2 phenotype, respectively.

$CD8^+$ Tc1 cells have stronger therapeutic effect in eradication of well-established tumors than Tc2 cells in animal model

To compare the therapeutic efficiency of OVA-specific $CD8^+$ Tc1 and Tc2 cells, mice bearing OVA-expressing EG7 tumors with ~4 mm or ~6 mm in diameter were *i.v.* injected with $CD8^+$ Tc1 or Tc2 cells. Our data showed that both OVA-specific $CD8^+$ Tc1 and Tc2 cells had a tumor eradication effect. Adoptive transfer of 5×10^6 Tc1 or Tc2 cells cured 8/8 (100%) and 2/8 (25%) mice bearing EG7 tumors in the early stage (~4 mm in diameter) (Figure 5A), respectively. In mice bearing well-established EG7 tumors (~6 mm in diameter), $CD8^+$ Tc1 cell treatment cured 50% (4/8) mice, whereas all (8/8) mice treated with $CD8^+$ Tc2 cells died of tumor growth (Figure 5B) ($p < 0.05$), indicating that $CD8^+$ Tc1 cells are significantly more effective in treatment of well-established EG7 tumors than Tc2 cells.

Discussion

Many experimental T cell therapy studies using tumor-specific $CD8^+$ Tc1 and Tc2 cells were conducted in the OVA-transfected B16 melanoma lung metastasis model (4, 33, 37-39). $CD8^+$ Tc1 cells were 5-fold more effective than Tc2 cells, though both adoptively transferred OVA-specific $CD8^+$ Tc1 and Tc2 cells eradicated OVA-expressing B16 melanoma lung metastasis (33). Both OVA-specific $CD8^+$ Tc1 and Tc2 cells also eradicated *s.c.* growth of OVA-expressing EG7 tumors (37). Again, $CD8^+$ Tc1 cells were 5-10 fold more effective than Tc2 cells. We have recently demonstrated that the therapeutic efficiency of adoptively transferred OVA-specific Tc1 cells can be greatly enhanced in the treatment of mice bearing well-established EG7 tumors when the mice were co-treated with adenovirus-mediated lymphotactin/IP-10 transgene expression (40), adenovirus-mediated lymphotactin transgene expression and $CD4^+$ Th cell transfer (35) to increase $CD8^+$ Tc1 cell tumor localization,

respectively. In this study, we directly compared the therapeutic efficiency of OVA-specific $CD8^+$ Tc1 and Tc2 cells in the treatment of well-established EG7 tumors. We demonstrated that both OVA-specific $CD8^+$ Tc1 and Tc2 cells had eradicative effect of tumors. However, $CD8^+$ Tc1 are more effective on treatment of well-established EG7 tumors than Tc2 cells. It has been previously shown that, following the adoptive transfer of equal amounts of $CD8^+$ Tc1 and Tc2 cells into tumor-bearing mice, the Tc1 cells tended to localize more in tumors than Tc2 cells (33). In this study, we demonstrated that, after adoptive T cell transfer, $CD8^+$ Tc1 cells had a higher capacity for *in vivo* survival than Tc2 cells. Therefore, we are inclined to think that the more efficient therapeutic effect of $CD8^+$ Tc1 cells seen in this study may be partly derived from the longer survival of Tc1 cells *in vivo*, which in turn leads to greater localization of Tc1 cells in the tumors.

The molecular mechanism of *in vivo* Tc1 and Tc2 cell-mediated tumor eradication still remains controversial, though it has been extensively studied. For example, Winter et al. originally demonstrated that, following adoptive T cell transfer, tumor regression is independent of perforin or Fas/TasL interactions (41). Dobrzanski et al. showed that $CD8^+$ Tc1 cell-derived IFN-γ and $CD8^+$ Tc2 cell-derived IL-4 and IL-5 are responsible for Tc1 and Tc2-mediated tumor eradication and animal survival (4). However, Kemp et al. demonstrated that Tc cell-secreted IFN-γ remains the only most critical antitumor effector mechanism *in vivo* (29). Recently, it has been illustrated that the host-derived TNF-α and IFN-γ are both involved in Tc1 and Tc2 cell-mediated tumor eradication (39, 42). More recently, Hollenbaugh et al have further elucidated that the ability of the host to respond to Tc1-secreted IFN-γ is critical for promoting acute tumor rejection, while the host production of IFN-γ is not important (43).

DCs play a central role in modulation of immune responses. Different DC subsets stimulate $CD4^+$ Th1 and Th2 or Tr1 responses (44-46). DCs in different maturation (immature and mature) stages induce active and tolerogenic immune responses, respectively (28, 47). However, T cells can also modulate DC phenotype and function. For example, T cells down-regulate peptide/MHC complexes on DCs after DC activation (48). $CD4^+$ T cells can condition DCs *via* CD40L/CD40 interactions. Subsequently, the "conditioned" DCs stimulate $CD8^+$ CTL responses (49, 50). It has been demonstrated that $CD4^+$ Tr cells and anergic T cells can down-regulate the expression of DC's MHC II and costimulatory molecules and inhibit DC's function (51, 52). It has also been demonstrated that both $CD4^+$ Th2 and $CD8^+$ Tc2 cells modulate DCs favoring induction of Th2 responses (53, 54). In this study, we demonstrated that $CD8^+$ Tc1 and Tc2 cells were able to skew the phenotype of OT II $CD4^+$ T cells toward IFN-γ-secreting Th1 and IL-4-secreting Th2 type, respectively. However, the molecular mechanism responsible for this immune modulation is not very clear. It has recently been reported that both type 2 $CD4^+$ and $CD8^+$ T cells can modify the function of DCs by favoring type 1 and 2 responses *via* secretion of IL-4, IL-10 and IL-13 (53-55).

Taken together, we demonstrate that $CD8^+$ Tc1 cells secreting IFN-γ are more effective in perforin-mediated cytotoxicity to tumor cells than $CD8^+$ Tc2 cells secreting IL-4, IL-5 and IL-10. $CD8^+$ Tc1 cells have prolonged *in vivo* survival after adoptive T cell transfer. $CD8^+$ Tc1 cells also have a stronger therapeutic effect in the eradication of well-established tumors in animal models than $CD8^+$ Tc2 cells. In addition, $CD8^+$ Tc1 and Tc2 cells skew the phenotype of $CD4^+$ T cells toward Th1 and Th2 type, respectively. Therefore, the information regarding the differential effector function, survival and immune modulation of type 1 and 2 $CD8^+$ cytotoxic T cells may prove useful when preparing *in vitro* DC-activated $CD8^+$ T cells for adoptive T cell therapy of cancer.

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References

1. Plautz GE, Touhalisky JE, Shu S. Treatment of murine gliomas by adoptive transfer of *ex vivo* activated tumor-draining lymph node cells. Cell Immunol. 1997;178:101-107.
2. Peng L, Shu S, Krauss JC. Treatment of subcutaneous tumor with adoptively transferred T cells. Cell Immunol. 1997;178: 24-32.
3. Tanaka H, Yoshizawa H, Yamaguchi Y, et al. Successful adoptive immunotherapy of murine poorly immunogenic tumor with specific effector cells generated from gene-modified tumor-primed lymph node cells. J Immunol. 1999;162:3574-3582.
4. Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and type 2 $CD8^+$ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. J Immunol. 2000;164:916-925.
5. Becker C, Pohla H, Frankenberger B, et al. Adoptive tumor therapy with T lymphocytes enriched through an IFN-γ capture assay. Nat Med. 2001;7:1159-1162.
6. Yee C, Thompson JA, Byrd D, et al. Adoptive T cell therapy using antigen-specific $CD8^+$ 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 U S A. 2002;99:16168-16173.
7. Bollard CM, Aguilar L, Straathof KC, et al. Cytotoxic T lymphocyte therapy for Epstein-Barr virus$^+$ Hodgkin's disease. J Exp Med. 2004;200:1623-1633.
8. Rosenberg SA, Dudley ME. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. Proc Natl Acad Sci U S A. 2004;101 Suppl 2: 14639-14645.
9. Vignard V, Lemercier B, Lim A, et al. Adoptive transfer of tumor-reactive Melan-A-specific CTL clones in melanoma patients is followed by increased frequencies of additional Melan-A-specific T cells. J Immunol. 2005;175:4797-4805.
10. Lewko WM, Hall PB, Oldham RK. Growth of tumor-derived

activated T cells for the treatment of advanced cancer. Cancer Biother Radiopharm. 2000;15:357-366.
11. Dudley ME, Wunderlich J, Nishimura MI, et al. Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. J Immunother. 2001;24:363-373.
12. Mukai S, Kjaergaard J, Shu S, Plautz GE. Infiltration of tumors by systemically transferred tumor-reactive T lymphocytes is required for antitumor efficacy. Cancer Res. 1999;59:5245-5249.
13. Ogawa M, Umehara K, Yu WG, et al. A critical role for a peritumoral stromal reaction in the induction of T-cell migration responsible for interleukin-12-induced tumor regression. Cancer Res. 1999;59:1531-1538.
14. Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol. 2003;3:269-279.
15. Huster KM, Busch V, Schiemann M, et al. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct $CD8^+$ memory T cell subsets. Proc Natl Acad Sci U S A. 2004;101:5610-5615.
16. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol. 2003;4:1191-1198.
17. Sprent J, Tough DF. T cell death and memory. Science. 2001; 293:245-248.
18. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.
19. Carter LL, Dutton RW. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. Curr Opin Immunol. 1996;8: 336-342.
20. Cerwenka A, Morgan TM, Harmsen AG, Dutton RW. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. J Exp Med. 1999;189:423-434.
21. Prezzi C, Casciaro MA, Francavilla V, et al. Virus-specific $CD8^+$ T cells with type 1 or type 2 cytokine profile are related to different disease activity in chronic hepatitis C virus infection. Eur J Immunol. 2001;31:894-906.
22. Accapezzato D, Francavilla V, Paroli M, et al. Hepatic expansion of a virus-specific regulatory $CD8^+$ T cell population in chronic hepatitis C virus infection. J Clin Invest. 2004;113: 963-972.
23. Ito N, Nakamura H, Tanaka Y, Ohgi S. Lung carcinoma: analysis of T helper type 1 and 2 cells and T cytotoxic type 1 and 2 cells by intracellular cytokine detection with flow cytometry. Cancer. 1999;85:2359-2367.
24. Anichini A, Mortarini R, Romagnoli L, et al. Skewed T-cell differentiation in patients with indolent non-Hodgkin lymphoma reversed by *ex vivo* T-cell culture with γc cytokines. Blood. 2006;107:602-609.
25. Ochi H, Wu XM, Osoegawa M, et al. Tc1/Tc2 and Th1/Th2 balance in Asian and Western types of multiple sclerosis, HTLV-I-associated myelopathy/tropical spastic paraparesis and hyperIgEaemic myelitis. J Neuroimmunol. 2001;119:297-305.
26. Inaoki M, Sato S, Shirasaki F, Mukaida N, Takehara K. The frequency of type 2 $CD8^+$ T cells is increased in peripheral blood from patients with psoriasis vulgaris. J Clin Immunol. 2003;23:269-278.
27. Gilliet M, Liu YJ. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. J Exp Med. 2002;195:695-704.
28. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med. 2001;193:233-238.
29. Kemp RA, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. J Immunol. 2001;167: 6497-6502.
30. Dobrzanski MJ, Reome JB, Dutton RW. Therapeutic effects of tumor-reactive type 1 and type 2 $CD8^+$ T cell subpopulations in established pulmonary metastases. J Immunol. 1999;162:6671-6680.
31. Xia D, Hao S, Xiang J. $CD8^+$ cytotoxic T-APC stimulate central memory $CD8^+$ T cell responses *via* acquired peptide-MHC class I complexes and CD80 costimulation, and IL-2 secretion. J Immunol. 2006;177:2976-2984.
32. Xiang J, Huang H, Liu Y. A new dynamic model of $CD8^+$ T effector cell responses *via* $CD4^+$ T helper-antigen-presenting cells. J Immunol. 2005;174:7497-7505.
33. Lenardo M, Chan KM, Hornung F, et al. Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. Annu Rev Immunol. 1999;17:221-253.
34. Shi M, Hao S, Chan T, Xiang J. $CD4^+$ T cells stimulate memory $CD8^+$ T cell expansion *via* acquired pMHC I complexes and costimulatory molecules, and IL-2 secretion. J Leukoc Biol. 2006;80:1354-1363.
35. Huang H, Bi XG, Yuan JY, Xu SL, Guo XL, Xiang J. Combined $CD4^+$ Th1 effect and lymphotactin transgene expression enhance $CD8^+$ Tc1 tumor localization and therapy. Gene Ther. 2005;12:999-1010.
36. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annu Rev Immunol. 2002;20:323-370.
37. Helmich BK, Dutton RW. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. J Immunol. 2001;166:6500-6508.
38. Dobrzanski MJ, Reome JB, Dutton RW. Role of effector cell-derived IL-4, IL-5, and perforin in early and late stages of type 2 CD8 effector cell-mediated tumor rejection. J Immunol. 2001;167:424-434.
39. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol. 2004;172: 1380-1390.
40. Huang H, Xiang J. Synergistic effect of lymphotactin and interferon γ-inducible protein-10 transgene expression in T-cell localization and adoptive T-cell therapy of tumors. Int J Cancer. 2004;109:817-825.
41. Winter H, Hu HM, Urba WJ, Fox BA. Tumor regression after adoptive transfer of effector T cells is independent of perforin or Fas ligand (APO-1L/CD95L). J Immunol. 1999;163:4462-4472.
42. Poehlein CH, Hu HM, Yamada J, et al. TNF plays an essential role in tumor regression after adoptive transfer of perforin/IFN-γ double knockout effector T cells. J Immunol. 2003;170: 2004-2013.
43. Hollenbaugh JA, Dutton RW. IFN-γ regulates donor CD8 T cell expansion, migration, and leads to apoptosis of cells of a solid tumor. J Immunol. 2006;177:3004-3011.
44. Iwasaki A, Kelsall BL. Unique functions of $CD11b^+$, $CD8\alpha^+$, and double-negative Peyer's patch dendritic cells. J Immunol. 2001;166:4884-4890.
45. Gordon JR, Li F, Nayyar A, Xiang J, Zhang X. $CD8\alpha^+$, but not $CD8\alpha^-$, dendritic cells tolerize Th2 responses *via* contact-

dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma. J Immunol. 2005;175:1516-1522.
46. Zhang X, Huang H, Yuan J, et al. CD4-8- dendritic cells prime $CD4^+$ T regulatory 1 cells to suppress antitumor immunity. J Immunol. 2005;175:2931-2937.
47. Chen Z, Dehm S, Bonham K, et al. DNA array and biological characterization of the impact of the maturation status of mouse dendritic cells on their phenotype and antitumor vaccination efficacy. Cell Immunol. 2001;214:60-71.
48. Kedl RM, Schaefer BC, Kappler JW, Marrack P. T cells down-modulate peptide-MHC complexes on APCs *in vivo*. Nat Immunol. 2002;3:27-32.
49. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature. 1998;393:478-480.
50. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a $CD4^+$ T-helper and a T-killer cell. Nature. 1998;393:474-478.
51. Vendetti S, Chai JG, Dyson J, Simpson E, Lombardi G, Lechler R. Anergic T cells inhibit the antigen-presenting function of dendritic cells. J Immunol. 2000;165:1175-1181.
52. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Cutting edge: human $CD4^+CD25^+$ T cells restrain the maturation and antigen-presenting function of dendritic cells. J Immunol. 2004;172:4676-4680.
53. Webb DC, Cai Y, Matthaei KI, Foster PS. Comparative roles of IL-4, IL-13, and IL-4Rα in dendritic cell maturation and $CD4^+$ Th2 cell function. J Immunol. 2007;178:219-227.
54. Iezzi G, Boni A, Degl'Innocenti E, Grioni M, Bertilaccio MT, Bellone M. Type 2 cytotoxic T lymphocytes modulate the activity of dendritic cells toward type 2 immune responses. J Immunol. 2006;177:2131-2137.
55. Alpan O, Bachelder E, Isil E, Arnheiter H, Matzinger P. 'Educated' dendritic cells act as messengers from memory to naive T helper cells. Nat Immunol. 2004;5:615-622.