Distinct Role of Antigen-specific T Helper Type 1 (Th1) and Th2 Cells in Tumor Eradication In Vivo

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Summary

The role of T helper type 1 (Th1) and Th2 cells in tumor immunity was investigated using Th cells induced from ovalbumin (OVA)-specific T cell receptor transgenic mice. Although Th1 cells exhibited stronger cytotoxicity than Th2 cells, both cell types completely eradicated tumors when transferred into mice bearing A20 tumor cells transfected with the OVA gene (A20-OVA). Th1 cells eradicated the tumor mass by inducing cellular immunity, whereas Th2 cells destroyed the tumor by inducing tumor necrosis. Both Th1 and Th2 cells required $CD8^+$ T cells to eliminate tumors, and neither of these cells were able to completely eliminate A20-OVA tumors from T and B cell–deficient $RAG2^{-/-}$ mice. Mice cured from tumors by Th1 and Th2 cell therapy rejected A20-OVA upon rechallenge, but $CD8^+$ cytotoxic T lymphocytes were induced only from spleen cells prepared from cured mice by Th1 cell therapy. Moreover, we demonstrated that Th1 and Th2 cells used distinct adhesion mechanisms during tumor eradication: the leukocyte function-associated antigen (LFA)-1–dependent cell–cell adhesion step was essential for Th1 cell therapy, but not for Th2 cell therapy. These findings demonstrated for the first time the distinct role of antigen-specific Th1 and Th2 cells during eradication of established tumors in vivo.

Key words: Th1 • Th2 • tumor • adoptive immunotherapy • cytokine

The ultimate goal of tumor immunotherapy is to induce tumor-specific T cell–mediated immunity that can block the growth and metastasis of malignant tumor cells in a tumor-bearing host (1). However, because of low immunogenicity and strong immunosuppression, this goal has been difficult to achieve (2, 3). To overcome this problem, it is essential to activate helper function at the tumor site (4). Many strategies have been devised to introduce local help: (a) introduction of cytokines or cytokine genes into the host (5, 6); (b) adoptive transfer of antigen-specific or nonspecific $CD4^+$ Th cells into tumor-bearing mice (7–9); (c) activation of cytokine production in vivo by immunopotentiators (10, 11); (d) dendritic cell–based vaccine therapy (12, 13); and (e) targeting of MHC class II–binding superantigens into the tumor site (14, 15).

$CD4^+$ Th cells can be subdivided into Th1 and Th2 cells. The balance of these cells (Th1/Th2 balance) has been shown to be critically important in various immune responses, including antitumor immune responses (16). Since Th1 cells producing IFN-γ and IL-2 are essential for the induction of cellular immunity, whereas Th2 cells producing IL-4, IL-5, and IL-10 play a key role in humoral immunity, it seems likely that the activation of Th1-dominant local help could facilitate the induction of antitumor immunity (17). Indeed, IL-12 has been demonstrated to be effective in inducing antitumor immunity in vivo through the activation of Th1 immunity (17–19). However, both Th1-derived cytokines (IL-2, IFN-γ) and Th2-derived cytokines (IL-4, IL-6, IL-10) have been demonstrated to be useful for cancer gene therapy (20–23). Moreover, tumor-specific Th2 clones were demonstrated to exhibit a strong antitumor activity in vivo (24). These controversial results have made it difficult to understand which cells, Th1 or Th2, are critical for antitumor immune responses.

To understand the precise role of Th1 and Th2 cells in tumor eradication, it is necessary to prepare a large number of monoclonal Th1 and Th2 cells from the same naive Th precursor cells in short-term cultures. Moreover, it is essential to perform these experiments with Th1 and Th2 cells that are specific for the same tumor-rejection antigen (TRA).[1] These requirements have been hard to achieve

[1]*Abbreviations used in this paper:* ICAM, intercellular adhesion molecule; OVA-pep, $I\text{-}A^d$–binding $OVA_{323-339}$ peptide; RAG, recombination activating gene; Tg, transgenic; TRA, tumor-rejection antigen.

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because of difficulties in inducing large numbers of Th1 and Th2 cells that recognize the same tumor antigen in short-term cultures. Using long-term cultures it may be possible to generate Th1 and Th2 clones that recognize the same tumor antigen, but the properties of these clones may not necessarily reflect the in vivo situation.

To overcome this problem, we induced Th1 and Th2 cells from transgenic (Tg) mice expressing TCR α/β chain genes, derived from a chicken OVA–specific I-A^d–restricted $CD4^+$ Th clone (25). We used these cells in conjunction with I-A^d–positive tumor cells expressing OVA antigen (A20-OVA), as a model TRA in order to examine the role of OVA-specific Th1 and Th2 cells in tumor eradication in vivo. The in vivo antitumor activity of Th1 and Th2 cells was compared by adoptive cell transfer into mice bearing an established A20-OVA tumor mass. Using this novel adoptive tumor immunotherapy model, we demonstrated for the first time the distinct role of antigen-specific Th1 and Th2 cells for tumor eradication in vivo.

Materials and Methods

Animals. BALB/c mice were obtained from Charles River Japan. BALB/c background recombination activating gene (RAG)$2^{-/-}$ mice were donated by Dr. M. Ito (Central Institute for Experimental Animals, Kanagawa, Japan). $OVA_{323-339}$-specific I-A^d–restricted TCR-Tg mice (DO11.10) maintained on the BALB/c background were donated by Dr. K.M. Murphy (Washington University School of Medicine, St. Louis, MO [25]). All the mice were female and were used at 5–6 wk of age.

Cytokines, mAbs, and Antigens. IL-12 was donated by Genetics Institute. Anti–IL-12 mAbs (C15.1 and C15.6) were a gift from Dr. G. Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia, PA). PMA, brefeldin A, recombinant murine IL-4, and antiasialo GM1 Ab were purchased from Wako Pure Chemical Industries, Ltd. Anti–IL-4 mAb (11B11) was purchased from American Type Culture Collection. PE–anti-CD4 mAb, peridinine chlorophyll protein (PerCP)–anti-CD4 mAb, FITC–anti-CD45RB mAb, FITC–anti-CD8 mAb, purified anti-CD3 mAb, purified anti–very late antigen (VLA)-4, purified anti–intercellular adhesion molecule (ICAM)-1 mAb, and recombinant mouse IFN-γ and anti–IFN-γ mAb (R4-6A2) were purchased from PharMingen. Anti–LFA-1 mAb was produced by our established KBA hybridoma clone (26). $OVA_{323-339}$ peptide was supplied by Dr. H. Tashiro (Fujiya Co. Ltd., Hadano, Japan).

Generation of Th1 and Th2 Cells. $CD4^+CD45RB^+$ naive T cells were isolated from nylon-passed spleen cells from DO11.10 TCR-Tg mice using cell sorting (FACS Vantage™; Becton Dickinson) as described previously (27). Purified $CD4^+CD45RB^+$ cells were stimulated with 10 μg/ml $OVA_{323-339}$ peptide in the presence of mitomycin C–treated BALB/c spleen cells, 20 U/ml IL-12, 1 ng/ml IFN-γ, 50 μg/ml anti–IL-4 mAb, and 20 U/ml IL-2 for Th1 development. Th2 cells were induced from the same naive Th cells in the presence of 1 ng/ml IL-4, 50 μg/ml anti–IFN-γ mAb, 50 μg/ml anti–IL-12 mAbs, and 20 U/ml IL-2. At 48 h, cells were restimulated with $OVA_{323-339}$ under the same conditions, and used at 9–12 d of culture.

Induction of LFA-1/ICAM-1–dependent Homotypic Aggregation. Th1 or Th2 cells (2×10^6 cells/well) were cultured in 12-well plates and stimulated with 2 μg/ml of anti-CD3 mAb or 20 ng/ml of PMA for 1–2 h. The ability of Th1 or Th2 cells to form homotypic aggregation was determined by counting the number of cell aggregates under the microscope as described previously (28). The blocking effect of mAbs was determined by adding 50 μg/ml of each mAb into the culture.

Cytokine Activity. IFN-γ or IL-4 activities of culture supernatants were measured using ELISA kits (Nycomed Amersham plc). The IFN-γ activity was determined using Biotrak IFN-γ ELISA kits (RPN2717), and IL-4 activity was determined using Biotrak IL-4 ELISA kits (RPN2712). The mean of triplicate samples was calculated.

Intracellular Cytokine Expression. For the detection of cytoplasmic cytokine expression, cells stimulated with immobilized anti-CD3 mAb for 6 h in the presence of brefeldin A were first stained with PerCP–anti-CD4 mAb, fixed with 4% paraformaldehyde, and treated with permeabilizing solution (50 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 0.5% Triton X-100, pH 7.5), then the fixed cells were stained with PE-conjugated anti–IL-4 mAb and FITC-conjugated anti–IFN-γ for 45 min on ice. The percentage of cells expressing cytoplasmic IL-4 or IFN-γ was determined by flow cytometry (FACSCalibur™; Becton Dickinson).

Production of OVA Gene Transfectants. A20 B lymphoma cells were transfected with chicken OVA cDNA, which was donated by Dr. M.J. Bevan, Research Institute of Scripps Clinic, La Jolla, CA (29). Transfectants were designated as A20-OVA tumor cells.

Cytotoxicity. The cytotoxicity mediated by Th1- or Th2-dominant cells was determined by 4-hr ^{51}Cr-release assays as described previously (30). A20 parental cells, A20-OVA cells, or $OVA_{323-339}$ peptide–pulsed A20 cells were used as target cells. The cytotoxicity (as a percent) was calculated by the method described previously (30).

Adoptive Tumor Immunotherapy. A20-OVA cells (2×10^6) were intradermally inoculated into BALB/c mice. When the tumor mass became palpable (6–8 mm), Th1 or Th2 cells (2×10^7) were intravenously transferred into the tumor-bearing mice. The antitumor activity mediated by the transferred cells was determined by measuring changes over time of the means of two perpendicular diameters of the tumor mass. The mean of six mice per group was indicated. In all experiments, tumor-free mice were followed for >90 d.

Results

OVA-specific Th1 and Th2 Cells Elicited from TCR-Tg Mice Can React with A20-OVA Tumor Cells. Th1 and Th2 cells were induced from $CD4^+CD45RB^+$ naive Th cells isolated from DO11.10 TCR-Tg mice, which recognize $OVA_{323-339}$ peptide (OVA-pep) bound on I-A^d molecules. Th1 cells were generated from naive Th cells by culture with OVA-pep in the presence of IL-2, IL-12, IFN-γ, and anti–IL-4 mAb, whereas Th2 cells were derived from naive Th cells by culture with OVA-pep in the presence of IL-2, IL-4, anti–IFN-γ, and anti–IL-12 mAb. As summarized in Fig. 1, 99% of the cells cultured under Th1 conditions for 10 d consisted of $CD4^+$ T cells, and ~70% of the cells expressed intracellular IFN-γ but not IL-4 (Fig. 1, A and B). In contrast, 99% of the cells cultured under Th2 conditions for 10 d consisted of $CD4^+$ T cells, and ~60% of the cells expressed intracellular IL-4 but not IFN-γ (Fig. 1, C and D). To apply these OVA-specific Th1 and Th2 cells to tumor immunotherapy, we generated I-A^d–positive A20-

OVA tumor cells expressing OVA antigen by transfection with the OVA gene. The A20-OVA tumor cells secreted OVA protein into the culture supernatant, which was detectable by ELISA (data not shown). A20-OVA cells stimulated OVA-specific Th1 and Th2 cells to induce the production of IFN-γ and IL-4, respectively (Fig. 1, E and F). Moreover, Th1 cells lysed A20-OVA tumor cells, although Th2 cells exhibited a negligible cytotoxicity (Fig. 1, G and H). Th1 cells appeared to lyse A20-OVA tumor cells mainly mediated by perforin, but not by TNF-α and the Fas/FasL pathway, because inhibition of granular exocytosis (30) caused almost complete inhibition of the cytotoxicity, whereas this blocking effect was not demonstrated by anti–TNF-α mAb plus anti-FasL mAb (data not shown). In some cases, Th2 cells showed low but significant cytotoxicity against A20-OVA, but this appeared to be derived from the contamination of IFN-γ–producing Th1 or Th0 cells (data not shown), because IFN-γ–nonproducing pure Th2 cell populations exhibited negligible cytotoxic activity (Fig. 1, F and H). The same stimulatory effect against Th1 and Th2 cells was also demonstrated by OVA-pep–pulsed A20 tumor cells, but not untreated A20 tumor cells (Fig. 1, E–H). These results demonstrated that the OVA peptide fragment from A20-OVA bound to I-A^d and was able to stimulate class II–restricted $OVA_{323-339}$-specific Th1 and Th2 cells to trigger their immunological functions.

Establishment of an Adoptive Tumor Immunotherapy Model Using Antigen-specific Th Cells. To determine the precise role of Th1 and Th2 cells in antitumor immunity, we designed a novel adoptive tumor immunotherapy model using OVA-specific Th1 and Th2 cells in conjunction with A20-OVA tumor-bearing mice. Fig. 2 A shows the experimental set-up for these experiments. BALB/c mice were inoculated with 2×10^6 A20-OVA cells, and when the tumor mass became palpable (6–8 mm), 2×10^7 Th1 or Th2 cells were transferred into the tumor-bearing mice. As shown in Fig. 2 B, the established A20-OVA tumor mass was completely rejected by adoptive transfer of OVA-specific Th1 cells, but not nonspecific Th1 cells, which were induced from splenic Th cells from wild-type BALB/c mice by activation with anti-CD3 mAb under Th1-inducing conditions. However, the growth of parental A20 tumor cells was not inhibited by transfer of OVA-specific Th1 cells (Fig. 2 C). These results clearly demonstrated that this adoptive tumor immunotherapy model allowed us to investigate the role of antigen-specific Th cells in antitumor immunity.

The Distinct Role of Th1 and Th2 Cells for Antitumor Immune Responses In Vivo. As shown in Fig. 3 A, both Th1 and Th2 cells exhibited strong antitumor activity in vivo and completely eradicated the tumor mass after adoptive transfer. All the mice cured from the tumor by Th1 or Th2

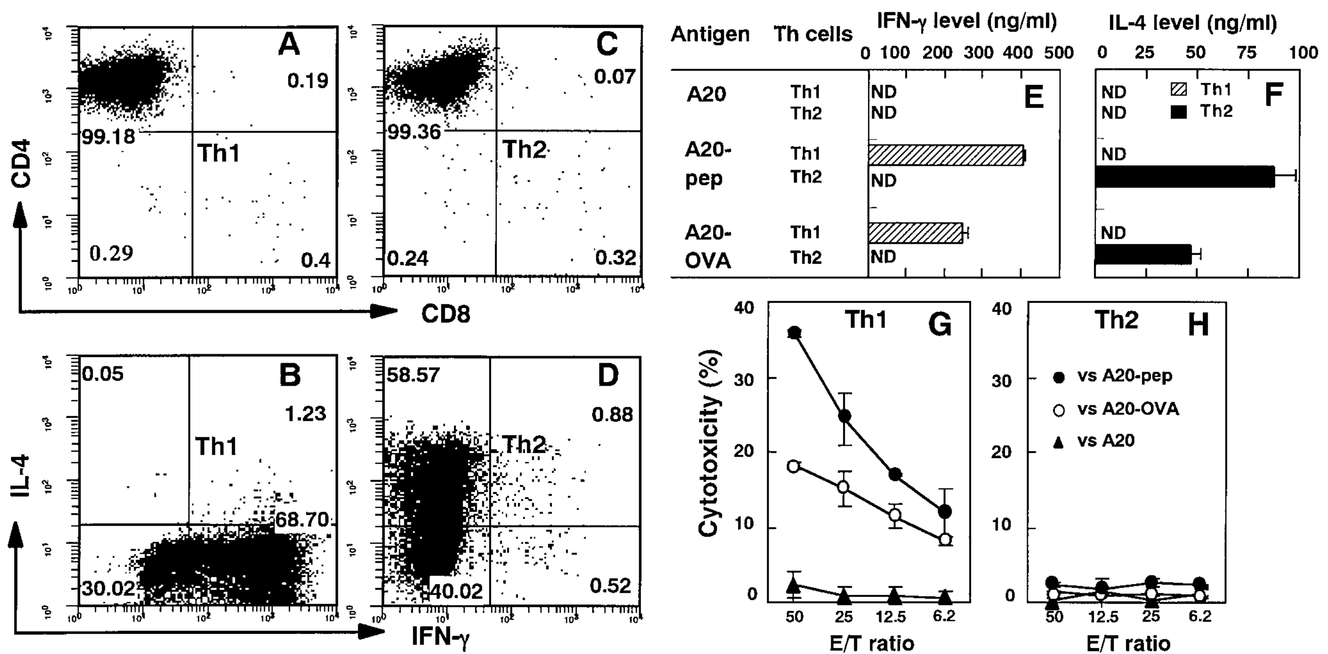

Figure 1. Characteristics of Th1 and Th2 cells from DO11.10 TCR-Tg mice and their activation by A20-OVA tumor cells. Th1 and Th2 cells were induced from CD4$^+$CD45RB$^+$ naive Th cells obtained from DO11.10 TCR-Tg mouse spleen cells under the conditions described in Materials and Methods. The expression of CD4/CD8 antigen (A and C) and cytoplasmic cytokine (IL-4/IFN-γ) expression (B and D) in Th1 (A and B) and Th2 cells (C and D) was determined by flow cytometric analysis. The cytokine-producing ability of Th1 (hatched bars) and Th2 cells (black bars) was determined by measuring the amounts of IFN-γ (E) or IL-4 (F) in culture supernatants after coculture with A20 tumor cells, A20 tumor cells pulsed with OVA-pep (A20-pep), or A20-OVA tumor cells, for 12 h. ND, not detected. The cytotoxic activity of Th1 (G) and Th2 cells (H) against A20 tumor cells (▲), A20 tumor cells pulsed with OVA-pep (A20-pep) (●), or A20-OVA tumor cells (○) was measured by 4-h ^{51}Cr-release assays. The bars represent mean ± SE of triplicate samples.

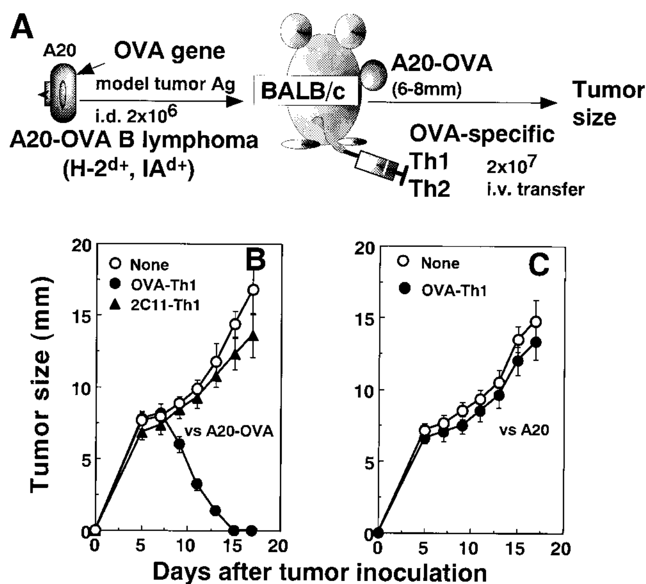

Figure 2. Establishment of an adoptive tumor immunotherapy model using antigen-specific Th cells. (A) The protocol for adoptive tumor immunotherapy using OVA-specific Th1 or Th2 cells. A20-OVA tumor cells were intradermally inoculated into wild-type BALB/c mice. When the tumor formed a mass of 6–8 mm in diameter, 2×10^7 OVA-specific Th1 or Th2 cells, induced from DO11.10 TCR-Tg mice, were transferred into the tumor-bearing mice. The therapeutic effect of Th1 or Th2 cell transfer was determined by measuring tumor size. According to the above protocol (A), OVA-specific Th1 cells (OVA-Th1, ●), antigen-nonspecific 2C11-activated Th1 cells (2C11-Th1, ▲), or saline (None, ○) were intravenously injected into A20-OVA–bearing BALB/c mice (B) or parental A20-bearing BALB/c mice (C). The antitumor activity of Th1 cells was determined by measuring changes over time of the means of two perpendicular diameters of tumor mass. Results are presented as mean ± SE of six mice. The tumor-free mice were followed for >90 d.

cell transfer were free of tumor for >90 d (data not shown). These experiments were repeated >10 times, and identical results were obtained. For the complete cure of tumor-bearing mice, the transfer of $>10^7$ cells was required for both Th1 and Th2 cell therapy (data not shown). Although both Th1 and Th2 cells exhibited antitumor activity in vivo, totally distinct processes of tumor rejection appeared to be included. The typical pattern of tumor growth or tumor rejection in control, Th1-transferred, or Th2-transferred mice is shown in Fig. 3 B. Interestingly, the tumor mass of mice that received Th1 cells gradually changed into a small, white mass and completely disappeared 7–10 d after cell transfer. In contrast, in tumor-bearing mice that received Th2 cells, the tumor mass changed to a red color 7–10 d after cell transfer, and a strong tumor necrosis was observed. This clear difference suggested that Th1 and Th2 cells eradicate tumors using distinct immunological mechanisms. To understand these distinct antitumor mechanisms, we studied the tumor tissue by histological analysis (Fig. 4). Although no significant lymphocyte infiltration was observed in control tumor tissue (Fig. 4 A), a marked lymphocyte infiltration was present in tumor tissue of mice that received Th1 cells (Fig. 4 B). However, in mice that received Th2 cells, a marked infiltration of inflammatory cells such as eosinophils and neutrophils was observed around the center of the tumor mass (Fig. 4 C). These results suggested that both Th1 and Th2 cells exhibited a strong antitumor activity in vivo, but that these cells used distinct tumor rejection mechanisms.

Tumor Rejection Mechanisms Mediated by Th1 and Th2 Cells. Fig. 5 A shows that intravenous injection of anti-CD4 mAb, anti-CD8 mAb, or anti–IFN-γ mAb completely inhibited the therapeutic effect of Th1-mediated adoptive immunotherapy. These results demonstrated that Th1 cells by themselves are not enough to induce complete tumor eradication and that the interaction between Th1 cells and $CD8^+$ T cells through cytokines such as IFN-γ may be essential to induce successful tumor rejection. The requirement for $CD8^+$ T cells in Th1-cell therapy was confirmed by demonstrating that the therapeutic effect of Th1 cell transfer was not induced in DO11.10 TCR-Tg

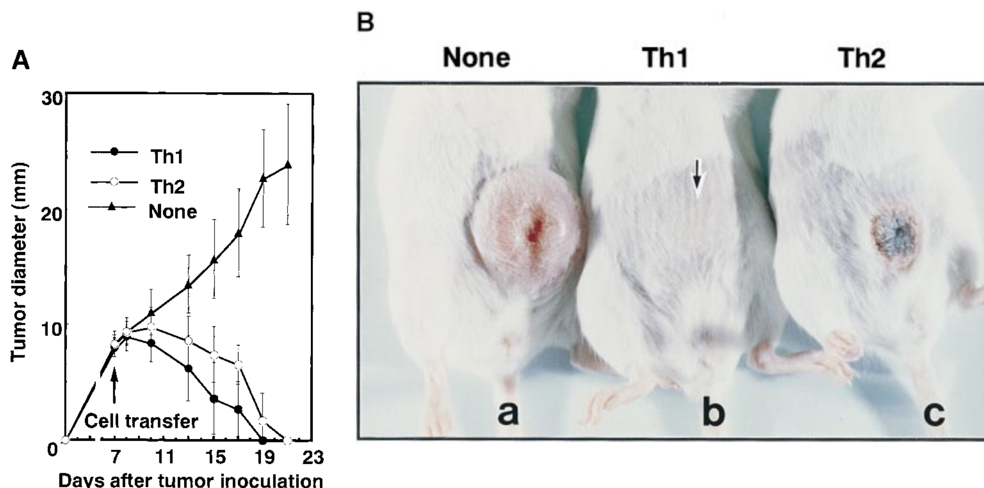

Figure 3. The distinct role of Th1 and Th2 cells for eradication of established tumors in vivo. (A) OVA-specific Th1 cells (●), OVA-specific Th2 cells (○), or saline (▲) were intravenously injected into BALB/c mice bearing A20-OVA tumors. The antitumor activity of Th1 and Th2 cells was determined by measuring changes over time of the means of two perpendicular diameters of the tumor mass. Results are presented as mean ± SE of six mice. The tumor-free mice were followed for >90 d. (B) Typical tumor growth or regression pattern in saline- (a), Th1- (b), or Th2-treated mice (c). Similar results were obtained in >10 independent experiments. The arrow indicates the site of tumor inoculation in Th1-treated mice.

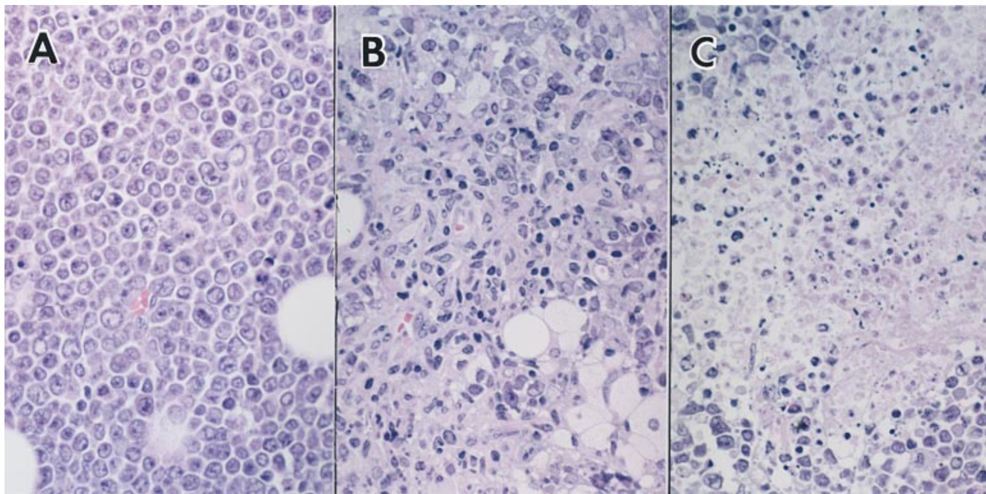

Figure 4. Histochemical analysis of the cellular mechanisms underlying Th1- and Th2-mediated tumor eradication in vivo. Tumor tissues were removed from tumor-bearing mice 7 d after treatment with saline (A), Th1-cell transfer (B), or Th2-cell transfer (C). The tissues were stained with hematoxylin and eosin.

mice that have I-A^d–restricted OVA-reactive $CD4^+$ T cells, but not $CD8^+$ T cells (Fig. 5 B). To further extend these findings, we carried out adoptive transfer experiments using tumor-bearing $RAG2^{-/-}$ mice that lack T, NKT, and B cells. A20-OVA tumors were inoculated into BALB/c background $RAG2^{-/-}$ mice, and when these tumors became palpable, the mice received OVA-specific Th1 or Th2 cells. As shown in Fig. 6, the growth of A20-OVA tumors in $RAG2^{-/-}$ mice was initially strongly inhibited by both Th1 and Th2 cells, but the tumor cells finally grew out in both types of mice, although regrown tumor cells expressed OVA antigen and could stimulate cytokine production of Th cells (data not shown). Thus, a single transfer of Th1 or Th2 cells by itself cannot completely cure tumor-bearing mice. These results strongly suggested that OVA-reactive $CD8^+$ T cells, activated in wild-type BALB/c mice early after tumor inoculation, play an important role in Th1- and Th2-mediated adoptive immunotherapy. To further investigate the role of $CD8^+$ T cells in Th1 and Th2 cell therapy, OVA-reactive $CD8^+$ T cells (2×10^5 cells) obtained from A20-OVA–immunized mice were transferred into $RAG2^{-/-}$ mice 7 d before the experiment. The small number (2×10^5 cells) of $CD8^+$ T cells transferred into $RAG2^{-/-}$ mice revealed marked expansion in vivo, and they made up >10% of spleen cells of $RAG2^{-/-}$ mice 1 wk after the cell transfer (data not shown). The transfer of $CD8^+$ T cells alone did not inhibit the growth of A20-OVA tumor cells (Fig. 6 A). However, when A20-OVA–bearing $RAG2^{-/-}$ mice first received $CD8^+$ T cells from A20-OVA–immunized mice and then received OVA-specific Th1 or Th2 cells, all of the mice were completely cured from the tumor. A significant but incomplete tumor growth inhibition was also observed when unprimed $CD8^+$ T cells were transferred into $RAG2^{-/-}$ mice (Fig. 6 B). These results clearly demonstrated the critical role for antigen-specific $CD8^+$ T cells in complete tumor eradication induced by adoptive transfer of antigen-specific Th1 or Th2 cells.

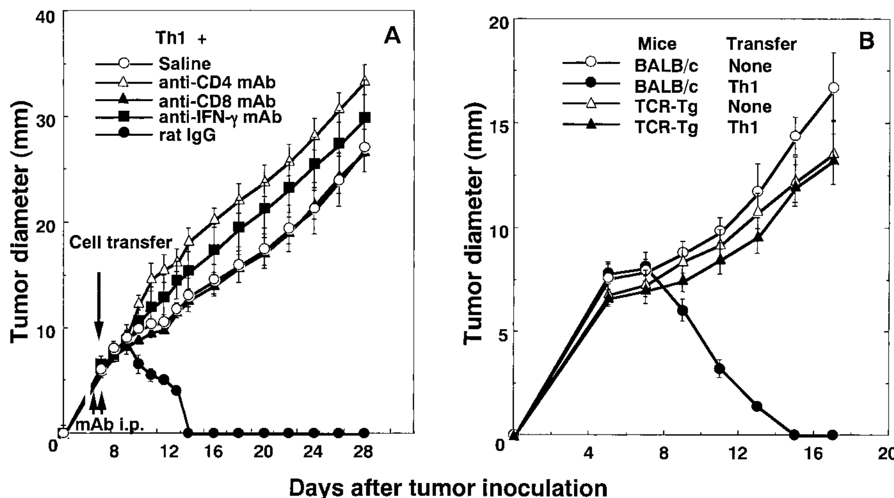

Figure 5. Tumor eradication mechanisms mediated by Th1 and Th2 cells. Th1 cell therapy was carried out using wild-type BALB/c mice and DO11.10 TCR-Tg mice according to the protocol described in the legend to Fig. 2 A. (A) At days −1 and 0 before Th1 cell transfer, either saline (○), anti-CD4 mAb (△), anti-CD8 mAb (▲), anti–IFN-γ mAb (■), or rat IgG (●) was intravenously injected into tumor-bearing BALB/c mice. (B) A20-OVA tumor cells were intradermally inoculated into wild-type BALB/c mice (○, ●) or BALB/c background TCR-Tg mice (△, ▲). When the tumor mass became palpable, Th1 cells (●, ▲) were transferred into the tumor bearing mice. As a control, mice were treated with saline (○, △). The antitumor activity of Th1 cells was determined by measuring changes over time of the means of two perpendicular diameters of the tumor mass. Results are presented as mean ± SE of six mice. The tumor-free mice were followed for >90 d.

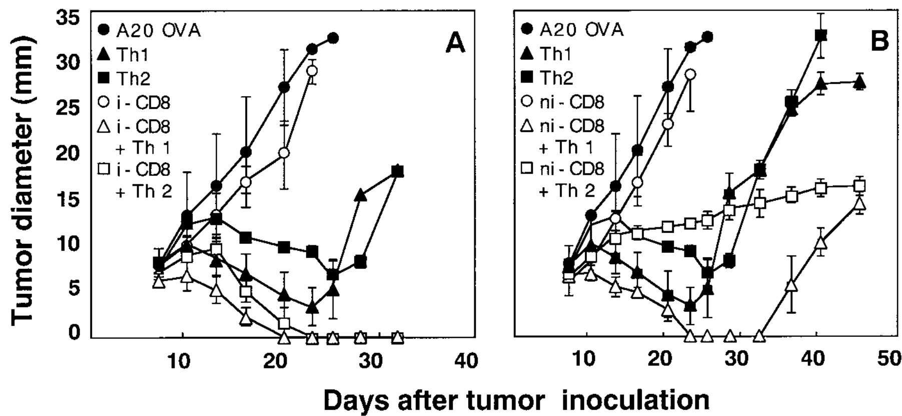

Figure 6. Requirement for $CD8^+$ T cells in inducing complete elimination of transplanted tumors in response to Th1 or Th2 cell therapy. The tumor therapy model was carried out according to the legend to Fig. 2 A except that aliquots (2×10^5/mouse) of $CD8^+$ T cells from immunized mice (i-CD8, A) or unprimed mice (ni-CD8, B) were transferred into the mice 7 d before the inoculation of A20-OVA tumor cells. $CD8^+$ T cells were isolated by cell sorting from A20-OVA–immunized BALB/c mice or untreated BALB/c mice. (A) When the tumor mass became palpable, the mice were treated with intravenous injection of saline (A20-OVA, ●), Th1 cells (▲), or Th2 cells (■). The tumor-bearing mice transferred with $CD8^+$ T cells from tumor-immunized mice (i-CD8) were also treated with intravenous injection of saline (○), Th1 cells (△), or Th2 cells (□). (B) When the tumor mass became palpable, the mice were treated with intravenous injection of saline (A20-OVA, ●), Th1 cells (▲), or Th2 cells (■). The tumor-bearing mice transferred with $CD8^+$ T cells from unprimed mice (ni-CD8) were also treated with intravenous injection of saline (○), Th1 cells (△), or Th2 cells (□). The antitumor activity of Th1 and Th2 cells was determined by measuring changes over time of the means of two perpendicular diameters of the tumor mass. Results are presented as mean ± SE of six mice. The tumor-free mice were followed for >90 d.

Adoptive Tumor Immunotherapy by Th1 Cells Can Induce Immunological Memory for CTL Generation. Next, we examined the therapeutic mechanisms of Th1 and Th2 cell therapy in terms of acquisition of immunological memory beneficial for CTL generation. Mice cured from tumors by Th1 and Th2 cell therapy were rechallenged with A20-OVA tumor cells. Fig. 7 A shows that both types of mice rejected A20-OVA tumors, but were unable to reject syngeneic Meth A tumor cells (data not shown). These results indicated that mice cured from A20-OVA tumors had acquired immunological memory. However, these memory cells, elicited after restimulation with mitomycin C–treated A20-OVA cells, produced totally different patterns of cytokines and differed in their cytotoxic potential (Fig. 7, B and C). Spleen cells from mice cured by Th1 cell therapy produced IFN-γ and revealed high cytotoxicity against A20-OVA but low cytotoxicity against A20 tumor cells (Fig. 7 B). In contrast, spleen cells from mice cured by Th2 cell therapy produced high levels of IL-4 but little IFN-γ (Fig. 7 B), and had little cytotoxicity against A20-OVA (Fig. 7 C). Thus, these findings strongly suggested that immunotherapy using adoptive transfer of Th1 cells appeared to be more effective than Th2 cells for inducing immunological memory suitable for the generation of CTL response. It was also demonstrated that some of the cured mice from A20-OVA by Th1 cell therapy, but not by Th2 cell therapy, showed resistance against rechallenged parental A20 tumor cells (data not shown).

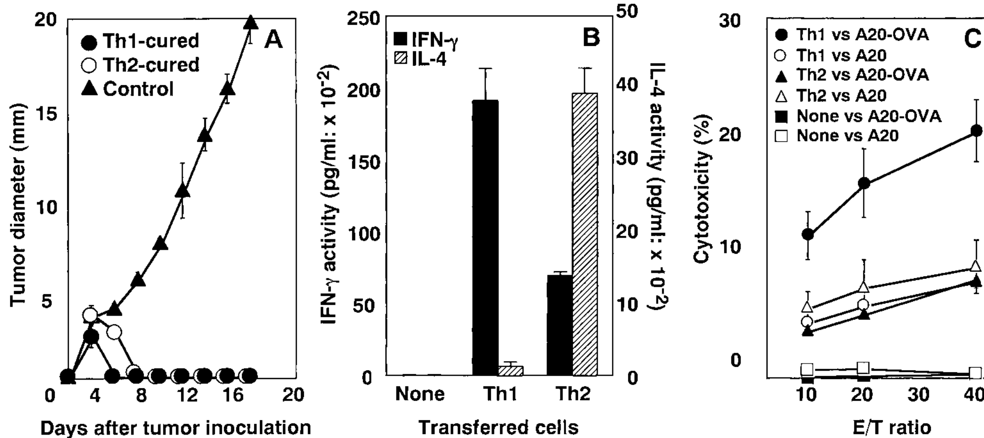

Figure 7. Adoptive transfer of Th1 cells but not Th2 cells induces cellular immunological memory. (A) Mice cured from A20-OVA tumors after Th1 (●) or Th2 cell therapy (○) were rechallenged by intradermal injection of 2×10^6 viable A20-OVA tumor cells. Untreated mice (▲) were used as controls. Tumor growth was determined by measuring changes over time of the means of two perpendicular diameters of the tumor mass. Results are presented as mean ± SE of six mice. The tumor-free mice were followed for >90 d. (B) Mice cured from A20-OVA tumors after Th1 or Th2 cell therapy were killed, and their spleen cells were restimulated in vitro with mitomycin C–treated A20-OVA by mixed lymphocyte tumor reaction (MLTR). As control, untreated BALB/c mice (None) were used for MLTR. After culture for 2 d, the levels of IFN-γ (black bars) and IL-4 (hatched bars) in culture supernatants were measured by ELISA. The bars represent mean ± SE of three mice. (C) The cells were harvested from MLTR cultures from mice that had received Th1 cells (●, ○), Th2 cells (▲, △), or no cells (None, ■, □), and their cytotoxicity was measured using ^{51}Cr-labeled A20-OVA (●, ▲, ■) or A20 tumor cells (○, △, □).The results are presented as mean ± SE of three mice.

Th1 and Th2 Cells Use Distinct Cell–Cell Adhesion Interactions during Eradication of Tumors In Vivo. Finally, we examined the role of distinct cell adhesion molecules in Th1 and Th2 cell therapy. While investigating the expression and function of adhesion molecules involved in cell migration, we found that Th1 cells, in response to stimulation with anti-CD3 mAb and phorbol ester, show strong LFA-1/ICAM-1–dependent homotypic adhesion (28; Fig. 8 C). However, no significant cell aggregation was observed for similarly activated Th2 cells (Fig. 8 D). This homotypic cell–cell aggregation was strongly blocked by anti–LFA-1 or anti–ICAM-1 mAb, but not by control rat Ig or anti–VLA-4 mAb (data not shown). From these results, we concluded that Th1 and Th2 cells have distinct capacity to use LFA-1/ICAM-1 cell adhesion interactions. This differential ability to form homotypic cell–cell aggregates did not result from a defect in LFA-1 or ICAM-1 expression, because both Th1 and Th2 cells expressed high levels of LFA-1 and ICAM-1 at the cell surface (Fig. 5, A and B). To investigate whether LFA-1/ICAM-1 interactions are important for the induction of antitumor activity in vivo, we examined the effect of anti–LFA-1 mAb administration on Th1 and Th2 cell therapy. Mice were inoculated with A20-OVA tumor cells, and when tumors became palpable, mice were treated by intravenous injection of anti–LFA-1 mAb (500 μg/mouse) twice at days −1 and 0 before adoptive transfer of Th1 or Th2 cells. As shown in Fig. 5 E, the therapeutic ability of Th1 cells was completely abrogated by anti–LFA-1 mAb administration. However, no significant inhibitory effect by anti–LFA-1 mAb was observed for Th2-mediated antitumor activity (Fig. 5 F). These results indicated that Th1 and Th2 cells showed distinct requirements for LFA-1–dependent cell–cell interactions in vitro and in vivo: LFA-1–dependent cell migration appeared to be critical for Th1-cell therapy but not for Th2-cell therapy.

Discussion

Several lines of evidence have indirectly demonstrated a role for the Th1/Th2 balance in antitumor immunity. First, the cytokine IL-12, which stimulates Th1-dominant immunity in vivo, was shown to have strong in vivo antitumor activity against a variety of tumors, including primary tumors (18, 19, 31, 32). Second, in vivo neutralization of IFN-γ caused the inhibition of the antitumor effect of IL-12, suggesting that IFN-γ–producing Th1 cells may play an important role in tumor rejection. However, this

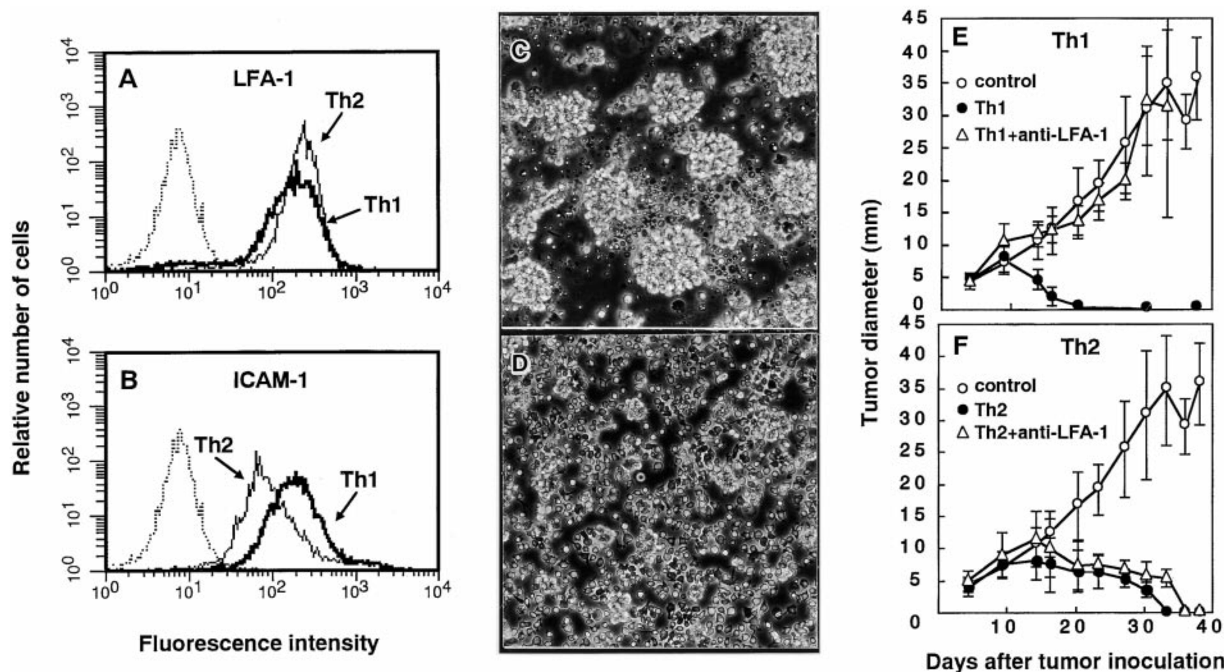

Figure 8. Th1 and Th2 cells use distinct cell adhesion interactions during eradication of tumors in vivo. (A) Expression of LFA-1 antigen on Th1 and Th2 cells. (B) Expression of ICAM-1 on Th1 and Th2 cells. Dotted line, unstained control cells. (C and D) LFA-1/ICAM-1–dependent homotypic cell–cell aggregation. Th1 (C) and Th2 cells (D) (2×10^6 cells) were stimulated with 20 ng/ml of PMA. After culture for 1 h, homotypic aggregates were formed. Typical cell–cell aggregates were photographed. We confirmed that the homotypic aggregation was strongly blocked by addition of either anti–LFA-1 mAb or anti–ICAM-1 mAb (data not shown). (E and F) The effect of anti–LFA-1 mAb administration in vivo on the therapeutic ability of Th1 (E) or Th2 cells (F) was examined using the protocol described in the legend to Fig. 2 A. (E) When the A20-OVA tumor mass became palpable, the mice were treated with saline (○), Th1 cell transfer (●), or Th1 cell transfer after anti–LFA-1 mAb administration (△). (F) When the A20-OVA tumor mass became palpable, the mice were treated with saline (○), Th2 cell transfer (●), or Th2 cell transfer after anti–LFA-1 mAb administration (△). The anti–LFA-1 mAb (500 μg/mouse) was intravenously injected into the mice at days −1 and 0 before cell transfer. The antitumor activity of Th1 and Th2 cells was determined by measuring changes over time of the means of two perpendicular diameters of the tumor mass. Results are presented as mean ± SE of six mice. The tumor-free mice were followed for >90 d.

conclusion is weakened by the observation that other T cells, including NKT cells and $CD8^+$ T cells, can produce IFN-γ in response to IL-12, and that these cell types can activate Th1-dominant immunity (33, 34). Indeed, it was recently demonstrated that the antitumor activities of IL-12 are mediated, in large part, by NKT cells (34). Moreover, the finding that Th2-derived cytokines (IL-4, IL-5, IL-6, IL-10) show antitumor activities in vivo that are as strong as the antitumor activities of Th1 cytokines (20–23, 35) has made it difficult to conclude which cell type is the most effective for eliciting complete tumor regression in vivo.

To address this issue, we designed a new adoptive tumor immunotherapy model using tumor antigen–specific Th1 or Th2 cells. As tumor cells, we used OVA gene–transfected tumor cells, and Th1 and Th2 cells were induced from mice transgenic for an OVA-specific TCR. Our results demonstrated that both Th1 and Th2 cells show potent antitumor activities in vivo (Fig. 3). Interestingly, Th1 cells induced a marked lymphocyte infiltration into the tumor mass and eradicated the tumor mass via cellular immunity. In sharp contrast, Th2 cells induced inflammatory responses at the tumor site and induced tumor necrosis (Fig. 4). The finding that Th2 cells, which can produce high levels of IL-4, induced the inflammation characterized by eosinophils and neutrophils is consistent with previous results with IL-4 gene–transfected tumors, which were rejected by inflammatory cells that included eosinophils and neutrophils (22, 36). However, it remains unclear how Th2 cells induce tumor necrosis.

Another important difference between Th1 and Th2 cell therapy is that Th1 therapy was able to induce a strong immunological memory suitable for the generation of CTLs, whereas Th2 cells did not induce the immunological memory for CTL generation very well (Fig. 7). Some of the cured mice from A20-OVA by Th1 cell therapy but not Th2 cell therapy showed resistance against rechallenged parental A20 tumor cells (data not shown), indicating that Th1 cell therapy might also be beneficial for the generation of CTLs, which recognize unknown tumor-rejection antigen expressed on parental A20 tumor. Based on our observation that in vivo administration of anti-CD4 mAb, anti-CD8 mAb, or anti–IFN-γ mAb blocked the therapeutic effect of Th1 cells against tumors (Fig. 5 A), we suggest that transferred Th1 cells migrate into local tumor sites, produce IFN-γ, and facilitate the induction of antitumor $CD8^+$ CTLs in vivo. The requirement for $CD8^+$ T cells in Th1 cell therapy is also demonstrated by our finding that DO11.10 TCR-Tg mice that have OVA-reactive $CD4^+$ T cells, but not $CD8^+$ T cells, were unable to permanently clear the tumor by Th1 cell therapy (Fig. 5 B). Direct evidence for the requirement for $CD8^+$ T cells was demonstrated using adoptive transfer of $CD8^+$ T cells into $RAG2^{-/-}$ mice (Fig. 6). This experiment also indicated that NKT cells are not involved in Th1- and Th2-induced antitumor activity in vivo because NKT cells are not differentiated in $RAG2^{-/-}$ mice, which are deficient in NKT cells, mainstream T cells, and B cells. Th2 cells contained $<2\%$ of IFN-γ–producing cells (Fig. 1 D), which might exhibit negligible cytotoxicity against A20-OVA (Fig. 1 H). Indeed, in some cases, Th2 cells exhibited low but significant cytotoxicity against A20-OVA in parallel with the increased production of IFN-γ. However, even in such cases, the IFN-γ produced by contaminating cells (Th1 or Th2) appeared not to be involved in the triggering of antitumor activity of Th2 cells, because administration of anti–IFN-γ mAb caused no significant blocking of the Th2-mediated therapeutic effect (data not shown). In mice that were cured from A20-OVA tumors by Th2 therapy, A20-OVA–specific CTLs were not detected. These findings suggest that antigen-nonspecific $CD8^+$ killer T cells are involved in Th2-mediated adoptive immunotherapy. Alternatively, $CD8^+$ TC2 cells (33) induced by IL-4 may contribute to tumor eradication in Th2 cell therapy. Since the immunological memory in mice cured by Th2 cell transfer may be mediated by humoral immunity, we are currently investigating whether Th2 immunological memory can be transferred into normal mice by serum isolated from tumor-cured mice.

The distinct antitumor immunity mediated by Th1 and Th2 cells may be due to the distinct cell adhesion interactions involved in the migration of these cells into tumor tissues across endothelia. Consistent with previous results (37), we found that Th1 cells express higher levels of P-selectin ligands and produced higher amounts of chemokines compared with Th2 cells (data not shown). In addition, we found that Th1 cells exhibit strong LFA-1/ICAM-1–dependent cell–cell interactions (Fig. 8 C), which are critical for lymphocyte activation, cell-mediated cytotoxicity, and transmigration of lymphocytes into inflammatory tissues (38, 39). In contrast, Th2 cells were defective in LFA-1/ICAM-1–mediated cell–cell interactions (Fig. 8 D), but were able to interact with the extracellular matrix on endothelia through the integrin $\alpha V\beta 3$ (data not shown). These results suggest that Th1 cells express adhesion molecules that facilitate transmigration into tumor tissues across the tumor vessels. Indeed, antitumor therapeutic activity of Th1 cells was completely blocked by administration of anti–LFA-1 mAb, whereas the activities of Th2 cells were not affected by anti–LFA-1 mAb injection (Fig. 8, E and F). From these results, we speculate that, at the tumor local site, Th1 cells actively respond to tumor cells and produce cytokines, which recruit other effector cells such as $CD8^+$ T cells, NKT, or NK cells into the tumor tissue. In contrast, Th2 cells, which are unable to enter tumor tissue because of a defect of adhesion mechanisms, may accumulate on the endothelial cells around the tumor mass and induce tumor necrosis via molecules such as TNF-α that damage tumor vessels (40). However, we have recently demonstrated that in vivo administration of anti–IL-4, anti–IL-10, or anti–TNF-α was unable to block the tumor necrosis induced by Th2 cell therapy (data not shown). Therefore, unknown mechanisms appear to be involved in Th2-induced tumor necrosis. One possibility would be that Th2-derived cytokines activated other inflammatory cells and the products of these cells damage endothelial cells to induce tumor necrosis. This hypothesis is strongly supported by recent

findings by Hung et al. (41) that CD4$^+$ T cells play an important role in inducing antitumor activity in vivo through activation of eosinophils and macrophages that produce superoxide and nitric oxide.

The finding that transfer of $>10^7$ Th1 or Th2 cells with CD8$^+$ T cells is required for the complete rejection of tumor (data not shown) means that, at an early phase of tumor rejection, the bursting of a strong cytokine storm derived from Th1 or Th2 cells may be essential for overcoming a strong suppression in the tumor-bearing host and for induction of CD8$^+$ CTL–mediated antitumor protective immunity in tumor-bearing mice. The present data demonstrate that Th1 and Th2 cells use distinct tumor eradication mechanisms. However, based on the following considerations, Th1 cells may be more suitable for adoptive tumor immunotherapy in the future: (a) Th1 cell therapy, but not Th2 cell therapy, induces strong immunological memory beneficial for CTL generation (Fig. 7); (b) Th2 cells produce high levels of IL-6, which can contribute to cachexia in late stage tumor-bearing hosts (42); and (c) in our experience, IFN-γ–producing Th1 cells are easily expanded from total spleen or peripheral blood cell populations, while it is hard to induce pure Th2 cells producing IL-4 but not IFN-γ from total spleen or peripheral blood cells in humans and mice (data not shown). In a previous report (43), we demonstrated that culture of tumor-infiltrating lymphocytes (TILs) with IL-2 plus IL-12 results in a profound increase in the development of autologous tumor-reactive CTLs. Moreover, we showed that this protocol enhanced the generation of autologous tumor-reactive Th1-dominant cells (our unpublished data). Therefore, if we can develop a large scale culture system for the generation of autologous tumor-reactive Th1 cells from TILs or PBLs of tumor patients, the adoptive tumor immunotherapy using tumor-specific Th1-dominant cells may be possible. The cytokine IL-12 shows great promise for the development of tumor immunotherapy (18, 19, 31, 32). However, recent findings have demonstrated that IL-12 also has adverse effects owing to overstimulation of Th1-dominant immunity (44). In terms of side effects, adoptive transfer of in vitro IL-12–activated Th1-dominant cells may minimize side effects, and could make the management of side effects easier. Thus far, IL-12 has been suggested for cytokine therapy and gene therapy of cancer. This paper further indicates that IL-12 may be a useful tool for application to a novel tumor immunotherapy protocol using the adoptive transfer of Th1-dominant T cells and/or CTLs (Th1 helper/killer therapy).

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