Critical role of the Th1/Tc1 circuit for the generation of tumor-specific CTL during tumor eradication *in vivo* by Th1-cell therapy

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Th1 and Th2 cells obtained from OVA-specific T cell receptor transgenic mice completely eradicated the tumor mass when transferred into mice bearing A20-OVA tumor cells expressing OVA as a model tumor antigen. To elucidate the role of Tc1 or Tc2 cells during tumor eradication by Th1- or Th2-cell therapy, spleen cells obtained from mice cured of tumor by the therapy were re-stimulated with the model tumor antigen (OVA) for 4 days. Spleen cells obtained from mice cured by Th1-cell therapy produced high levels of IFN-γ, while spleen cells from mice cured by Th2-cell therapy produced high levels of IL-4. Intracellular staining analysis demonstrated that a high frequency of IFN-γ-producing Tc1 cells was induced in mice given Th1-cell therapy. In contrast, IL-4-producing Tc2 cells were mainly induced in mice after Th2-cell therapy. Moreover, Tc1, but not Tc2, exhibited a tumor-specific cytotoxicity against A20-OVA but not against CMS-7 fibrosarcoma. Thus, immunological memory essential for CTL generation was induced by the Th1/Tc1 circuit, but not by the Th2/Tc2 circuit. We also demonstrated that Th1-cell therapy is greatly augmented by combination therapy with cyclophosphamide treatment. This finding indicated that adoptive chemoimmunotherapy using Th1 cells should be applicable as a novel tool to enhance the Th1/Tc1 circuit, which is beneficial for inducing tumor eradication *in vivo*. (Cancer Sci 2003; 94: 924–928)

Since genes encoding tumor-rejection antigens (TRA) were cloned, it has been accepted that $CD8^+$ CTL (Tc) obtained from tumor-bearing hosts recognize MHC-binding TRA and directly destroy tumor cells.[1–3] This encouraged us to develop an efficient method to induce tumor-specific CTL *in vivo*, as a promising approach for tumor immunotherapy. It may be possible to induce complete cure of tumor-bearing hosts, if we could induce a high frequency of TRA-specific CTL *in vivo*.[4–6] However, recently, it was reported that the increase of CTL frequency by tumor vaccine therapy was not always consistent with the therapeutic effect.[7] The inability of CTL to destroy tumor cells in the tumor-bearing host may be due to a defect of the helper arm, because of strong immunosuppression.[8–10] Therefore, it is of great importance to investigate what factors are essential for inducing strong antitumor immunity in tumor-bearing hosts.

It has been demonstrated that, in addition to CTL, many immunoregulatory cells, such as DC, NK, NKT and Th, are involved in the generation of anti-tumor immunity *in vivo*.[11–14] In particular, Th cells, which can produce various cytokines essential for growth and differentiation of CTL, play an important role in inducing anti-tumor effector cells in immunosuppressed tumor-bearing hosts.[15–17] Recently, both Th and Tc cells have been subdivided into type 1 and type 2 cells based upon their differential cytokine production patterns.[18–20] Type 1 Th (Th1) or Tc (Tc1) cells produce IL-2 and IFN-γ, while type 2 Th (Th2) or Tc (Tc2) cells produce IL-4 and IL-5. The former plays a critical role in cellular immunity, while the latter is involved in humoral immunity. A key role of Th1/Th2 immunity in immune diseases has been demonstrated by many investigators.[21–23] On the other hand, the correlation between Tc1/Tc2 immunity and immune diseases remains unclear.[24]

In a previous paper,[25] we initially demonstrated that both anti-tumor specific Th1 and Th2 cells exhibited strong anti-tumor activity through distinct mechanisms. Th1 eradicated tumor mass via cellular mechanisms, but Th2 cells eradicated tumor mass via induction of necrosis. Although we showed that both Th1 and Th2 cells required $CD8^+$ T cells to induce complete cure of tumor-bearing mice, it remains unclear how Th1 or Th2 interact with Tc cell subsets *in vivo* during tumor eradication. To elucidate this issue, we analyzed the function of Tc cells obtained from mice cured of tumor by Th1- or Th2-cell therapy. Here, we demonstrate that Th1-cell therapy produces a strong anti-tumor immunity *in vivo*, concomitantly with induction of IFN-γ-producing cytotoxic T cells (Tc1). On the other hand, Th2-cell therapy induced tumor necrosis *in vivo* in parallel with the induction of IL-4-producing noncytotoxic T cells (Tc2). Thus, the Th1/Tc1 circuit appeared to be crucial for the induction of CTL with killing activity, which is beneficial for tumor eradication. We also demonstrated that cyclophosphamide (CY) treatment, which has been used for combined immunotherapy,[26] augmented the therapeutic effect of Th1-cell therapy, indicating that adoptive chemoimmunotherapy represents a new strategy for tumor cell therapy.

Materials and Methods

Animals. BALB/c mice were obtained from Charles River Japan (Yokohama, Japan). BALB/c-background $RAG2^{-/-}$ mice were kindly donated by Dr. M. Ito (Central Institute for Experimental Animals, Kanagawa). $OVA_{323-339}$-specific I-A^d-restricted T cell receptor-transgenic mice (DO11.10) maintained on the BALB/c background were kindly donated by Dr. K. M. Murphy (Washington University School of Medicine, St. Louis, MO.[25]) All the mice were female and used at 5–6 weeks of age.

Cytokines, mAbs and antigens. IL-12 was kindly donated by the Genetics Institute (Cambridge, MA). Anti-IL-12 mAbs (C15.1 and C15.6) were kind gifts from Dr. G. Trinchieri (Schering-Plash Dardilly, France). Recombinant murine IL-4 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Anti-IL-4 mAb (11B11) was purchased from ATCC (Rockville, MD). PE-anti-CD4 mAb, FITC-anti-CD45RB mAb, FITC-anti-CD8 mAb, purified anti-CD3 mAb, recombinant mouse IFN-γ and anti-IFN-γ mAb (R4-6A2) were purchased from PharMingen (San Diego, CA). $OVA_{323-339}$ peptide was kindly supplied by Dr. H. Tashiro (Fujiya Co., Ltd.,

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Hadano). Cyclophosphamide (CY) was purchased from Sigma (St. Louis, MO).

Generation of Th1 and Th2 cells. $CD4^+$ $CD45RB^+$ naive T cells were isolated from nylon-passed spleen cells from DO11.10 TCR-transgenic mouse using a FACS Vantage (Becton Dickinson, San Jose, CA) as reported previously.[25] 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 re-stimulated with $OVA_{323-339}$ under the same conditions, and used at 9–12 days of culture.

Cytokine activity. IFN-γ or IL-4 activities of culture supernatants were measured using an ELISA kit (Amersham International, Buckinghamshire, England). The IFN-γ activity was determined using a Biotrack IFN-γ ELISA kit (RPN2717) and IL-4 activity was determined using a Biotrack IL-4 ELISA kit (RPN2712). All data are presented as mean values of triplicate samples.

Production of *OVA*-gene transfectant. A20 B lymphoma cells were transfected with chicken OVA cDNA (kindly donated by Dr. M. J. Bevan[25]) subcloned into the expression vector BCGMSNeo. The transfectants were designated as A20-OVA tumor cells.

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 i.v. transferred into the tumor-bearing mice. In combination therapy with CY and Th1 or Th2 cells, mice with an established tumor mass (6–8 mm) were treated with CY (i.p.; 0.4 mg/mouse) and subjected to i.v. transfer of Th1 (5×10^6). The antitumor activity was determined by measuring the time course of changes in the mean product of two perpendicular diameters of the tumor mass. Data are presented as the mean values of 6 mice per group.

Generation of Tc1 and Tc2 by *in vitro* re-stimulation. Spleen cells obtained from untreated tumor-bearing mice or mice cured by Th1- or Th2-cell therapy were re-stimulated *in vitro* with the model tumor antigen OVA (50 μg/ml) for 4 days. IFN-γ and IL-4 levels in culture supernatants were measured at 1–4 days after culture. After 4-day culture, $CD4^+$ T cells and $CD8^+$ T cells were isolated from culture by the use of MACS beads. The cytotoxicity mediated by unfractionated cells, purified $CD4^+$ T cells or $CD8^+$ T cells was determined by 4-h ^{51}Cr-release assay as described previously.[27] A20 parental cells, A20-OVA cells CMS-7 fibrosarcoma were used as target cells.

Results and discussion

In accordance with previous findings,[25] both OVA-specific Th1 and Th2 cells exhibited a strong tumor growth-inhibitory activity in mice bearing an established A20-OVA tumor mass (6–8 mm) (Fig. 1A). All the tumor-bearing mice that received Th1 or Th2 cells (2×10^7 cells) were completely cured and survived over 60 days (Fig. 1B). As reported previously,[25] such complete cure was not induced in T cell-deficient $RAG2^{-/-}$ mice. However, if $CD8^+$ T cells were transferred into $RAG2^{-/-}$ mice, both Th1 and Th2 cells showed a strong antitumor activity *in vivo*, indicating that Th/Tc cell-cell communication is crucial to induce an efficient anti-tumor immunity in tumor-bearing mice.

To elucidate how transferred Th cells interact with host Tc cells during eradication of the tumor mass *in vivo*, spleen cells obtained from mice cured of A20-OVA tumor by Th1- or Th2-cell therapy were re-stimulated with the model tumor antigen OVA, and the functional characteristics of activated $CD4^+$ T cells and $CD8^+$ T cells were examined. The kinetics of IFN-γ and IL-4 production during culture is illustrated in Fig. 2. Spleen cells from mice given Th1-cell therapy (Th1 mice) produced higher levels of IFN-γ on OVA re-stimulation compared with those from mice given Th2-cell therapy (Th2 mice). Conversely, spleen cells from Th2 mice produced higher levels of IL-4 compared with Th1 mice. To characterize the cytokine-producing cells at the single cell level, we analyzed the charac-

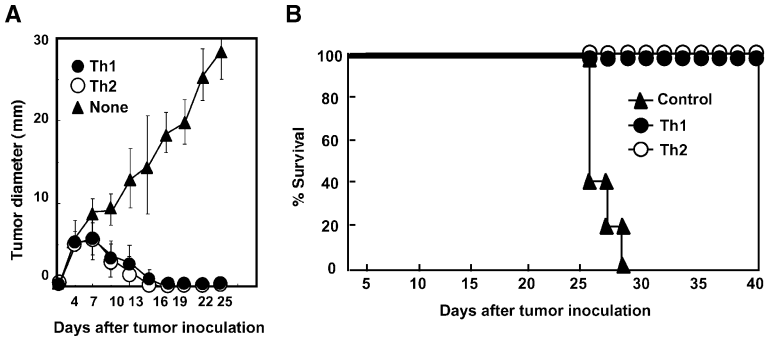

Fig. 1. Adoptive tumor immunotherapy with Th1 or Th2 cells. A20-OVA tumor cells were intradermally inoculated into wild-type BALB/c mice. After A20-OVA tumor had formed a tumor mass (6–8 mm in diameter), 2×10^7 OVA-specific Th1 (●) or Th2 cells (○) induced from DO10 TCR-Tg mice were i.v. transferred into the tumor-bearing mice. Control mice (▲) were treated with saline. (A) The therapeutic effect of Th1 or Th2 cells was determined by measuring tumor size. (B) Survival days of tumor-bearing mice treated with or without cell therapy was measured. Mice that were tumor-free for over 60 days were considered as completely cured. The data represent mean±SE of 5 mice.

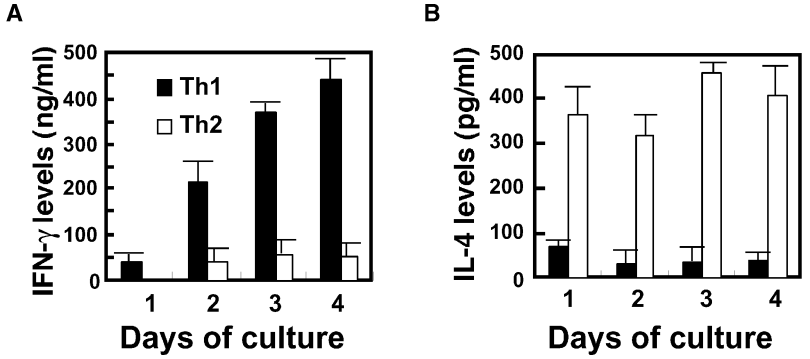

Fig. 2. Cytokine-producing ability of mice cured of tumor by Th1 or Th2 cell therapy. Spleen cells obtained from mice cured of A20-OVA tumors by cell therapy with Th1 (■) or Th2 cells (□) were re-stimulated *in vitro* with OVA (50 μg/ml) for 4 days. Culture supernatants were harvested every day and their IFN-γ (A) and IL-4 (B) levels were measured by ELISA. The data represents mean±SE of 3 mice.

teristics of IFN-γ- or IL-4-producing cells by means of an intracellular staining technique (Fig. 3). $CD4^+$ T cells induced from spleen cells of Th1 mice produced high levels of IFN-γ, but not IL-4. $CD4^+$ T cells derived from Th2 mice produced high levels of IL-4, but not IFN-γ. Most IFN-γ- or IL-4-producing $CD4^+$ T cells were stained with clonotypic mAb, KJ.126, indicating that the cytokine-producing $CD4^+$ T cells were derived from transferred cell populations (data not shown). Interestingly, it was also demonstrated that $CD8^+$ T cells derived from Th1 mice contained more IFN-γ-producing cells (34.6%) than IL-4-producing cells (8.0%). Conversely, $CD8^+$ T cells derived from Th2 mice contained more IL-4-producing cells (49.1%) than IFN-γ-producing cells (15.1%).

The cytotoxicity of the Tc1-rich population derived from Th1 or the Tc2-rich population derived from Th 2 mice was also examined. As is clear from Fig. 4, unfractionated cells derived from Th1 mice showed higher cytotoxicity against A20-OVA tumor cells than did unfractionated cells derived from Th2 mice. Although unfractionated cells derived from Th2 mice showed marginal cytotoxicity against A20-OVA, purified $CD4^+$ T cells (Th2) and $CD8^+$ T cells (Tc2-rich population) showed no significant cytotoxicity against A20-OVA tumor. In contrast, the cytotoxicity of unfractionated cells derived from Th1 mice was greatly enriched in $CD8^+$ T cells (Tc1-rich population) though $CD4^+$ T (Th1) showed slight cytotoxicity. The Tc1-rich population showed tumor-specific cytotoxicity against A20-OVA, but not against CMS-7 fibrosarcoma cells.

Thus, our results suggested that Th1-cell therapy could prime $CD8^+$ Tc1 cells, which then produced IFN-γ and exhibited tumor-specific cytotoxicity, leading to eradication of the tumor mass *in vivo*. We confirmed the previous finding[28] that Th1 cells expressed higher levels of IFN-γ receptor, but not IL-4 receptor, than did Tc2 cells (data not shown). Thus, Th1 cells might facilitate the induction of Tc1 cells through upregulation of IFN-γ-mediated signaling and downmodulation of IL-4-mediated signaling. On the other hand, Th2-cell therapy could not prime cytotoxic Tc1 cells, though it stimulated IL-4-producing Tc2 cells without a specific antitumor cytotoxicity. This finding is consistent with our previous result[25] that Th2 cells eradicated the tumor mass by inducing necrosis, with infiltration of inflammatory cells into the tumor mass. Recently, it was demonstrated that eosinophils induced by STAT-6-dependent signaling are crucial for inducing tumor necrosis.[29] Moreover, it was reported that IL-4 production in tumor-bearing mice caused the production of IgG1 Ab, but tumor-reactive IgG1 Ab is not important for tumor eradication, in contrast to Th1-dependent IgG2a Ab.[30] Therefore, IL-4-producing Th2 cells in tumor-bearing mice might be important for inducing tumor necrosis mediated by eosinophils, rather than Ab production.

Recently, however, Dobrzanski *et al.*[18–20] reported that both Tc1 and Tc2 cells derived from OT-1 transgenic (Tg) mice exhibited anti-tumor activity *in vivo* and prolonged the survival of tumor-bearing mice. Moreover, they demonstrated that OT-1-derived Tc2 cells showed strong cytotoxicity against antigenic tumor cells, as did Tc1 cells. We also confirmed that Tc2 cells induced from OT-1 or 2C TCR-Tg mice showed a strong cytotoxicity, like Tc1 cells (data not shown). Moreover, Tc2 cells induced in the presence of IL-4, anti-IFN-γ mAb and anti-IL-12 mAb should have the ability to produce IFN-γ in addition to IL-4 during maturation in culture for 10 days. Therefore, our Tc2

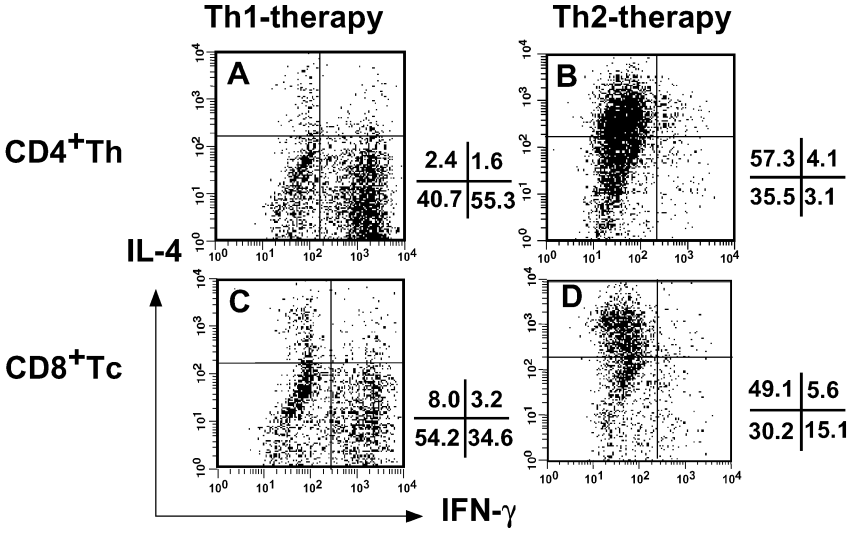

Fig. 3. Generation of Tc1 or Tc2 cells in mice cured of tumor by Th1 or Th2 cell therapy. BALB/c mice bearing A20-OVA tumors were cured of tumor by Th1 or Th2 cell therapy as described in Fig. 1. Spleen cells obtained from mice cured of A20-OVA tumors by cell therapy with Th1 or Th2 cells were re-stimulated *in vitro* with OVA for 4 days. The cytokine-producing ability of $CD4^+$ (A, B) or $CD8^+$ T (C, D) cells derived from mice treated with Th1 cell therapy (A, C) or Th2-cell therapy (B, D) was determined by intracellular cytokine staining analysis. The numbers represent the percentage of positive cells.

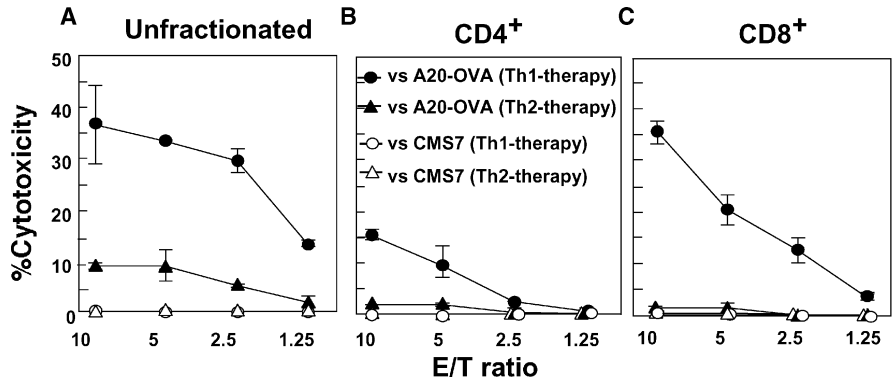

Fig. 4. Generation of $CD8^+$ CTL with killing activity in mice treated with Th1-cell therapy, but not Th2-cell therapy. Spleen cells obtained from mice cured of A20-OVA tumors by cell therapy with Th1 (●, ○) or Th2 cells (▲, △) were re-stimulated *in vitro* with OVA for 4 days. Then, $CD4^+$ Th1 or Th2 cells or $CD8^+$ Tc1 or Tc2 cells were isolated from cultured cells and their cytotoxicity against A20-OVA (●, ▲) or CMS7 tumor cells (○, △) was determined by 4-h ^{51}Cr release assay. The data represent mean±SE of 3 mice.

cells induced from *in vivo* circulating CD8$^+$ T cells appeared to show different functional properties from OT-1-derived Tc2 cells. This might be because of (i) higher sensitivity of Tg mouse-derived T cells to antigen stimulation compared with wild-type T cells or (ii) different induction condition; OT-1-derived Tc2 are induced *in vitro* by stimulation with Ag+IL-4+IL-2, whereas our Tc2 are induced in tumor-bearing mice by physiological Th2/Tc2 interaction with tumor antigen stimulation. Thus, Tc2 cells induced from TCR-Tg mice *in vitro* produce IFN-γ in addition to IL-4, and exhibit a strong anti-tumor cytotoxicity. However, our present data suggest that Tc2 cells that interact with Th2 cells under tumor-bearing conditions produce IL-4, but not IFN-γ, and they showed no significant cytotoxicity against tumor cells.

Our results demonstrated that both Th1 and Th2 cells could eradicate an established tumor mass via distinct mechanisms. However, Th1-cell therapy may be preferable to induce tumor-specific anti-tumor immunity *in vivo*.[14, 25] This hypothesis is supported by other results[31, 32] which show that immunodeviation toward Th1-dominant immunity is preferable to overcome immunosuppression of tumor-bearing host and to induce tumor-specific immunity *in vivo*. So far, many investigators have focused on the induction of TRA-specific CTL *in vivo* by TRA-peptide or TRA-pulsed DC to cure tumors.[32, 33] However, the increase of CTL frequency is not consistent with the increase of therapeutic potential.[7] This might be because of immunosuppression mechanisms of tumor-bearing hosts that inhibit growth and function of CTL.[15–17] To overcome this problem, it might be effective to enhance DC1/Th1 cell-cell interaction, which would accelerate the induction of Th1-dominant immunity *in vivo* and enable the induction of activation of CTL at the tumor local site.[34] Here, we have demonstrated that adoptive tumor immunotherapy using Th1 cells can overcome immunosuppression of tumor-bearing mice, leading to the induction of tumor-specific IFN-γ-producing Tc1 cells from host cells during tumor eradication. Tc1, but not Tc2, showed tumor-specific cytotoxicity in our experiments. Therefore, it might be important to develop therapeutic methods which can accelerate host Th1-dominant immunity.

As shown in Fig. 5, the therapeutic effect of Th1 cell therapy was greatly enhanced by combination therapy with CY treatment. Generally, over 2×10^7 cells are required to induce completely cure A20-OVA-bearing BALB/c mice, and tumor growth is not inhibited by less than 5×10^6 cells (data not shown). However, if tumor-bearing mice were treated with CY injection (40 mg/kg) before Th1-cell transfer, Th1 cells inhibited tumor growth even when only 5×10^6 Th1 cells were transferred into mice. In accordance with previous results,[25] the tumor mass in mice treated with combination therapy with CY and Th1 cells gradually changed to a small, white mass, and was completely rejected by around 6–8 days after cell transfer. Thus, the efficacy of Th1 cell therapy is greatly augmented by combination with CY treatment. Although it has reported that CY augmented the efficacy of immunotherapy,[35, 36] the precise

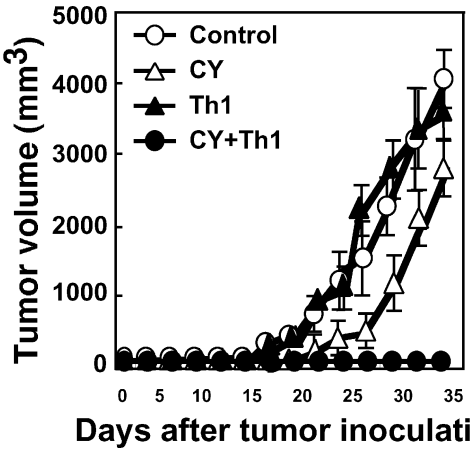

Fig. 5. Augmentation of the efficacy of Th1-cell therapy by combination with CY treatment. A20-OVA tumor cells were intradermally inoculated into wild-type BALB/c mice. After A20-OVA tumor had formed a tumor mass (6–8 mm in diameter), the mice were treated with none (○), CY (40 mg/kg, △), Th1 cells (5×10^6 cells, ▲) or CY+Th1 cells (●). The data represent mean±SE of 5 mice.

mechanism of CY-induced immunomodulation remains unclear. However, recently, it was reported that CY treatment of tumor-bearing mice caused conversion of tumor-infiltrated Mϕ from IL-10-producers to IFN-γ-producers, resulting in destruction of tumor vasculature.[37, 38] Moreover, it was suggested that CY treatment facilitates homeostatic expansion of transferred effector cells.[39] These findings support our result that combination therapy with Th1 and CY is beneficial for inducing anti-tumor immunity *in vivo*. As some anti-cancer drugs other than CY have also been reported to augment the therapeutic effect of immunotherapies,[40] we are planning to investigate whether anti-cancer drugs such as adriamycin and cisplatin are able to potentiate the efficacy of Th1-cell therapy.

In conclusion, we have demonstrated that Th1-cell therapy is preferable to Th2-cell therapy to induce tumor-specific IFN-γ-producing CD8$^+$ cytotoxic T cells (Tc1), which are critical for preventing tumor metastasis and tumor recurrence. We believe that adoptive chemoimmunotherapy using Th1 cells and CY could be an effective strategy for tumor therapy in the near future.

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