A Tumor-specific Th2 Clone Initiating Tumor Rejection via Primed CD8+ Cytotoxic T-Lymphocyte Activation in Mice

Yuan Shen and Shigeyoshi Fujimoto
Department of Immunology, Kochi Medical School, Nankoku-shi, Kochi 783, Japan

ABSTRACT

We established a CD4+ T-cell clone specific for syngeneic methylcholanthrene-induced sarcoma, S1509a raised in an A/J mouse, involved in tumor regression. The phenotype of the T-cell clone was CD3+ TCR-β+, CD4+, CD45RB+, LFA-1+, ICAM-1+, CD44+, and VLA-4+. The CD4+ T-cell clone specifically proliferated following antigen stimulation with attenuated S1509a in the presence of syngeneic accessory cells, and this antigen-induced proliferation was inhibited with anti-CD4 and anti-IL-4a monoclonal antibodies. The CD4+ T-cell clone designated YS1093 secreted interleukin (IL) 4, IL-5, and IL-6, but not IFN-γ, tumor necrosis factor α, or IL-2, thus indicating that the clone belongs to the Th2 type. YS1093 cells and their culture supernatant after antigen stimulation augmented the primed cytotoxic T lymphocyte killing activity at the effector phase. YS1093 cells having Th2-type characteristics made the homologous growing tumor regress in the tumor-bearing syngeneic mice when YS1093 cells were transferred into the tumor-bearing mice i.v.

The in vivo tumor regression initiated by YS1093 cell transfer essentially required the presence of CD8+ T cells in the tumor-bearing hosts, thus suggesting that some specific Th2 cells are positively involved in tumor regression by activating primed CD8+ cytotoxic T lymphocytes against the homologous tumor in situ.

INTRODUCTION

Analysis of immune mechanisms of tumor rejection by passive transfer of tumor-specific immune lymphocytes into the corresponding growing tumor-bearing hosts in syngeneic mouse models provides ideas for the development of a consistent, specific immunotherapy directed to autologous cancer in human patients. Rejection of tumors has been shown to be mediated by immune T cells with CD4+ Th2/CTLs and/or CD8+ CTLs in many experimental syngeneic tumor regression by activating primed CD8+ cytotoxic T lymphocytes bearing hosts through i.v. passive cell transfer to establish cancer-specific passive immunotherapy for cancer patients.

MATERIALS AND METHODS

Mice. Five-week-old male mice of specific pathogen-free A/J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). BALB/c and BALB/c-nu/nu mice were propagated in our specific pathogen-free animal facilities. All mice were bred in our facilities and used at 6–8 weeks of age for these experiments.

Tumors. Methylcholanthrene-induced sarcoma S1509a (16) and S713a raised in an A/J mouse origin and methylcholanthrene-induced sarcoma MS61391c of a BALB/c mouse were used in these experiments. These tumor cells were maintained in vitro in stationary culture with RPMI 1640 (Life Technologies, Inc., Long Island, NY) supplemented with 5% FCS (Filtron, Brooklyn, Australia) and 100 μg/ml of Kanamycin (Meiji Pharmaceutical Co., Tokyo, Japan) designated a complete RPMI medium.

Preparation of ALCs in Vitro. Fifteen million lymphoid cells of spleens and lymph nodes from either normal A/J mice or A/J mice that had been inoculated with 2 × 10⁶ viable S1509a cells 12 days before as tumor-bearing hosts or A/J mice that had been immunized s.c. with 1.5 × 10⁶ cells of MMC-(Koyawahako, Tokyo, Japan) treated S1509a about 10 days before as immune hosts were restimulated in vitro with 5 × 10⁴ MMC-treated homologous tumor cells at a responder:stimulator ratio of 300:1 in 1 ml of DMEM (Life Technologies, Inc.) supplemented with 5% FCS, 20 mm HEPES, 100 μg/ml of Kanamycin, and 5 × 10⁻⁴ M 2-ME (designated a complete DMEM medium) for 5 days. For MMC treatment, 1 × 10⁷ S1509a cells were treated with 100 μg/ml of MMC in 1 ml of complete RPMI medium at 37°C for 40 min, then washed with PBS three times, and resuspended in either PBS for in vivo use or complete DMEM medium for in vitro stimulation. After a 5-day culture, lymphoid cells were harvested and tested for their cytotoxic activity.

Establishment of the CD4+ T-Cell Clone. ALCs were further cultured for 5 more days and then restimulated with MMC-treated S1509a in the presence of syngeneic feeder cells prepared from normal A/J spleen cells irradiated with 23 Gy of γ-rays by a 137Cs from a gamma irradiator (Gammacell 1000, Nordion, Canada) in a 6-well-type microplate (Nunc, Kamstrup, Denmark). The re-stimulation of ALCs from immune spleen cells was performed three times at 2-week intervals in RPMI 1640 supplemented with 5% FCS, 5 × 10⁻⁵ M 2-ME, 100 μg/ml of Kanamycin, and 5–10% concanavalin A (Pharmacia, Uppsala, Sweden) stimulated rat spleen cell supernatant (concanavalin A supernatant) as a T-cell growth factor designated a maintaining medium. After a 2-month culture, proliferative cells were found in one well among six wells. Cell cloning was performed by a single-cell manipulation of the long-term proliferative culture cells from the ALCs and made in four 96-well flat-bottomed microplates. Only four CD4+ T clones came out, but finally one of four clones was specifically reactive to S1509a and was established as a CD4+ T-cell clone. The established CD4+ T-cell clone (1 × 10⁵) specific for S1509a having an in vivo effect on tumor growth suppression was designated YS1093 and maintained in stationary culture with the maintaining medium by the monthly restimulation with MMC-treated S1509a at a responder:stimulator ratio of 100:1 in the presence of irradiated normal A/J spleen cells (2.5 × 10⁶ cells/ml). The cultures were maintained at 37°C in air containing 7% CO₂.
Cell Lines. Other T-cell clones 1E12 and C5.3 are CD8+ CTL clones established from BALB/c immune spleen cells to a syngeneic methylcholanthrene-induced sarcoma MS61391c in our laboratory. HR2 is a CD4+ T-cell (Th2) line specific for bml2 established from C57BL/6 immune spleen cells in our laboratory (17). 2C3.21 is a Th1-type CD4+ T-cell clone derived from BALB/c immune spleen cells by stimulation with MMC-treated MS61391c in our laboratory. J774A.1, a macrophage tumor cell line, was obtained from the Japanese Cell Research Bank (Tokyo, Japan).

Cell Culture Supernatant. YS1093 cells were harvested from stationary culture and washed three times with PBS, and 10^5 cells/ml of YS1093 were restimulated with 5 x 10^5/ml of MMC-treated S1509a and irradiated syngeneic T-cell-depleted spleen cells (1 x 10^6/ml) in the complete RPMI medium for 48 h. After a 48-h incubation, the culture supernatant was harvested by centrifugation at 1500 rpm for 10 min and filtered using a 0.22-μm Millipore filter. The culture supernatant was used for further analysis.

Abs. Rat anti-CD-4 (L3T4; GK1.5), rat anti-CD8 (Ly-2; 3.155 and 2.43), rat anti-Ig-2 (S4B6), mouse anti-I-Ak (26-7-11S), rat anti-IL-4 (11B11), mouse anti-I-Ek (17-3-35), and hamster anti-CD-3 (145-2C11) were kind gifts from Dr. N. Shinojara (Cellular Immunology Division, Mitsubishi Life Science Institute, Kanagawa, Japan). Rat anti-ICAM-1(Y71/174), rat anti-CD45RA (14.8), rat anti-CD45RB (MB23G2), rat anti-CD4-3 (PS2), hamster anti-TCR-β (H57-597), rat anti-mouse IFN-γ (R4-6A2), and rat anti-IL-6 (05-114) were purchased from American Type Culture Collection (Rockville, MD). Mouse anti-Thy-1.2 (HO-13-4) was kindly supplied by Dr. H. Ishikawa (Department of Microbiology, Keio University, Tokyo, Japan). Rat anti-CD44 (KM114) and rat anti-IL-5 (TB13) were kindly supplied by Dr. A. Tominaga (Department of Biology, Kochi Medical School, Kochi, Japan). Rat anti-IL-10 (SXC.1 and SXC.2) was kindly supplied by Dr. A. Sehon (Department of Immunology, Faculty of Medicine, University of Manitoba, Winnipeg, Canada). Rat anti-mouse IFN-γ (XMG1.2) was purchased from Endogen, Inc. These MAbs were purified from either culture supernatants or ascitic fluids of these hybridoma cell lines by 40% ammonium sulfate precipitation and then by protein G-Sepharose 4B fast-flow chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden) in the case of the IgG class. The IgM class such as anti-Thy-1.2 and anti-IL-10 (SXC.1 and SXC.2) were purified using agar block electrophoresis with 0.1 m barbitual buffer (pH 8.6) in our laboratory.

Cell Proliferation. Antigenic proliferation of YS1093 was performed as follows. YS1093 cells were harvested from culture dishes (Nunc), washed with PBS three times, and then resuspended in RPMI 1640 supplemented with 5% FCS, 20 mM HEPES, 5 x 10^{-5} m 2-ME, and 100 μg/ml of Kanamycin. The cells were plated in a 96-well round-bottomed microplate (Nunc) at a cell concentration of 1 x 10^5 cells/0.2 ml in each well and 10-Gy irradiated T-cell-depleted normal A/J spleen cells were added as feeder cells at a cell concentration of 5 x 10^5/0.2 ml/well, and MMC-treated S1509a cells or irrelevant tumor S713a cells were added at a cell concentration of 5 x 10^5/0.2 ml/well and were cultured for 3 days. The cells were pulsed with 0.5 μCi/ml of [3H]TdR for the last 16 h of a 3-day culture. Three days after the incubation, the cells from each well were harvested and washed separately. Then their radioactivity was counted using a liquid scintillation counter (LKB-Wallac Betaplate 1205-012; Finland).

Cytotoxic Assay. Cytotoxic activity was measured using a 16-h 51Cr release assay according to the method described previously (16). Briefly, 1.5 x 10^5 cells of 51Cr-labeled target cells and various numbers of effector cells at different E:T ratios in a total volume of 0.2 ml were assigned to each well of 96-well round-bottomed microplates in quadruplicate and incubated for 16 h at 37°C in 5% CO2. A 0.1-ml aliquot of the supernatant was taken from each well, and the radioactivity was measured in a Packard Auto-Gamma 5000 gamma scintillation counter. Target cell lysis was expressed as the percentage of specific 51Cr release calculated using the following formula:

\[
\% \text{ specific } 51\text{Cr release} = \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}} \times 100
\]

The maximum releasable counts amounted to 85–90% of the total radioactivity incorporated into target cells with 1.25% Saponin (Nacalai Tesque, Inc., Kyoto, Japan). The spontaneous release from the target cells in the wells averaged less than 25% of the maximum release.

Cytokine Assay. Cytokine production by YS1093 was detected using the following methods. IL-2, IL-4, IL-5, IL-6, and TNF were detected using a bioassay. IL-2- and IL-4-dependent cell line C5.3, which was established as a CD8+ CTL clone from BALB/c spleen cells in our laboratory, and IL-6-dependent cell line MH60 (kindly supplied by Dr. A. Okano, Ajinomoto Co. Ltd., Yokohama, Japan; Ref. 18) were plated in a 96-well microplate at a concentration of 5 x 10^5 in 0.2 ml/well in quadruplicate with serially diluted culture supernatant to be tested. The cells were pulsed with 0.5 μCi/ml of [3H]Tdr during the last 16 h of a 48-h culture. Cells of the IL-5-dependent cell line BCL1 (supplied by the Japanese Cell Research Bank; Ref. 19) were plated at 2 x 10^4/0.2 ml of cell concentration in a well of a 96-well microplate and pulsed with 0.5 μCi/ml of [3H]Tdr during the last 6 h of a 24-h incubation (20). After the cells were washed and harvested, their radioactivities were measured using the liquid scintillation counter. TNF was detected using a bioassay according to a method described elsewhere (21). IFN-γ and IL-10 of YS1093 culture supernatant were measured using a sandwich ELISA. Briefly, anti-mouse IFN-γ (XMG1.2) and anti-IL-10 (SXC.1) were used as coating MAbs. Alkaline phosphatase-conjugated anti-mouse IFN-γ (R4-6A2) and biotinylated anti-mouse IL-10 (SXC.2) and alkaline phosphatase-conjugated avidin were used as detection reagents, respectively.

Flow Cytometry. YS1093 cells were washed once with cold staining buffer (0.1% PBS containing 2% BSA and 0.1% sodium azide) and incubated with various primary MAbs for 30 min at 4°C, washed once with staining buffer, and then stained with a FITC-conjugated secondary MAbs for 30 min at 4°C. After washing, stained cells were analyzed with EPICS752 (Coulter Electronics, Hialeah, FL).

Adaptive Cell Transfer. YS1093 cells were harvested from 50-ml culture flasks (Nunc) and washed three times with PBS. After the tumors had grown...
TUMOR-SPECIFIC T
K2 CLONE INDUCES TUMOR REGRESSION

In Vivo Evaluation of the YS1093 Effect on Tumor Growth. A/J mice were inoculated s.c. with 1.5 × 10⁶ cells of S1509a in the middle of their backs. Five days after the tumor cell inoculation, when the tumor had grown 5–7 mm in diameter, the tumor-growing syngeneic mice were divided into various groups including control and experimental groups. The evaluation of the in vivo YS1093 effect on the corresponding tumor growth in the TBHs by the passive i.v. cell transfer was performed by measuring tumor size with Vernier calipers in terms of two diameters at right angles after depletion of the tumor sites with chemical hair remover until 30 days after the tumor cell inoculation. As control groups, five tumor-growing mice were given injections of PBS and four tumor-growing mice were given injections of a nonreactive CD8⁺ T-cell line instead of YS1093 cells. The tumor growth was expressed as a tumor area by mm².

Treatment of Animals with Anti-CD8 MAB (2.43). To deplete the CD8⁺ T-cell population in S1509a-bearing hosts, 1 mg of a γ-globulin fraction partially purified by 40% saturated ammonium sulfate precipitation from ascitic fluid of BALB/c-nu/nu mice i.p. inoculated with 2.43 hybridoma was given i.v. to each S1509a-bearing A/J mouse in the experimental group five times daily. Flow cytometric analysis of spleen cells from a treated mouse proved that the CD8⁺ T-cell population was not detected in the spleen cells.

RESULTS

Establishment of the CD4⁺ T-Cell Clone (YS1093) and Its Phenotype. To establish specific T-cell clones reactive to syngeneic S1509a tumor cells, A/J immune spleen cells were repeatedly restimulated in vitro with MMC-treated S1509a in the presence of feeder
cells 10 days after the first in vitro restimulation with the attenuated homologous tumor cells. Two months later, the proliferating cells were cloned by single manipulation and only one clone was extraordinarily proliferated by tumor cell stimulation. The phenotype of the established clone designated YS1093 was examined using flow cytometric analysis. As shown in Fig. 1, YS1093 expressed CD3, TCR-β, CD4, CD45RB, ICAM-1, LFA-1, CD44, and VLA-4 molecules on its cell surface.

**Antigen Specificity of YS1093 Cells.** Since YS1093 was determined to be a CD4+ T-cell clone, specificity of YS1093 was examined by antigen-induced proliferation of the cell. YS1093 clearly showed proliferation by the stimulation with S1509a tumor cells in the presence of accessory cells but not by irrelevant syngeneic tumor S713a cell stimulation as shown in Fig. 2a. Furthermore, this proliferative response was inhibited by MAbs directed to both CD4 (GK1.5) and I-Ek (17-3-3) but not by a MAb to I-Ak (26-7-11) as shown in Fig. 2b. These results clearly indicate that YS1093 specifically proliferated by S1509a stimulation through MHC class II I-Ek molecules of accessory cells.

**Cytokine Production of YS1093 Cells.** Cytokine production of YS1093 was examined in the culture supernatant using either a bioassay or ELISA. Only IL-4, IL-5, and IL-6 were detected in the supernatant, as shown in Fig. 3.

**In Vitro Biological Activity of YS1093 and Its Factor(s) to CTLs.** CD8+ CTLs directed to S1509a can be induced either in the S1509a immune spleen cells or in the homologous TBH spleen cells by in vitro stimulation with the MMC-treated homologous tumor cells. As shown in Fig. 4, the cytotoxic activities of S1509a of the induced CTLs in both immune spleen cells and TBH spleen cells were clearly abrogated by treatment with anti-Thy-1.2 (HO-13-4) as well as anti-CD8 (3.155) MAbs and rabbit complement but not with the anti-CD4 (GK1.5) MAb. Furthermore, the cytotoxic activities were shown to be specific for S1509a and irrelevant syngeneic S713a was not lysed by the CTLs (Fig. 5). These results indicated that the induced CTLs in both immune and TBH spleen cells were CD8+ T cells and specific for S1509a. When YS1093 cells or their factors (culture supernatant) were added to the immune spleen cells at the in vitro proliferation phase, their killing activity was not augmented at all (data not shown). Interestingly, however, when YS1093 cells or their factors were added to the induced CTLs at the effector phase, YS1093 cells and their factors (Fig. 6, A and B) did augment the CTL killing activity against S1509a, although they did not have any cyto-
The other group of mice was given i.v. injections of 1 x 10^8 cells of YS1093 in PBS. When the inoculated tumor cells grew to about 7 mm in diameter in the backs of mice, the transferred experimental group of mice (S) were depicted. Each line refers to tumor effector phase (Fig. 6E). However, this augmenting activity of toxic effect themselves on S1509a (Fig. 6D and F). Moreover, their factors also augmented the cytotoxic activity of an allogeneic CD8^+ CTL clone (1E12) established from BALB/c immune spleen cells to syngeneic methylcholanthrene-induced sarcoma S1509a raised in an A/J mouse was shown to initiate the homologous tumor regression in vivo via activation of CD8^+ CTLs in the TBHs by i.v. cell transfer of YS1093. Because YS1093 cells secreted IL-4, IL-5, and IL-6, the CD4^+ T-cell clone was classified as a Th2-type T-cell subset from the profile of this cytokine production. Effector cells having a specific antitumor activity have been generally documented to be a Th1-type subset secreting IL-2, IFN-γ, and TNF-α and IL-β (12–14). Furthermore, since it was recently reported by several investigators that IL-12 augments the IFN-γ-producing Th1-type immune response to tumor, resulting in tumor regression (22, 23), and suppresses IL-4-producing Th2-type immune response (6, 14, 24).

**DISCUSSION**

In this study, we demonstrated that a Th2-type CD4^+ T-cell clone (YS1093) specific for a syngeneic methylcholanthrene-induced sarcoma S1509a raised in an A/J mouse was shown to initiate the homologous tumor regression in vivo via activation of CD8^+ CTLs in the TBHs by i.v. cell transfer of YS1093. Because YS1093 cells secreted IL-4, IL-5, and IL-6, the CD4^+ T-cell clone was classified as a Th2-type T-cell subset from the profile of this cytokine production. Effector cells having a specific antitumor activity have been generally documented to be a Th1-type subset secreting IL-2, IFN-γ, and TNF-α and IL-β (12–14). Furthermore, since it was recently reported by several investigators that IL-12 augments the IFN-γ-producing Th1-type immune response to tumor, resulting in tumor regression (22, 23), and suppresses IL-4-producing Th2-type immune response (6, 14, 24).
our present data are contradictory to the general Th1/Th2 cross-regulation concept. On the other hand, it was reported that IL-4 is a potent cytokine for CTL differentiation and very effective in inducing antigen-specific CTLs in some in vitro systems (24). Moreover, antitumor activity of IL-4 in some animal model experiments in vivo via activation of cytotoxic eosinophils was observed (10, 25, 26). In our system, Th2-type YS1093 cells that had no direct cytotoxic activity against the homologous tumor S1509a cells also showed clearly specific antitumor activity in vivo. However, this in vivo antitumor effect initiated by i.v. transfer of YS1093 essentially required the presence of CD8+ T cells in the TBHs. Moreover, YS1093 cells and their culture supernatant augmented the primed CD8+ CTL activity induced in not only immune host spleen cells but also the homologous TBH spleen cells against target tumor cells at the effector phase in a 16-h 51Cr release assay, although they did not show remarkable enhancing activity in the CTL induction at the induction phase when they were added to immune spleen cells at the antigen restimulation (data not shown). Since this augmenting activity was not inhibited by any neutralizing MAb to IL-4, IL-5, or IL-6 separately, thus it is suggested that the augmenting activity is mediated by either combinational activity of these cytokines or a novel factor(s) released from YS1093 cells. These results postulate that tumor regression in vivo by YS1093 cell transfer will be initiated by the specific migration of CD4+ T cells from intravasculance into tumor sites, and when the migrated CD4+ T cells are specifically restimulated by antigen-presenting cells in tumor tissue, they secrete various cytokines and activate the primed CD8+ CTLs and probably some of the inflam-

Fig. 9. In vivo specificity of YS1093-suppressive effect on S1509a tumor growth. Double TBHs were prepared in A/J mice inoculated with 1.5 × 10^6 cells of S1509a in the left side of the back and of irrelevant syngeneic tumor S713a in the right side of the back in each mouse. When these tumors grew to about 7 mm in diameter, the mice were divided into two groups. The control group (n = 3) was untreated; ■, S1509a tumor growth curve in the control group; ◇, S713a tumor growth curve in the control group. The experimental group (n = 4) was transferred i.v. with YS1093 several times: □, the S1509a tumor growth curve in the experimental group. ○, S713a tumor growth curve in the experimental group. Bars, SE.

Fig. 10. The data on day 20 from Fig. 9 are presented. A, control group; B, experimental group.

Finally, our present findings propose that once we can establish the same Th2-type CD4+ T-cell clone as YS1093 cells directed to autologous tumor cells in human cancer patients, this type of CD4+ T-cell clone can be a very important key lymphocyte in the establishment of anticancer-specific passive immunotherapy by cell transfer.

ACKNOWLEDGMENTS

We thank Dr. Masaru Takata and Sayo Kataoka for their skillful techniques for flow cytometric analysis and ELISA. We extend our sincere gratitude to Miho Kimura for her devoted secretarial assistance and preparation of the manuscript.

REFERENCES


A Tumor-specific Th2 Clone Initiating Tumor Rejection via Primed CD8⁺ Cytotoxic T-Lymphocyte Activation in Mice

Yuan Shen and Shigeyoshi Fujimoto

Cancer Res 1996;56:5005-5011.

Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/21/5005

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.