Combinations of tumor-specific CD8+ CTLs and anti-CD25 mAb provide improved immunotherapy

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Received October 9, 2007; Accepted December 20, 2007

Abstract. One new approach to cancer therapy is based on the adoptive transfer of tumor-specific cytotoxic T cells and anti-CD25 antibodies. In the present study, CD8+ and IFN-γ secreting T lymphocytes (CTLs) were enriched as tumor-specific cytotoxic T cells from spleen lymphocytes of mice bearing the Renca tumor (a murine renal carcinoma line originating from a BALB/c mouse) after stimulation with tumor cells. An anti-CD25 IL-2Rα(anti-CD25) mAb from hybridoma PC61 was used for depletion for CD4+CD25+ regulatory T (Treg) cells. Treatment-efficacy for tumor-bearing mice was compared using 4 systems: 1, whole spleen lymphocytes stimulated with tumor cells *in vitro* from tumor-bearing mice; 2, CTLs; 3, anti-CD25 mAbs; 4, CTLs and anti-CD25 mAbs. At the 50th day after tumor inoculation, in the group which received anti-CD25 mAb for depletion of T cells and inoculation of CTLs, tumors had disappeared and no re-growth was observed. In contrast, all mice of the non-treated and other three groups, treated with whole spleen cells alone, CTLs alone and anti-CD25 mAb alone, had died. These results showed that a combination of Treg cell-depletion using anti-CD25 mAbs and CTL administration is a feasible approach for treatment of cancers which warrants further exploration in the clinical setting.

Introduction

Since the initial cloning of the interleukin-2 (IL-2) gene (1), adoptive-immunotherapy for treatment of cancer using lymphokine-activating killer cells, tumor-infiltrating cells and cytotoxic T lymphocytes has been performed by many researcher groups to improve therapeutic efficacy (2-4). Unfortunately, results were limited in most cases by the weak immunogenicity of many tumors. However, Sakaguchi *et al* have shown that a population of lymphocytes expressing CD4 and CD25 antigens under non-activated conditions suppress functions of the immune system, thus, now being renamed regulatory T cells (Treg) (5), and a single administration of anti-CD25 mAbs can cause regression of tumors in syngeneic mice when given soon after tumor inoculation (6). These reports indicated that administration of T cells activate with lymphokines, anti-CD3 antibodies and autologous tumors or anti-CD25 mAb can rspectively enhance anti-tumor immunity. However, these treatments used separately do not show sufficient efficacy. For improved outcomes, more tumor-specific CTLs should be used, and, before administration of CTLs, Tregs should be eliminated.

It is known that lymphocytes rapidly express and secrete cytokines after stimulation with appropriate antigens and the quantity of IFN-γ produced determines the cytotoxicity. Recently, a new system to detect and separate IFN-γ secreting cells at the single cell level was developed (7), allowing isolation of tumor-specific T cells even if present at low frequencies. The number of tumor-specific T cells may also be enhanced by repetitive stimulation with autologous tumor cells *in vitro*.

In the present study, we compared the therapeutic efficacy of anti-CD25 mAbs, whole spleen cells stimulated with tumor cells for 4 days and tumor-specific CD8+ CTLs separated from stimulated spleen cells. Our results showed that a combination of Treg-depletion using anti-CD25 mAbs and CTL administration is a feasible approach for cancer treatment warranting further exploration in the clinic.

Materials and methods

Mice and the cell line. For all experiments, four-week old female BALB/c mice were purchased from Japan SLC Co. (Shizuoka, Japan) and maintained in the Animal Center of the Aichi Medical University School of Medicine in accordance with guidelines for animal experimentation.

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Key words: CTL, regulatory T cells, anti-CD25, immunotherapy

The Renca murine renal cell carcinoma line of spontaneous origin in a BALB/c mouse (8-10) was maintained in RPMI-1640 medium (Sigma Aldrich Japan, Tokyo, Japan), supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria) 50 units/ml penicillin and 50 μ/ml of streptomycin (Gibco BRL, Tokyo, Japan). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Antibodies. The anti-CD25 IL-2Rα mAb (PC61) (11), a rat IgG1 antibody, was provided by Dr K. Tsujimura (Aichi Cancer Center, Aichi, Japan). For *in vivo* administration, the anti-CD25 mAb was used after purification from ascites using a HiTrap Protein G HP column (GE Healthcare UK Ltd., UK). An FITC labeled anti-mouse CD4 rat mAb (isotype:rat IgG2b, clone GK1.5), an FITC labeled anti-mouse CD8α rat mAb (isotype:rat IgG2a, clone 53-6.7) and a phycoerythrin (PE)-labeled anti-mouse CD25 rat mAb (isotype:rat IgM, kappa; clone 7D4) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were also purchased.

Flow cytometric analysis of regulatory T cells. Mouse spleen cells were incubated for 20 min in the dark at 4˚C with FITC labeled anti-mouse CD4 and PE labeled anti-mouse CD25 antibodies and washed. They were then resuspended in FACS buffer (PBS, pH 7.2 containing 0.5% BSA and 2 mM EDTA) and immediately analyzed on a FACS Calibur (Becton-Dickinson Biosciences, San Jose, CA) using CellQuest software.

IFN-γ secretion assays. Measurement of IFN-γ^+ secretion by tumor-specific T lymphocytes was performed using a Mouse IFN-γ Secretion Assay-Detection Kit PE (Miltenyi Biotec GmbH). Mouse spleen cells were labeled for 15 min at 4˚C with an IFN-γ-specific high-affinity capture matrix, i.e., a bi-specific Ab-Ab conjugate directed against CD45 and IFN-γ. Afterwards, cells were transfered into 37˚C warmed medium containing tumor cells for 90 min to permit secretion of IFN-γ, washed and stained for 20 min at 4˚C with FITC labeled anti-CD8α and PE labeled anti-IFN-γ antibodies. After washing, cells were resuspended in FACS buffer and immediately analyzed on a FACSVantage™ SE (Becton-Dickinson Biosciences).

^{51}Cr release cytotoxicity assays. Cytotoxic activity of spleen cells from tumor-bearing mice and enriched CTLs were measured by ^{51}Cr release assay. CTLs were enriched by flow cytometric cell sorting after staining as described above. Target Renca cells were labeled with Na_2CrO_4 (CJS11, GE Healthcare UK Ltd.) and incubated with effector cells at 37˚C in a humidified atmosphere containing 5% CO_2. Supernatants were obtained after incubation and subjected to gamma-counting with a 1272 Clinigamma counter (LKB Wallac, Perkin-Elmer, Japan). Maximum and spontaneous release levels defined were as counts from samples incubated with 2% NP-40 (Nonident P-40) and medium alone, respectively. The means of triplicate samples were averaged and the percentage specific ^{51}Cr release was determined as follows: percentage of specific lysis = [(experimental ^{51}Cr release-spontaneous ^{51}Cr release)/(maximum ^{51}Cr release-spontaneous ^{51}Cr release)] x 100.

Generation of tumor-stimulated whole spleen cells and purification of CTLs for administration. Renca cells ($2x10^6$/mouse) were intradermally transplanted in the back of 4-week-old BALB/c mice. Two weeks later, spleen cells from tumor bearing mice and irradiated Renca cells were co-cultured to activate CTLs against tumor cells in RPMI-1640 medium supplemented with 10% FCS, 50 units/ml penicillin-streptomycin and $5x10^{-5}$ M 2-mercaptoethanol. After a 4-day incubation, CD8$^+$ cells were concentrated using a CD8α$^+$ T Cell Isolation Kit and a VarioMACS Separator (Miltenyi Biotec GmbH), and then separated CTLs using a Mouse IFN-γ Secretion Assay-Detection Kit PE and a cell-sorting system (BD FACSVantage SE, Becton-Dickinson) by the method described above. Following collection, the purity of the cells (>90%) was confirmed by FACS.

Adoptive immunotherapy. Renca cells (in 0.2 ml) were transplanted intradermally into the backs of BALB/c mice with a 27-guage needle. After 14 days, tumor-bearing mice were divided into 5 groups each containing 5 mice: group 1, no therapy as control; group 2, administration of whole spleen cells ($1x10^6$/mouse) stimulated with irradiated Renca cells for 4 days via the heart; group 3, administration of CTLs ($1x10^6$/mouse) via the heart; groups 4 and 5, intraperitoneally injection of anti-CD25 mAb (PC61, 250 μg/mouse). Group 5, this was followed by inoculation of CTLs via the heart 7 days thereafter. Anti-tumor activity was determined by measuring tumor size with Vernier calipers. Tumor volume was calculated by formula: tumor volume = 0.4 x length (mm) x [width (mm)]^2 (12).

Results

CD4$^+$CD25$^+$ cells (Treg) increase with tumor development. At first, we investigated the relation between numbers of T cells and tumor growth, and then the effects of *in vivo* administration of the anti-CD25 mAb on CD25$^+$ cells in the spleen. CD25$^+$ cells comprised ~10% CD4$^+$ cells and 2.9±0.2% total spleen lymphocytes in non-treated mice, as shown in Fig. 1. However, this population was increased to 3.7±0.5% on day 14 after transplantation of Renca cells. After a single administration of 0.25 mg of the anti-CD25 mAb to tumor-bearing mice, CD4$^+$CD25$^+$ cells reduced day by day, to reach 0.5% by day 8.

Maximum expansion of CD8$^+$ T cells reactive with Renca. Optimal culture conditions for obtaining maximum numbers of CTLs for adoptive transfer in immunotherapy were examined. It has been shown that CTLs can be expanded by stimulation using autologous tumor cells, therefore whole spleen cells from tumor-bearing mice were stimulated with irradiated Renca cells. On days 1-4 thereafter, the percentages of CD8$^+$/IFN-γ^+ cells in collected cultured lymphocytes were analyzed as CTLs by flow cytometry. The largest population (34%) of CD8$^+$/IFN-γ^+ cells was detected on day 4 after stimulation (Fig. 2). So that a 4-day stimulation was concluded as a suitable period for antigen stimulation to expand CTLs.

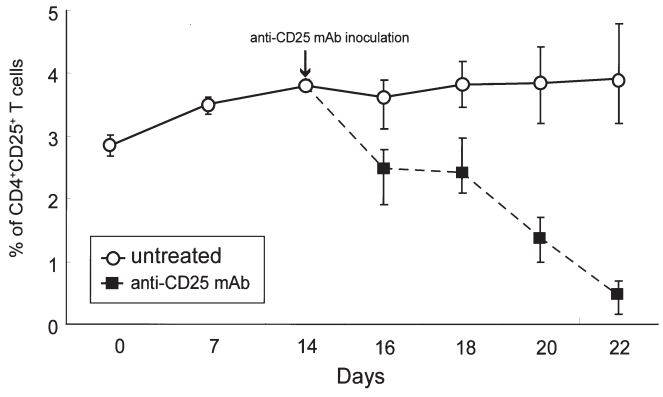

Figure 1. $CD4^+CD25^+$ cells (%) after transplantation of $2x10^6$ Renca cells and administration of 0.25 mg PC61 to tumor-bearing mice. Assays were performed by FACSCalibur as described in Materials and methods. On day 0, $CD4^+CD25^+$ cells accounted for an average of 2.95%, this rising to 3.82% (n=3) two weeks later. Eight days after a single administration of anti-CD25 mAb, $CD4^+CD25^+$ cells were depleted and the observed average was 0.47%. In contrast, $CD4^+CD25^+$ cells of untreated mice increased gradually to 3.91%.

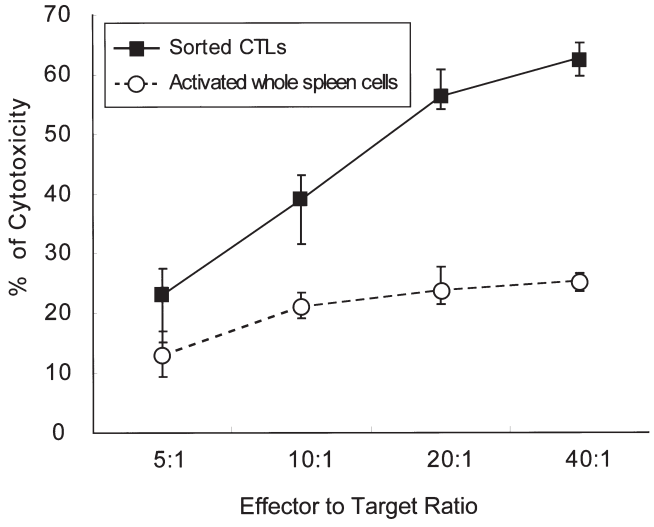

Figure 3. Cytotoxic activity of purified tumor-specific CTLs. The cytotoxic activity of purified CTLs and activated whole spleen cells were measured by ^{51}Cr release cytotoxicity assay as described in Materials and methods; $p<0.001$.

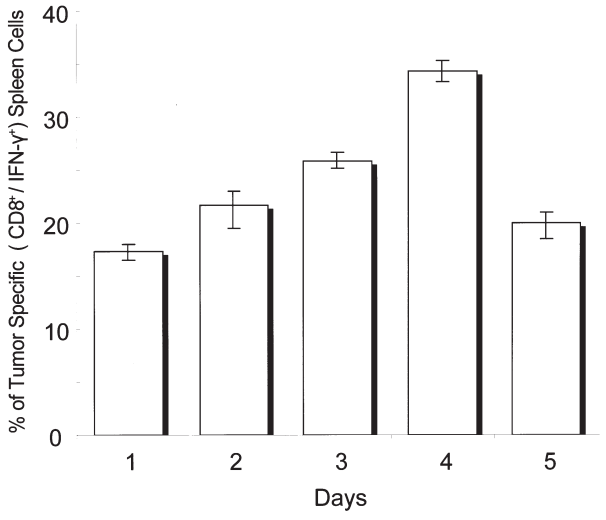

Figure 2. Tumor-specific ($CD8^+$ and IFN-γ-secreted) cells (%) 1, 2, 3, 4 and 5 days after stimulation with Renca cells. $CD8^+$ and IFN-γ-secreted cells were analyzed by FACS Calibur as described in Materials and methods. After a 4-day incubation, the largest percentage of tumor specific ($CD8^+$ and IFN-γ-secreted) cells was induced, at approximately 34.4%.

Cytotoxic activity of purified CTLs. To examine the cytotoxic activity of separated $CD8^+$/IFN-γ^+ cells as CTLs, we performed ^{51}Cr release cytotoxicity assays using stimulated whole spleen cells and separated CTLs as detailed in Materials and methods. Separated CTLs showed higher cytotoxic activity than whole spleen cells at all effector-to-target (E/T) ratios (Fig. 3). At an E/T ratio of 40:1, CTLs showed about 3-fold higher cytotoxicity.

Multimodal therapy of renal cancer xenograft tumors. Two weeks after transplantation of Renca cells, therapy was initiated using the anti-CD25 mAb, whole spleen cells stimulated with Renca cells for 4 days and CTLs separated from those spleen cells. At this time, the tumors were approximately 5.5-8.0 mm in diameter with a mean volume of tumor about 110 mm^3. The percentage of $CD8^+$/IFN-γ^+ cells (CTLs) in whole spleen cells before stimulation with Renca cells was about 0.7% (Fig. 4A). After stimulation for 4 days and separation for inoculation, purity cells was over 90% (Fig. 4B).

A dramatic survival benefit was seen with the anti-CD25 mAb and CTL combination. In the mice of this group, tumors had disappeared and the animals survived at 50 days after transplantation (Figs. 5 and 6). All non-treated mice died within 27 days of tumor transplantation, while, animals receiving whole spleen cells alone, CTLs alone and anti-CD25 mAb alone survived until days 27-30, 35-42 and 42-50, respectively (Fig. 7).

Discussion

To improve the efficacy of immunotherapy for cancer, many researchers have focused on development of antibodies against tumor cells and strategies for activation of lymphocytes with cytotoxic activity against tumor cells *in vitro* and *in vivo*. For humoral immunity, recombinant technology allows use of antibodies originating from mice for treatment of cancer after changing to chimeric and humanized antibodies and established drugs such as Rituxan for lymphomas and Herceptin for breast cancer (13).

In the cellular immunology area, two discoveries have generated particular interest. One is that many antigen-peptides are recognized by T cells as tumor antigens whose amino acid sequences can be determined (14,15). Clinical studies using synthesized peptides as cancer vaccines have already been reported and in several cases were confirmed (16,17). The other is that phenotypic characteristics ($CD4^+CD25^+$) of cells with immunological suppressive functions have been clarified, the cells being named regulatory T cells. These T cells prevent autoimmune diseases by controlling the activity of auto-reactive T cells (5). Several

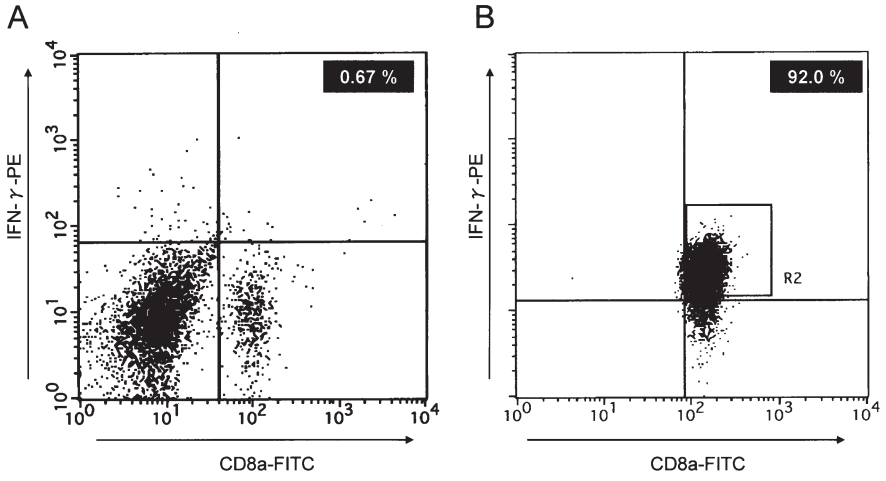

Figure 4. Detection and purification of *in vitro*-stimulated tumor-specific CTLs. Stimulation, purification and detection of IFN-γ-secreting CD8+ T cells were performed as described in Materials and methods. Two weeks after transplantation of Renca cells to BALB/c mice, IFN-γ-secreted CD8+ T cells accounted for approximately 0.6-0.8% of the total (A). After purification, it was over 90% (B).

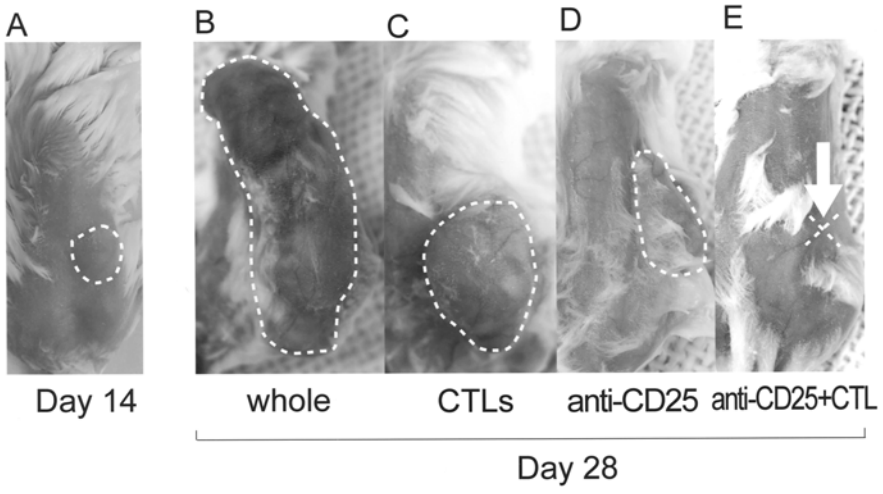

Figure 5. Transplanted tumors (dotted lines in each photograph) after administration of lymphocytes and the anti-CD25 mAb. At transplantation of Renca ($2x10^6$ cells) at day 14 (A). (B-D) show tumors at day 28. In group 2, $1x10^6$ of whole spleen cells were inoculated via the heart (B). In group 3, $1x10^6$ CTLs were inoculated via the heart (C). In group 4, mice were inoculated with 250 μg of anti-CD25 mAb only (D). In group 5, mice were treated with 250 μg of anti-CD25 mAb and $1x10^6$ CTLs by heart puncture.

reports have suggested that T cells are closely related with immune tolerance of cancer (18,19). Onizuka *et al* reported that $CD4^+CD25^+$ cells reduced maximally on days 3-4 and fully recovered by day 9 after a single *in vivo* administration of anti-CD25 mAb and a single *in vivo* administration of anti-CD25 mAb caused the regression of tumors that grew progressively in syngeneic mice. Furthermore, their kinetic analysis showed that the administration of anti-CD25 mAb later than day 2 after tumor inoculation caused no tumor regression (20). Recently, increase of T cells was found to be associated with high mortality in ovarian carcinoma patients, suggesting a generalized role in disease progression (19). Furthermore, circulating $CD4^+CD25^+$ Treg cell numbers are reported to correlate with tumor stage in patients with gastric or esophageal cancers and hepatocellular carcinomas (21,22).

In this study, we therefore attempted to overcome immunosuppressive conditions in tumor-bearing hosts and increase anti-tumor activity by adoptive transfer of tumor-reacted $CD8^+$ CTLs.

First, we confirmed that tumor-transplanted mice are indeed immunosuppressed. In tumor bearing mice, it was observed that Tregs showed a tendency to increase day by day (Fig. 1). Since similar observation have been made for human cancer patients (22), our system using syngenic tumor-transplanted mice was validated as a useful model for analysis of immunological treatment for cancer.

Next, we injected the anti-CD25 mAb into tumor-bearing mice and confirmed decrease in numbers of Treg, but in contrast to the study of Onizuka *et al*, in our case maximal decrease was observed at days 7-8 after the single injection of anti-CD25 mAb. In both experiments, a strain of mice and monoclonal antibody used was the same. However, Onizuka

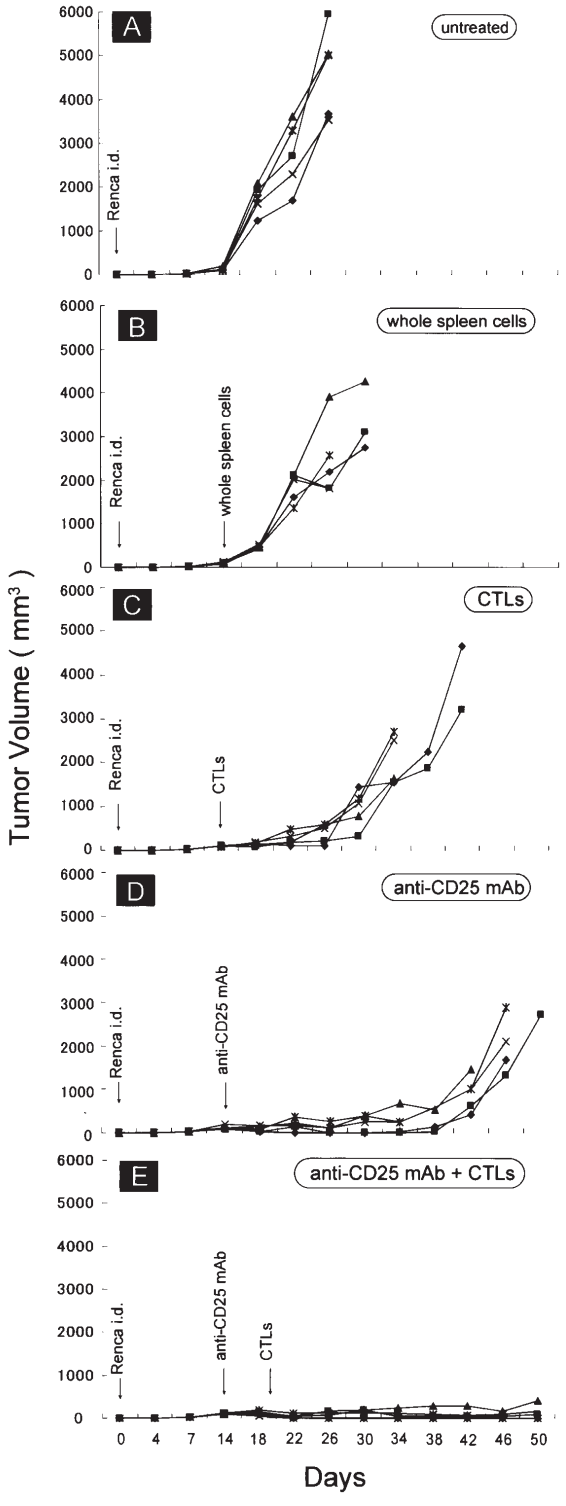

Figure 6. Effects of *in vivo* administration of lymphocytes and anti-CD25 mAb on tumor growth. Renca cells ($2x10^6$) were transplanted into the backs of the mice. Treatments were performed as described in Materials and methods using 5 mice in each groups. (A), untreated mice; (B), mice inoculated with $1x10^6$ whole spleen cells on day 14; (C), mice inoculated with $1x10^6$ purified CTLs on day 14; (D), mice inoculated with 250 μg of anti-CD25 mAb on day 14; (E), mice inoculated with anti-CD25 mAb followed by administration of purified CTLs.

et al used standard mice and we employed tumor-bearing animals and this could conceivably have influenced the

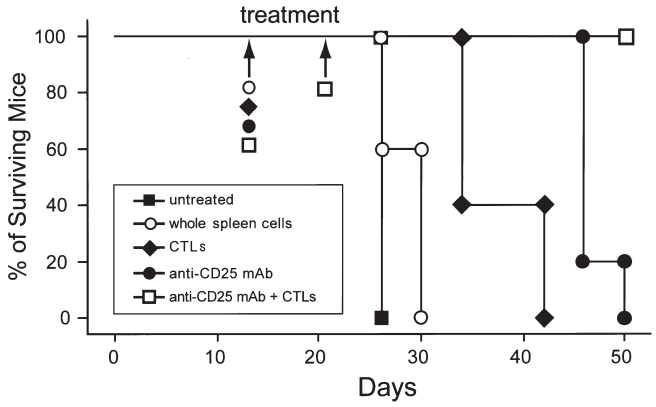

Figure 7. Combination immunotherapy using the anti-CD25 antibody and purified tumor-specific CTLs completely suppressed growth of transplanted tumors.

efficacy. Clearly this is an important consideration for future clinical studies.

Finally, we compared treatment efficacy of inoculation of CTLs with or without Treg depletion. Usually, when we need to know the numbers of cytotoxic T cells against particular antigens, we use ELISPOT assays which can also detect secreted IFN-γ. In analysis of cytotoxic activities of the cells, IFN-γ secreted $CD8^+$ cells showed 3-fold the cytotoxic activity of whole spleen cells stimulated with tumor cells (Fig. 3), pointing to real tumor specificity. On administration alone, however, CTLs showed lower anti-tumor activity than the single inoculation of anti-CD25 mAb. Thus, in tumor-bearing mice with T cells, the cytotoxic activity of CTLs is completely suppressed.

In conclusion, our results provided evidence that depletion of T cells and inoculation of CTLs are both necessary for treatment of cancers, this combination therapy offering a promising strategy for immunotherapy.

Acknowledgements

This study was supported by Aichi Cancer Research Foundation and Grant-in-Aid for General Scientific Research, a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Science, Sports and Technology and Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare, Japan. We thank Dr Kunio Tsujimura for kindly providing the anti-CD25 mAb and for helpful advice. We thank Dr Nobuyoshi Ohta of Aichi International Hospital, Aichi. We thank Ms. Norica Yamada, Ms. Sino Kojima in Department of Pathology, and Mr. Makoto Naruse in Central Research Laboratory, Aichi Medical University, for technical assistance.

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