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Original Article

A tritherapy combination of inactivated allogeneic leukocytes infusion and cell vaccine with cyclophosphamide in a sequential regimen enhances antitumor immunity

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Abstract

Background: Tumor-induced immunosuppression can impede tumor-specific immune responses and limit the effects of cancer immunotherapy. The aim of this study was to investigate the possible effects of sequential chemoimmunotherapeutic strategies to enhance antitumor immune responses.

Methods: Using the E7-expressing tumor TC-1 as the tumor model, the treatment groups were divided into the following groups: (1) inactivated allogeneic leukocyte infusion (ALI), (2) ALI + MMC-inactivated TC-1 cell vaccine, and (3) ALI + MMC-inactivated TC-1 cell vaccine + cyclophosphamide (CTX).

Results: In our study, we demonstrated that treatment with immune-modulating doses of CTX results in a beneficial tumor microenvironment with the suppression of Tregs. ALI has a limited therapeutic effect, as does the MMC-inactivated TC-1 cell vaccine. Our results showed that CTX preconditioning and persistent ALI treatment along with the MMC-inactivated TC-1 cell vaccine resulted in significant inhibition of tumor growth and extended survival.

Conclusion: Our study illustrated the effects of immune-modulating doses of a sequential chemoimmunotherapeutic strategy targeting the tumor and its microenvironment. The results suggest potential clinical effects for the immunotherapy of HPV-associated malignancies.

Keywords: Alloantigen; Cyclophosphamide; Immunotherapy; Regulatory T cells; Tumor microenvironment

1. Introduction

Human papillomavirus (HPV) accounts for approximately 5.3% of cancers throughout the world and consists of cervical cancer and subsets of genital and head and neck cancer.[1,2] It is estimated that 50 million women carry HPV, and approximately 500,000 women develop cancer yearly,[3] posing a threat to human health worldwide. Thus, there is a significant need to develop better strategies to treat HPV-induced lesions.

Tumor-induced immunosuppression plays a critical role in preventing tumor-specific immune responses and decreasing the effect of cancer immunotherapy.[4,5] There is growing acknowledgment that there may be significant advantages to eliciting immune responses against a broad spectrum of antigens expressed by cancer cells rather than targeting a single antigen.[6,7]

Allogeneic transplantation (including hematopoietic stem cell transplantation) has been used as a successful therapeutic method for tumor treatment.[8] It is well recognized that

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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alloantigens can induce powerful cellular and humoral immune responses and play an important role in graft vs. host (GVH) or host vs. graft (HVG) response. However, both GVH and HVG biology have many antitumor mechanisms in common, such as antigen presenting cell (APC) activation,[9] fas- and perforin-based cytolysis[10] and IFN-γ-secreting type 1 T cells.[11] Therefore, it is possible to use these characteristics as therapeutic methods.

Over the past 20 years, the efforts to create antitumor immune responses have been based on the sophisticated mechanisms of T cell activation and antigen presentation. However, in an allogeneic transplantation model, the phenomenon in which the powerful host T cell immune reaction to allogeneic major histocompatibility complex (MHC) molecules has drawn little attention. A recent study illustrated the application potential for the allogeneic response to induce weak self-restricted T cell responses to tumor Ags.[12] This study raised the prospect of a novel allogeneic immunotherapy for patients with malignant tumors.

The tumor microenvironment plays an important role in impeding the clinical responses.[13] Novel strategies should be based on targeting the tumor and its microenvironment. It is widely believed that immune-modulating doses of chemotherapeutic drugs induce a powerful response of the immune cells in the tumor microenvironment and exert an antitumor reaction.[14–16] Therefore, the combination of effective chemotherapeutic agents with immunotherapy may elicit the optimal clinical response in cancer patients.

In the present study, to explore the antitumor effect, we combined cyclophosphamide (CTX), MHC-unmatched allogeneic leukocytes and inactivated TC-1 tumor cells in a mouse subcutaneous TC-1 tumor model. The purpose was to establish the theoretical and experimental basis for a novel sequential biological treatment for HPV-induced lesions. We hope that this will be a promising strategy for clinical study as a cure of HPV-related cancer in the near future.

2. Methods

2.1. Mice and cell lines

Inbred female C57BL/6 (B6, H-2) mice (8–10 weeks) were purchased from the Experimental Animal Institute of Peking Union Medical College. All animals were maintained under specific pathogen-free conditions, and all procedures were performed according to approved protocols and in accordance with recommendations for the proper care of laboratory animals.

TC-1 tumor cells derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV-16 E6, E7 and c-Haras oncogenes were provided by Dr. T.C. Wu from Johns Hopkins University. The following standard experimental mouse tumor cell lines were used in vitro and in vivo: B16-F10 (H-2b) melanoma and YAC-1 lymphoma.

TC-1, B16-F10 and YAC-1 cells were cultured in RPMI 1640 (Gibco-BRL, Carlsbad, CA) supplemented with 10% fetal calf serum containing 10% fetal bovine serum (HyClone, Logan, UT) in the presence of 200 μg/mL of Geneticin (G418) at 37 °C with 5% CO_2.

2.2. Cell vaccine preparation

Murine splenocytes were separated from freshly spleens of BALB/c mice. For inactivating splenocytes, 25 μg/mL mitomycin C (MMC) were taken into the cell suspension in a concentration of 1×10^7 cells/mL. Meanwhile, 100 μg/mL mitomycin C (MMC) were taken into the TC-1 cell suspension in a concentration of 1×10^6 cells/mL. And the cells were incubated for 60 min. At last the cell were washed with phosphate-buffered saline (PBS) and resuspended.

2.3. Reagents

CTX (Cat. C0768; Sigma–Aldrich, Milwaukee, WI) was dissolved at 10 mg/mL in deionized water.

2.4. Therapeutic tumor experiment protocol

On Day 0, mice were injected subcutaneously (s.c.) on the right flank with 1×10^5 TC-1 tumor cells. On Day 10, mice bearing tumors approximately 60 mm^3 were arbitrarily assigned to six groups as follows: the PBS control group; TC-1 cell vaccine group; ALI groups (4×10^7, 2×10^7, 1×10^7); CTX group; ALI and TC-1 cell vaccine group; and the CTX, ALI and TC-1 cell vaccine group. CTX was given intraperitoneally (i.p.) at a dose of 50 mg/kg on Day 10 after tumor challenge. On Day 11, the MMC-inactivated allogeneic leukocytes were administered intratumorally (i.t.) and repeated twice every 3 days. On Day 12, the 2×10^6 MMC-inactivated cancer cells were injected subcutaneously (s.c.) and then boosted twice every 3 days. Tumors were monitored every 3 days, and the survival of mice was recorded. Tumor dimensions were measured with calipers, and the values were inserted into the following formula: tumor volume (mm^3) = 0.52 × (length × $width^2$). The number of deaths was assessed at each interval. All measurements were obtained in a strictly blinded fashion.

2.5. Flow cytometry

TC-1 cancer cells were incubated for 30 min with an optimal concentration of FITC-conjugated anti-mouse MHC Class I (Cat. 11-5999; eBioscience, San Diego, CA) on ice and then washed twice with cold phosphate buffered saline (PBS).

For the samples obtained from fresh TC-1 carcinoma masses, separation of the tumor-infiltrating mononuclear cells was carried out by differential gradient centrifugation, and the tumor-infiltrating mononuclear cells were found at the interface of 75% and 100% Ficoll-Hypaque. For samples obtained from spleens, erythrocytes were removed by suspending the cells in lysis buffer and then rinsing the cells twice with RPMI 1640. Cells were incubated for 30 min with an optimal concentration of antibodies on ice and then washed twice with cold phosphate buffered saline (PBS). The following

antibodies were used in the experiments: Alexa FluorVR 488 anti -mouse/rat/human FOXP3 antibody (Cat. 320011; Biolegend, San Diego, CA), PE/Cy5 anti-mouse CD4 antibody (Cat. 100409; Biolegend), PE-conjugated anti-mouse CD25 (Cat. 102007; Biolegend). Analysis was performed on a FACScan (EPICS ELITE ESP model; Beckman Coulter, Fullerton, CA), and data were analyzed with the Expo32 software (Beckman Coulter).

2.6. Cytotoxicity assays

TC-1-bearing mice were treated according to the treatment protocol described earlier. Three days after the third administration of the combined cell vaccine, splenocytes from treated mice were prepared as effector cells. Cytotoxicity assays were performed using a CytoTox 96® Nonradioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. B16-F10, TC-1 and YAC-1 cells were used as target cells. Effector cells were added to target cells at a ratio of 60:1, 30:1 and 15:1 (tested in triplicate).

2.7. Statistical analysis

For comparison, ANOVA was used for the comparisons among 3 or more groups of individual time points. Student's t-test was used to compare means between the 2 groups. Log-rank test were analyzed in the survival data. Differences were considered to be significant when $p < 0.05$. Statistical analysis was performed using commercially available software (SPSS 11.0).

3. Results

3.1. Subcutaneous injection of the MMC-inactivated TC-1 cell vaccine has a limited therapeutic effect

To investigate the antitumor immunity, we challenged mice with 1×10^5 TC-1 tumor cells/mouse, then immunized mice with MMC-inactivated TC-1 tumor cells. As shown in Fig. 1, compared with the PBS control group, the therapy group showed no significant difference ($p > 0.05$). This result demonstrated that subcutaneous injection of MMC-inactivated TC-1 tumor cells as a vaccine has limited effects.

3.2. Allogeneic leukocyte infusion (ALI) has a limited therapeutic effect

We used the TC-1 tumor model to examine the antitumor effects of allogeneic leukocyte infusion (ALI). As shown in Fig. 2A, TC-1 tumors from mice were treated with 1×10^7, 2×10^7, 4×10^7 MMC-inactivated BALB/c leukocytes/mouse. The average sizes of tumors on Day 14 were 906 ± 377.47 mm^3 in the 1×10^7 group, 747.91 ± 370.39 mm^3 in the 1×10^7 group, 507.39 ± 208.56 mm^3 in the 4×10^7 group (Day 14), whereas those given PBS injections grew to an average size of 708.04 ± 397.83 mm^3. The 4×10^7 group appeared to have some effect, but its effect was not significantly different from that of the PBS group ($p > 0.05$). Fig. 2B shows that compared with PBS, ALI was unable to increase the lifespan of tumor-bearing mice ($p > 0.05$).

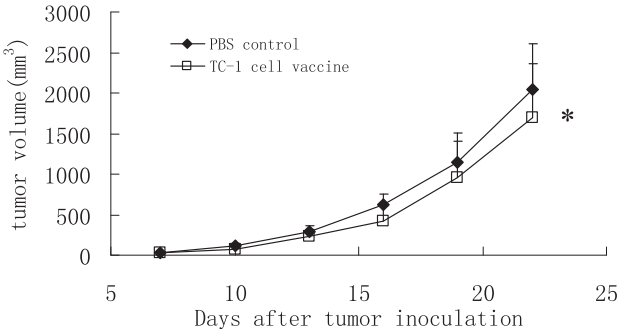

Fig. 1. Subcutaneous injection of MMC inactivated TC-1 cell vaccine has limited therapy effect. C57BL/6 mice inoculated s.c. with 1×10^5 TC-1 cells. When the tumors reached an average diameter of approximately 5 mm on Day 10, the mice were randomized and treated with 2×10^6 MMC inactivated TC-1 cell vaccine 0.1 mL PBS or PBS alone. The same treatments were repeated on Day 13 and Day 16. Tumor volumes were monitored for 25 days until the control mice began to die. Data are presented as mean tumor volumes ± SD of five mice per group in a representative experiment. * indicates $p > 0.05$, compared to PBS. These data are from two experiments with similar results. $n = 8$ mice/group.

3.3. Sequential immune-modulating doses of CTX and combined vaccine treatment dramatically improved the antitumor effect

We then hypothesized that the sequential strategy of CTX, ALI and TC-1 cell vaccine would overcome immune evasion and induce a powerful antitumor immune response. The tri-therapy has a greater effect on inhibiting tumor growth than the single treatments or bitherapy strategy. On Day 24, tumor growth was inhibited 56% in the CTX + ALI + TC-1 cell vaccine group (804.36 ± 229.24 mm^3) compared with that in the control group (1835.01 ± 533.96 mm^3, $p < 0.05$; Fig. 3B). No significant inhibition was observed in the ALI + TC-1 cell vaccine group or the CTX-treated group. Compared with the administration of PBS, the administration of CTX, ALI and TC-1 cell vaccine also increased the lifespan of tumor-bearing mice ($p < 0.05$). The mean survival time (MST) of the PBS group was 45.6 days, whereas the CTX + ALI + TC-1 cell vaccine group was 54.4 days (Table 1). Compared with the PBS control group, the ALI and TC-1 cell vaccine group could also increase the MST by 5.2 days (50.8 vs. 45.6, Table 1), which demonstrated that the administration of the ALI and TC-1 cell vaccine may have some therapeutic effect.

3.4. TC-1 cancer cells express normal levels of major histocompatibility complex (MHC) class I molecule

One example of tumor evasion mechanisms is the loss of MHC-I expression. We determined the level of MHC-I expression using FACS. Fig. 4 illustrates that TC-1 cancer cells express normal levels of major histocompatibility

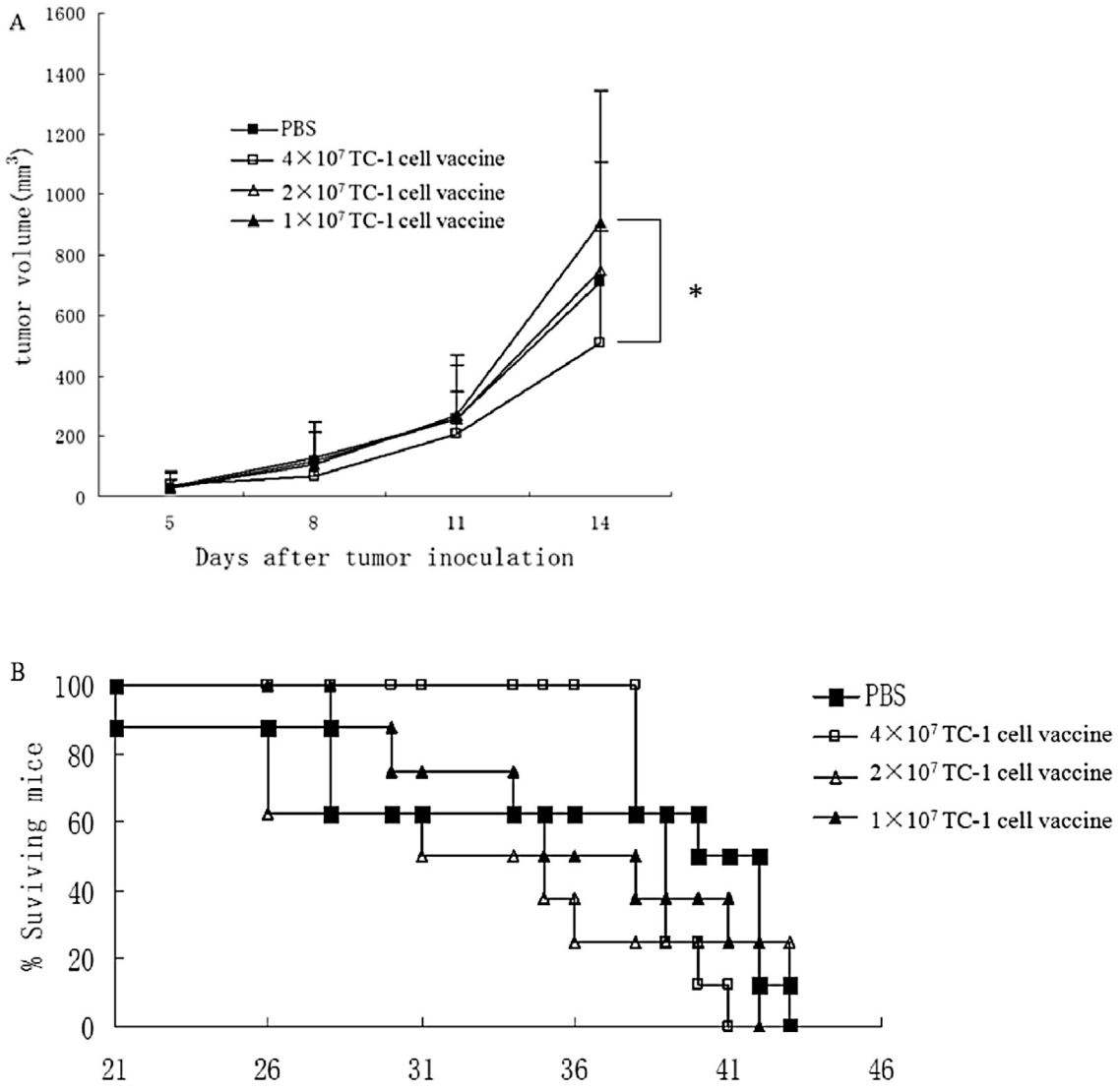

Fig. 2. The antitumor effects of ALI C57BL/6 mice were inoculated s.c. with 1×10^5 TC-1 cells. When the tumors reached an average diameter of about 5 mm mice were randomized and treated with ALI or PBS on Day 10. The same treatments were repeated on Day 13 and Day 16. (A) Tumor sizes of B6 mice treated with 1, 2 or 4×10^7 inactivated BALB/c leukocytes infusion. The results are demonstrated as mean ± SD. *$p > 0.05$ compared with PBS group on Day 14. (B) Survival of mice per treatment group. $n = 8$ mice/group.

complex (MHC) class I molecule, which demonstrated that TC-1 cancer cells had another way to escape the host immune response. This result also showed that the TC-1 cancer antigen could be cross-presented by the MHC-I pathway to elicit an antitumor effect.

3.5. Tregs were suppressed by immune-modulating doses of CTX and combined vaccine treatment in C57BL/6 mice bearing TC-1 tumors

To determine the effect of CTX and combined vaccine treatment on Tregs, we analyzed the proportion of CD4 + CD25 + FoxP3+ Tregs when administering immune-modulating doses of CTX and combined vaccine treatment as described in the Methods. Splenocytes and tumor-infiltrating mononuclear cells were assessed for the proportion of Tregs on Day 19. The combination of CTX and TC-1 cell vaccine suppressed Treg populations. The proportion of CD4 + CD25 + FoxP3 + /CD4+ lymphocytes in the combined treatment were 7.7 ± 3.1% in spleens and 11.9 ± 1.5% in tumors compared with 17.1 ± 1.8% and 33.2 ± 5.7% in the PBS group ($p < 0.05$), respectively. Compared with PBS, CTX primarily decreased Tregs in spleens ($p < 0.05$) and tumors ($p < 0.05$).

3.6. Tritherapy in established tumors induces innate and adaptive antitumor response

In the current study, we asked whether antitumor cytotoxicity was detectable after tritherapy in established tumors. Splenocytes from animals in which TC-1 tumors were inhibited by tritherapy demonstrated cytolysis activity toward TC-1 tumor cells in vitro rather than B16-F10 (Fig. 5), whereas splenocytes from PBS treated mice failed to

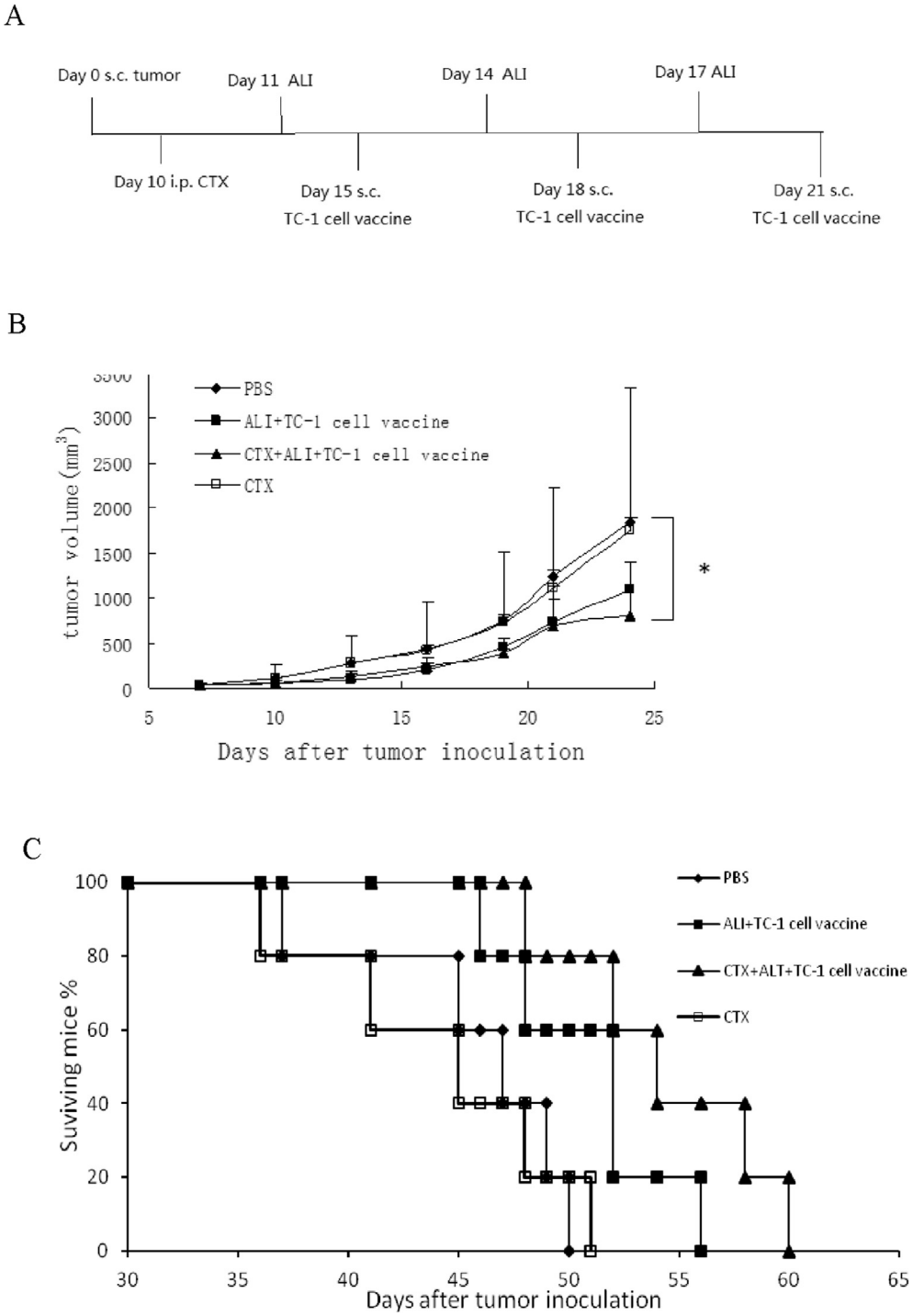

Fig. 3. Anti-tumor responses were enhanced by tritherapy in established tumors. C57BL/6 mice were inoculated s.c. with 1×10^5 TC-1 cells. The tumors were allowed to reach an average diameter of about 5 mm before the mice received our vaccination protocol as described in Methods. (A) Tumor sizes (mm^3). (B) Survival curve of mice. (C) Diagram depicting the tritherapy strategy. Each group included 7–10 mice. Tumor incidence was monitored after tumor inoculation. *$p < 0.05$ compared with PBS group.

demonstrate lysis toward either TC-1 or B16-F10. Thus, it appears that a measurable CTL response was activated in a tumor-bearing mouse only when the tumor was inhibited by tritherapy treatment. Further, tritherapy treatment also resulted in significant cytotoxicity against NK-sensitive YAC-1 cells (see Fig. 6).

4. Discussion

Tumor microenvironments consist of proliferating cancer cells, infiltrating inflammatory cells, tissue cells, and blood vessels. The tumor modifies the function of infiltrating cells to create a microenvironment favorable for tumor progression

Table 1
Median survival time of mice.

Group	Median survival time (days)
PBS	45.6
ALI + TC-1 cell vaccine	50.8
CTX + ALI + TC-1 cell vaccine	54.4
CTX	44.2

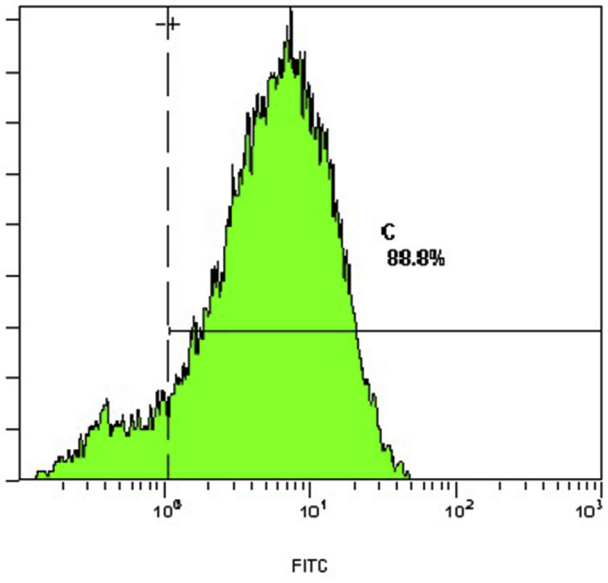

Fig. 4. The level of MHC-I expression on TC-1 cancer cell. Flow cytometric analysis of MHC-I expression on TC-1 cancer cell.

and immune system evasion. One of the successful ways of tumor-induced immune evasion is the activation of CD4 + CD25 + FoxP3+ regulatory T cells (Tregs). There are naturally low numbers of Tregs. However, this study demonstrated that Tregs have been induced in cancer patients and suppress immune responses in the microenvironment through IL-10 and TGF-β secretion.[17] Recent research suggests that oncologic therapies, such as chemotherapy, surgery, and radiation, can induce Tregs and strengthen their suppressing functions.[18–20] Tregs in the host immune system produce an imbalance that could not be corrected by immunotherapies targeting only activation of antitumor responses.[21] Another study illustrated a large number of tumor-infiltrating and splenic Tregs activated during the growth of TC-1 tumors.[22,23] Cyclophosphamide (CTX) is a non-specific cytotoxic drug, which exerts its biological effects by interfering with cellular DNA synthesis. CTX could decrease the number of Tregs and suppress the remaining Tregs.[24] The studies reported that CTX enhanced the effect of tumor vaccines and adoptive transfer of antigen-specific lymphocytes. CTX is known as an immunosuppressive agent and studies have shown that patients who were given CTX had depleted Treg cells, which were involved in cancer-induced immune tolerance.[25] Moreover, CTX administration downregulated the expression of glucocorticoid-induced TNF receptor (GITR) and Foxp3.[24–27] Another study illustrated that the timing between CTX administration and vaccine injection is essential to induce the antitumor response.[28] CTX administration regulated the immune system in the priming vaccination phase. Immune-modulating doses of CTX could elicit the selective effect against Tregs, and we did not find the autoimmune signs such as decrease in weight, lymphopenia and other toxicities in each group (data not shown).

In the present study, we reported the antitumor potential of MHC-unmatched allogeneic leukocytes which had been mitotically inactivated to prevent GVHD. This situation mimics one-way allogeneic mixed leukocyte reaction (MLR) in vivo. Other researchers have illustrated that both one-way and two-way MLR elicit an intense release of cytokines,

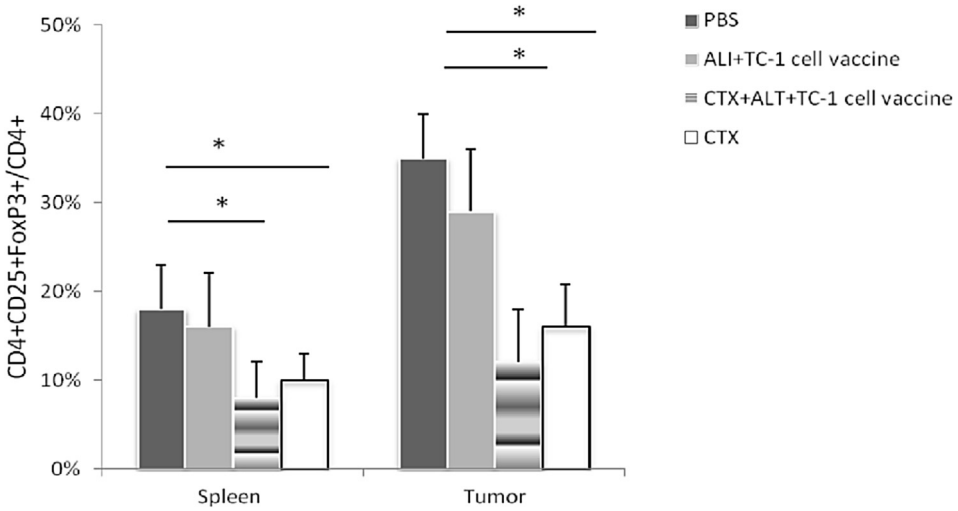

Fig. 5. The effect of immune-modulating doses of CTX and combined vaccine treatment on the proportion of Tregs. C57BL/6 mice were inoculated s.c. with 1×10^5 TC-1 cells. The tumors were allowed to reach an average diameter of about 5 mm before the mice received our vaccination protocol as described in Methods. Splenocytes and tumor-infiltrating mononuclear cells were assessed for the proportion of Tregs on Day 19. The proportions of CD4 + CD25 + FoxP3 + / CD4+ cells in spleen lymphocytes and tumor-infiltrating mononuclear cells in the indicated groups were shown. $n = 5$ mice/group.

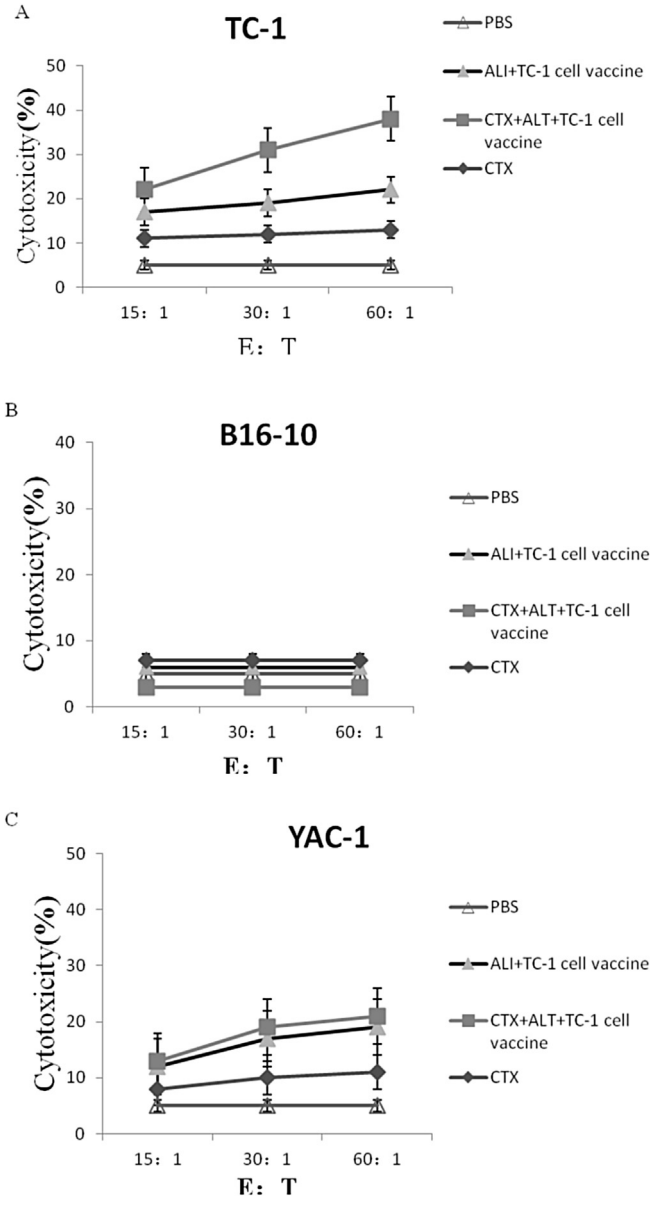

Fig. 6. Tritherapy in established tumors induces innate and adaptive antitumor response. TC-1 tumor model and vaccination protocol as described in Methods. On Day 24, 3 days after the third combined cell vaccine, splenocytes were collected and used as effector cells against TC-1 (A), B16-F10 (B), and YAC-1 (C) tumor cells in cytotoxic assay at the indicated E:T ratio. $n = 5$ mice/group.

especially type 1 cytokines (e.g. TNF-α and IFN-γ).[29,30] Therefore, we assumed that an in vivo MLR may lead to a proinflammatory, adjuvant effect of the host immune cells and break the autologous immune tolerance of tumor cells. The ability of graft rejection to elicit antitumor reaction was illustrated in a clinical test of skin transplantation from HLA-unmatched donors.[31] Measurable disease responses were reported in patients with hormone-refractory prostate cancer. This study illustrated antitumor effects induced by HLA-unmatched donor skin transplantation.

In the tritherapy strategy, we first used CTX to decrease the Tregs in the tumor microenvironment, followed by ALI to break the immune tolerance, and finally, we injected inactivated TC-1 tumor cells subcutaneously to provide the tumor-specific antigen. The efficient cross-presentation of tumor-specific antigen (TSA) is pivotal for the effects of cancer immunotherapy. Cross-presentation comprises antigen internalization, processing, and the presentation of peptides on MHC-I molecules by DCs.[32] As the TC-1 tumor can express high levels of MHC-I molecules (Fig. 4), the cross-presentation would be effective.

Combining chemotherapy to immunotherapy has been demonstrated effective in some preclinical trials and in a clinical model. In this study, we demonstrated a novel potent tritherapy of CTX, ALI and a cancer cell vaccine and showed that it was effective for the treatment of TC-1 tumors when administered in a sequential regimen. The addition of CTX is feasible and well tolerated, and it is familiar to clinicians.[33]

In conclusion, the sequential combined chemotherapy to immunotherapy scheme induces a beneficial host microenvironment. It will be essential to test this combination scheme on tumors that express associated tumor antigens. Reversal of tumor-mediated immunosuppression will be necessary for the success of immunotherapies. These findings are very encouraging for subsequent clinical development in the field of HPV therapeutic vaccines.

Acknowledgments

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