International Journal of Immunopathology & Pharmacology

Letter to the Editor

Cyclophosphamide eradicates murine immunogenic tumor coding for a non-self-antigen and induces antitumor immunity

International Journal of Immunopathology and Pharmacology
Volume 32: 1–5

DOI: 10.1177/2058738418796591
journals.sagepub.com/home/iji
SAGE

Mohamed L Salem,[1,2] Sabry A El-Naggar,[1,2] Heba A Mahmoud,[3] Rehab M Elgharabawy[4] and Abeer M Bader[5]

Abstract

Although the majority of cancers respond to chemotherapy, most cancer types relapse, at least in part, due to the poor immunogenicity of most tumor. We have reported before that treatment of tumor bearing mice with a combination of the anti-cancer chemotherapy cyclophosphamide (CTX) and immunotherapy can result in complete tumor regression using T-cell receptor (TCR) transgenic $CD8^+$ T cells specific to antigens. This study aimed to determine whether chemotherapy can cure immunogenic tumor which expresses non-self-tumor antigen and result in antitumor immunity. Either EL4 cell line, a poorly immunogenic thymoma, or EG7, a clone of EL4 cells transfected with ovalbumin (OVA), as a non-self-antigen were inoculated subcutaneously into wild type or splenectomized C57BL/6 mice and then treated once with intraperitoneal (i.p.) injection of 4 mg CTX/mouse. In certain experiments, the mice were rechallenged with the same tumor type 1–2 months after the primary challenge. Treatment of EL4 bearing mice with CTX induced transient antitumor effect followed by tumor progression. Interestingly, however, treatment of EG7-bearing mice with CTX resulted in regression of early and advanced tumors. EG7 tumor-free mice rejected the second and the third challenges with EG7 cells, but not with challenge EL4 cells. These antitumor effects did not require spleen, since splenectomized mice showed similar antitumor effects of CTX on EG7 cells. Taken together, these data indicate that expression of non-self-antigen by poorly immunogenic tumor might be a reliable means to increase its immunogenicity and its response to chemotherapy.

Keywords

cancer, chemotherapy, cyclophosphamide, EL4, EG7, immunotherapy, lymphoma

Date received: 25 December 2017; accepted: 23 July 2018

Introduction

Several approaches have been applied to overcome the resistance to harness antitumor immunity toward self-tumor antigens with main goal to develop memory response.[1] For instance, adoptive transfer of in vitro activated T-cell receptor (TCR) transgenic $CD8^+$ T cells after chemotherapy can mount robust antitumor responses.[2] Furthermore, certain immunotherapeutic strategies in combination with chemotherapy could successfully also induce antitumor immunity in non-TCR transgenic animal models.

[1]Immunology and Biotechnology Unit, Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt
[2]Center of Excellence in Cancer Research, New Teaching Hospital, Tanta University, Tanta, Egypt
[3]Department of Pharmacology, College of Medicine, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia
[4]Pharmacology and Toxicology Department, Faculty of Pharmacy, Tanta University, Tanta, Egypt
[5]Zoology Department, Faculty of Science, Cairo University, Giza, Egypt

Corresponding author:
Mohamed L Salem, Immunology and Biotechnology Unit, Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt.
Email: Cecr@unv.tanta.edu.eg; mohamed.labib@science.tanta.edu.eg

These effects were suggested to be due, in part, to the immunomodulatory effects of chemotherapy, indicating that chemotherapy itself possesses paramount immunomodulatory effects when the tumor microenvironment is immunogenic.[3]

Cyclophosphamide (CTX) is used alone or in combination with other chemotherapeutic drugs for the treatment of several human malignancies.[4] It has shown that preconditioning of a recipient tumor-bearing host with CTX-induced lymphopenia significantly improves the activation, proliferation, and functions of adoptively transferred $CD8^+$ T cells.[5] Even in the absence of adoptive T-cell transfer, CTX preconditioning regimen was also found to enhance T-cell responses to antitumor vaccination, including dendritic cell–based vaccination.[5] We have also reported that preconditioning with CTX enhances the antitumor effects of adoptive T-cell therapy in a non-transgenic tumor mouse models.[2] Taken together, CTX is capable of expressing beneficial immunomodulatory effects besides its direct antitumor effects. Whether the antitumor effects of CTX can generate antitumor memory immune responses toward poorly immunogenic and chemoresistant tumor is of a great interest. To address this questions, we compared the antitumor effect of CTX on EL4, a murine thymoma cell line, which is poorly immunogenic and resistant to CTX,[6] and EG7, which is an EL4 cell line that is genetically engineered to express the non-self-antigen ovalbumin (OVA) to make them more immunogenic.[7] The obtained results concluded that CTX acts on EG7 cells, but not on EL4 cells, and induced regression associated with the development of memory response.

Materials and methods

Animals, reagents, and antibodies

Female adult C57BL/6 mice (6–8 weeks) were used in all experiments. All animals were housed in accordance with the institutional and federal guidelines. Ovalbumin (OVA) was purchased from Sigma (St Louis, MO, USA), dissolved in 10% dimethyl sulfoxide (Sigma), and diluted in phosphate-buffered saline (PBS). CTX (Sigma) was stored at −70°C and reconstituted in PBS before use.

Cell lines

EL4 is a cell line (T-cell thymoma) and EG7 is a clone of EL4 cells that is transfected with cDNA gene coding for chicken egg ovalbumin (OVA), and upon transfection, EG7 cells express ova in a membranous form.[8] Both EL4 and EG7 cells express only class-I molecules but not class-II molecules. All cell lines were purchased originally from the American Type Tissue Collection (ATCC, Rockville, MD, USA).

Tumor challenge and CTX treatments

The cell lines were inoculated (2.5×10^5 per mouse) via subcutaneous (s.c.) injection into the left flank of wild type or splenectomized B6 mice. The tumor growth was monitored by measuring the length and width of the tumor twice a week using digital caliper. The tumor surface area was calculated by multiplying the two diameters and expressed in millimeter square. In certain experiment, the mice were rechallenged with the tumor 1–2 months after the primary tumor inoculation. After 10 days of tumor inoculation, the mice were treated with intraperitoneal (i.p.) injection of 4 mg/mouse CTX in 0.1 mL PBS according to our previous studies[2,5] while the control groups received 0.1 mL PBS.

Statistical analysis. Numerical data obtained from each experiment were expressed as mean ± SD. Statistical differences between the experimental groups were assessed using Student's t-test. *P* values less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed using SPSS statistical version 16 software package (SPSS® Inc., USA).

Results

Transient antitumor effects of CTX on EL4 cells

First, we tested the effect of CTX treatment on EL4 cells, which are known to poorly respond to treatment with CTX. As shown in Figure 1(a), EL4 cells showed progressive growth from day 10 to day 20. CTX treatment of EL4 bearing mice on day 10 induced a transient regression of EL4 cells between days 12 and 14 and then followed by progressive tumor growth.

Therapeutic antitumor effects of CTX against early and advanced EG7 tumor

To test whether the transient effect of CTX treatment on EL4 cells shown in Figure 1(a) can be more effective after transfection of these cells with OVA

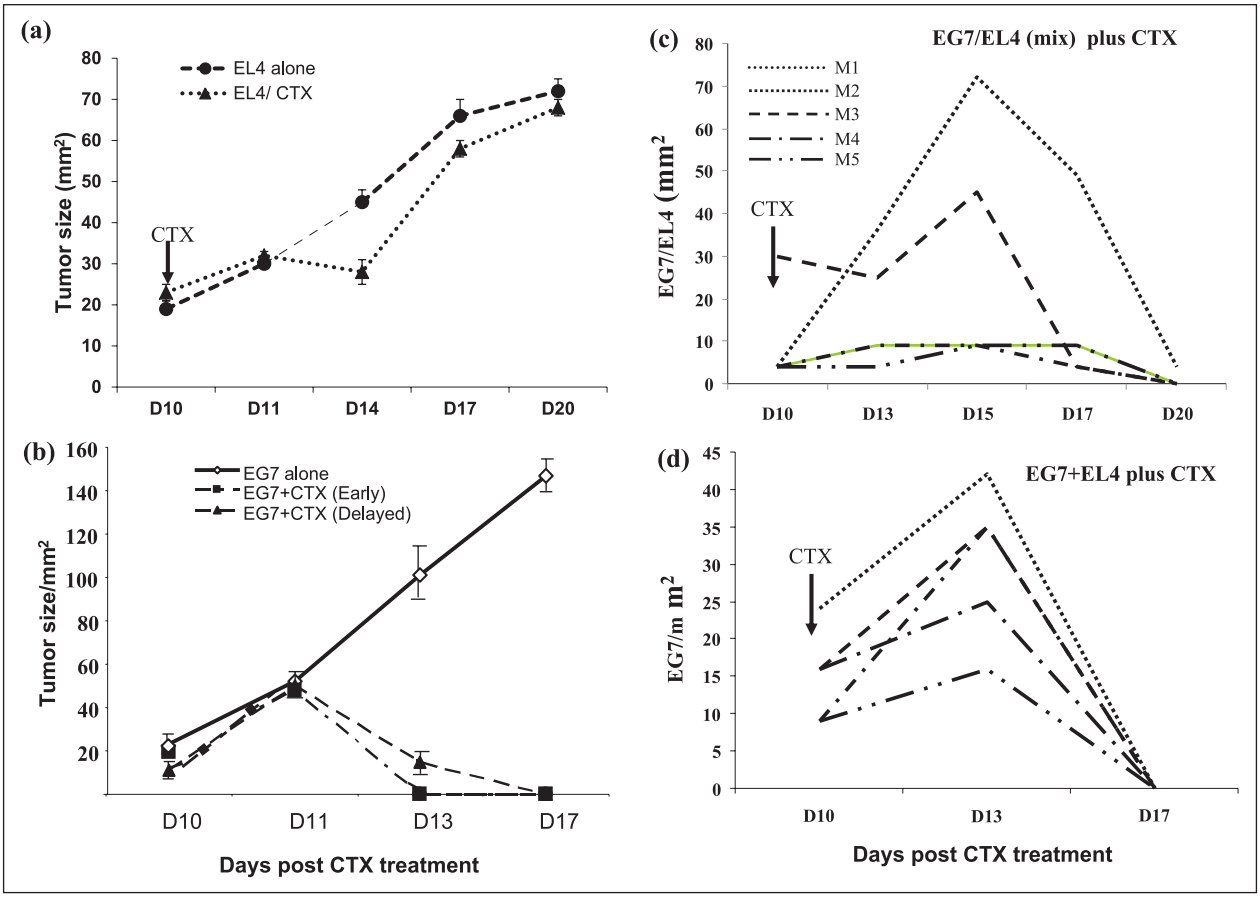

Figure 1. Treatment with CTX at early time points cured advanced EG7 cells coinciding with specific memory response. B6 mice were subcutaneously injected in their flanks with viable (a) EL4 or (b) EG7 at 2.5×10^5 cells/mouse; 10 days after tumor inoculation, the mice were treated with intraperitoneal injection of 200 µL of free PBS or containing 4 mg/mouse CTX. (c) EL4 cells (2.5×10^5) were admixed with 2.5×10^5 EG7 cells (1:1 ratio) and subcutaneously injected into the left flank of naïve B6 mice. (d) Naïve B6 mice were subcutaneously inoculated into their left flank with 2.5×10^5 EL4 cells and into their right flank with 2.5×10^5 EG7 cells. The tumor size was monitored twice a week until day 17 of inoculation.

as a non-self-antigen (i.e. more immunogenic), mice were inoculated into their flank with 2.5×10^5 EG7 viable cells and then were treated with CTX. As shown in Figure 1(b), EG7 cells alone (without any treatment) showed progressive growth from day 10 to day 17. However, CTX treatment on day 10 after tumor inoculation resulted in rapid tumor eradication by day 13 (Figure 1(b)). The same results were observed when CTX treatment was delayed to day 11 of tumor inoculation (Figure 1(b)).

The primary antitumor effect of CTX against EG7 tumor

To test whether separate inoculation of EL4 cells but simultaneously with EG7 cells inoculation or mixing EL4 and EG7 cells together alters the resultant effect of CTX on EG7 growth, we inoculated groups of B6 mice (n=5) with 2.5×10^5 EL4 cells admixed with 2.5×10^5 EG7 cells in the left flank (Figure 1(c)). Another group of mice (n=5) were inoculated in the left flank with EG7 (2.5×10^5) cells and in the right flank with the same number of EL4 cells. The mice were treated with CTX on day 10, and the tumor growth was monitored and measured by caliper on days 11, 13, 17, and 20 after inoculation. As shown in Figure 1(c), CTX treatment of mice bearing EL4/EG7 cells induced regression of the mixed tumor starting on day 15 followed by complete regression on day 20. CTX treatment on day 10 (early treatment) of mice bearing EG7 and EL4 tumors in their contralateral flanks was still able to induce complete regression of EG7 tumor (Figure 1(d)). Interestingly, EL4 in the same mice showed only transient regression (data not shown), similar to those shown in Figure 1(a).

Table 1. Antitumor effects of CTX against primary EG7 tumor resulted in generation of EG7-specific memory response against tumor rechallenge.

Groups	Tumor size (mm^2)—10 days after rechallenge (EL4 lymphoma)	Tumor size (mm^2)—13 days after rechallenge	Tumor size (mm^2)—17 days after rechallenge
EG7/CTX/EL4	100% tumor-bearing mice, all mice showed 4–10 mm^2	100% tumor-bearing mice (25, 25, 6, 42, and 20 mm^2)	100% tumor-bearing mice (121, 80, 20, 64, 100 mm^2)
EG7/CTX/EG7 (second challenge)	100% tumor-bearing mice, all mice showed 4–10 mm^2	All mice showed complete tumor regression	All mice cured
EG7/CTX/EG7/EG7 (third challenge)	100% tumor bearing mice, all mice showed 4–10 mm^2	20% tumor-bearing mice (0, 0, 0, 0, and 20 mm^2)	40% tumor-bearing mice (0, 0, 0, 80, and 16 mm^2)

Curing advanced EG7 tumor by CTX associates with specific anti-EG7 memory response

To address whether eradication of primary EG7 cells after CTX treatment associates with generation of antitumor memory responses, we repeated the experiment as above in Figure 1(b). Treatment of EG7-bearing mice with CTX resulted in regression of the tumor in all mice. The mice were then rechallenged with the same number of EG7 or EL4 cells in their flank. Then, the growth of the secondary tumors was measured. It was found that only EG7 but not EL4 cells were regressed when inoculated into EG7-bearing mice previously treated with CTX (Table 1). Upon the second challenge with EG7 cells, all EG7 tumor inoculated into mice were completely regressed measured at days 13 and 17 after rechallenge (Table 1). Interestingly, after the third challenge with EG7 cells of the mice that showed regression of the first and second EG7 cells, only 20% and 40% of mice showed small tumors on days 13 and 17 (Table 1), and then, the tumors were completely cured (data not shown). Taken together, these data indicate that the antitumor effect of CTX against EG7 tumor is specific and associates with sustained memory responses.

Splenectomy did not alter the antitumor effect of CTX against EG7 tumor

Given that spleen harbors a large pool of T-cells and that CTX induces mobilization of immune cells, in particular myeloid cells, into spleen and induces their activation in particular dendritic cells,[4] we asked whether the absence of spleen interferes with the effects of CTX on EG7 cells. To address this question, splenectomized mice were subcutaneously inoculated in their right flank with EG7 cells and then treated after 10 days with CTX (4 mg/mouse). In wild-type mice, EG7 tumor reached $36 \pm 8\,mm^2$ and $83.0 \pm 8.6\,mm^2$ on days 13 and 17, respectively, of tumor inoculation. Interestingly, splenectomized mice showed similar tumor growth profile, when the tumor size reached $45 \pm 8.9\,mm^2$ and $74.5 \pm 8.1\,mm^2$ on days 13 and 17, respectively. Treatment of EG7-bearing mice wild or splenectomized mice with CTX, however, cured the tumor in all mice measured on days 13 and 17 (data not shown). These results indicate that spleen had no major effect on the response of EG7 tumor to CTX treatment.

Discussion

We addressed whether increasing the immunogenicity of tumor cells can enhance their responses to effects of chemotherapy and induce generation of antitumor immunity, and we compared the antitumor effects of CTX, as a model of anti-cancer chemotherapy, on EL4 and EG7 thymoma as models for poorly and immunogenic tumors, respectively. We found that single treatment with CTX induced transient antitumor effect on EL4 cells, while induced complete regression of advanced EG7 tumors coincided with specific antitumor memory immunity.

Prior studies reported that combination of CTX with doxorubicin, interleukin (IL)-2, interferons (IFNs), and tumor necrosis factor (TNF)-α or vaccination with tumor antigens can induce regression of poorly immunogenic tumors such as colon and EG7 through induction of specific antitumor immunity.[9,10–12] These effects were mediated by making tumor cells more susceptible to lysis by T-lymphocytes as well as induction of bystander apoptosis of the neighboring tumor cells.[13,14] Uniquely, however, the results of this study show that CTX by itself and in the absence of any other modalities induced specific regression of advanced poorly immunogenic thymoma after making them

immunogenic. We found that absence of spleen, which may mediate tumor-induced tolerance,[15] did not alter these antitumor effects of CTX. Given that both EG7 and EL4 cells similarly express class-I and very low class-II molecules[15] and that EG7 but not EL4 express OVA, these antitumor effects of CTX could be mediated by OVA-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) against OVA expressed in EG7 cells. The effects of CTX can also be suggested to its capability of depleting T_{reg} cells and inducing bystander inflammatory microenvironment, including type-1 IFN, dendritic cell (DC) mobilization and activation, and Th1-type cytokines.[5,12] Together, these bystander effects may mediate inhibition of the regulatory molecules B7-2 and PD-1/PD-L1[3] and upregulate the expression of the co-stimulatory molecules B7-1 and major histocompatibility complex (MHC) class-II on EG7 tumor and dendritic cells, resulting in overall enhancement of antitumor immunity. In conclusion, our results indicate that chemotherapy following induction of immunogenic tumor microenvironment can result in tumor regression and antitumor immunity.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Salem ML, Diaz-Montero CM, Chen Y, et al. (2007) The TLR3 agonist poly (I: C) acted directly on mouse CD8 T cells and augmented their antigen-specific responses upon adoptive transfer into naïve recipient mice (48.31). *The Journal of Immunology* 178: S80–S81.
2. Salem ML, Kadima AN, El-Naggar SA, et al. (2007) Defining the ability of cyclophosphamide preconditioning to enhance the antigen-specific CD8+ T-cell response to peptide vaccination: Creation of a beneficial host microenvironment involving type I IFNs and myeloid cells. *Journal of Immunotherapy* 30: 40–53.
3. Bracci L, Schiavoni G, Sistigu A, et al. (2014) Immune-based mechanisms of cytotoxic chemotherapy: Implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death & Differentiation* 21: 15–25.
4. Jurado García JM, Sánchez A, Pajares B, et al. (2008) Combined oral cyclophosphamide and bevacizumab in heavily pre-treated ovarian cancer. *Clinical and Translational Oncology* 10: 583–586.
5. Salem ML, Al-Khami AA, El-Naggar SA, et al. (2010) Cyclophosphamide induces dynamic alterations in the host microenvironments resulting in a Flt3 ligand-dependent expansion of dendritic cells. *The Journal of Immunology* 184: 1737–1747.
6. Han S, Knoepp SM, Hallman MA, et al. (2007) RasGRP1 confers the phorbol ester-sensitive phenotype to EL4 lymphoma cells. *Molecular pharmacology* 71: 314–322.
7. Reddy R, Zhou F, Huang L, et al. (1991) pH sensitive liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition of a soluble protein. *Journal of Immunological Methods* 141: 157–163.
8. Moore MW, Carbone FR and Bevan MJ (1988) Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* 54: 777–785.
9. Tongu M, Harashima N, Yamada T, et al. (2010) Immunogenic chemotherapy with cyclophosphamide and doxorubicin against established murine carcinoma. *Cancer Immunology, Immunotherapy* 59: 769–777.
10. Kato M, Nakamura Y, Suda T, et al. (2011) Enhanced anti-tumor immunity by superantigen-pulsed dendritic cells. *Cancer Immunology, Immunotherapy* 60: 1029–1038.
11. He L-Z, Prostak N, Thomas LJ, et al. (2013) Agonist anti-human CD27 monoclonal antibody induces T cell activation and tumor immunity in human CD27–transgenic mice. *The Journal of Immunology* 191: 4174–4183.
12. Schiavoni G, Sistigu A, Valentini M, et al. (2011) Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. *Cancer Research* 71: 768–778.
13. Ramakrishnan R, Assudani D, Nagaraj S, et al. (2010) Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. *The Journal of Clinical Investigation* 120: 1111–1124.
14. Maccubbin DL, Wing KR, Mace KF, et al. (1992) Adriamycin-induced modulation of host defenses in tumor-bearing mice. *Cancer Research* 52: 3572–3576.
15. Zhou F, Rouse T and Huang L. (1992) Prolonged survival of thymoma-bearing mice after vaccination with a soluble protein antigen entrapped in liposomes: A model study. *Cancer Research* 52: 6287–6291.