Cancer Letters 366 (2015) 93–99

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

# High-dose cyclophosphamide induces specific tumor immunity with concomitant recruitment of LAMP1/CD107a-expressing CD4-positive T cells into tumor sites

Tatsushi Naito [a,b], Tomohisa Baba [a], Kazuyoshi Takeda [c], Soichiro Sasaki [a], Yasunari Nakamoto [b], Naofumi Mukaida [a,*]

[a] Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan
[b] Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui, Eiheiji-cho, Fukui 910-1193, Japan
[c] Division of Cell Biology, Biomedical Research Center, Graduate School of Medicine, Juntendo University, Bunkyo-ku, Tokyo 113-8421, Japan

ARTICLE INFO

*Article history:*
Received 7 April 2015
Received in revised form 1 June 2015
Accepted 15 June 2015

*Keywords:*
CC chemokine
CD4+ T cell
Cytolytic T cell
LAMP1/CD107a

ABSTRACT

Cancer chemotherapy regimens, particularly those employing high-dose cytotoxic drugs such as cyclophosphamide (CTX), have been considered to be immune suppressive. However, we observed that a single administration of high-dose CTX abolished tumors arising from subcutaneous injection of a mouse hepatoma cell line and subsequently induced specific tumor immunity. Depletion of T cells, specifically CD4+ T cells, abrogated the CTX-mediated tumor regression. CTX treatment induced the rapid recruitment of CD4+ T cells into the tumors, and these recruited cells initiated expression of LAMP1/CD107a, a cytotoxic granule molecule, and granzyme B in the absence of antigen presentation at draining lymph nodes and proliferation in the tumor tissues. Moreover, CTX enhanced the expression of a CC chemokine, CCL3, in tumor tissues, and CTX-mediated tumor regression was attenuated in mice deficient in CCR5, the receptor for this chemokine. Consistently, less CTX-induced accumulation of intratumoral LAMP1/CD107a-expressing CD4+ T cells was observed in mice receiving splenocytes derived from CCR5-deficient mice than in those receiving splenocytes derived from WT mice. Thus, CTX induces the expression of CCL3, which induces the intratumoral migration of CD4+ T cells expressing cytotoxic molecules, leading to tumor eradication and subsequent specific tumor immunity.



## Introduction

Cyclophosphamide (CTX) is oxidized to 4-hydroxycyclophosphamide [1], which enters cells and spontaneously decomposes to phosphoramide mustard. At a physiological pH of 7.4, this component causes covalent linkage of DNA alkyl groups [2]. The resultant inter-strand cross-link creates denaturation-resistant DNA fractions, thereby inhibiting DNA replication and leading to subsequent apoptosis [3]. The cytotoxic action of CTX is exerted against highly proliferative cells, particularly lymphocytes and cancer cells, and causes the depletion of lymphocytes from the peripheral blood and tissue [4]. Consequently, CTX, particularly at high doses (100–200 mg/kg), has been employed as one of the most potent immune suppressive drugs to combat life-threatening autoimmune diseases and prevent graft-versus-host disease after allogenic bone marrow transplantation [5].

Regulatory T cells (Tregs), characterized by the expression of CD4, CD25, and Foxp3, accumulate in tumor tissues of both patients and mice and can contribute to immune tolerance to cancer cells [6]. Low doses of CTX can deplete Tregs in the blood and lymphoid organs of tumor-bearing mice and cause a decrease in the number of Tregs that infiltrate tumor tissues [7,8]. This decrease in infiltrating Tregs can improve cytotoxic T cell- and/or NK cell-mediated antitumor immunity, not only in tumor-bearing mice but also in advanced cancer patients [9]. Moreover, low-dose CTX can induce the polarization of Th2 to Th1 and eventually exert anti-metastatic effects [10]. Recent observations have suggested that a single high dose of CTX can induce specific tumor immunity [11]. Hence, we examined the effects of a single high dose of CTX on tumors arising from subcutaneous injection of a mouse hepatoma cell line. We demonstrate that high-dose CTX can eradicate tumors in a T cell-dependent (particularly CD4+ T cell) manner. Our results contrast with the widely held view that high-dose CTX can be immune suppressive.

*Abbreviations:* APC, allophycocyanin; CFSE, carboxyfluorescein diacetate, succinimidyl ester; CTLs, cytolytic T lymphocytes; CTX, cyclophosphamide; dLN, draining lymph node; FCM, flow cytometry; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HPRT, hypoxanthine phosphoribosyltransferase; MFI, mean fluorescence intensity; Tregs, regulatory T cells.

* Corresponding author. Tel.: +81 76 264 6735; fax: +81 76 234 4520.
*E-mail address:* mukaida@staff.kanazawa-u.ac.jp (N. Mukaida).

http://dx.doi.org/10.1016/j.canlet.2015.06.009
0304-3835/

## Materials and methods

### Mice

Specific pathogen-free male five- to seven-week old BALB/c mice (WT mice) and BALB/c-nu mice (nude mice) were purchased from Charles River Japan (Yokohama, Japan). CCR5-deficient ($CCR5^{-/-}$) mice were backcrossed with BALB/c mice for more than eight generations [12]. CD45.1 BALB/c congenic mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions. All animal experiments were approved and performed according to the Guideline for the Care and Use of Laboratory Animals of Kanazawa University.

### Tumor cell line

A murine hepatocellular carcinoma (HCC) cell line, BNL 1ME A.7R.1 (BNL), was purchased from the American Type Culture Collection (Manassa, VA) and maintained at low passage throughout the study. The cells were cultured as previously described [13].

### Tumor injection

The left flanks of eight-week old male WT, nude, or $CCR5^{-/-}$ mice were subcutaneously (s.c.) inoculated with $5 \times 10^5$ BNL cells in 100 μL of PBS. Tumor sizes were evaluated three times a week using calipers, and tumor volumes were calculated using the following formula: Tumor volume ($mm^3$) = (the longest diameter) × (the shortest diameter) × (depth)/2. When the tumor volume reached 40–80 $mm^3$, the mice were intraperitoneally (i.p.) injected with 150 mg/kg CTX (Fig. 1A). In some experiments, mice received two i.p. injections of anti-CD4 (GK1.5, 100 μg/animal) or anti-CD8 antibody (53.6.7, 200 μg/animal) on Days 1 and 14 (Fig. 1D). In another series of experiments, at 30 days after the first inoculation of BNL cells into the mice, the animals whose tumors had completely regressed after treatment were inoculated again with $5 \times 10^5$ BNL cells and $2.5 \times 10^5$ colon 26 (Col26, a murine colon carcinoma cell line) cells into the right and left flanks, respectively. As a control, WT mice were inoculated with 100 μL of PBS into the left flank instead of the first BNL inoculation. The day of the second BNL inoculation was designated as day 0.

### Adoptive transfer of splenocytes

Single cell populations were obtained from the spleens of CD45.1 congenic WT mice, then the cells were labeled with 2 μM of carboxyfluorescein diacetate, succinimidyl ester (CFSE, Life Technologies Inc., Grand Island, NY). Ten million CFSE-labeled cells in 200 μL of PBS were intravenously transferred to CD45.2 WT mice via the tail vein one day after CTX administration (Fig. 3A). Similarly, $1 \times 10^7$ CFSE-labeled splenocytes from $CCR5^{-/-}$ mice were intravenously injected into the tail vein one day after CTX administration (Fig. 5A).

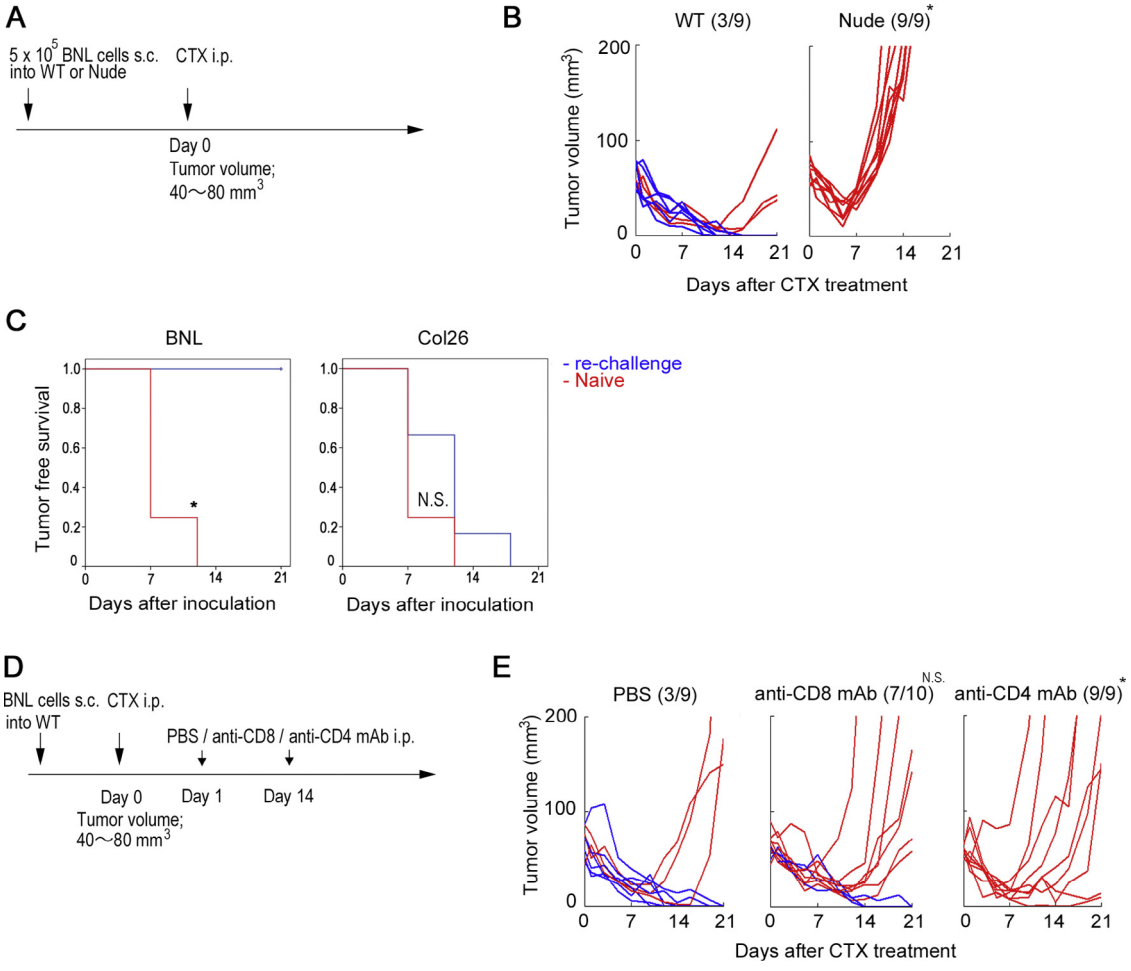


**Fig. 1.** BNL tumors are eradicated by single high-dose CTX administration. (A) Schematic representation of the experimental procedures. Five hundred thousand BNL cells were inoculated into the left flank of WT or nude mice. CTX was intraperitoneally injected at a dose of 150 mg/kg into the mice when the tumor volume reached 40–80 $mm^3$. Tumor volumes were measured every two to three days after CTX treatment. (B) Tumor volumes were measured after CTX treatment in BNL tumor-bearing WT (left panel) or nude mice (right panel). The blue and red lines indicate the tumor volumes in the animals whose tumors regressed after CTX treatment and those whose tumors recurred after treatment, respectively. The parentheses indicate the numbers of mice with recurrent tumors among the total number of mice. *p* values were calculated using Fisher's exact test. $^*p < 0.05$. (C) WT mice whose BNL tumor disappeared after CTX treatment or naïve WT mice subcutaneously received BNL and Col26 cells into the right and left flanks, respectively. Tumor-free time intervals were determined until 21 days after injection. The blue and red lines indicate re-challenged and naïve mice, respectively. Each group consisted of six animals. *p* values were calculated using the log-rank test. $^*p < 0.05$. (D and E) Mice were treated, as shown in Fig. 1A. The mice were intraperitoneally injected with anti-CD4 mAb (100 μg/animal), anti-CD8 mAb (200 μg/animal), or PBS one day and 14 days after CTX treatment, as shown in D. Tumor volumes were measured in mice injected with PBS (left panel), anti-CD8 mAb (middle panel), or anti-CD4 mAb (right panel) for 21 days after CTX injection. The blue and red lines indicate the animals whose tumors regressed after CTX treatment and those whose tumors recurred after treatment, respectively (E). The parentheses indicate the numbers of mice with recurrent tumors among the total number of mice. *p* values were calculated using Fisher's exact test. $^*p < 0.05$; N.S., no significant difference.