# Beta-(1-3),(1-6)-D-glucan Enhances the Effect of Low-dose Cyclophosphamide Treatment on A20 Lymphoma in Mice

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**Abstract.** *Background: Beta-(1-3),(1-6)-D-glucans demonstrate antitumor effects in vivo due to the activation of innate immune cells. Cyclophosphamide (CY) enhances natural or therapeutically induced antitumor immune responses by reducing the number and activity of regulatory T (Treg) cells. Materials and Methods: We tested whether oral administration of soluble beta-glucan augmented the inhibitory effect of intraperitoneally injected low-dose CY (30 mg/kg) on subcutaneously growing A20-lymphoma in Balb/c-mice. Results: Administration of CY one week after tumor inoculation significantly diminished tumor growth (p=0.009) and the absolute number of Treg cells in the peripheral blood compared with phosphate buffered saline-treated mice (p=0.036). Treatment of CY pre-conditioned lymphoma-bearing mice with daily beta-glucan (400 μg/mouse) between day 9 and day 13 after tumor injection significantly delayed onset of tumor growth, compared to mice which received only CY (p=0.01). Conclusion: Beta-(1-3),(1-6)-D-glucan could be useful in the treatment of lymphoma after low-dose chemotherapy with CY.*

The treatment of tumor-bearing hosts with a combination of chemotherapeutic and immune-stimulatory agents receives increasing attention and is tested in numerous pre-clinical and clinical studies. Cyclophosphamide (CY) is a commonly used chemotherapeutic agent for the treatment of lymphomas and solid tumor malignancies. Besides its direct cytotoxic function on tumor cells, CY was shown to exert pronounced immune-modulatory effects by decreasing T suppressor cells, resetting dendritic cell (DC) maturation and strong induction of immune-activatory cytokines in the 'rebound phase' of transiently drug-induced lymphocytopenia (1-4). Thus, the dosage and administration schedule of CY to improve the efficacy of natural or therapeutically induced anti-tumor immune responses was evaluated in several preclinical and clinical studies (2, 4-6).

Beta-(1-3),(1-6)-D-glucans are branched glucose polymers derived from the cell wall of a variety of microorganisms and plants, which were shown to exert tumor-inhibitory effects due to pleiotropic activation of the innate immune system including macrophages, DC and granulocytes (7-10). In mouse tumor models and in cancer patients, the therapeutic effect of antineoplastic agents was enhanced by beta-glucans of different origins (11-13). However, the knowledge about the antitumor activity of β-glucans in CY-treated tumor-bearing hosts is limited (14, 15). This study evaluated whether an orally administered beta-(1-3),(1-6)-D-glucan derived from the cell wall of *Saccharomyces cerevisiae* enhances inhibition of subcutaneously (*s.c.*) inoculated A20-lymphoma in mice treated with low-dose CY.

## Materials and Methods

*Mice and cell line.* Female, 8-week-old Balb/c-mice were purchased from Charles River (Sulzendorf, Germany) and were maintained in the pathogen-free animal facility of epo GmbH (Berlin-Buch, Germany) following institutional guidelines and with approval from the responsible authorities. A20, a Balb/c-derived B lymphoma cell line, was kindly provided by Genethor GmbH (Berlin, Germany) and was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (all from Biochrom, Berlin, Germany).

*Reagents and antibodies.* Cyclophosphamide was obtained from Baxter Oncology GmbH (Halle, Germany). An aqueous preparation of underivatized soluble beta-(1–3), (1–6)-D-glucan (20 mg/ml), derived from the cell wall of *S. cerevisiae* was provided by Biotec Pharmacon (Tromsø, Norway). Endotoxin level was <0.05 EU/ml.

*In vivo experiments.* To evaluate the therapeutic effect of CY on the growth of the A20 lymphoma, mice were intraperitoneally injected with CY (30 mg/kg) dissolved in 100 μl phosphate-buffered saline (PBS), or received the same volume of PBS as control (both groups n=6), one week after *s.c.* inoculation of $1\times10^5$ tumor cells. To

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determine the effect of beta-glucan on A20 lymphoma growth in CY-treated mice, the carbohydrate (400 μg) was dissolved in 100 μl PBS and administered intragastrically, once daily for 5 days, starting 48 hours after injection of CY, as described above. CY-treated control mice received no further treatment (both groups n=6). The tumor volume was determined by measuring the width and length twice weekly using an electronic caliper. A tumor volume greater than 0.005 $cm^3$ indicated a detectable tumor, and mice were sacrificed when the tumor volume reached 1.5 $cm^3$. All animal experiments were performed according to the German animal protection law and with approval from the responsible authorities.

*Quantification of Treg cells*. CD4+CD25(high) T-cells were quantified in the peripheral blood of individual mice by flow cytometry. In brief, 50 μl peripheral blood was taken from all mice by retro-orbital puncture, before and 2 or 7 days after administration of CY. Whole blood cells were stained with FITC-labeled anti-mouse CD4 (L3T4) and PE-labeled anti-mouse CD25 (PC61.5) antibodies, or relevant isotype control antibodies (all from eBioscience, Frankfurt, Germany), on ice. Using a FACS-Calibur (Beckton Dickinson, Heidelberg, Germany), $3\times10^5$ cells were measured and data was analysed using the CellQuest program (BD Biosciences, Heidelberg, Germany).

*Statistical analysis*. Data is presented as mean±the standard error of the mean (SEM). Statistical significance of the data was calculated by two-sided, paired Student's *t*-test. A significance level of $p<0.05$ was chosen.

## Results

The administration of 0.6 mg CY into A20 lymphoma-bearing mice one week after inoculation of the lymphoma significantly diminished tumor growth ($p=0.009$) (Figure 1A). Furthermore, one week after injection of CY, the number of $CD4^+$CD25(high) T-cells in the peripheral blood was reduced on average by 69±13% ($p=0.01$), compared to the pre-treatment number, whereas in the same period, PBS-treated mice showed a decrease of Treg cells of 30±31% ($p=0.26$) (Figure 1B). The ratio of $CD4^+$CD25(high)/$CD4^+$ T-cells lacked a notable change within both treatment groups (data not shown), due to a concomitant significant decrease of the number of $CD4^+$ T-cells of 68±8% and 44±18% in the peripheral blood of CY- and PBS-treated tumor-bearing mice, respectively ($p=0.004$ and $p=0.026$, respectively) (Figure 1C).

To determine the effect of beta-glucan on the tumor growth in mice pre-treated with low-dose CY, the carbohydrate was consecutively administered over 5 days, starting 2 days after injection of the chemotherapeutic agent. Co-treatment with beta-glucan delayed transiently the development of a detectable tumor ($p=0.01$) (Figure 2).

## Discussion

CY is routinely used in the treatment of patients with lymphomas and solid tumors. In accordance to reports on the treatment of A20 and other murine B-cell lymphomas, CY demonstrated a tumor-inhibitory effect when administered at a low dose in the present study (16-18). A additional tumor inhibition by the beta-glucan after pre-treatment with CY was similarly reported with the subsequent or simultaneous treatment of tumor-bearing hosts with this chemotherapeutic agent, or different anticancer drugs and beta-glucan, and was likely mediated by the immune-stimulatory activity of the carbohydrate (7-15). Furthermore, the antitumor activity of both consecutively administered agents appear to be synergistic, considering the previously shown weak tendency of the beta-glucan alone to diminish the growth of *s.c.* inoculated A20 lymphoma (9). Indeed, several studies demonstrated that a pre-conditioning of tumor-bearing hosts with CY restored naturally occurring immune responses and significantly enhanced the generation of subsequently therapeutically induced antitumor immune responses (1-6, 14, 15). However, the additional tumor-inhibitory effect of the beta-glucan was transient, suggesting that CY may establish a less immunosuppressed state, which could have alleviated the activation of innate and adaptive immune responses by the carbohydrate only temporarily (2, 4-6). Tumor-induced immune-suppression *via* expansion of Treg cells, myeloid-derived suppressor cells and altered cytokine production, may have down-regulated antitumor immune responses (19-22). Recently, the treatment of tumor-free mice with a similar amount of CY as used in our study was shown to suppress Treg cell numbers in the spleen over 4 weeks (2). However, intratumoral Treg cells may have recovered earlier than Treg cells in the peripheral blood or they could have lacked a significant reduction by low-dose CY, as reported for mammary tumor-bearing mice (23). Furthermore, a beta-(1-3),(1-6)-glucan was shown to reduce a strong decrease of $CD4^+$ and $CD8^+$ T-cells by CY in the murine spleen, and this protective effect could also encompass Treg cells (24). The failure of low-dose CY to notably change the ratio of Treg cells and total $CD4^+$ T-cells in tumor-bearing hosts was similarly reported in the spleen of colon-carcinoma-bearing Balb/c-mice (6), being due to a concomitant decrease of total $CD4^+$ T-cells in the peripheral blood, as described for murine spleen and lymph nodes (1, 2). The absence of a decrease of the Treg cell number until day 3 of treatment with CY is in contrast to reports by others (1, 2). However, this might have little impact on the activation of innate immune cells by the carbohydrate, since its delivery started at the time point and its uptake and processing into bioactive fragments was shown to require 3-4 days (25). Taken together, we provide evidence that orally administered beta-(1-3),(1-6)-D-glucan enhances the inhibitory effect of low-dose CY on *s.c.* growing A20 lymphoma in mice, which could have implications for a chemo-immune therapy of cancer patients.

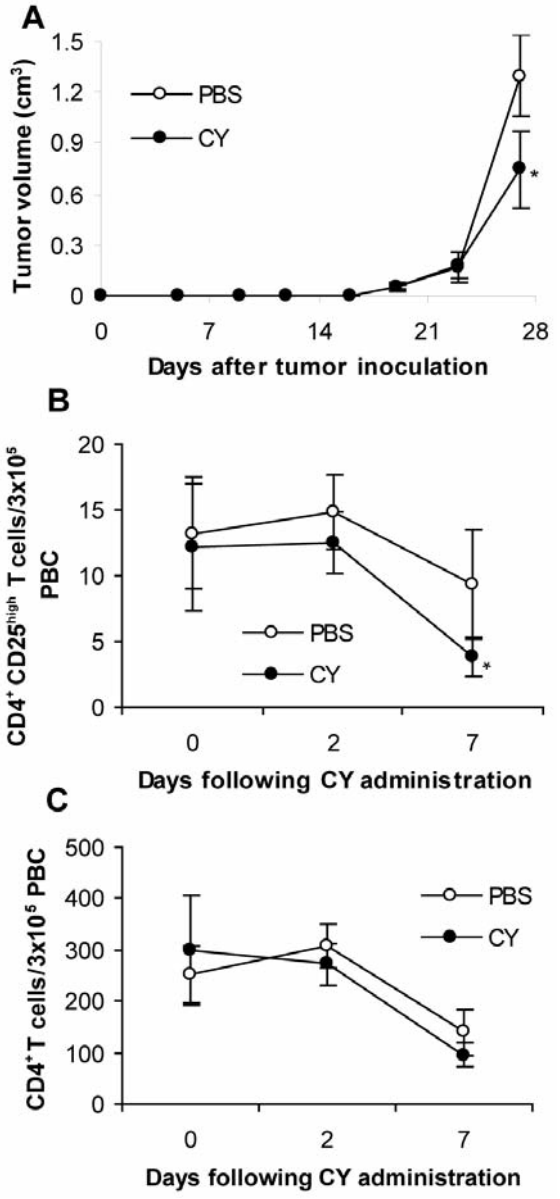


Figure 1. *Effect of CY on A20 lymphoma growth and Treg cells in vivo. A: Tumor volume measured twice weekly in Balb/c-mice which were injected i.p. with CY (30 mg/kg) or PBS (each group n=6) one week after s.c. inoculation of $1\times10^5$ tumor cells. B and C: Number of $CD4^+CD25^{high}$ and $CD4^+$ T-cells in the peripheral blood of A20-lymphoma-bearing mice. Whole blood cells from CY- or PBS-treated mice taken at the indicated time points were stained with fluorescence-labeled antibodies against CD4 and CD25 and analyzed by flow cytometry. Data show the mean±SEM of all mice of a treatment group and statistical significance was determined by two-sided, paired Student's t-test and is indicated by *p<0.05.*

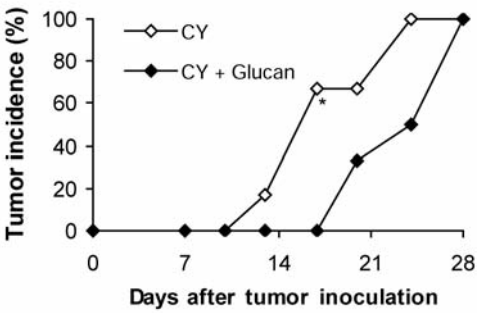


Figure 2. *Effect of beta-glucan on A20 lymphoma growth in CY-treated Balb/c-mice. CY (30 mg/kg) was injected into Balb/c-mice on day 8 of the inoculation of $1\times10^5$ tumor cells, followed by the oral administration of beta-glucan or no agent (each group n=6) once daily over five days starting 48 hours after injection of CY. Data show the percentage of mice exhibiting a detectable tumor at the indicated time point and are representative of a single experiment. Statistical significance was determined by two-sided, paired Student's t-test and is indicated by *p<0.05.*

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